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A New Theoretical Approach to Single-Molecule Fluorescence Optical Studies of RNA Dynamics

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Abstract. Single-molecule fluorescence spectroscopy in condensed phases has many important chemical and biological applications. The single-molecule fluorescence measurements contain information about conformational dynamics on a vast range of time scales. Based on the data analysis protocols methodology proposed by X. Sunney Xie, the theoretical study here mainly focuses on the single-molecule studies of single RNA with interconversions among different conformational states, to with a single FRET pair attached. We obtain analytical expressions for fluorescence lifetime correlation functions that relate changes in fluorescence lifetime to the distance-dependent FRET mechanism within the context of the Smoluchowski diffusion model. The present work establishes useful guideline for the single-molecule studies of biomolecules to reveal the complicated folding dynamics of single RNA molecules at nanometer scale.

1. Introduction

In the past few years, single-molecule fluorescence resonant energy transfer (FRET) spectroscopy is undergoing an increasingly popular method of probing the complex dynamics of single biomolecules [1-12]. Site-specific labeling of biomolecules with appropriate dyes is a prerequisite for these experiments. Fluorescently labeled single biomolecules can be detected in two configurations, either immobilized by a surface attachment or freely diffusing in solution. These two detection formats provide different and complementary kinds of information. Single-molecule FRET measurements of surface immobilized RNA have been a powerful approach for studying equilibrium conformational dynamics of DNA and RNA [5-9, 12-14]. The ability to watch one biomolecule at a time helps us to obtain unique information on relevant observables, resolve subpopulations in a heterogeneous sample and record asynchronous time trajectories of observables that would otherwise be hidden during biochemical reactions [1-16]. This ability is crucial in many biological contexts, in which triggering is
not possible or ensembles moving stochastically on complex reaction landscape quickly lose their coherence, resulting in averaged behavior.

In a single-molecule FRET experiment, one uses a single pair of dye molecules attached on a biomolecule. If the donor is excited by an incident laser, the excitation energy can be transferred from the donor to the acceptor, provided that the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor. This leads to a reduction in the donor’s fluorescence intensity and excited state lifetime, and an increase in the acceptor’s emission intensity. The fluorescence excitation energy from the donor to the acceptor then depends on the FRET rate \[ \Gamma_{FRET} = \gamma_D \left( \frac{R_0}{r} \right)^6 \]  

where \( R_0 \) is the Förster distance at which 50% of the energy is transferred, \( \gamma_D \) is the fluorescence decay rate of the donor in the absence of the acceptor, and \( r \) the distance between the donor and the acceptor.

The FRET rate is thus very sensitive to \( r \) which could be used as a “nano-ruler” that allows one to infer the distance changes of the probed molecule to monitor the conformational changes. Thus, the energy transfer rate, in turn, depends sensitively on the donor and acceptor distance \( r \). The detection of fluorescence from single pair of dye molecules involves repeated cycling of the molecule between ground and excited states and detection of the series of emitted photons; nonradiative relaxation of the excited state results in a reduction of the maximum photon flux. When diffusing or flowing single molecules in a liquid traverse the laser excitation volume, fluorescence photon bursts are generated. Although short, the bursts can be analyzed for their duration, brightness, spectrum, and fluorescence lifetime, thereby providing molecule information on identity, size, diffusion coefficient, and conformation dynamics. Because a large number of events (photon bursts) can be collected in a relatively short time, statistical analyses of these data are possible, and histograms can be constructed. Most studies of single-molecule fluorescence bursts were to measure distributions in burst size or in fluorescence lifetime. For example, two-color burst analysis and multiple-lifetime approaches for identification and separation were suggested and implemented.

Fluorescence lifetime spectroscopy at the single molecule level allow the measurement of translational, rotational, and conformational dynamics of biomolecules over a wide range of time scales. In combination with fluorescence resonant energy transfer (FRET), intermolecular and intramolecular distances between about 2nm to 10 nm can be probed. Indeed, single-molecule FRET analysis, which is based on fluorescence lifetime, is an alternative to the popular intensity-based methods. The advantages include the less susceptibility to variation in the excitation intensity, photon-detection efficiency, or spectral cross talk. The related techniques, fluorescence correlation spectroscopy and two-color cross-correlation spectroscopy have been used to analyze, sort, and detect conformational states of single biomolecules in the excitation volume.

Figure 1. Schematic representation of the single-molecule FRET experiments, in which a single FRET pair attached to a RNA molecule. (A stands for acceptor dye molecule and D stands for donor dye molecule, respectively).

By using a laser probe as an excitation source, fluorescence of a single molecule has been detected. The emission spectra [17] and excited state lifetime [17–20] of a single molecule have also been measured. Examples for single-molecule fluorescence lifetime measurements include tetramethylrhodamine (TMR) dye molecules linked to tRNA\(^{\text{tme}}\) and DNA. The fluctuations of the
single-molecule lifetime contain detailed dynamic information. In this light, single-molecule fluorescence lifetime holds the promise of serving as a sensitive measure for conformational dynamics. We note that time resolved fluorescence lifetime measurements provide more information than just the single quantity $E_{\text{obs}}$. Consequently, time resolved data—when available—can be used to constrain the underlying distance distribution $p(R)$ more accurately [11]. Moreover, such experiments can be used to obtain information about chain dynamics [12]. To gain insights from the measured single-molecule fluorescence lifetime correlations, Xie et al. have presented the data analysis protocols that afford an efficient extraction of conformational dynamics on a broad range of time scales from single-molecule fluorescence lifetime trajectories photon by photon [1,2], in cases where fluorescence quenching is either due to Förster fluorescence resonance energy transfer or due to the excited-state electron transfer. It is concluded that the method not only extends the fast measurable time scale to a microsecond or less, but also allows the disclosure of information from the limited number of detected photons of a single molecule over the time scales that are important in characterizing the function of a biological macromolecule. A similar detection scheme has been used by Seidel and co-workers for single-molecule identification and conformational dynamics studies [26]. Their data were processed by averaging over every 80 photons, from which the distribution of fluorescence lifetime is constructed and analyzed. Photon statistics, however, demands that a number of photons be obtained, or “binned”, in order to reach a statistically reasonable estimate of the lifetime [1, 2, 16-20]. The time evolution of the experimental observable, or trajectory, is analyzed to extract the information on molecular dynamics [1,2,6]. The stochastic delay times of individual photons require that many photons be binned in order to obtain the fluorescence lifetime. Therefore, in combination with single-molecule FRET technique, fluorescence lifetime measurement using time-correlated single-photon counting (TCSPC) is oblivious to events that occur on a time scale faster than or comparable to the bin time [1,2,16] circumventing such a shortcoming. It has been recognized that there is more information available should the single-molecule trajectory be analyzed photon by photon.

However, several challenges need to be overcome to further study the single-molecule FRET related techniques. To improve the time resolution and statistics for single-molecule FRET studies, we have proposed a versatile strategy for studying conformational dynamics of a single biomolecule [27]. In this work, we focus on the lifetime fluctuation-correlation analysis of single RNA molecule using single-molecule FRET experiments, in which the lifetime of single FRET pair of dyes is dependent on donor-acceptor distance. We present a Smoluchowski diffusion model that affords an efficient extraction of conformational dynamics from lifetime trajectories of single-molecule FRET, based on the fluctuation-correlation analysis of observables.

2. Model and Results
Consider a RNA molecule that undergoes a Smoluchowski diffusion in the free-energy potential $V(r)$ with interconversions among several conformational states with different fluorescence lifetimes, and a single FRET pair attached to it undergoes the FRET mechanism. The fluorescence lifetime of the donor fluorophore is $\gamma(t) = \left[ \gamma_D + \gamma_{\text{FRET}}(r(t)) \right]^{-1}$, where $\gamma_D$ is the fluorescence decay rate of the donor in the absence of acceptor and $\gamma_{\text{FRET}}$ is the energy transfer rate from the donor to acceptor. Conformational dynamics or structural fluctuations of a RNA molecule may cause changes in the donor-acceptor distance $r$, which, in turn, may result in a variation of the energy transfer rate. In this light, fluorescence lifetime can serve as a sensitive and distance-dependent probe for conformational dynamics.

Firstly, briefly review the data analysis methodology proposed by X. Sunney Xie [1, 2]. Given a kinetic scheme of interconversion, all the dynamic information is contained in the time-dependent state propagator $G(f, t_f; i, t_i)$, which describes the conditional probability of the system arriving at state $f$ at time $t_f$ given the system being at state $i$ at time $t_i$. The structure of $G(f, t_f; i, t_i)$ is determined by the rate constants that characterize the kinetic scheme. One goal of statistical analyses
of single molecule trajectories is therefore to determine the interconversion rates from experimental observables – which, in the present case, are the time-stamped photon sequences from TCSPC. The approach utilizes correlation analyses of the observables. Specifically, we calculate the two-time fluctuation-correlation function, or autocorrelation, of two events separated by period \( t \) \[ C_{2}(t) = \langle \delta \gamma(t)^{-1} \delta \gamma(0) \rangle = \langle \delta \gamma^{-2} \rangle \hat{C}_{2}(t) \] (2)

where \( \delta \gamma(t)^{-1} = \gamma(t)^{-1} - \langle \gamma^{-1} \rangle \), and \( \hat{C}_{2}(t) \) is the normalized autocorrelation function, defined as

\[
\hat{C}_{2}(t) = \frac{\langle \gamma(t)^{-1} \gamma(0)^{-1} \rangle - \langle \gamma^{-1} \rangle^2}{\lim_{t \to 0} \langle \gamma(t)^{-1} \gamma(0)^{-1} \rangle - \langle \gamma^{-1} \rangle^2}
\] (3)

Similarly, we define a three-time fluctuation-correlation function of three events separated by periods \( t_{1} \) and \( t_{2} \) \[ C_{3}(t_{1}, t_{2}) = \langle \delta \gamma(t_{1} + t_{2})^{-1} \delta \gamma(t_{1})^{-1} \delta \gamma(0) \rangle = \langle \delta \gamma^{-3} \rangle \hat{C}_{3}(t) \] (4)

where the normalized three-time correlation function is

\[
\hat{C}_{3}(t_{2}, t_{1}) = \frac{\langle \delta \gamma(t_{1} + t_{2})^{-1} \delta \gamma(t_{1})^{-1} \delta \gamma(0) \rangle}{\lim_{t_{1} \to 0} \lim_{t_{2} \to 0} \langle \delta \gamma(t_{1} + t_{2})^{-1} \delta \gamma(t_{1})^{-1} \delta \gamma(0) \rangle}
\] (5)

where

\[
\langle \delta \gamma(t_{1} + t_{2})^{-1} \delta \gamma(t_{1})^{-1} \delta \gamma(0) \rangle = \langle \gamma(t_{1} + t_{2})^{-1} \gamma(t_{1})^{-1} \gamma(0) \rangle
\]

\[
- \langle \gamma(t_{1})^{-1} \gamma(0)^{-1} \rangle + \langle \gamma(t_{1})^{-1} \gamma(0)^{-1} \rangle + \langle \gamma(t_{1} + t_{2})^{-1} \gamma(0)^{-1} \rangle \langle \gamma^{-1} \rangle + 2 \langle \gamma^{-1} \rangle^3
\] (6)

The temporal correlation comes from either the \( \langle \gamma(t)^{-1} \gamma(0)^{-1} \rangle \) or the \( \langle \gamma(t_{1} + t_{2})^{-1} \gamma(t_{1})^{-1} \gamma(0)^{-1} \rangle \) terms. They can be expressed in terms of the propagator \( G(f, t_{j}; i, t_{i}) \) as

\[
\langle \gamma(t)^{-1} \gamma(0)^{-1} \rangle = \sum_{f, i} \gamma^{-1}_{f} G_{\beta}(t) \gamma^{-1}_{i} \rho_{i}^{\text{eq}}
\] (7)

And

\[
\langle \gamma(t_{1} + t_{2})^{-1} \gamma(t_{1})^{-1} \gamma(0)^{-1} \rangle = \sum_{f, i, j} \gamma^{-1}_{f} G_{\beta}(t_{2}) \gamma^{-1}_{i} G_{\beta}(t_{1}) \gamma^{-1}_{i} \rho_{i}^{\text{eq}}
\] (8)

where \( G_{\beta}(t) = G(f, t_{j}; i, t_{i}) \), \( t = t_{j} - t_{i} \) and \( \gamma^{-1}_{i} \) and \( \rho_{i}^{\text{eq}} \) are the fluorescence lifetime and equilibrium probability for finding the system at state \( i \), respectively.

Next, we turn to considering a single RNA molecule that undergoes the Smoluchowski diffusion in a free-energy potential \( V(r) = \frac{1}{2}m\omega^{2}r^{2} = \frac{1}{2}\kappa r^{2} \), where \( m \) is the reduced mass of RNA molecule, and \( \kappa \) is the potential constant, with interconversions among several conformational states with different fluorescence lifetimes, and a single FRET pair attached to it undergoes the fluorescence resonance energy transfer. The fluorescence lifetime of the donor fluorophore is \( \gamma^{-1} = \left( \gamma_{D} + \Gamma_{\text{FRET}} \right)^{-1} \), where \( \gamma_{D} \) is the fluorescence decay rate of the donor in the absence of the acceptor and \( \Gamma_{\text{FRET}} \) is the energy transfer rate from the donor to the acceptor. Conformational dynamics or structural fluctuations of a RNA molecule may cause changes in the donor-acceptor distance \( r \), which, in turn, may result in a variation
of the energy transfer rate. In this light, fluorescence lifetime can serve as a sensitive and distance-dependent probe for conformational dynamics. It is then possible to probe directly the fluctuation of donor-acceptor distance \( r(t) \). The rate of widely used FRET mechanism can be expressed as

\[
\Gamma_{\text{FRET}}(t) = \gamma_D \left( R_0 / r(t) \right)^6,
\]

where \( r \) is the center-to-center donor-acceptor distance. As a result of the structural fluctuations, the fluorescence lifetime of donor varies in time \( \gamma(t)^{-1} = [\gamma_D + \gamma_{\text{FRET}}(r(t))]^{-1} \).

To illustrate the trajectories \( r(t) \) of this motion, we perform diffusion dynamics simulation for a RNA chain using the velocity Verlet algorithm \([6, 28]\). The instantaneous value of the distance \( r(t) \) in Fig.2 determines, at each moment, the probability of excitation transfer from the donor (D) to the acceptor (A), and thus the fluorescence lifetime according to equation 1.

**Figure 2.** The simulated trajectories \( r(t) \) of Smoluchowski diffusion motion for a RNA chain using the velocity Verlet algorithm.

As we all know, the corresponding dynamic equation can be described by the fractional Fokker-Planck equation (FFPE) developed by Klafter and co-workers \([29]\). Now we use the FFPE solutions to calculate the fluorescence lifetime correlation functions for single-molecule FRET measurements. The definitions of and the solutions to FFPE are given in Refs. 29 and 30. The FFPE has the form \([1, 29, 30]\)

\[
\frac{\partial}{\partial t} H_{\alpha}(r, t | r_0, t_0) = \int_0^1 \frac{d\lambda}{\Gamma(\lambda)} \left[ \lambda \frac{dV(r)}{dr} + \lambda \frac{d^2 \theta}{dr^2} \right] H_{\alpha}(r, t | r_0, t_0), \tag{9}
\]

where \( \alpha \) is the fractional drift coefficient with the dimension \( s^{-\alpha} \) with \( \theta = k_B T / \kappa \) characterizes the magnitude of the fluctuation at room temperatures (fluctuation–dissipation theorem). The Riemann–Liouville fractional operator is defined by \([1, 29, \text{and } 30]\)

\[
\int_0^t H_{\alpha}(r, t' | r_0, t_0) dt' = \frac{1}{\Gamma(\alpha)} \frac{d}{dt} \int_0^t H_{\alpha}(r, t' | r_0, t_0) dt'. \tag{10}
\]

The solution to equation (9) is

\[
H_{\alpha}(r, t | r_0, t_0 = 0) = \frac{1}{\sqrt{2\pi \theta}} \sum_{n=0}^\infty \frac{1}{2^n n!} E_{\alpha}(-n\bar{r}^\alpha) h_\kappa \left( \frac{\bar{r}_0}{\sqrt{\lambda}} \right) h_\kappa \left( \frac{\bar{r}}{\sqrt{\lambda}} \right) e^{-\bar{r}^2/2} \tag{11}
\]

where the reduced parameters are \( \bar{r} = r / \sqrt{\theta} \) and \( \bar{t} = t / \sqrt{\lambda} \). The \( h_\kappa \)’s are Hermite polynomials and their eigenvalues here are \( \kappa = n \). The Mittag–Leffler function is defined through \([29]\)
\[ E_{\alpha}(-nt^\alpha) = \sum_{m=0}^{\infty} \left(\frac{-n}{m+1}\right)^m \]  

where \( \Gamma(z) = \int_0^\infty y^{-1} e^{-y} \, dy \) is the gamma function. The analytical expressions for the probability density function \( H_{\alpha} \) at short-to-intermediate times and \( H_{\alpha} \) at long times is given by, respectively\([1,21]\)

\[
H_{\alpha,S}(r,t; r_0, t_0 = 0) = \left[ 2\pi\theta \left(1 - e^{-2\lambda_\alpha r^\alpha / \Gamma(1+\alpha)} \right) \right]^{-1/2} \exp \left\{ -\frac{\left( r - r_0 e^{-\lambda_\alpha r_0^\alpha / \Gamma(1+\alpha)} \right)^2}{2\theta \left(1 - e^{-2\lambda_\alpha r_0^\alpha / \Gamma(1+\alpha)} \right)} \right\}
\]

\[
H_{\alpha,L}(r,t; r_0, t_0 = 0) = \left[ 2\pi\theta \left(1 - \lambda_\alpha^2 r^{-2\alpha} / \Gamma(1-\alpha)^2 \right) \right]^{-1/2} \exp \left\{ -\frac{\left( r - r_0 \lambda_\alpha r_0^{-\alpha} / \Gamma(1-\alpha)^2 \right)^2}{2\theta \left(1 - \lambda_\alpha^2 r_0^{-2\alpha} / \Gamma(1-\alpha)^2 \right)} \right\}
\]

The donor-acceptor displacement correlation functions in these two limiting cases show a stretched exponential

\[ \langle r(t) r(0) \rangle_{\alpha,S} = \theta e^{-\lambda_\alpha r^\alpha / \Gamma(1+\alpha)} \]

and a power-law behavior

\[ \langle r(t) r(0) \rangle_{\alpha,L} = \theta \lambda_\alpha r^{-\alpha} / \Gamma(1-\alpha) \]

respectively. Using equation (10)-(14), the fluorescence lifetime fluctuation correlation at short-to-intermediate time for FRET dominated lifetime mechanism is given by \([1,29]\)

\[
\langle \delta r(t) \delta r(0)^{-1} \rangle_{FRET} = \gamma_D^2 \frac{6\theta}{R_0^2} \sum_{n=1}^{6} c_n \exp \left[ -n\lambda_\alpha r^\alpha / \Gamma(1+\alpha) \right]
\]

At long time, the fluorescence lifetime correlation function becomes \([1,29]\)

\[
\langle \delta r(t)^{-1} \delta r(0)^{-1} \rangle_{FRET} = \gamma_D^2 \frac{6\theta}{R_0^2} \sum_{n=1}^{6} c_n \left[ \lambda_\alpha^2 r^{-\alpha} / \Gamma(1-\alpha)^2 \right]^n
\]

where \( c_n \) (n=1,2,..,6) are given by \( c_1 = 6 \left( x_{\alpha}^5 + 10 x_{\alpha} x_{\alpha}^3 \theta + 15 x_{\alpha}^2 \theta^2 \right) \), \( c_2 = 75 \theta \left( x_{\alpha}^4 + 6 x_{\alpha}^2 \theta + 3 \theta^2 \right)^2 \), \( c_3 = 400 x_{\alpha}^2 \theta^2 \left( x_{\alpha}^2 + \theta \right)^2 \), \( c_4 = 900 \theta^3 \left( x_{\alpha}^2 + \theta \right)^2 \), \( c_5 = 7200 x_{\alpha}^2 \theta^2 \) and \( c_6 = 1200 \theta^5 \). Equation (17) and (18) clearly show that the time scale of the Smoluchowski diffusion dynamics spans several decades. In this case, the dynamics stretch across a greater span of time scales as the value becomes smaller higher degree of anomaly in diffusion.

The three-time fluorescence lifetime correlation function of equation (4) for this model at short-to-intermediate time for FRET dominated lifetime mechanism is

\[
C_3(t_1,t_2) = \langle \delta r(t_1 + t_2)^{-1} \delta r(t_1)^{-1} \delta r(0)^{-1} \rangle_{FRET} = \gamma_D^3 \frac{6\theta}{R_0^3} \sum_{n=1}^{6} \sum_{n=1}^{6} c_n \exp \left[ -n\lambda_\alpha (t_1 + t_2)^\alpha / \Gamma(1+\alpha) \right] \times \exp \left[ -n\lambda_\alpha (t_1 + t_2)^\alpha / \Gamma(1+\alpha) \right] \times \sum_{n=1}^{6} c_n \exp \left[ -n\lambda_\alpha (t_1 + t_2)^\alpha / \Gamma(1+\alpha) \right] \times \sum_{n=1}^{6} c_n \exp \left[ -n\lambda_\alpha (t_1 + t_2)^\alpha / \Gamma(1+\alpha) \right] \times \sum_{n=1}^{6} c_n \exp \left[ -n\lambda_\alpha (t_1 + t_2)^\alpha / \Gamma(1+\alpha) \right]
\]

At long time, the three-time correlation function of equation (4) becomes
\[ C_3(t_1, t_2) = \left\{ \delta \gamma(t_1 + t_2)^{-1} \delta \gamma(t_1)^{-1} \delta \gamma(0)^{-1} \right\}_{\text{FRET}} = \gamma_D^3 \frac{6 \theta}{R_0^6} \sum_n^6 c_n \left[ \gamma_n^0 \left( t_1, t_2 \right)^{\text{rad}} \Gamma(1-\alpha)^n \right] \]

\[ -\sum_{n=1}^6 c_n \left[ \gamma_n^+ \left( t_1, t_2 \right)^{\text{rad}} \Gamma(1-\alpha)^n \right] - \sum_{n=1}^6 c_n \left[ \gamma_n^- \left( t_1, t_2 \right)^{\text{rad}} \Gamma(1-\alpha)^n \right] \]

\[ = \sum_{n=1}^6 c_n \left[ \gamma_n^0 \left( t_1 + t_2 \right)^{\text{rad}} \Gamma(1-\alpha)^n \right] \]

It is obvious that three-time correlation function analysis is capable of handling models of degenerate state observables and especially handling more complicated conformational dynamics of a single RNA molecule. The analytical expressions that relate changes in fluorescence lifetime to the distance-dependent fluorescence resonance energy transfer within the context of Smoluchowski diffusion model have been obtained. The results corroborate the notion that a methodology that allows characterization of structural dynamics over several decades of time scales is crucial for a full understanding of such a complicated biomolecule system. The dynamics of such a RNA molecule that undergoes a diffusion dynamics would be fully understood with strategies that are capable of characterizing single-molecule dynamics on a wide range of time scales based on single-molecule FRET experiments.

3. Conclusions

The single-molecule FRET experiments provide a unique opportunity for exploring the relationship between structure and dynamics of a single biomolecule. The ultimate challenge is to measure conformational dynamics in vivo. The theoretical study here mainly focused on the aspects of single-molecule FRET experiments that utilize the data analysis methodology proposed by X. Sunney Xie used in single-molecule spectroscopy. To this end, we’ve derived analytical expressions of two-time and three-time correlation functions that relate changes in fluorescence lifetime to the distance-dependent FRET mechanism within the context of the Smoluchowski diffusion model. Our study establishes potentially useful guidelines for the experimental study of monitoring the dynamical processes in real time and revealing the mechanism of complex reaction of a single RNA at molecule scale. We hope that this subject will pave its way to the understanding of different biochemical systems at the molecule level.

References