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RAPID COMMUNICATION

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Theoretical study on single-molecule spectroscopy

Abstract The photon-by-photon approach for single molecule spectroscopy experiments utilizes the information carried by each detected photon and allows the measurements of conformational fluctuation with time resolution on a vast range of time scales, where each photon represents a data point. Here, we theoretically simulate the photon emission dynamics of a single molecule spectroscopy using the kinetic Monte Carlo algorithm to understand the underlying complex photon dynamic process of a single molecule. In addition, by following the molecular process in real time, the mechanism of complex biochemical reactions can be revealed. We hope that this theoretical study will serve as an introduction and a guideline into this exciting new field.

Keywords single-molecule spectroscopy (SMS), kinetic Monte Carlo, dynamical process, conformation, nanotechnology

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1 Introduction

In the final decades of the last century, much scientific research turned toward the great dream and imagination proposed by Richard Feynman in 1959 and that is to manipulate and control matter at an atomic and molecular scale. Attaining the ultimate limit of exploring single atoms or molecules on surfaces and subsequently modifying their behavior has been achieved in some sense, using nanometer-scale interactions with tunneling electrons or forces from sharp tips, in scanning tunneling microscopy (STM) or atomic force microscopy (AFM). On the other hand, optical probing of single molecules, atoms or ions in complex condensed matter environments, offers the advantages of operating at a distance, which can result in significant challenges as well as exciting opportunities. The challenges generally derive from the limitations posed by diffraction and the difficulty of detecting a single entity in the interfering background arising from the host crystal, polymer, liquid, or protein. This new approach provides significant new insights into the interaction of light with matter, behavior of single particles, and their interaction with their nanoenvironments. The opportunities result from the wealth of new information obtained regarding the interactions of the single entity with its native environment, unobscured by the ensemble averaging that characterizes conventional experiments.

Single molecule spectroscopy (SMS) is a method for studying the chemical transformations of one large molecule, such as a protein or an enzyme, immobilized on a support. The chemical transformations may involve a large molecule alone or a large molecule interacting with smaller molecules [1－7]. Recent experimental advances made in optics and microscopy now allow SMS in many different systems. Observations of spectra from single molecules have become routine in many laboratories around the world. For single molecule systems, intramolecular and molecular fluctuations are large and thus the deterministic mass action laws of chemical kinetics do not hold and are replaced by statistical random evolution equations involving two different types of stochastic processes. Moreover, SMS is sensitive to temporal heterogeneity. In general, condensed phase systems are not static, but fluctuating in time. In a typical ensemble experiment, different dynamical behaviors of individual sys-

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tems are averaged over a large number of systems to yield an average value of dynamical parameters, for example, a rate constant, and it is hard to probe spontaneous temporal fluctuations occurring in an ensemble of systems in a direct way. However, in the SMS experiment, one can directly follow the time-dependent fluctuation phenomena occurring at the level of an individual molecule along a time trajectory. SMS experiments make it possible to have a direct comparison between the theoretical description and experimental measurements. In the usual theoretical descriptions of the dynamics of a system, a certain hypothetical single system is presumed that is supposed to follow an averaged dynamics of the ensemble of systems. The state of that hypothetical system is described by a certain single molecular quantity, such as the “wave function” or the “state” of the system even though those theoretical models are applied to an ensemble of systems. It is implicitly assumed that an appropriately chosen single hypothetical system well represents the dynamics of an ensemble of samples. In an SMS experiment, however, one does not need to suppose such a hypothetical single system in a theoretical description. In addition, it is possible to make a direct comparison between the theoretical description and experimental measurements. Due to those distinct features in SMS, or more specifically, in SMS measurements, many theoretical concepts and models usually adopted in an ensemble measurement may not be appropriate in describing SMS. Indeed, as SMS is applied to the studies of dynamical processes in a wide range of systems, a variety of novel phenomena that have not been conceived of in traditional measurements are being peeled away from the ensemble averaging processes. Examples of new phenomena observed in SMS include the random variation in line shape of individual molecules, sudden spectral jump processes, fluorescence intermittency, and photon bunching and antibunching phenomena.

The outline of this paper is as follows. In Section 2, we present the concepts and methods of SMS experiments. In Section 3, we use a kinetic Monte Carlo procedure to simulate the photon emission dynamics of a single molecule. Lastly, in Section 4, we conclude with all the results.

2 Concepts, methods, and measurements of single-molecule spectroscopy

SM spectra are extremely sensitive to the local nanoenvironment in which the SM is situated, so that the spectra of the SM will fluctuate in time owing to changes in the local environments. SMS takes advantage of this sensitivity by using an SM as local probes. In Fig. 1, we show a schematic representation of an electronic energy level structure of an SM, interacting with a cw laser. In a typical experiment, a cw laser (frequency ω_L) excites the molecule. After the absorption of an exciting photon, the excited molecule quickly decays (generating phonons) to the first electronic-excited state, from which a fluorescence photon is emitted. This cycle restarts once the system relaxes to the electronic ground state. In most experiments involving an SM, the fluorescence is simply collected and little concern is given to the finer details of the fluorescence frequency and spatial distributions.

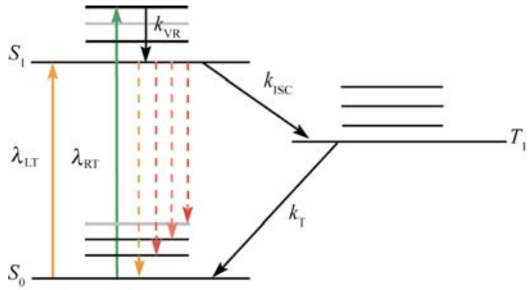

Fig. 1 The three-state model of a Single Molecule (SM). The molecule in its ground state is excited to a vibrational level of the excited singlet state, and after a very fast vibrational relaxation, the molecule is found in the ground vibrational of the first electronic-excited state (S_1). Then, either the molecule emits a photon (dotted line pointing downward) or the system relaxes to the typically long-lived triplet state (T_1). The fluorescent photons are Stokes-shifted with respect to the laser photons, owing to the vibrational levels. Hence, in an experiment, one may distinguish between incoming laser photons and fluorescent photons.

Several optical configurations have been demonstrated for single-molecule detection. At low temperatures, high resolution optical methods, such as laser frequency modulation spectroscopy (LFMS) [1, 21], or fluorescence excitation spectroscopy [2, 6—25], can be applied and coupled with optical microscopy. At room temperature, one can detect the fluorescent burst of a molecule passing through the focused laser beam, or one can use optical microscopy to observe the same single molecule for an extended period, measuring the signal strength, lifetime, polarization, and etc. At low temperatures, one usually excites the zero-phonon lines of single molecules, which are very narrow and intense. Their cross-sections (the typical order for σ is $\sim 10^{-11}$ cm^2 [8, 53]) being comparable to a fraction of a wavelength of light squared, the excited volumes, i.e., the small focused spot on the sample, can be as large as hundreds or even thousands of cubic microns, i.e., in the picoliter range. On the other hand, at room temperature, fluorescent molecules display only broad bands, with cross-sections which are typically of the order of 10^{-16} cm^2, about five to six orders of magnitude lower than those of zero-phonon lines. Therefore, the excited volumes must be reduced close to the femtoliter level or less to have an effective single-molecule excitation. In such small volumes, a few orders of magnitude lower than the excited volumes in low-temperature experiments can only be achieved either with high quality diffraction-limited microscope objectives or with near-field optics. Successful microscopic techniques include near-field scanning optical microscopy (NSOM) [27, 67] and confocal scanning optical microscopy (CSOM) [68], as well as the wide-field methods of epi-fluorescence and total internal reflection microscopy (TIR) [22—39]. In near-field imaging, an aluminum-coated optical fiber tip is used as the excitation source [7, 10, 62], which is positioned a few nanometers above the raster-scanned sample. The emission is collected through the transmissive substrate with a high numerical aperture microscope objective. The far-field images are generally taken with the same microscope, with the fiber tip removed. The excitation is then focused by the objective lens [29]. Near-field microscopy allows spectroscopic imaging at a resolution beyond the diffraction limit [62], while far-field images are diffraction-limited to approximately half the wavelength of the light. However, in a near-field microscope, the metal coating can perturb the optical measurements, mainly the emission lifetime and the fluorescence intensity. Many other new techniques are still evolving, for example, two-photon fluorescence detection [62, 63] and imaging [63, 64], surface-enhanced Raman spectroscopy [64—66], etc.

The major factors to determine the fluorescence yield of a single molecule can be identified by approximately modeling the label as a three-level system consisting of a couple of singlet states, namely, the ground state (S_0), the first excited state (S_1), and one triplet state (T_1). The molecule may be excited from S_0 to S_1 via laser or intermolecular resonance energy transfer. The excited state (S_1) may decay back to the ground state (S_0) via fluorescence emission, or occasionally to T_1 via intersystem crossing (ISC). Once trapped in the triplet state, the molecule pauses fluorescence until it gets back to the singlet ground state via ISC. Since the ISC rates are usually much smaller than the radiation decay rate, a temporary loss of fluorescence signal will occur, i.e., a phenomenon called blinking. The steady-state solution yields the fluorescence intensity

$$I_{\mathrm{s}} = \frac{\hbar\omega}{2\sigma_{\mathrm{p}}} \frac{\gamma + k_{\mathrm{ISC}}}{1 + k_{\mathrm{ISC}} / 2k_{\mathrm{T}}} \tag{1}$$

Here, ω is laser frequency in resonance with $S_1 \rightarrow S_0$ transition, and σ_{p} is the peak absorption cross section. γ is radiation decay rate, k_{ISC} is the rate for intersystem crossing (ISC) from singlet excited state (S_1) to triplet state (T_1) and k_{T} is ISC rate from T_1 back to the ground state (S_0). Once saturation occurs, a further rise in laser power will generate more noise than signal. To maximize the saturation intensity, it is crucial to have a large value of γ or a small value of ($k_{\mathrm{ISC}}/k_{\mathrm{T}}$), which are intrinsic photophysical properties of the dye molecules. When the excitation is weak ($\Gamma \ll \gamma$), the fluorescence intensity increases with excitation. However, saturation will occur when the excitation reaches a threshold, and I_F becomes almost independent of Γ. Then the fluorescence emission is limited by the ratio $k_{\mathrm{ISC}}/k_{\mathrm{T}}$. A molecule with a small value of the ratio has high fluorescence intensity. This amounts to a low ISC rate to the triplet state and a high rate out of it. This means that there is a low frequency for the occurrence of blinking and a short period for dark spectrum. In addition to blinking, the label dye may irreversibly lose its ability to absorb and emit photons after a great number of excitation cycles. This phenomenon, called photobleaching, is a consequence of long-term irradiation of intense lasers and is likely caused by chemical reactions between the excited dye and certain species in its environment. Besides, the fluorescence emission is also restricted, ultimately by the radiation decay rate γ. Indeed a molecule cannot emit photons faster than what the natural lifetime of its excited state allows even in the ideal case of no blinking and photobleaching. Thus, the radiation decay rate, the ISC rates, and also photobleaching are the major determinants of fluorescence intensity of a label dye, and thereby the time resolution of single-molecule spectroscopy measurement.

A common theme in SMS is a molecule in a fluctuating environment under a constant or pulsed laser illumination. In order to describe SMS in a rather simple, but general model, we imagine a single molecule as having two electronic states, ground and excited states, $\{g\}$, $\{e\}$. The electronic excited state is located at v_0 above the ground state. Since we consider a molecule in a condensed medium, i.e., a dynamic environment, the interaction between a molecule and its local environment causes the molecular properties, including the transition frequency to fluctuate in time. Also, the molecule is under illumination with a continuous wave laser-field, with monochromatic frequency ω_L, that causes the electronic transition of the molecule from the ground state to the excited state, which is usually via a transition dipole. As a zeroth order picture, we can write down the following Hamiltonian for the SMS:

$$H = \frac{\hbar}{2}\omega(t)\sigma_z - d \cdot E \cos(\omega_{\mathrm{L}} t) \tag{2}$$

where σ_z is the Pauli matrix. Here, the absorption frequency of the single molecule is time-dependent due to the interaction of the molecule with bath modes via a *diagonal coupling*. One may also consider an *off-diagonal* coupling of the molecule to the bath; however, for simplicity, we do not consider this here. The second term in Eq. (2) describes the interaction between the single molecule and the laser field, while d is the dipole operator with the real matrix element $\mathbf{d}$eg. Here, we have not included any intramolecular processes, for example, intersystem crossing, which involves other electronic states. They could be included when transitions between multiple electronic states are considered. Based upon the model Hamiltonian in Eq. (2), we describe the photon emission process in the SMS. When the molecule in a condensed media is illuminated via a laser field with a monochromatic frequency, ω_{L}, it makes a vibronic transition to a vibrationally excited level of the electronic excited state, from which it rapidly undergoes a nonradiative relaxation process to the lowest vibrational level of the electronic excited state via phonon emission process on a timescale of picoseconds. Finally, from the lowest vibrational level of the excited electronic state, the molecule relaxes to the ground electronic state within the natural lifetime, Γ^{1}, by emitting a single photon, and then the photon is detected in the experiment. Under continuous wave illumination, the whole process is repeated.

Assuming a nondegenerate three-state SM in an external classical laser field, the molecule can be described by a 3×3 density matrix ρ whose elements are ρ_{ee}, ρ_{gg}, ρ_{tt}, ρ_{ge}. Applying the electronic dipole and rotating-wave approximation, the stochastic Bloch equations of motion for the density matrix ρ are given by

$$\dot{\rho}_{gg} = \gamma\rho_{ee} + k_{\mathrm{T}}\rho_{ee} + \mathrm{i}\cdot\frac{1}{2}\Omega\,(\rho_{eg} - \rho_{ge})\,\mathrm{e}^{-\mathrm{i}\nu t} \tag{3a}$$

$$\dot{\rho}_{ge} = -\mathrm{i}\,(\omega + \delta\omega_{\mathrm{L}}(t))\rho_{ge} - \gamma\rho_{ge} - \mathrm{i}\left(\frac{1}{2}\right)\Omega\,(\rho_{gg} - \rho_{ee})\,\mathrm{e}^{-\mathrm{i}\nu t} \tag{3b}$$

$$\dot{\rho}_{tt} = \gamma\rho_{ee} - k\rho_{tt} \tag{3c}$$

$$\dot{\rho}_{ee} = -(\gamma + k_{\mathrm{T}})\rho_{ee} + \mathrm{i}\cdot\frac{1}{2}\Omega\,(\rho_{ge} - \rho_{eg})\,\mathrm{e}^{-\mathrm{i}\nu t} \tag{3d}$$

where $\omega = \omega_e - \omega_g$, $\Omega = -d_{eg}\cdot E/\hbar$ is the Rabi frequency, $\delta\omega_{\mathrm{L}}(t) = \omega_0 - \sum_i \Delta\omega_i(t)$, is the detuning frequency when the SM is coupled to the bath molecules, each of which contributes to the frequency shift of the SM.

3 Fluorescence intensity fluctuations in single-molecule spectroscopy

In a typical SMS experiment, in order for the experimental method to be useful, it is necessary that at least one of the chemical states of the single molecules is fluorescent and at least one other state is non-fluorescent. The experimental observable is the fluorescence signal (photons) $I(t)$ corresponding to the molecule studied and fluorescent photons that are collected in time bins. In an SMS experiment, we record in real time both the fluorescence intensity, i.e., number of detected photons, $I(t)$ in time bins and the chronological time t_p of each detected photon in time bins. The sequence of such "time stamped" photons is then subjected to statistical analysis. Such a scheme allows for measurements of fast dynamics or for measurements of dynamics over a broad range of time scales. Photon statistics, however, demands that a certain number of photons be obtained, or "binned," in order to reach a statistically reasonable estimate of the lifetime [24]. In these experiments [50—53], the initial state of an SM is randomly chosen as 1 or 2 state. After which, one applies a train of N laser pulses. The duration of the pulse should be much shorter but the separation between pulses much longer than the fluorescence lifetimes. The molecule is excited by a pulse and decays to the ground state by emitting a photon. The delay time between the excitation and the emission is measured. Then, the process is continued to collect the statistics of the lifetimes. When the many excitation-emission events along the N pulse trains are measured from an SM, one can build up a histogram of the lifetime distribution for that molecule, which will exhibit a bi-exponential form. To improve the time resolution and statistics for single-molecule studies, several new strategies have recently been introduced for studying the conformational dynamics [24, 29, 30]. The kinetic information is extracted from various functions computed from the time series $I(t)$ of fluorescence photons describing the evolution of such chemical transformations. A time dependent intensity $I(t)$, i.e., photon counts per bin time, is obtained when the bin timescale is shorter than the timescale of the underlying physical or chemical processes responsible for the intensity fluctuations. In this light, single-molecule fluorescence lifetime holds the promise of serving as a sensitive measure for conformational dynamics. The kinetic information is extracted from various functions computed from the time series $I(t)$ describing the evolution of these chemical transformations.

Fluorescence intensity fluctuations measured from single molecules are found in many different systems, for example, green fluorescence proteins [13], dendrimers [18], conjugate polymers [19, 20], enzymatic reactions [14], and dye molecules [27]. In many cases [45], the fluorescence intensity from single molecules shows two-state fluctuation behavior, "bright" or "on" state versus "dark" or "off" state. Depending on the nature of the dynamic processes involved, the characteristics of the fluorescence intensity fluctuations yield different information. In this case, various statistical measures have been utilized in order to obtain dynamic information from the single molecule intensity fluctuation trajectories. Further, one of those quantities which have proved useful is the fluorescence intensity correlation function, $g^{(2)}(\tau)$ in Eq. (4),

$$g^{(2)}(\tau) = \frac{\langle I(t+\tau)I(t)\rangle}{\langle I\rangle^2} \tag{4}$$

A theoretical description of the fluorescence correlation function, $g(2)$, in the context of the SMS has been given by Orrit and co-workers. [2, 3, 24, 60] Instead of listing the applications of $g^{(2)}(\tau)$ made from obtaining dynamic information from different systems, we discuss a basic idea underlying the connection between the experimental measurements and the theoretical model. When we measure photons emitted from a single molecule using a detector in experiments, we identify $I(t)$ to be the number of photon counts per bin time. In many cases, fluorescence intensity fluctuations are characterized as an alternating sequence of two different intensities, I_1 and I_2 . One can calculate the fluorescence autocorrelation function $g^{(2)}$ by counting the number of photon pairs separated by time t. Stationary emission processes are usually considered so that

$$g^{(2)}(t;\, t+\tau) = g^{(2)}(\tau) \tag{5}$$

which is t independent. Here, we assume that the single molecule system is ergodic and is in the steady state.

In order to calculate the correlation function theoretically, we start with an appropriate dynamic equation of the single molecule system under investigation; for example, a Bloch equation for the purpose of describing both coherence and population or a rate equation for populations only. In Fig. 2, we show a schematic representation of an electronic energy level structure of an isolated SM. Assuming a simple non-degenerate two-state SM in an external classical laser field as in Eq. (2), the molecule can be described by a 2×2 density matrix ρ whose elements are ρ_{ee}, ρ_{gg}, ρ_{eg}, ρ_{ge}. Using Eq. (2), the stochastic Bloch equations, in the rotating wave approximation, are

$$\dot{\rho}_{aa} = \gamma\rho_{bb} + \mathrm{i}\cdot\frac{1}{2}\Omega(\rho_{ba} - \rho_{ab})\,\mathrm{e}^{-\mathrm{i}\nu t} \tag{6a}$$

$$\dot{\rho}_{ab} = -\mathrm{i}\,(\omega + \delta\omega_{\mathrm{L}}(t))\rho_{ab} - \gamma\rho_{ab} - \mathrm{i}\cdot\frac{1}{2}\Omega\,(\rho_{aa} - \rho_{bb})\,\mathrm{e}^{-\mathrm{i}\nu t} \tag{6b}$$

$$\dot{\rho}_{bb} = -\gamma\rho_{bb} + \mathrm{i}\cdot\frac{1}{2}\Omega\,(\rho_{ab} - \rho_{ba})\,\mathrm{e}^{-\mathrm{i}\nu t} \tag{6c}$$

where $\omega = \omega_e - \omega_g$, $\Omega = -d_{eg}\cdot E/\hbar$ is the Rabi frequency, $\delta\omega_{\mathrm{L}}(t) = \omega_0 - \sum_i \Delta\omega_i(t)$, is the detuning frequency when the SM is coupled to the bath molecules, each of which contributes to the frequency shift of the SM.

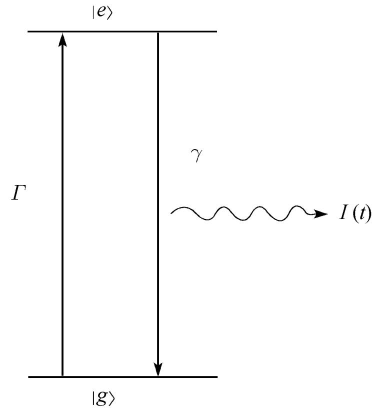

Fig. 2 Schematic illustration two-state model of energy diagrams of a single molecule (SM).

We apply a kinetic Monte-Carlo algorithm that simulates the dynamic evolution of Eq. (6) of the single molecule system as they undergo jumps among the available states [61, 13—19]. In this algorithm, stochastic "trajectories" of the system are generated according to the probabilities determined by Eq. (6). Specifically, if the system is found in state i at some time t, then, during a short time interval Δt, it can undergo a transition to the state j with a probability equal to $k_{ij}\,\Delta t$. And here, we use the following parameters γ=1 ns^{-1}, and Γ=2 ns^{-1}. These parameters are reasonably chosen and are close to those formulas the SMS used in Refs. [4—21, 38—49]. The single molecule is excited by continuous laser and fluorescence signals I (t), e.g., number of photons emitted from the single molecule in a fixed time window are counted. Then, we obtain $g^{(2)}(\tau) = \dfrac{I(t)I(t+\tau)}{\langle I\rangle^2}$. We notice that the fluorescent photons emitted by a coherently driven SM exhibit the nonclassical effect of photon antibunching, namely,

$$\lim_{\tau\to 0} g^{(2)}(\tau) = 0 \tag{7}$$

Physically, the emission of one photon from a single emitter makes the detection of a second photon, after a short delay, improbable. We notice that when the separation between two photons is infinite, t, all the correlation among photons is lost, that is,

$$\langle I(t+\tau)I(t)\rangle \to \langle I(t+\tau)\rangle\langle I(t)\rangle = \langle I\rangle^2, \quad \tau\to\infty \tag{8}$$

where we have used the fact at steady state the average fluorescence intensity is time-independent, $\langle I(t)\rangle = \langle I\rangle$. Therefore, when the time interval between the photon pair is much longer than all the correlation time scales, one expects that

$$\lim_{\tau\to\infty} g^{(2)}(\tau) = 1 \tag{9}$$

In other words, in the long time limit, we have Eq. (7). Because of the long delay times, the emission of two photons (not necessarily consecutive photons) is uncorrelated. Surprisingly, a single quantum-dot spectroscopy has revealed a new type of strongly nonstationary behavior where Eq. (7) does not hold. Thus, we found that, for a quantum optical regime, in the short time regime comparable to the inverse Rabi frequency, the emission of photons are strongly correlated because when one photon is detected at time zero, the system is projected into the ground state immediately. Thus, it takes a finite amount of time to detect the next photon (Fig. 3). The signature of this phenomenon appears as a dip

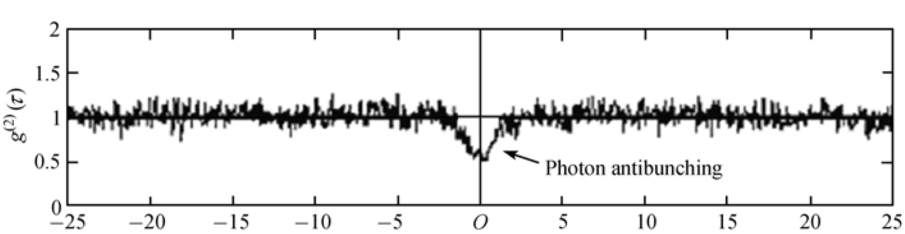

Fig. 3 Fluorescence emission fluctuation of a single molecule (SM), which reveals the photon antibunching at t = 0.

in the correlation function $g^{(2)}(t)$, typically in the nanosecond regime, and it is called photon antibunching [21]. This phenomenon is a true indication that one is indeed observing an SM since in an ensemble measurement, strong correlations between adjacent photons from an SM would be washed out by the average over many molecules. The first observation of the photon antibunching phenomena from an SM was observed and analyzed by Basche *et al.* [42]. Later it was found in many other systems including those at room temperature [31, 47—49].

4 Conclusions and prospects

In the last couple of decades, optical studies on single molecules have raised great hopes, not only in the biosciences, but also in physical chemistry and materials science. This is because of the unaveraged microscopic information, with minimal contact or perturbation of the system under study. Different measurement methods have been adopted in SMS to obtain more insights into the systems with improved detection limits, such as NSOM, FCS, TIRM, etc. With confocal arrangements, substantial reduction in the background signals has been achieved by the reduction in the detection volume.

The field of single molecule spectroscopy (SMS) has grown rapidly in recent years. Recent experimental advances made in SMS and observations of spectra from single molecules have become routine in many laboratories around the world. We expect that the SMS studies will continue to provide more novel phenomena in condensed phases in the future, and it is certain that many of them will require developments of new theoretical concepts as well as sophistication of theoretical methods in order to have a better microscopic understanding of dynamics of molecules in condensed media. We hope that this theoretical study will serve as an introduction and a guideline into this exciting new field.

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