Introduction to Single molecule fluorescence spectroscopy
(CHM409 Physical Methods in Chemistry, Course teacher: Dr. Biplab Kumar Kuila)

Till now, what we have studied is the study of fluorescence response from a collection of molecules present in a solution. The concentration of the solutions is generally taken in micromolar (μmol) concentration. Considering, 1μmol concentration, the number of molecule present per liter will be ~ 6 X 10^{17}. So the fluorescence responses what we are getting is the collective response from such large number of fluorophores. The fluorescence property from a large collection of molecules is called ensemble average fluorescence property. The fluorescence property of single individual molecules may not be necessarily same with the ensemble average property due to averaging. Single-molecule fluorescence spectroscopy provides a fundamental advantage in direct measuring the fluorescence of individual molecules or sub-populations and give information of the molecular confirmations and processes. A detailed example for better understanding the difference between ensemble average fluorescence and single molecule fluorescence is given below.

Let consider oligonucleotide structures such as the hairpin loop of a single-stranded DNA which are called molecular beacon, shown in Figure 1.1 (a-b). These structures are not static and can fluctuate between fully closed states (a), open random coils (b) and possibly also between intermediate partially folded states. The dynamic behavior of these oligonucleotide structures probably plays an important role in their function since recognition events, such as those involved in regulation of gene expression, are likely to be affected by the kinetics of conformational changes.

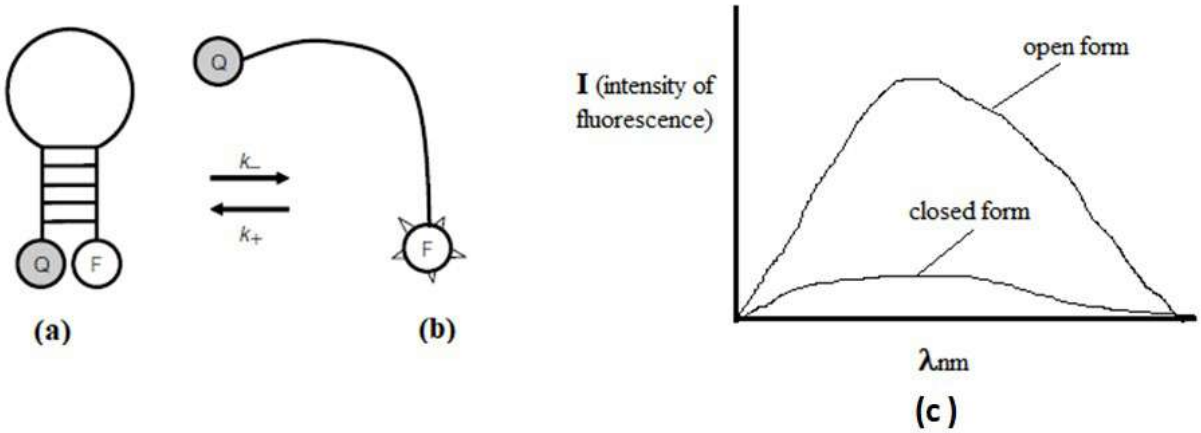

Fig. 1.1 shows schematic diagram of two conformations of a DNA molecular beacon (a &b) and their probable fluorescence spectra (c). The five bases at the two ends of the beacon are

complementary to each other. The size of the loop and its content are varied. The beacon flips between open and closed forms with the characteristic rates k_- and k_+.

The fluorophore (F) and the quencher (Q) are covalently linked to the two arms of the beacon. In the open state, the beacon fluoresces whereas in the closed state the fluorescence is quenched (Fig. 1.1c). Now consider a case where you have measured the fluorescence of this molecular beacon solution and get the fluorescence spectra experimentally as shown in figure 1.2 (experimentally observed, dotted line). The intensity of the experimentally observed fluorescence spectra is in between 100% open and 100% closed form. So from the experimental spectra one can conclude that the molecular beacon solution may contain ~50% closed and ~50 % open conformations which are in equilibrium. Now consider another situation, where molecular beacon may adopt a conformation favored by that particular solvent which is neither fully open nor fully closed but a partially open state (Fig. 1.2). In this partial open state, the quencher and the fluorophore are separated by a moderate distance and fluorescence is not quenched very significantly and beacon solution shows moderate fluorescence. So, the beacon solution where all the molecules are in partially open form may also results fluoresce similar to the 50:50 open – close mixture.

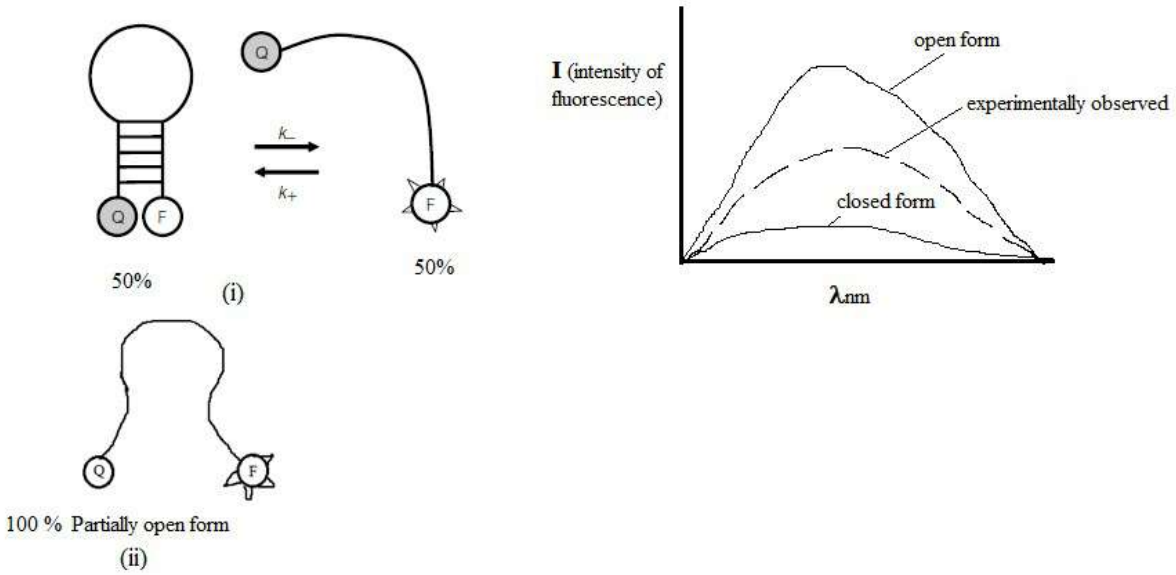

Fig. 1.2 shows schematic diagram of two possible states that the molecular beacon present in solution (i) 50% - 50% mixture of closed and open structures (ii) 100% partially open structures. Both the solution should result fluorescence as shown by dotted line in the fluorescence spectra (right side).

So, the experimentally observed fluorescence spectra will result either from a molecular beacon solution which contain 50% open and 50% close form or a solution which contain 100% partially open form. But the question will automatically arise which is correct? So the ensemble average fluorescence study cannot give concrete information of molecular conformation of the DNA molecular beacon in solution. So the only solution is to look into the molecules individually and study their individual fluorescence. Now if you consider an arrangement (Fig. 1.3) where molecules will enter one by one into a small observation volume and fluorescence will be measured. When the molecule is outside of the observation volume, there is no fluorescence but when it enters into the volume, fluorescence will be observed. The fluorescence intensity is again zero when it comes out from the volume. The intensity of the fluorescence for the molecular beacon will depends on its conformation, high for open, low for closed and medium for partially open structure.

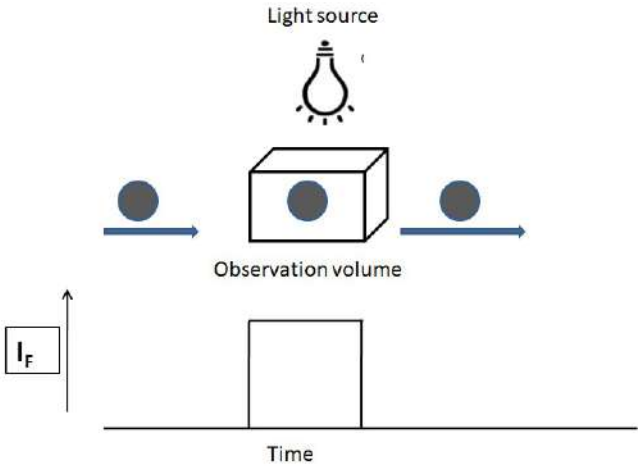

Fig. 1.3 simple schematic diagram of single molecule fluorescence measurement

For case one (50% open and 50% close conformation), individual or molecular fluorescence measurement will result the plots as shown in figure 1.4 (a). Whereas, the solution containing only one conformation of molecule (partially open structure) will result plot 1.4 (b). By differentiating the two observed plots in single molecule fluorescence measurement, one can easily conclude whether the solution has actually 50% open and 50% close conformation or 100% partially open conformation. In case of protein, it has been observed that the partially opened structures are not fixed they shows some breathing type motion in solution where the

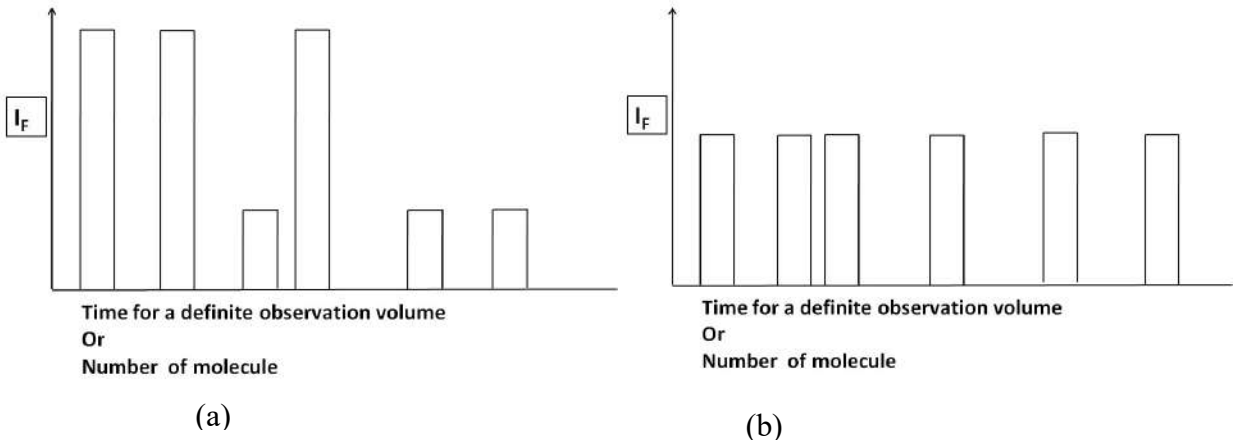

Fig. 1.4. plot of fluorescence intensity (I_F) vs time (for a definite observation volume) or number of molecules for single molecule fluorescence measurement for molecular beacon solution containing (a) 50% open and 50% close conformation (b) 100% partially open conformation. For simplicity we have assumed only six molecules.

time scale is slow in the order of micro second and these breathing type of motion will change the distance between fluorophore and quencher segment and results small fluctuation in fluorescence as shown in fig. 1.5. These conformational fluctuation dynamics can also be studied by single molecule fluorescence spectroscopy.

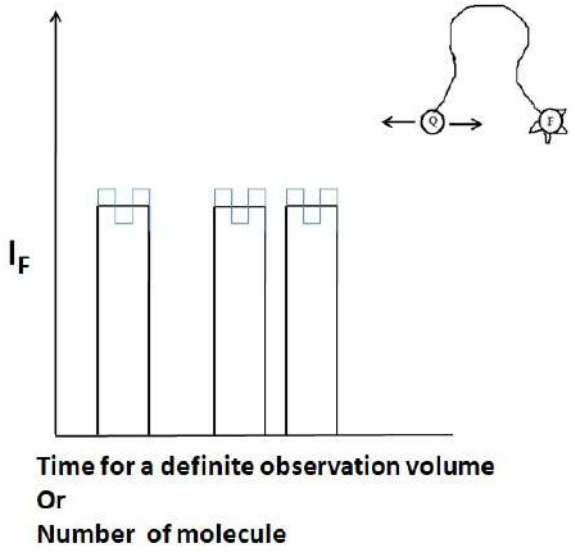

Fig. 1.5. Plot of fluorescence intensity (I_F) vs time showing conformational fluctuation dynamic.

Consider a simple enzymatic reaction as described in fig. 1.6. S-F (where S is the substrate attached with a fluorophore) is reacting with E-Q (where E is the enzyme attached with a quencher) to form a substrate enzyme complex S-F : E-Q and finally give the product free substrate and free fluorphore (S + F + E-Q). The study of the kinetic for this enzymatic reaction through fluorometric assay will results the plot as shown in the figure 1.6. Initially, enzyme (E-Q) solution will be taken in the cuvette and then S-F will be added. At the beginning, there will be no fluorescence and with progress of the reaction as more and more free fluorophore is formed the fluorescence intensity will increase with time. At the end of the reaction the fluorescence intensity will be constant. Form the plot one can easily determine the rate constant of the reaction. But the method using ensemble average fluorescence for kinetic study has certain draw back. For example, the mixing of S-F with E-Q requires some time which is called dead time of the reaction. Depending on rate of the reaction, it may be highly possible that some of the molecules may already form products during the mixing time. So the reaction kinetics will be influenced by this dead time. This method will also not tell us whether this reaction is passing through any intermediate states or not.

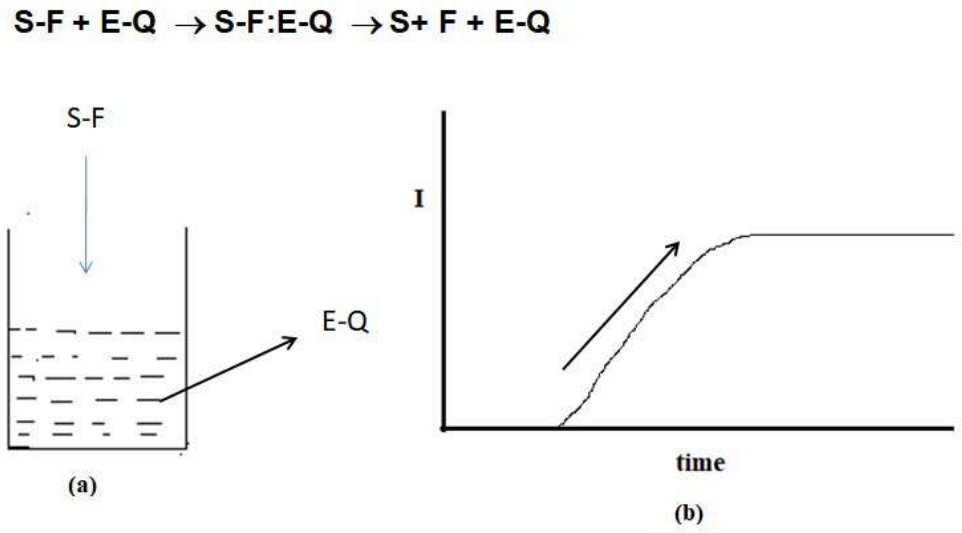

Fig. 1.6. showing enzymatic reaction, schematic of the reaction procedure and probable ensemble average fluorescence plot

Now if we perform single molecular fluorescence spectroscopy we can overcome these draw backs and go further insight into the reaction kinetics. Now imagine a situation where you are only monitoring the fluorescence of a single S-F molecule which is present in the observation

volume as described in the fig. 1.7a. One E-Q molecule will come to S-F, interact and finally go away. Now if we monitor the fluorescence of this S-F molecule with time, we will get a plot similar as described in fig. 1.7b. Initially, the fluorescence will be weak as F is attached with S. After complex formation, the intensity will further decrease as it is more close to quencher and remain constant throughout the life time of the complex. When the product will form, the fluorescence intensity will increase as F is free and finally become constant. After, single molecule fluorescence studies on many molecules; one can get an idea of the rate of the reaction. Here we also can avoid the dead time of the reaction as well as get idea of intermediate complex formation. For example, if one get a plot similar to fig. 1.7c for an enzymatic reaction that indicates two intermediate states in the enzymatic reaction.

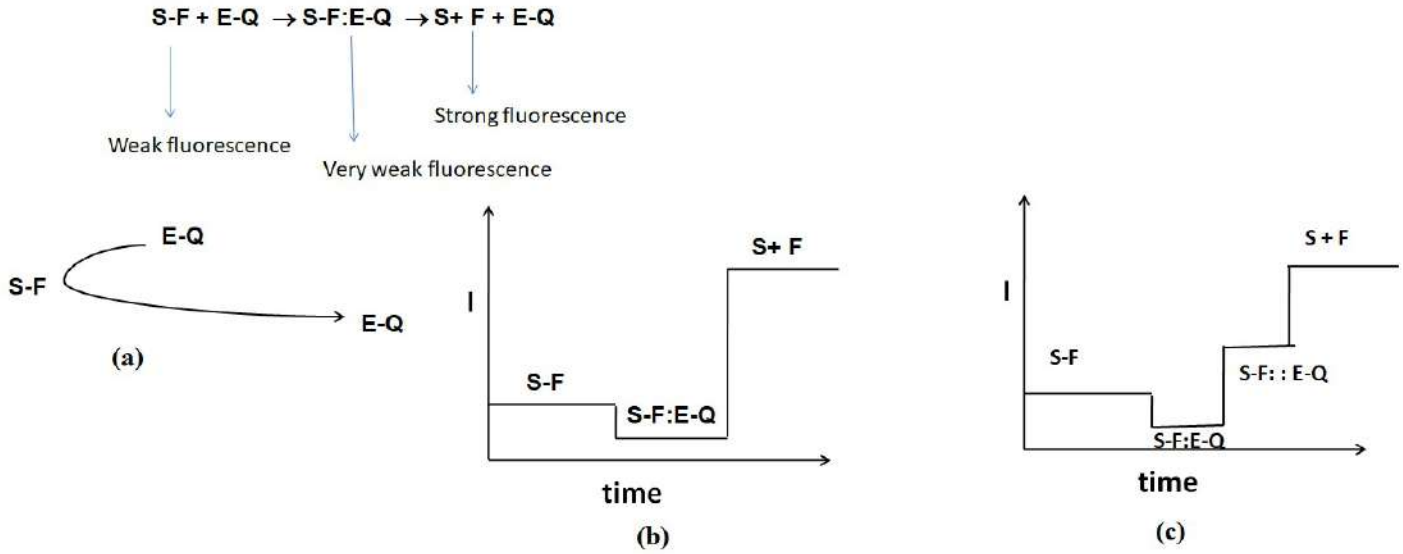

Fig. 1.7. (a) showing schematic diagram for single molecule fluorescence measurement involving S-F. (b) and (c) depicting fluorescence intensity (I) vs time plot for single molecule fluorescence measurement of enzymatic reactions involving one and two intermediate states respectively.

Instrumentation: In case of single molecular spectroscopy, we measure the fluorescence of a single molecule and it is a very difficult task. As the number of photon emitted for a single molecule is very low so we have to consider a number of parameters which need to be improved before measurement.

(i) The number of emitted photon coming out from the single molecule should be more than the back ground signal. Back ground signal should be extremely low. The sources of the back ground may be emission form impurities, several optical components (like coating), scattered light. Impurities should be very very low, virtually there will be no impurities in the solvent.
(ii) Dark count of the detector which is mainly coming from the thermal electrons present in the detector should be extremely low (near to zero) so that the detector can measure very small number of emitted photon coming out from a single molecule.
After taking care of all these above parameter, there is still one parameter which will make us unable to observe fluorescence from a single molecule is the Raman scattering. As the number of solvent molecule is very high compared to fluorophore molecule (which is single in number), the Raman scattering intensity due to solvent molecules in the entire wavelength range will be very high compared to fluorescence intensity coming from a single molecule (Fig. 1.8) and we

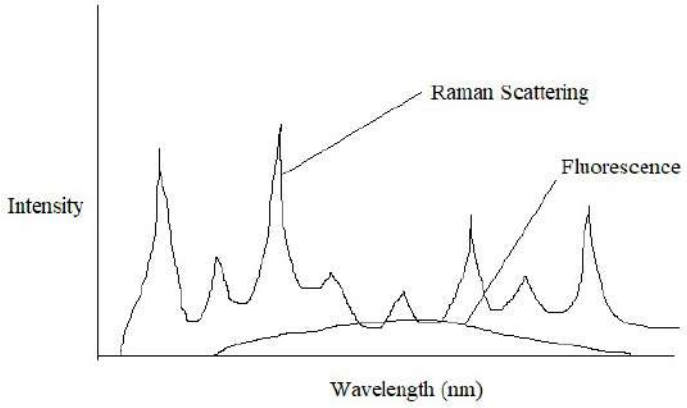

Fig. 1.8 schematic of the plot showing relative intensities of Raman scattering of solvent and fluorescence of the fluorophore.

cannot observe single molecule fluorescence. The only way to decrease the intensity of Raman scattering is to decrease the number of solvent molecules into the observation volume. From the idea of Raman cross section of the solvent molecule and absorption cross section of the fluorophore, it can be shown that if we can create a system **where one fluorophore molecule is present in ~ 1 femto liter (1fl) of solvent, then the Fluorescence intensity will be much higher than the Raman scattering intensity of the solvent ($I_F >>> I_{Raman}$). The solution should be diluted enough so that one molecule will be present in 1fl observation volume.**

One way to achieve such small volume is total internal reflection method.

Total internal reflection method: We know that evanescent waves are formed when sinusoidal waves are (internally) reflected off an interface at an angle greater than the critical angle so that total internal reflection occurs. If you have two medium with refractive index n_1 and n_2 ($n_1 > n_2$) and incident light beam falls through medium of refractive index n_1 towards the medium of refractive index n_2 with an incident angle θ_i which is greater than the critical angle (θ_c), there will be total internal reflection. The light will be reflected at the interface by satisfying the condition of total reflection as shown in the fig. 1.9. We know that an evanescent field will be generated at the interface region as shown in figure 1.9. The strength of the evanescent filed will exponentially decay in the z direction as we go away from the interface. The nature of decay can be expressed by the equation

$I_e(z) = I_e^0(z)\, e^{-z/d}$ where $d = (\lambda/4\pi)(n_1^2 \sin^2\theta_i - n_2^2)^{-1/2}$

For example, let value of $n_1 = 1.6$, and $n_2 = 1.3$, $\theta_c = 55^0$, let $\theta_i = 70^0$

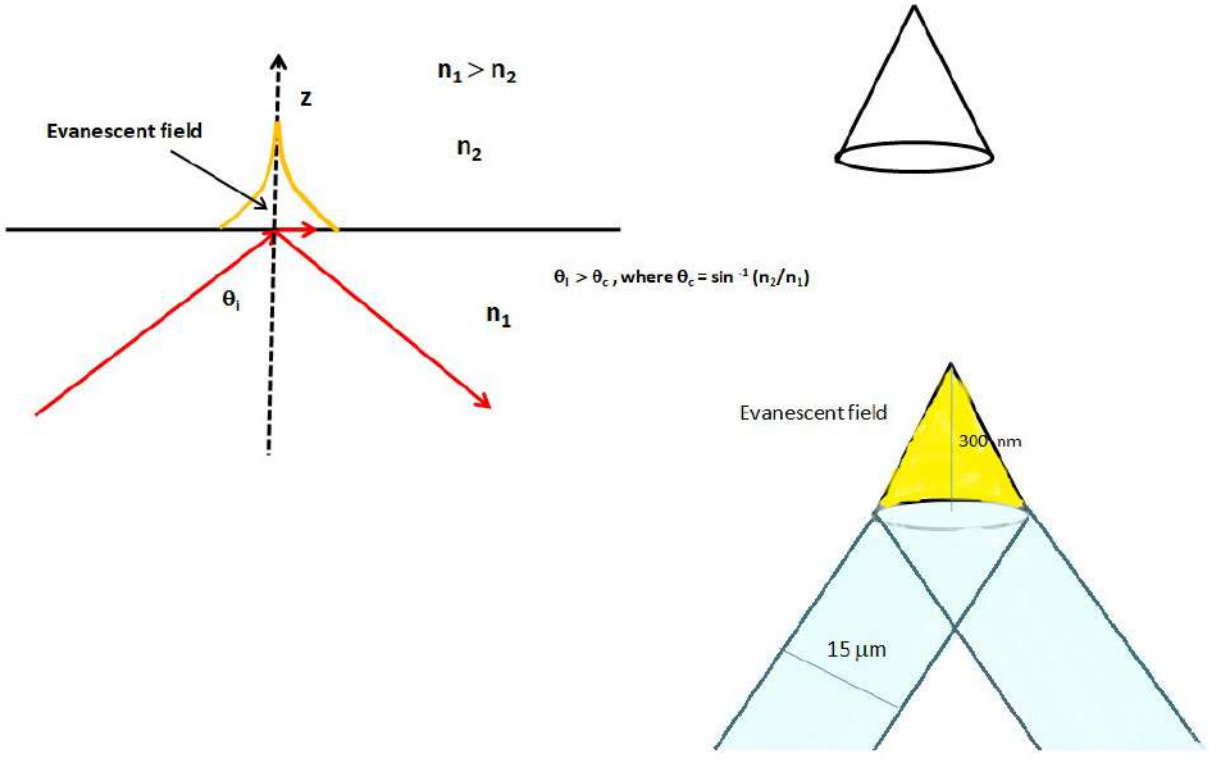

Fig. 1.9 Optical geometry for total internal reflection (TIR).

So for a light of λ = 500 nm, the d value will be ~ 65 nm. The intensity of the field will exponentially decrease depending the value of d (here d ~ 65 nm) and will almost vanish at 4.5 time of the d value, I_e (z) ~ 0, when z ~ 4.5 X 65 = 260 nm. So the volume where the evanescent field exists can be described as a cone (as described in fig. 1.9). if you know the dimeter of your excitation light you will know the diameter of the cone. The volume of the cone where evanescent field exists can be calculated using the equation $v = (1/3)\ \pi r^2 h$, where r is the dimeter and h is the height of the cone. Let consider the dimeter of the light is 15 μm, and height of the cone is 300 nm, the volume of the cone will be around 17.6 fl. So using the principle of total reflection method we can achieve observation volume of 17.6 fl.

Now to realize the device, a schematic of the device is shown in the figure.1.10. The device is made of glass with a refractive index let n_1 = 1.5, sample cell is placed on the glass and attached. The fluorophore solution is taken in the cell and the solution is sufficiently dilute but the fluorophores are distributed uniformly throughout the solution. The fluorophores undergo Brownian motion and changes their position throughout the solution.

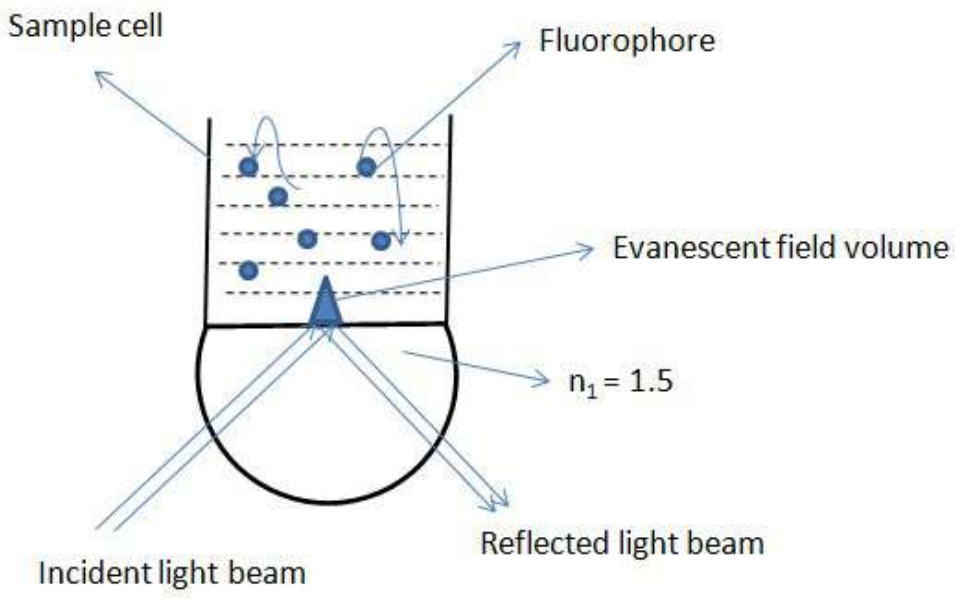

Fig. 1.10 Schematic of the device used for total internal reflection (TIR).

The light wave which is present in the evanescent field volume has the same excitation wavelength with the incident beam. So any molecule due to its Brownian motion when enters into the small observation volume, we can observe its fluorescence.

Confocal detection method: Another way to achieve such observation volume is the confocal detection method. A lens is used in this method to tightly focus the incoming excitation light into very tiny spot. The size of the spot can be controlled by diffraction limited focusing using a laser.

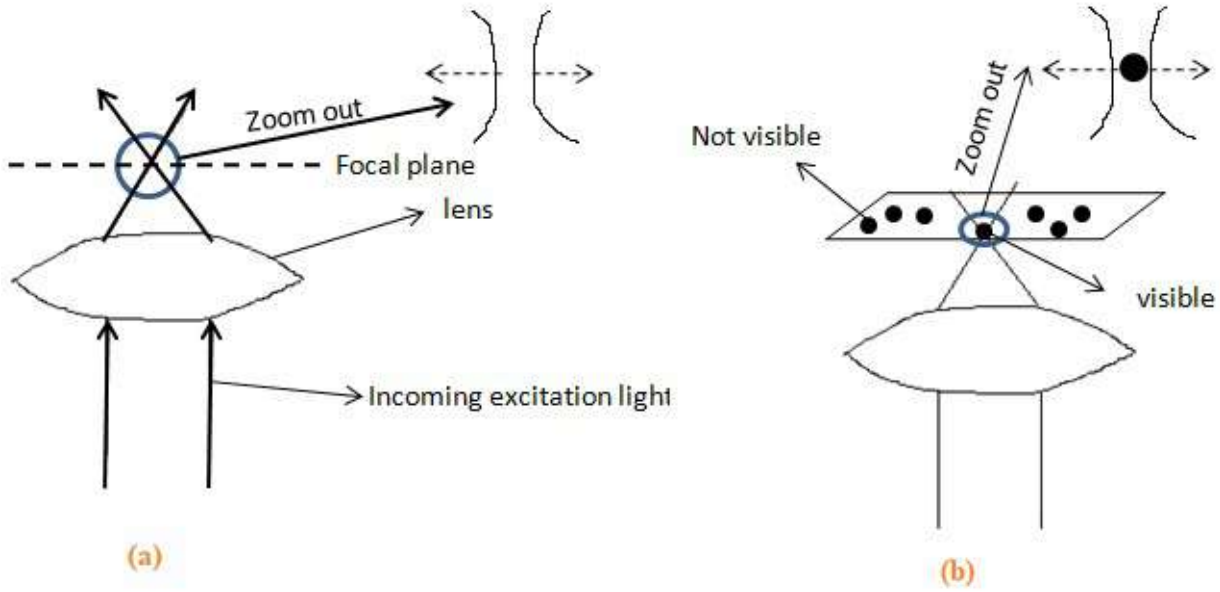

Fig. 1.11 simple schematic diagram of the optical detection setup. (b) shows that many molecules are present in the focal plane but the only molecule present inside the tiny spot which is focused will be visible or its fluorescence can be measured.

If the space nears the focal plane is zoomed out as shown in the figure 1.11, you will see that a small observation volume can be really achieved and the diameter of the volume in the focal plane is shown in the figure 1.11. Now spot size or diameter at the focal plane can be decreased by just increasing the diameter of the excitation beam as sown in figure 1.12. If we assume that the molecules which under investigation are only present in the focal plane (Figure 1.11b), then within these molecules which is only present inside the tiny spot in the focal plane will be visible. The volume of the tiny spot will depends on many parameters like numerical aperture of the focusing lens, dimeter of the exciting light beam. But this principle cannot be applied in case

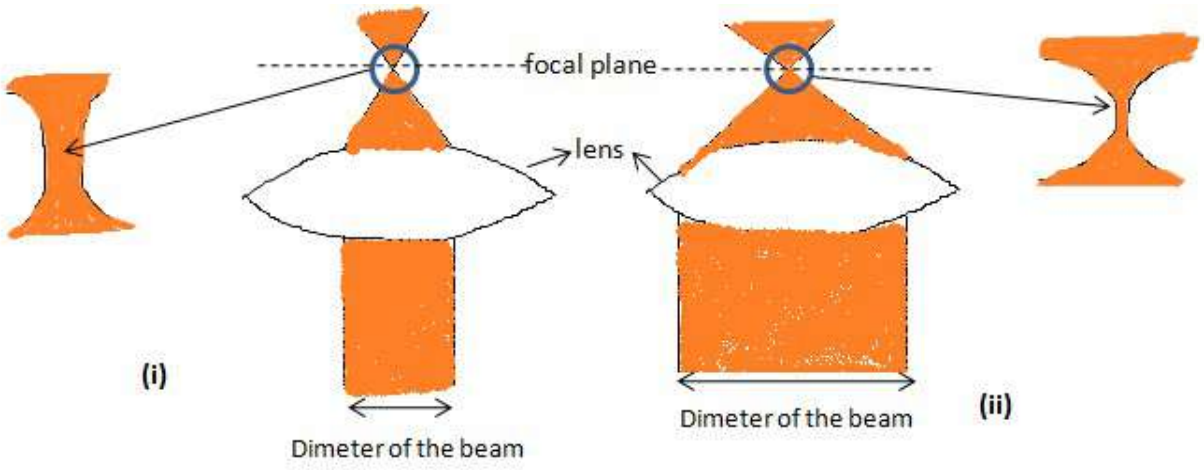

Fig. 1.12 showing schematic diagram of the optical detection setup with red laser. (i) smaller dimeter of the beam (ii) larger diameter of the beam. In case of larger diameter of the beam the diameter of the spot at the focus plane is very small

of bulk solution because all the fluorophores present in the illuminated cone as shown in the figure 1.13 will only be excited and give fluorescence. But other molecules which are present outside the illuminated cone will not give fluorescence (Fig. 1.13). Illumination through the objective is called epi-illumination.

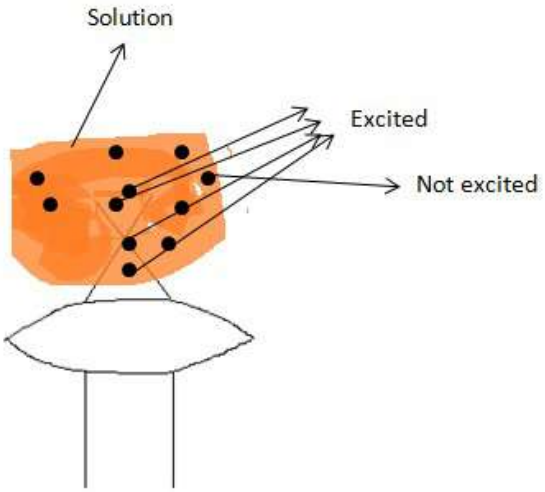

Fig. 1.13 *schematic diagram of the optical setup with red laser*

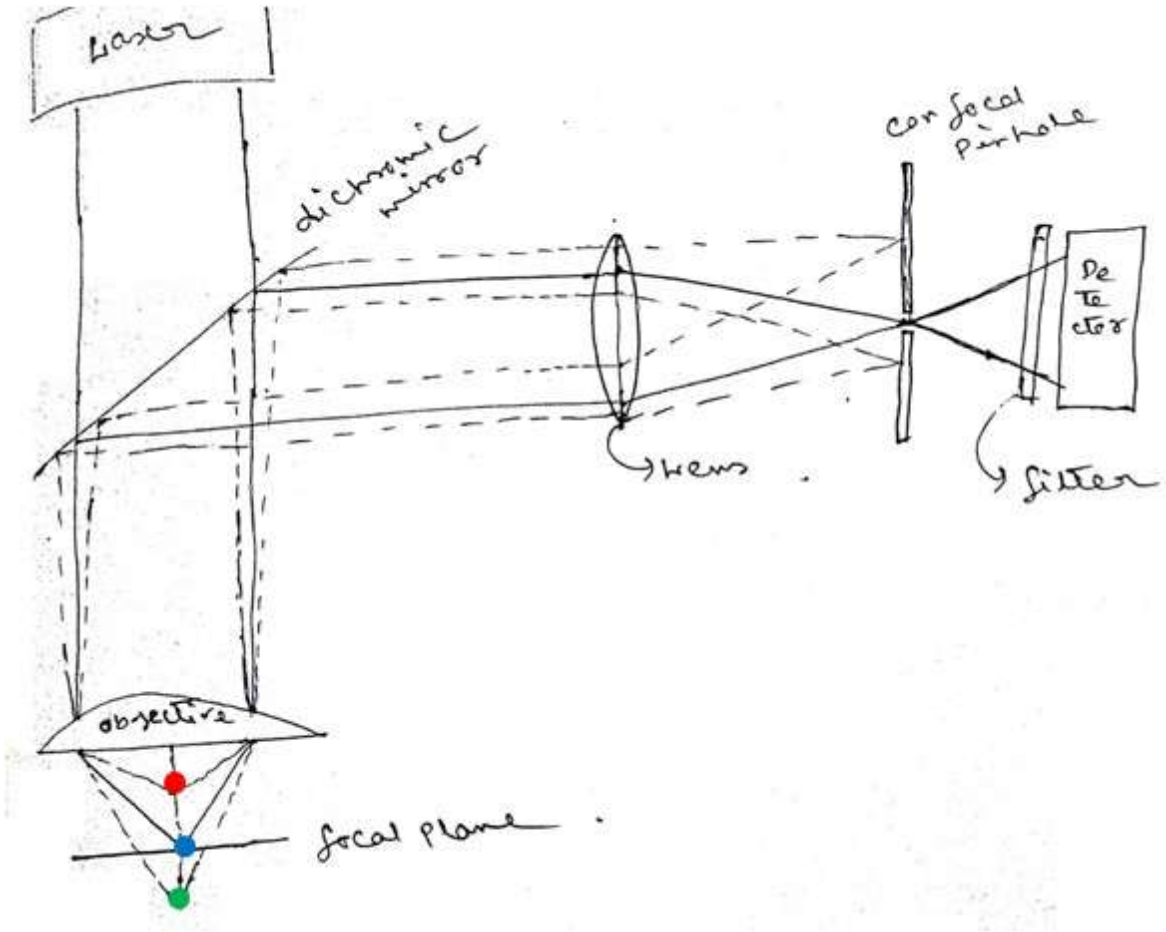

Fig. 1.14 schematic diagram describing the principle of epi-fluorescence with confocal setup.

Emission is collected back through the objective, which is called the epifluorescence configuration. Emission is separated from light at the incident wavelength by a dichroic filter that reflects wavelengths longer than the incident light to a detector. A dichroic filter is a device that transmits some wavelengths and reflects others. In this case the dichroic filter transmits the excitation and reflects the emission wavelengths. In case of solution as shown in the figure 1.13, the observation volume of the illuminated cone will be really large which results high Raman scattering from the large number of solvent molecules and single molecule fluorescence cannot be observed. Now, the problem of these unwanted signals can be solved by using confocal optics. This means that a small pinhole aperture is placed at a focal point in the light path. By ray tracing of the light path one can see that light from above or below the focal plane is not focused on the pinhole, and does not reach the detector. A simple schematic of the set up is shown in the figure 1.14. As shown in the figure, we need to measure the fluorescence of the molecule

(represented as blue dot) which is present in the tiny observation volume on the focal plane. For any molecule above or below the focal plane will result high observation volume and for that Raman scattering will be high and we will not be able to measure the fluorescence of that single molecule (red or green dot). When excitation laser beam will pass through the objective lens, all the molecules (red, blue and green) will be excited. But, as shown from the diagram, the emitted light originated from the molecules which are present below and above of the focal plane (green and red dot) will be blocked by the pin hole. Only the emitted light originated from the molecule which is present in the focal plane (blue dot) will be passed through the pin hole and will be detected by the detector. As the observation volume of that tiny space in the focal plane is around in the order of femto liter and its fluorescence can be easily detected.

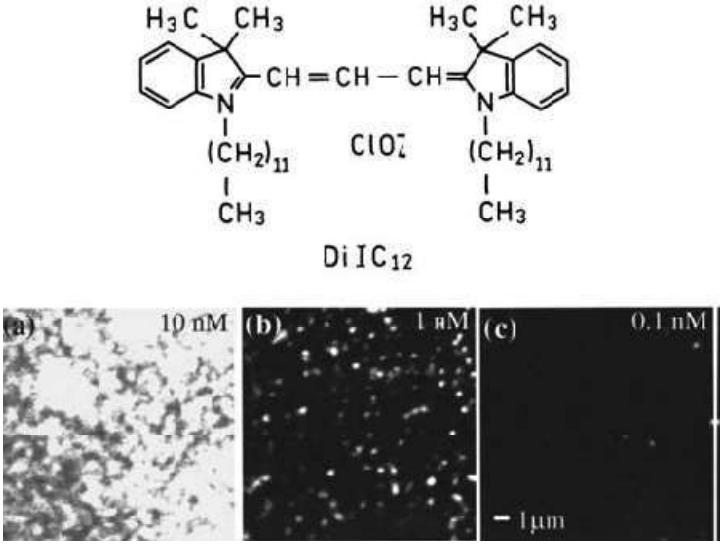

Fig. 1.15 Single-molecule images of DiIC12 on glass.

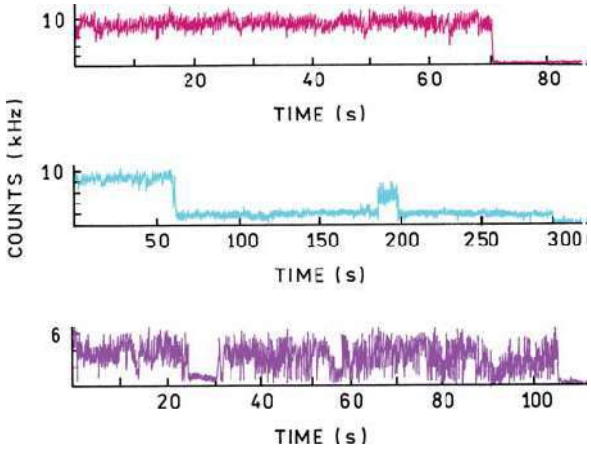

Fig. 1.16 Time-dependent fluorescence intensities of DiIC12 on glass.

Figure 1.15 shows images of the fluorophore DiIC12 that was spin coated on glass from a toluene solution. If the initial solution is too concentrated the signals from the molecules overlap, resulting in a spatially continuous intensity (left). At a lower initial concentration, individual DiIC12 molecules can be seen (middle), and at lower concentrations only few molecules are seen (right). The spots for a single molecule are about 300 nm across, reflecting the limited resolution available with light microscopy. The bright spots were assigned to single molecules based on the proportionality of number density to concentration and observation of single-step photobleaching. Figure 23.18 for three different DiIC12 molecules. In each case the intensity fluctuates dramatically, and eventually the emission stops when the molecule undergoes permanent photodestruction. The middle panel is the most typical blinking profile: a relatively constant intensity, followed by a rapid drop in intensity, followed by a return of the intensity.