Dual-Channel Single-Molecule Fluorescence Resonance Energy Transfer to Establish Distance Parameters for RNA Nanoparticles

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The increasing interest in RNA nanotechnology and the demonstrated feasibility of using RNA nanoparticles as therapeutics have prompted the need for imaging systems with nanometer-scale resolution for RNA studies. Phi29 dimeric pRNAs can serve as building blocks in assembly into the hexameric ring of the nanomotors, as modules of RNA nanoparticles, and as vehicles for specific delivery of therapeutics to cancers or viral infected cells. The understanding of the 3D structure of this novel RNA dimeric particle is fundamentally and practically important. Although a 3D model of pRNA dimer has been proposed based on biochemical analysis, no distance measurements or X-ray diffraction data have been reported. Here we evaluated the application of our customized single-molecule dual-viewing system for distance measurement within pRNA dimers using single-molecule Fluorescence Resonance Energy Transfer (smFRET). Ten pRNA monomers labeled with single donor or acceptor fluorophores at various locations were constructed and eight dimers were assembled. smFRET signals were detected for six dimers. The tethered arm sizes of the fluorophores were estimated empirically from dual-labeled RNA/DNA standards. The distances between donor and acceptor were calculated and used as distance parameters to assess and refine the previously reported 3D model of the pRNA dimer. Distances between nucleotides in pRNA dimers were found to be different from those of the dimers bound to procapsid, suggesting a conformational change of the pRNA dimer upon binding to the procapsid.

KEYWORDS: DNA packaging motor · RNA 3D structure · nanotechnology · nanobiotechnology · nanomedicine · distance measurement of nanoparticles
Computer modeling of the 3D structure of pRNA monomer, dimer, and hexamer has been reported. Generation of these models was based on indirect methods including photoaffinity cross-linking, chemical modification interference, complementary modification, cryo-AFM, mutagenesis, ribonuclease probing, primer extension, and oligo targeting. Recently, our lab has assembled a customized single-molecule dual-viewing TIRF imaging system (SMDV-TIRF) with single fluorophore sensitivity and dual-color imaging ability. In this report, we evaluate its efficacy to establish more accurately measured distance parameters for the pRNA dimer based on the results from single-molecule FRET, for refinement of the 3D model.

**RESULTS AND DISCUSSION**

**Determination of the Arm Length of the Fluorophores.** The distance range between the donor and the acceptor to achieve measurable energy transfer is approximately 1–7.5 nm for a Cy3/Cy5 pair. The labeling of RNA with fluorescent dyes generates tethered arms whose length is an important factor in distance determination by FRET. However, it is difficult to obtain a solid value of arm size for each fluorophore due to other factors such as orientation, folding and flexibility in molecular arrangement. Empirical determination is one feasible approach to estimate the length for both the donor and acceptor arms. Hence, we used five standard RNA/DNA hybrids with known distances between the donor and acceptor arms. Therefore, we used five standard RNA/DNA hybrids with known distances between the donor and acceptor arms (12bp, 14bp, 16bp, 18bp, and 20bp) (Figure 2A) to determine the empirical arm size of the fluorophore and to evaluate the feasibility of using our dual-viewing single molecule imaging system to study pRNA structure. The RNA/DNA hybrids were chosen here as the labeled pRNA dimers studied in this report were partially constructed through RNA/DNA annealing.

The FRET pair Cy3(green)/Cy5(red) (Figure 2A) was selected because of its fluorescence stability. A 532 nm green laser was used for Cy3 excitation. Both donor and acceptor signals were recorded simultaneously during continuous Cy3 excitation using the SMDV-TIRF dual channel imaging system constructed in the lab. A typical time trajectory of fluorescence intensity for a FRET event is shown in Figure 2B. The Cy5 was photobleached before Cy3 due to its less stability, and the intensity of Cy3 increased due to the loss of FRET. The FRET event can therefore be confirmed based on a sudden drop in acceptor intensity accompanied with a sudden increase in donor intensity: in another words, the anticorrelated donor and acceptor signals. Changes of donor fluorescence intensity in the presence ($I_{DA}$) and absence ($I_{D}$) of the acceptor were measured. The FRET efficiency ($E$) can be obtained based on the change of acceptor emission in the presence ($I_{DA}$) and absence ($I_{D}$) of the acceptor eq 1 (Figure 2B).

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Figure 1. Illustration of (A) sequence and (B) structure of the pRNA dimer. The same letters in upper and lower case indicate complementary sequences for the pRNA loop-loop interaction, while different letters indicate noncomplementary loops. For example, pRNA Ab represents pRNA where right loop A (5'G45G46A47C48) is complementary to left loop a' (3'C85C84U83G82) of pRNA Ba; and left loop b' (3'U85G84C83G82) is complementary to right loop B (5'A45C46G47C48). The RNA1 and RNA2 were assembled into dimer via the interlocking loops. Numbers in green represent the six donor positions in subunit Ab and numbers in red represent the two acceptor positions in subunit Ba' in two different dimers (see Table 2). The mechanism of viral DNA packaging and ejection has been of great interest and been studied extensively. Bacteriophage phi29 DNA-packaging motor contains a hexameric pRNA ring. The pRNA molecule contains two hand-in-hand interlocking loops, identified as the right- and left-hand loops marked as an upper- and a lower-case letter, respectively (Figure 1). It has been shown that there are two functional domains in pRNA — one (bases 23–97 at the central region) for intermolecular interaction and formation of the pRNA multimers, the other for DNA packaging. Dimeric or trimeric pRNA have been demonstrated as the building blocks in pRNA hexamer assembly, and can be constructed for applications in nanotechnology. The pRNA dimers or trimers derived from the phi29 pRNA system have been demonstrated as polyvalent vehicles for delivery of a variety of therapeutic molecules such as siRNA, ribozymes, or aptamers to specific cells via ligand directed specific targeting. Further application in nanotechnology and nanomedicine will require elucidation of the 3D structure of the pRNA dimer.
Unlike FRET efficiency calculations using acceptor emission,\textsuperscript{15,24,56} this equation does not need to take into account of factors such as the leakages of Cy3 signal to Cy5 channel, direct excitation of Cy5 by Cy3 excitation wavelength, the different quantum yields and detection efficiency for Cy3 and Cy5, and thus simplifies the calculation. The distance ($R$) between the FRET pair was derived from $E$ by eq 2:

$$R = R_0(1/E - 1)^{1/6}$$

The Förster distance $R_0$ was experimentally measured to be 5.3 nm for the Cy3/Cy5 pair, in agreement with published result.\textsuperscript{57}

The FRET efficiency ($E$) and distance ($R$) deduced from $E$ for each of the individual molecules were summarized in histograms (Figure 2C and D) and fitted with a Gaussian curve to obtain the mean value of $E$ and $R$ for each hybrid (Figure 2C and D, Table 1).

The RNA backbone contains ribose rather than the deoxyribose found in DNA. Unlike DNA, RNA is unable to coil into the B-Type double helix because the hydroxyl group at the 2'-carbon of the sugar ring in RNA makes the ring too large to fit into the allotted space for the B-Type duplex due to steric hindrance.\textsuperscript{58} Therefore,
double-stranded RNA molecules form either an A-helix, or Z-helix with specific sequences. In RNA/DNA hybrids, both RNA and DNA polynucleotide chains resist general conversion into the B-form, but rather adopt the A-conformation.59 Because of the ionic strength used in our studies (100 mM NaCl, 10 mM Mg²⁺, 50 mM Tris-HCl, pH 8), the RNA/DNA hybrids have the following structural parameters: 10.9 bp per helix turn, a pitch of 3.0 nm and a helix rise of 0.275 nm per bp60 (http://www.whatislife.com/reader/dna-rna/dna-rna.html). The relationships between the $E$ and $R$ values, with basepair numbers are plotted (Figure 3). The calculated distances were compared with theoretical distances (number of bp multiplied by 0.275 nm) (Figure 3B). These differences were used as estimates of the arm size of the two fluorophores (Table 1). Thus, the average arm size of 0.9 nm was applied in the distance measurement in the pRNA dimer structural studies.

Selection of Nucleotide Positions for Fluorescence Labeling. The feasibility of applying FRET for RNA structure studies is a case by case issue. RNAs will refold partially or completely when a new chemical group, dye or nucleotide is introduced into the RNA sequence. It is not feasible to simply introduce a pair of dyes to the RNA and measure the FRET to derive the distance. However in this work, after each dye introduced into each pRNA molecule, we assess their structures and folding by at least one of the following approaches: 1. dimer formation;36,61 2. procapsid binding;31,62,63 3. DNA packaging activity;64,65 4. in vitro phi29 virion assembly activ-
The mutated or truncated RNA with incomplete pRNA sequence would be inactive in DNA packaging or in virion assembly. Thus, using biological activity to assess their folding is not possible. In such cases, we only tested their binding to procapsid using \[3H\] RNA (Figure 4C). If the pRNA failed in any one of the functions, we regarded the pRNA as structurally re-folded and did not consider the data comparable to the wild type pRNA.

In addition, it has been reported that FRET efficiency is affected by the orientation of the fluorophore placed in double helical nucleic acids, and a cylindrical model that accounts for the orientation factor and the helical structure has been used.\(^{15, 17, 68, 69}\) It is also suggested that introduction of structural flexibility for the freely rotating of the fluorophores would reduce the orientation effect.\(^{17, 69}\) In this study, we provided flexibility to the fluorophore by placing it at the terminal of the pRNA as an unfolded structure and the length of the fluorophore arm was deduced empirically with RNA/DNA hybrid standards (see first section).

**Determination of Nucleotide Distance within the pRNA Dimers.**

The smFRET was utilized to obtain distances between two nucleotides (Nt) of two pRNAs in dimers (Figure 1). To simplify the description of RNA construction and multimer assembly, uppercase letters are used to represent the right-hand loop and lowercase letters to represent the left-hand loop of pRNA (Figure 1A).

The labeling of pRNA for single molecule imaging has been challenging, as single fluorophore labeling at desired nucleotide position is required. Utilizing our unique single labeling strategy coupled with the circular permutation pRNA (cpRNA) technique,\(^{48, 70}\) labeling with only one fluorophore at the desired base is ensured. The circular permutation technique generates new \(3'/5'\) openings at desired base locations along the pRNA sequence.\(^{48, 70-72}\) The correct folding of pRNAs after this rearrangement has been extensively tested.\(^{39, 48, 44, 70, 73}\) The single labeling at the new \(5'\)-ends was then achieved by \textit{in vitro} transcription with a fluorescent AMP.\(^{74}\) Various RNA molecules (Figure 1B, RNA1 in the dimer) were constructed using the circular permutation pRNA approach and labeled with a single Cy3 at various nucleotides (Nt). Each labeled RNA was paired with its partner RNA2 (Figure 1B) containing both a Cy5 and a biotin moiety. The biotin label allows for immobilization to a streptavidin coated quartz slide surface for TIRF imaging. The distances between two inter-molecular bases within the dimer (Figure 1B) were studied by smFRET. Dimers were assembled with high efficiency when pRNA Ab\(^{-}\) was mixed with an equal amount of Ba\(^{+}\), as confirmed by native PAGE gel (Figure 4A and B), procapsid binding (Figure 4C) and AFM imaging (Figure 5).

Eight partner pairs, a–h, were assembled into dimers (Table 2). Dimers a–f displayed FRET signals (Figure 6). No fluorescence energy transfer was detected for dimer g or h, indicating that the distance between the donor/acceptor pair in these dimers was out of the detectable range. This result was expected, as the data from previous 3D computer model\(^{39}\) generated a distance of 11.1 nm between Nt1 of one pRNA and Nt21 of the second pRNA, and 9.3 nm between Nt30 of one pRNA and Nt1 of the second pRNA in the dimer. Such distances do not fall within the 1–7.5 nm range and are therefore undetectable by FRET. The calculated FRET distance included the length of the fluorophore arms. The distances after arm-size correction were used in refining the previous 3D structure of the pRNA dimer model.\(^{39}\)

Without RNA2 (biotin-Cy5-Ba\(^{+}\)), nonspecific binding of monomer RNA1 (Cy3-Ab\(^{-}\)) itself to the streptavidin surface was negligible, indicating that the Cy3 signals observed in our experiments were truly from the pRNA dimers. To ensure that the FRET observed was not between monomers in two adjacent dimers attached to streptavidin, a dimer composed of Cy3-Ab\(^{-}\) and biotin-Ba\(^{+}\), was mixed with another monomer Ba\(^{+}\) plus monomer biotin-Cy5-Ab\(^{-}\). No overlapped signals of Cy3 and Cy5, and no measurable FRET were found for the mixture immobilized to the streptavidin surface. This indicates that in our experiment conditions, the possibility of two dimers or one dimer and one monomer residing close enough to produce cross FRET signals between them is very low.
Refining the 3D Computer Model of the pRNA Dimer. The 3D computer model of the pRNA dimer reported previously (PDB code 1L4Q) was refined with a new set of distance parameters derived from smFRET. Explicit modeling of the fluorescent dyes Cy3/Cy5 was not feasible due to the lack of well-tested force-field parameters. Instead, harmonic parameters were applied between the backbone phosphorus atoms. Specifically, starting with the model of the pRNA dimer (PDB code 1L4Q), six distance parameters, labeled a, b, c, d, e, f (Table 2), were imposed. In addition, based on previous study, the nucleotide 82 from one pRNA was imposed to be within 1.2 nm from nucleotides 39–41, 49, and 62–64 of the second pRNA of the dimer. Geometry optimization and further stability analysis were performed using the NAMD simulation package for molecular dynamics, with the CHARMM27 force-field. The initial structure was subjected to 10,000 conjugate-gradient energy minimization steps with the above-mentioned parameters and a spring constant of 20 kcal/mol/Å².

As can be seen from Figure 7A, the resulting structure that incorporates the new parameters from smFRET is qualitatively in agreement with the previously published model. The rmsd (Root Mean Square Deviation) between the backbone atoms of the optimized structure (Figure 7A, blue) and the previous model used as the starting structure (Figure 7A, red) was 3.1 Å. The sum of squared differences (SSD) between the distances observed in the final model and the imposed distances was 107 Å². Most of the adjustment from initial 700 Å² to the final 107 Å² occurred within the first 5000 steps of the simulations, followed by a slow decrease up to 10,000 steps. To further test the sensitivity of the results, additional minimization runs with varying values of the spring constant were performed, yielding

| Name of dimers | Label position | Number of spots analyzed (N) | FRET efficiency (E) | Distance (R, nm) | Distance with arm size subtracted (R, nm) | Distance from the original model (nm) | Typical FRET traces for dimers a-f |
|---------------|----------------|-----------------------------|---------------------|-----------------|-----------------------------------------|--------------------------------------|----------------------------------|
| a             | 30 21          | 125                         | 0.46 ±0.09          | 5.4 ±0.4        | 4.5                                     | 4.6                                  | ![image](image1)                  |
| b             | 75 21          | 88                          | 0.45 ±0.06          | 5.5 ±0.2        | 4.6                                     | 4.8                                  | ![image](image2)                  |
| c             | 79 21          | 119                         | 0.47 ±0.10          | 5.4 ±0.4        | 4.5                                     | 3.7                                  | ![image](image3)                  |
| d             | 40 21          | 92                          | 0.69 ±0.09          | 4.7 ±0.3        | 3.8                                     | 5.2                                  | ![image](image4)                  |
| e             | 36 21          | 109                         | 0.51 ±0.05          | 5.2 ±0.2        | 4.4                                     | 3.5                                  | ![image](image5)                  |
| f             | 21 21          | 126                         | 0.48 ±0.08          | 5.4 ±0.3        | 4.5                                     | 5.8                                  | ![image](image6)                  |
| g             | 1 21           | No FRET                     |                     |                 |                                         |                                      | ![image](image7)                  |
| h             | 30 1           | No FRET                     |                     |                 |                                         |                                      | ![image](image8)                  |

Refined 3D model of the pRNA dimer (PDB code 1L4Q) was refined with new distance parameters derived from smFRET. Explicit modeling of Cy3/Cy5 was not feasible due to lack of well-tested force-field parameters. Harmonic parameters were applied between backbone phosphorus atoms. Six distance parameters (a, b, c, d, e, f) were imposed. Nucleotide 82 from one pRNA was imposed within 1.2 nm of nucleotides 39–41, 49, and 62–64 of the second pRNA of the dimer. Geometry optimization and stability analysis were performed using NAMD and CHARMM27. Initial structure underwent 10,000 conjugate-gradient energy minimization steps with parameters and a spring constant of 20 kcal/mol/Å². Resulting structure qualitatively agrees with previously published model. Rmsd between backbone atoms was 3.1 Å, SSD between distances was 107 Å². Sensitivity to spring constant was tested, showing further minimization steps with varying spring constants.
similar results (although more minimization steps were required for weaker harmonic parameters).

Comparison of the pRNA Head Loop Structure in Our Published 3D Model with a Newly Published pRNA Head Loop Structure by NMR. An NMR structure of the pRNA head loop with base 51–61 was recently reported. Superposition of bases 51–61 from our published computer models (yellow in Figure 8, which remained unchanged in the refined structure) with the NMR structure (PDB code 2KVN) (blue in Figure 8) revealed that the two structures agreed quite well, with a rmsd of 3.3 Å for the backbones. The differences mostly appear at positions G55,

![Figure 6](image_url)

Figure 6. Distance determination of pRNA dimers corresponding to pRNA dimer a–f shown in Table 1. (A) Distribution of FRET efficiencies. (B) Summary of calculated distances of different pRNA dimers.

![Figure 7](image_url)

Figure 7. Refined 3D model of pRNA dimer. (A) Comparison of the original (red) and the refined (blue) models. (B) Refined structure showing positions of nucleotides used for labeling. (C) Elucidation of the location of two nucleotides that were labeled with the donor and the acceptor, respectively, in individual dimer (a–h), with one pRNA subunit in light green ribbon and the other subunit in light red. The bases used for distance measurements are in dark red spacefill format in RNA1 and blue in RNA2. Numbers represent the sequences of each nucleotide. The distances measured from FRET efficiency are indicated.
A56, and G57 in the tip loop region (red circle) itself, where the structure was flexible in the NMR ensemble with a range of conformations observed. Thus, we can conclude that the refined model is consistent with the high resolution empirical ensemble within the head-loop fragment.

Conformational Shift of pRNA Dimer upon Binding to Procapsid. It has been reported that the dimer is the binding unit of the pRNA hexamer on the procapsid.36 It has also been reported that there is no pRNA sequence specificity for interaction between pRNA and the hub of the DNA-packaging motor. Instead, the specificity relies on the formation of the RNA static ring of appropriate size to fit on to the contour shape of the connector.79 The mechanism in converting the dimer, which is expected to contain two closed hands, into a hexamer, which is expected to contain two open hands, is a very intriguing but still unsolved question. In comparing the FRET of labeled nucleotides in dimers to their FRET after dimer binding to the procapsid, we found a significant difference. For example, the FRET was almost undetectable in dimers composed of pRNA Ab (Cy3 at Nt21) and Ba (Cy5 at Nt1). However, stronger signal of Cy5 (emission around 670 nm) due to FRET was found after they bound to the procapsid (Figure 9). In another case, strong and relatively homologous FRET was detected in dimers composed of pRNA Ab (Cy3 at Nt21) and Ba (Cy5 at Nt21), while the FRET was not assessable when they bound to the procapsid. In addition, the distance between two Nt21 in the pRNA Ab’/Ba’ dimer was 5.4 nm derived from FRET. However, the distance between the Nt21s of two pRNA Ab’ that bound to the procapsid is 15.4 nm as determined by Single Molecule High Resolution Imaging with Photobleaching (SHRimP).11 Due to the current impossibility of obtaining a single FRET pair in the hexamer, which is composed of three dimers, it is not possible to obtain the distance information for the hexamer using smFRET. Nevertheless, extensive studies31,36,80,81 reveal that the structure of the dimer in solution is completely different from that on the procapsid, suggesting a significant conformational change upon binding to the procapsid.

CONCLUSION

Our previous 3D structure was derived using data obtained from multiple experiments, including photo-affinity cross-linking,32,40–42 chemical modification interference,43,44 complementary modification,33,45,46 cryo-AFM,36,43,44,47 mutagenesis,70 ribonuclease probing,31,41 primer extension,41 and oligo targeting.50,51 In this report, smFRET was used for the first time to determine the RNA distance parameters and to enable further refinement of the 3D structure of the pRNA. The results reported here support the conclusion that smFRET data can be successfully used as a basis for deriving a high resolution 3D model of pRNA and that the previous low-resolution pRNA model is consistent with the new smFRET distance constraints.

METHODS

Preparation of Dual-Labeled RNA/DNA Hybrids. RNA oligos of different lengths with 5’ Cy3 label were made by in vitro transcription using ADO F550/570 AMP, similar as described before.3,4,74 The Cy3 RNA oligos were then annealed with a complementary biotin-Cy5-DNA oligo (IDT) to form RNA/DNA hybrids with different lengths between the Cy3 and Cy5 pair.

In vitro Synthesis of Fluorescent pRNA. To achieve single fluorophore labels at the desired positions on RNA1 in the dimer, the circular permutation strategy was applied to give a new 5’ opening site at different locations.70 Cy3-labelling at the 5’ end of RNA was achieved by in vitro transcription with T7 RNA polymerase using dsDNA templates containing the T7 class II promoter (O.25) in the presence of 2 mM ADO F550/570 AMP (AdenGenix, Inc.).74,82 RNA2 of the dimer was made by the hybridization of a reconstructed pRNA molecule with a biotin-Cy5-DNA oligo, resulting in a label at either the corresponding nucleotide 21 or at nucleotide 1 on the pRNA molecule.
Article

Constructs of pRNA Dimers. The pRNA dimers were constructed by mixing the paired pRNA molecules (Ab and Ba) at equal concentrations in the presence of 10 mM MgCl2.

Single Molecular FRET Assay. Streptavidin-coated chamber was prepared by incubation of the quartz chamber with 1 mg/ml biotin-BSA (Sigma) for 15 min, and subsequently 0.33 mg/ml streptavidin (Prozyme) for 15 min. The biotin-labeled samples were immobilized to the chamber surface through streptavidin–biotin linkage with concentrations adjusted to give discrete fluorescent spots. Excess sample was flushed out by native polyacrylamide (PAGE) gel electrophoresis and the oxygen scavenger system (0.5% D585/30 m) and Cy5 (Chroma, D680/30 m). The signals were recorded by Kinetic Imaging (Andor Technology).

The pRNA dimers were constructed by mixing the paired pRNA molecules (Ab and Ba) at equal concentrations. Fluorescence signals were collected through the objective (NA = 1.4, oil immersion) and were recorded by Andor iXon 887 V electron multiplied camera. To prevent fast photobleaching, the flow cell was infused with an oxygen scavenger system (0.5% β-γ-glucose, 10 mM β-mercaptoethanol, 0.2% Glucose Oxidase (Roche), and 0.25% Catalase (Sigma)) during imaging. The concentration of the samples was adjusted to show discrete fluorescent spots in the images. Sequential images were taken with an exposure time of 300 ms continuously. The recorded movie, with more than 2000 frames, was analyzed by Kinetic Imaging (Andor Technology).

Atomic Force Microscopy Imaging. The pRNA dimer was purified by native polyacrylamide (PAGE) gel electrophoresis and the AFM imaging of pRNA dimer was carried out as previously reported.47 Native Gel Electrophoresis and Binding Assay of Modified pRNAs to Procapsid. The native PAGE gel electrophoresis for dimer detection followed the reported procedure.46 The assay of the modified pRNA binding to procapsid was performed as previously published.46

The PDB code of the original 3D model of the dimer in Protein Data Bank is 1L4Q. The refined model is available at http://www.1eng.uc.edu/nanomedicine/peixuanguo/dimer.html.

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