Single-Molecule Spectroscopy

Every Molecule is Different!

Kankan Bhattacharyya

Three scientists (William E Moerner, Eric Betzig and Stefan W Hell) have been awarded the 2014 Nobel Prize in Chemistry for their contributions to single-molecule spectroscopy (SMS). This new branch of spectroscopy was invented 25 years ago in 1989. In this article, we give a brief outline of the history of this technique and some of the exciting applications.

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1. Introduction

Molecules of the same chemical composition are often assumed to have identical properties. In reality though, every molecule is different. For instance, from the kinetic theory of gases, we know that different molecules move with different velocities. As a result of this, different molecules absorb at different external frequencies because of the Doppler shift (see *Box* 1). Further, in a heterogeneous medium the local environment of each molecule may be different. This gives rise to large variations of those properties which depend on the medium (e.g., local polarity or viscosity). For instance, in a biological cell the local environment at the membrane may be drastically different from that in the cytoplasm or in the nucleus. Even within the cytoplasm, different organelles (mitochondria, lysosome or endosome, etc.) may have different properties.

For several decades, many scientists were excited by the prospect of recording high-resolution spectra[1] free from Doppler broadening. One popular strategy of eliminating Doppler effect is cooling of gases by adiabatic expansion and then selecting only one group of ultra-cold atoms/molecules having identical velocity.

[1] High-resolution spectrum refers to a spectrum consisting of very sharp lines. The sharp lines clearly display transitions to individual rotational, vibrational and electronic states.

Keywords
Single-molecule spectroscopy (SMS), confocal microscopy, FCS, sm-FRET, FLIM.

Box 1. Doppler Broadening

The condition of absorption of a photon is

$$\Delta E = E_2 - E_1 = h\nu_{\text{obs}}\,, \tag{i}$$

where ΔE represents the energy gap and ν_{obs}, the frequency of a photon observed by a molecule. Even if ΔE is the same for all the molecules, the observed frequency may vary from one molecule to another because of the Doppler shift. If a molecule moving with a velocity u is irradiated by an external frequency ν_{ext}, the frequency observed by a molecule is

$$\nu_{\text{obs}} = \nu_{\text{ext}}[1 \pm (u/c)]\,. \tag{ii}$$

In (ii) '+' corresponds to the situation when the molecule is moving towards the photon (source) and '−' corresponds to molecule moving away from the photon (source). Since the velocity of the molecules exhibits a broad distribution, different molecules absorb at different ν_{ext}. For each value of a velocity, there is a narrow absorption line. But the overall absorption spectrum of a molecule is a superposition of many such sharp lines corresponding to different velocities. The superposition gives rise to a broad spectrum (*Figure* A) which resembles the Maxwell–Boltzmann velocity distribution curve. Since the molecules have different absorption frequencies, the gas is inhomogeneous. Thus Doppler effect is an inhomogeneous broadening effect.

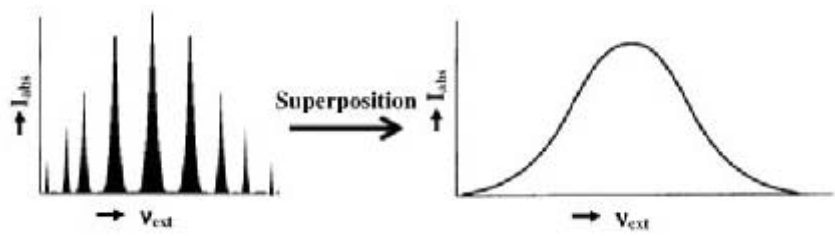

Figure A. Doppler broadening because of superposition of individual absorption lines.

This technique is widely used in atomic or molecular beams and has attracted the 1986 Nobel Prize in Chemistry. The other strategy is to completely eliminate motion by trapping a single ion in a quadrupole electric field (1989 Nobel Prize in Physics) or by momentum transfer from photon (laser cooling, 1997 Nobel Prize in Physics).

2. History of SMS: The Pioneers

In the 1980's, there were several attempts to record absorption spectra[2] of individual molecules at different sites (or defect centers) of a solid crystal, under Doppler-free condition. In a solid, the translational motion of the

[2] Absorption spectrum is a plot of optical density, OD = log (I_0/I), against wavelength.

molecules is eliminated. However, contributions of the rotational and vibrational levels broaden the spectrum. Cooling the molecule to ultra-low temperatures (1.6 K in superfluid helium) ensures that the molecules are in the lowest vibrational and rotational levels to yield the zero-phonon line (free from rotational and vibrational transitions).

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Recording an absorption spectrum under single molecule condition is an extremely difficult proposition because of the following reason. According to the Lambert–Beer's law, the absorbance or optical density (OD) is defined as

$$\mathrm{OD} = \log(I_0/I), \tag{1}$$

where I_o and I are the intensities of the light before and after the sample. If N denotes the number of photons striking a molecule, then after absorption of one photon by a single molecule, the number of transmitted photons is $N-1$, so that

$$\mathrm{OD} = \log[N/(N-1)]\,. \tag{2}$$

Since the change (N to $N-1$) is very small, one has to measure N(or intensity, I) very accurately. To do this, Moerner and his group, then working at the IBM Amaden Research Centre (California), pushed the technology to the limit using a frequency modulation (FM) technique. The FM technique was developed in 1980 by Bjorkland, at the same IBM lab [1]. This method is similar to the principle of capturing signals by a FM radio. In this method, the wavelength of the laser is varied (e.g., by varying the cavity length) around the central frequency, ν, at a low frequency, $\Delta\nu = \Omega$. The intensity of the light transmitted by the sample, I, contains both a part modulated at the frequency Ω and a randomly fluctuating one. Many things (such as, laser intensity fluctuations and instrumental noise) contribute to the random fluctuations. Using a lock-in amplifier, one can

Chemistry Nobel 2014

E Betzig

S Hell

W Moerner

filter out only the signal modulated at a frequency Ω and thus accurately measure the intensity.

In 1987, Moerner and Carter applied the FM technique to detect the fine structure of in homogeneously broadened absorption lines of individual pentacene molecules doped in a para-terphenyl crystals [2]. Two years later, using double modulation, Moerner and Kaldor advanced the FM technique further, to achieve single-molecule detection [3]. They showed that when they tuned their laser frequency at the peak of the absorption spectrum, a very large number ($\sim 10^5$) of molecules contribute to the absorption. However, by tuning the frequency to the wings or edges of the absorption spectrum, they could reduce the number of absorbed photons to nearly unity. This allowed them to achieve single-molecule detection (SMD). In the last sentence of this paper [3], they rightly predicted that, "*Such work (SMD)would open up a new frontier of spectroscopy of single defect centers in solids where no Doppler, recoil or multicenter averaging are present.*"

While Moerner used a quite complicated technique, in 1990, Oritt and Bernar (University of Bordeaux, France) proposed a simpler technique – many molecules emit fluorescence when excited [4]. Thus detection of fluorescence photon is indirect proof of absorption. This is now well-known as fluorescence-excitation spectroscopy. In the last sentence of this paper [4] they made a *modest* claim, "*This result is obviously of great significance for the low-temperature dynamics of the condensed phase.*"

Because of the simplicity of Oritt's approach, fluorescence excitation became a popular technique for single-molecule detection as Zare and many other workers earlier used laser-induced fluorescence (LIF) for detection of a few molecules in jets [5]. These two papers of Moerner and Oritt [3,4] are widely heralded as the foundation of single-molecule spectroscopy.

Box 2. Earlier Efforts at Single-Molecule Detection

It may be mentioned that before Moerner and Oritt, many others tried to detect single molecule, that too in a solution using fluorescence at room temperature. In 1961, Rotman studied hydrolysis of β-D-galactose catalyzed by β-D-galactosidase using a fluorescent probe 6-hydroxyfluoran (6-HF)bonded to galactose [6]. In the bonded state, 6-HF does not emit. On hydrolysis when free 6-HF is liberated, intense fluorescence is observed. In order to study hydrolysis by single-enzyme molecule, they sprayed a very dilute solution of the enzyme and the substrate over few drops of silicone oil placed on a microscopic slide and detected fluorescence using a microscope. This work is fore runner of the many beautiful experiments on single enzymology designed much later by Xie group and others [7]. In 1976, Hirschfeld applied fluorescence microscopy to detect single antibody molecules [8]. In 1983, Keller and co-workers, first used laser-induced fluorescence (LIF), to detect single molecule under flowing conditions [9]. Further examples of fluorescent detection of single molecule in solution were reported by Keller group and Mathies group [10–11]. As early as in 1972, Magde, Elson and Webb developed fluorescence correlation spectroscopy (FCS) to study a few molecules in solution [12]. Since all biological studies involve room temperature solutions, these studies eventually became the most popular applications of SMD.

Around 1986, Betzig, at AT&T Bell Labs, advanced the room temperature single-molecule detection (SMD)to break the Abbe limit. According to the principle, also called the Rayleigh criterion, (1873), the minimum spot size in a microscope cannot be smaller than half of the wavelength used (λ). Thus for the shortest wavelength of visible light (400 nm) the spatial resolution is $\lambda/2 \sim$ 200 nm. Using near-field scanning optical microscopy (NSOM), Betzig and co-workers broke the Abbe limit and demonstrated that one can achieve sub-wavelength spatial resolution [13–15]. The abstract of one of their papers [14] reads, "*In near-field scanning optical microscopy, a light source or detector with dimensions less than the λ is placed in close proximity ($\lambda/50$) to a sample to generate images with resolution better than the diffraction limit. A near-field probe has been developed that yields a resolution of approximately* 12 *nm (approximately $\lambda/43$).*"

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>
> – *Betzig et al*

Betzig played the role of a game changer and inspired many others to develop super-resolution microscopes with spatial resolutions better than the Abbe limit. In 1994,

"...We overcome the diffraction resolution limit by employing stimulated emission to inhibit the fluorescence process in the outer regions of the excitation point-spread function. In contrast to near-field scanning optical microscopy, this method can produce three-dimensional images of translucent specimens."

– Hell and Wichmann

Hell and Wichmann (then at University of Turku, Finland) developed stimulated emission depletion (STED) microscopy [16]. In the abstract of this paper [16], they wrote, "*We propose a new type of scanning fluorescence microscope capable of resolving* 35 *nm in the far field. We overcome the diffraction resolution limit by employing stimulated emission to inhibit the fluorescence process in the outer regions of the excitation point-spread function. In contrast to near-field scanning optical microscopy, this method can produce three-dimensional images of translucent specimens.*"

In a STED microscope, the exciting laser (of frequency ν_{abs}) produces a big focused spot of radius ($\lambda/2$). Then, a second laser of frequency ν_{em} equal to the emission frequency irradiates the outer periphery of the big spot. The second laser causes stimulated emission and hence, rapid transition of the molecules from the excited state to the ground state. This results in depletion of the molecules in the excited state and thus bleaches the peripheral region leaving a central bright region of much smaller radius $< \lambda/2$ (*Figure* 1).

In summary, within 5 years of the first demonstration of SMD by Moerner, there were major breakthroughs in

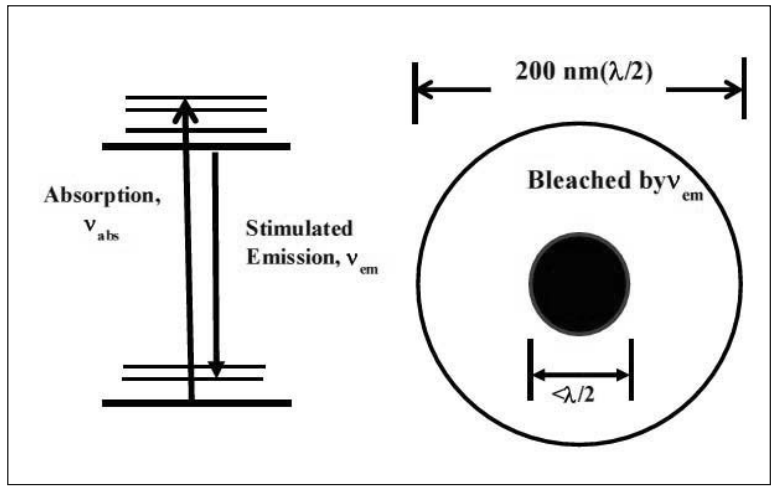

Figure 1. Schematic design of a STED microscope.

this field. Since a Nobel Prize cannot be given to more than three scientists, the Nobel Prize Committee finally chose Moerner for the first true demonstration of SMD and Betzig and Hell for super-resolution microscopy.

3. SMD in Solution and at Room Temperature: Confocal Microscopy

Though single-molecule detection (SMD) was first achieved at low temperature (1.6 K), most exciting applications of SMD involve real biological systems which remain active in solutions at room temperature. For this, Zare's group and others developed confocal microscopy [17–19]. In their first paper, Zare and coworkers wrote [17], "*Confocal fluorescence microscopy coupled with a diffraction-limited laser beam and a high-efficiency detection system has been used to study the diffusive movement and emission process of individual fluorescent molecules in the liquid phase at room temperature. The high detection sensitivity achieved at fast data acquisition speeds (greater than 1 kilohertz) allows real-time observation of single-molecule fluorescence without statistical analysis. The results show fluorescence-cycle saturation at the single-molecule level and multiple recrossings of a single molecule into and out of the probe volume as well as the triplet state.*"

"Confocal fluorescence microscopy coupled with a diffraction-limited laser beam and a high-efficiency detection system has been used to study the diffusive movement and emission process of individual fluorescent molecules in the liquid phase at room temperature."

– Zare et al

Though there are many variations of experimental strategies for SMD, confocal microscopy is by far the most popular. Therefore, we will describe confocal microscopy[3] in some detail (*Figure* 2). In a confocal microscope, the exciting laser is reflected by a dichroic mirror towards the sample through a lens (objective). The lens focusses the light on the sample (a liquid drop) kept on a microscopic slide. On excitation the sample emits fluorescence in all directions. A part of the fluorescence passes through the same objective lens and becomes a parallel beam. The dichroic mirror transmits the fluorescence. (It is called dichroic because it has a dual function –

[3] By controlling the optics, a confocal microscope can image a thick sample at different depths and hence give a 3D picture.

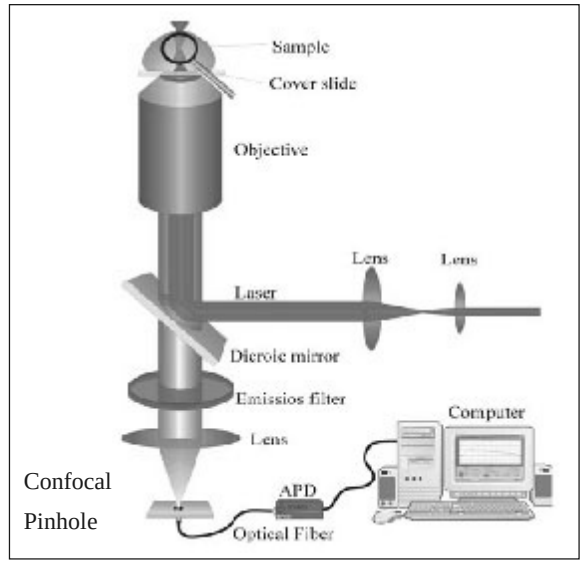

Figure 2. Confocal microscope.

reflection at one wavelength (laser excitation) and transmission at another (for fluorescence)). The parallel fluorescence beam is then focused by a lens on a pinhole, called confocal pinhole. It ensures that only fluorescence from the focused exciting light is collected. Thus it is the heart of the confocal microscope. After passing through the pinhole the fluorescence is collected by a detector. *Box* 3 describes in detail the focused spot.

Box 3. Focused Spot

As shown in *Figure* A, the shape of the focused spot is an ellipsoid of radius r_{xy} along x- and y-axes, and r_z along z-axis. For a simple ellipsoid the volume is given by $(4/3)\pi r_{xy}^2 r_z$. For a Gaussian beam profile of the laser, the brightness is given by $I_0 \exp[-2(x^2+y^2)/r_{xy}^2] \exp(-2z^2/r_z^2)$.

For such a beam profile, the boundary is defined as the surface where the intenisty is reduced to e^{-2} of its maximal value, I_0. In this case, the effective volume is $(\pi)^{3/2} r_{xy}^2 r_z$.

The typical volume of the focused spot is $\sim$ 1fL (femtoliter $= 10^{-15}$L). If one uses a nano-molar (10^{-9} M) solution the number of molecules in the focal volume would be around 0.6. Some useful units are: 1 yoktomole $= 10^{-24}$ M; 1 guacamole =1 molecule = 1/ (Avogadro Number) $= (1/6.023) \times 10^{-23}$M.

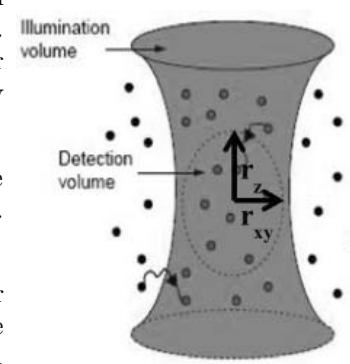

Figure A. Focused spot.

4. Fluorescence Correlation Spectroscopy (FCS)

In a confocal microscope, the intensity of the observed fluorescence fluctuates because of diffusion of the molecule in and out of the focal volume (*Figure* 3). FCS involves a correlation analysis of fluctuations in fluorescence intensity. This provides information about molecular diffusion and interactions. Specifically, FCS may be used to determine coefficient of translational diffusion (D_t). In FCS, we define a correlation function $G(\tau)$ of the fluorescence intensities as [11,16,18],

$$G(\tau) = < \delta F(0)\, \delta F(\tau) > / < F >^2 , \qquad (3)$$

where <F> denotes the average fluorescence intensity and δF (τ) is the fluctuation or deviation of the fluorescence intensity at a time delay, τ, from the mean value, i.e., δF (τ) = <F> − F(τ). The diffusion coefficient D_t is obtained by fitting $G(\tau)$ to suitable fitting functions as discussed elsewhere [12,17,19].

In a confocal microscope, the intensity of the observed fluorescence fluctuates because of diffusion of the molecule in and out of the focal volume. FCS involves a correlation analysis of fluctuations in fluorescence intensity.

4.1 *Size of Particles*

D_t is related to the viscosity of the medium (η) and the hydrodynamic radius of the diffusing fluorescent molecule (R_h) by the Stokes–Einstein equation as

$$D_t = k_B T / 6\pi R_h \eta . \qquad (4)$$

If the viscosity is known, one can calculate the value of R_h (i.e., size) from the D_t values. This is useful to determine the size of a protein in the native, denatured

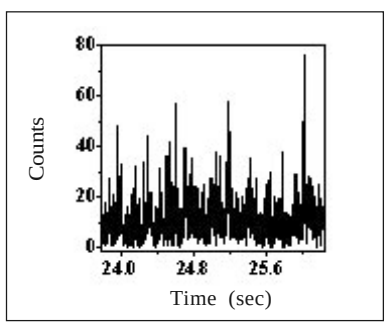

Figure 3. Fluctuations of fluorescence intensity observed in a confocal microscope.

The main triumph of single-molecule spectroscopy is the ability to determine the rate constant for individual molecules at different regions. This has dramatically improved our understanding of chemistry because all the chemical properties we have determined earlier are averages of exceedingly large number of molecules.

and molten globule state. Many diseases (e.g., Alzheimer's disease) involve aggregation of proteins and hence, in size. Such diseases may also be monitored from determination of D_t and subsequently, size of the protein aggregate. Very recently, FCS has been applied to monitor the size and growth of nanoparticles [20].

4.2 *Heterogeneity in Biological Systems*

Many natural and biological systems are essentially heterogeneous. In this case, the value of the viscosity, η (and hence, D_t) varies from one region to another. For such a medium, one gets multiple values of D_t instead of a single value [21]. The distribution curve (*Figure* 4) describes the variation of viscosity within a vesicle where each individual value corresponds to separate focused volumes, 200 nm in size. Since viscosity controls the rate of bi-molecular reactions, such a distribution indicates that the rate constant of a chemical reaction may be region dependent in a heterogeneous medium. The main triumph of single-molecule spectroscopy is the ability to determine the rate constant for individual molecules at different regions. This has dramatically improved our understanding of chemistry because all the chemical properties we have determined earlier are averages of exceedingly large number of molecules.

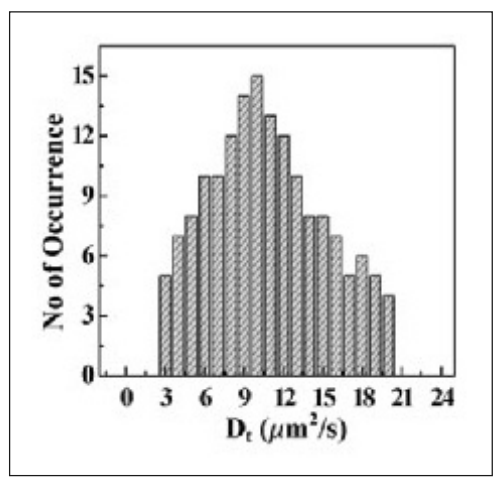

Figure 4. Distribution of D_1 in a vesicle.
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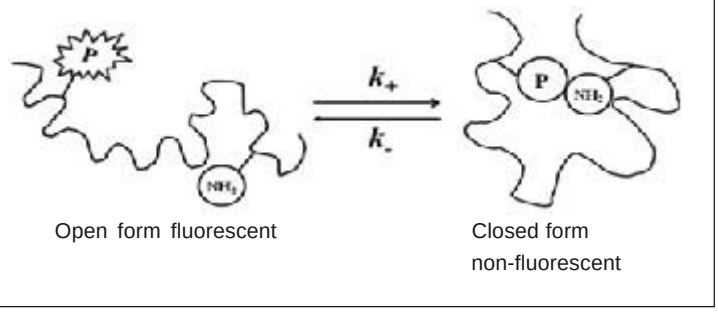

Figure 5. Conformation dynamics in a protein.

4.3 *Conformational Fluctuation*

Apart from diffusion, fluctuations in fluorescence intensity (*Figure* 5) are also caused by conformational fluctuation of a protein. To study conformational fluctuation, one attaches a fluorescent probe to a protein. Every protein has amino-containing residues. During conformational fluctuation, when the amino group comes into close contact with the fluorophore (within 1 Å distance, so that the two electron clouds overlap), rapid quenching occurs because of electron transfer (*Figure* 5) [22].

During quenching, the quencher, Q, transfers an electron to an excited molecule M* to produce an anion M^- and a cation Q^+ both of which are non-emisive. This reduces or quenches emission intensity.

5. Fluorescence-Lifetime Imaging Microscopy (FLIM)

The other advantage of confocal microscope is that one can record emission spectrum and lifetime of the flourophores within individual regions of size 200 nm. The plot of lifetime at different regions is known as a fluorescence lifetime image [19]. The fluorescence lifetimes of many probes are markedly dependent on the local polarity and visocisty. Thus, a FLIM image provides valuable information on the local environment of a heterogeneous medium.

Fluorescence Resonance Energy Transfer (FRET) is an elegant technique to measure the distance between a donor and an acceptor molecule. FRET refers to the simulataneous de-excitation of a donor molecule (D) from the excited to the ground state along with excitation of an acceptor molecule (A) from the ground to the excited state.

6. Single-Molecule Fluorescence Resonance Energy Transfer (sm-FRET)

Fluorescence Resonance Energy Transfer (FRET) is an elegant technique to measure the distance between a donor and an acceptor molecule. FRET refers to the simulataneous de-excitation of a donor molecule (D) from the excited to the ground state along with excitation of an acceptor molecule (A) from the ground to the excited state.

$$\mathrm{D}^* + \mathrm{A} \rightarrow \mathrm{D} + \mathrm{A}^* .$$

According to Forster mechanism, the rate of FRET is inversely proportional to the sixth power of the donor–acceptor distance (R_{DA}^6). Thus FRET is often called a molecular ruler [19].

In a single-molecule FRET (sm-FRET) experiment, a donor and an acceptor are covalently bonded to the molecule under study. The sm-FRET efficiency ($\varepsilon_{\mathrm{FRET}}$) is obtained from the emission intensity of a single donor (I_{D}) and single acceptor (I_{A}) using the following equation [19],

$$\varepsilon_{\mathrm{FRET}} = \frac{I_{\mathrm{A}}}{I_{\mathrm{D}} + I_{\mathrm{A}}}. \tag{5}$$

The distance between the donor and acceptor dye pairs (R_{DA}) is calculated from the relation [19],

$$R_{\mathrm{DA}} = R_0 \left[\frac{1 - \varepsilon_{\mathrm{FRET}}}{\varepsilon_{\mathrm{FRET}}} \right]^{\frac{1}{6}} . \tag{6}$$

The sm-FRET study provides a three-dimensional map of the biomolecules in terms of R_{DA} with the donor and acceptor attached to selected sites or residues.

7. Conclusion

Single-molecule spectroscopy (SMS) originated from a physicist's dream of recording high-resolution spectrum. Very soon, SMS captured the imagination of chemists and biologists. The most exciting applications of SMS

appear to be in the field of biology. Many new phenomena are being discovered in selected organelles in a live cell using SMS. These studies are deepening our understanding of how living things work. Through SMS we are approaching the dream, first described by Richard Feynman in 1963 as "everything the living things do can be understood in terms of jiggling and wiggling of atoms". It is nice to see that a single molecule is capable of uniting physicists, chemists and biologists.

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