Single Molecule Fluorescence Spectroscopy

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Single molecule spectroscopy can uncover fluctuation averaged out in ensemble measurements. In addition, single molecule spectroscopy naturally implies ultra-sensitive measurements, which should play important role in future proteomics and genomic research. Fluorescence method is the most commonly used single molecule detection. In this paper, we will use single conjugated polymer fluorescence spectroscopy to illustrate the power of this technique.

INTRODUCTION

Single molecule detection is an emerging field that is not only important from a purely scientific point of view but also in light of its significant technological impact.[1] The main advantage of single molecule experiments is the ability to observe phenomena otherwise obscured in ensemble measurements, such as the distribution of spectral positions and shapes, and discrete fluctuations in intensity. While most theoretical models are designed to describe the behavior of a single molecule, in most spectroscopic experiments, the average behavior of a huge number of molecules are observed. Comparison between experiment and theory is made by ensemble averaging of the theoretical predictions. With single molecule detection, it is possible not only to directly compare experiment with theory, but also to check the statistical assumptions used in the ensemble of molecules. In short, this technique provides a method to seek the relationship between the statistical ensemble and the individual identity. For example, how many molecules are needed to form an ensemble? Are the temporally averaged properties of a single molecule equivalent to the statistical average of an ensemble of molecules? (Egoric Principle) It is important to note that reducing time average is crucial for observing interesting single molecule fluctuation behavior. Long time averages of single molecule behavior are likely to obtain similar information as that obtained by studying ensemble of molecules.

To observe the spectroscopic behavior of a single molecule, one generally would like to have the molecule in question relatively immobilized – i.e. in a condensed rather than liquid or gaseous phase. This allows the same molecule to be studied as long as it remains active. Thus, various spectroscopic techniques can be applied to obtain far richer information than it is possible to obtain from molecules diffusing in solution. W. E. Mourner performed the first experiments in single molecules in the condensed phase at liquid Helium temperature.[2] The first room temperature work was performed in 1993 at AT & T Bell Laboratories by Eric Betzig and Robert Chichester.[3] They used the new technique of near-field (scanning) optical microscopy (NOM or NSOM) to observe the photoluminescence from a single dye molecule. Soon afterward it was shown that (far field) confocal microscopy is adequate – and far more convenient experimentally.[4] Since then single molecule microscopy and spectroscopy have attracted extensive attention. Novel phenomena have been observed in single molecule experiments[8,9,10] such as the fact that the photoluminescence intensity of a single dye molecule exhibits blinking behavior[11] at millisecond time scale. Although this has tentatively been assigned to intersystem crossing (ISC) between singlet and triple states, a complete understanding has not yet been provided. It should be noted, however, that most single molecule studies concentrate on one of two systems: dye molecules or dye molecule attached to large bio-molecules, such as proteins and DNA. In order to interpret correctly the information gained in the second class of experiments, considerable work is required to understand the behavior of the dye molecules themselves. Ensemble measurements remain essential for understanding and interpreting single molecule results.

SINGLE MOLECULE TECHNIQUES

In conducting single molecule spectroscopy and mi-

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croscopy, two main experimental challenges present themselves: sample preparation and fluorescence detection. In the following paragraphs, we will look at both these challenges, emphasizing the ways in which these challenges have been met in our lab.

Sample Preparation

Methods for preparing dye molecules on a substrate for single molecule experiments have been well documented[3,6] and basically involve sufficiently diluting the dye of interest (to ensure that only one molecule is in the excitation volume at one time) and spin-coating on a substrate that does not absorb light at the excitation wavelength (ie. fused silica). Much less work has been published, however, concerning the preparation of complex macromolecules such as polymers and proteins. In our lab the primary focus has been on understanding energy transfer in, and photo-physics of, luminescent conjugated polymers[13] – in particular PPV derivatives such as MEH-PPV and DOO-PPV. While on the one hand studying their properties as thin films,[14] considerable effort has been made to study single polymers of DOO-PPV[15,16,18] and MEH-PPV[19] in the condensed state. As such, while the following discussion of sample preparation has general application, we will concentrate specifically on the preparation of thin films in which polymers are embedded.

In our experiments, pristine DOO-PPV was first fractionalized by taking advantage of the limited solubility of its symmetrically substituted structure.[17] The powder dried to prevent breakage of the chain. In this form the polymer can be stored relatively indefinitely. Immediately prior to spin coating, the fraction to be examined was redissolved into a "good" solvent, chloroform, to a final concentration of $<10^{-4}$ M of monomer. In this case a "good" solvent has the meaning of a solvent in which the polymer readily dissolves. As the polymer is not very stable in chloroform, it is also important to prepare fresh solutions each time. Finally, it should be noted that even trace impurities in the solvent (especially chloroform) contribute to unwanted background photoluminescence. Thus only the highest purity solvents were purchased and then purified further in the lab. The sample was then further diluted (by factors of 10^3 to 10^6) in a polystyrene (PS) matrix (polystyrene:chloroform:toluene = 10 mg:18 mL:4 mL). The recipe for the PS is for two reasons. Firstly, toluene is included since chloroform is a solvent that is easily vaporized. Thus, if one uses only chloroform, during spin coating the solvent vaporization will lower the substrate temperature, resulting in the condensation of water on the substrate (and the polymer film) from the air. The result is a foggy film. Adding some toluene helps to prevent this. However, since toluene is not a "good" solvent for the DOO-PPV, one can only use a limited amount of this toluene. It should be noted that PMMA way also a good choice for the supporting matrix. As oxygen is extremely efficient quencher of fluorescence, dry-N_2 was bubbled through all solutions to purge dissolved oxygen from the solution. The solution was then spin-cast (15 s at 2000 rps followed by 10 s at 3000 rps) onto a fused silica cover slip substrate (Esco Products). The resulting film thickness is <100 nm containing ~20 of the molecules of interest in a 100 μm^2 region.[16] In single molecule work it is important to verify that one is looking at single molecules and not aggregates. One simple check, that we preformed is to prepare a number of thin films at the same time, varying the concentration of the polymer over a few orders of magnitude and ensuring that the density (of spots when observed with the confocal microscope) changes linearly with concentration. At this point further steps were taken to limit exposure to ambient conditions. Immediately following spin coating, some samples transferred to a nitrogen dry box and placed in a specially prepared N_2 cell before viewing.[15] Other samples were placed under vacuum (10^{-5} Torr) for a few hours to allow remaining oxygen to diffuse out of the film before a depositing layer of Aluminum to prevent its reentry.[18,19] Compared to unprotected films, both sealing methods allow for photoluminescence to be observed for considerably longer times (<1 sec without sealing, minutes in the case of sealing in N_2 cell, hours in the case of Al coating). Unfortunately, sealing with Al has the effect of reducing the overall photoluminescence (perhaps via an aluminum-quenching channel). Recently, we have had some success in overcoming this problem by incorporating a thin buffer layer between the film and the Al coating. The method of sample and substrate preparation is summarized in Fig. 1.

Photoluminescence Detection

Having discussed the problem of sample preparation, we now turn our focus onto the detection of fluorescence. The detection of a single molecule is difficult due to the extremely weak optical signal from one molecule compared to the huge background noise. Typically, a molecule will emit ~10000 photons/second with reasonable excitation power – to unambiguously identify and detect this small number of photons provides a major scientific challenge.[12] One is faced with the twin problems of maximizing the collection of the photons emitted by the molecule of interest and minimizing the collection of background light. (Electrical noise can be reduced by using detectors with low dark count rates.) Background light is generated from Raman scattering, auto-fluorescence of filters and the objective oil, scattered excitation light, as

well as photons emitted from neighboring molecules within the excitation volume. In order to minimize this noise component, one seeks to both reduce the generation of background light (If we want to see the stars, we go to Nantou not downtown Taipei!), and lower the collection efficiency for the background light. Of course, we need to do this while at the same time maximizing the collection efficiency for photons emitted by the molecule of interest!

Nearfield Optical Technique

One of the key methods of reducing the generation of background light is to seek to minimize the excitation volume. Initial experiments[3,7,21] in this field made use of the technique of near-field optical microscopy to do precisely this. An optical fiber with a sub-100 nm aperture was used to deliver excitation light to the molecule and the emitted light was collected by a high NA objective lens. After passing through appropriate filters to eliminate the excitation light, single molecule fluorescence was detected using an avalanche photo-diode operating in Single Photon Counting mode. Unfortunately, a key drawback with this method is the complex interaction between the molecule and the metal coated near field optical probe.[22] It has been observed (and verified theoretically) that the presence of the probe changes not only the angular emission properties of the molecule[20] but also its lifetime[5,12] by at least a factor of 3. As a result, the confocal microscope has become the workhorse in the area of single molecule spectroscopy with the application of NOM being limited to those areas in which its inherently higher resolution is absolutely required. For example, the recently developed near-field molecular scanner has potential applications in high resolution imaging of protein/DNA interactions in aqueous solution.[28]

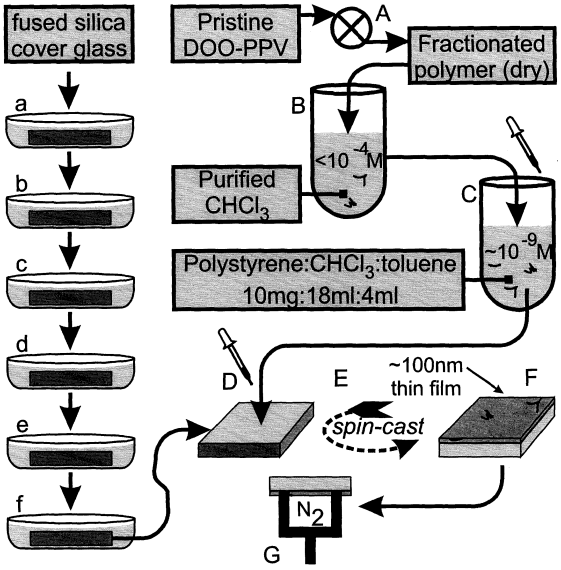

Fig. 1. Preparation of sample and substrate for single molecule spectroscopy. (A) Pristine DOO-PPV is first fractionalized[17] and dried. The fraction to be used is then (B) dissolved in purified chloroform and (C) diluted into the polystyrene matrix, before being (D) dropped onto a cleaned fused silica (0.5" square, 0.17 mm thick) substrate and (E) spin-coated to form a (F) film ~100 nm thick, with approximately 20 single polymers in a 100 micrometer square region. After a short time under low pressure, the sample is placed in a (G) nitrogen cell to protect the film from ambient conditions. The substrate is cleaned (a) first in chloroform (24 hours + ultrasound (US)), before being treated successively by (b) acetone (1 hour), (c) chromic acid (24 hours), and (d) KOH (2 hours with US) and finally stored in (e) nitric acid until ready to use. Finally, just before use, it is (f) immersed in ethanol for 2 hours with US before being first blow and then vacuum dried. DDW is used for rinsing between chemicals.

Confocal Technique

Fig. 2 presents a simplified schematic of a generalized confocal microscope in which the key components are labeled. In this technique a laser, after passing through a narrow band filter is reflected towards the sample by a dichroic mirror. A (objective) lens is used both to focus the excitation light on the sample and to collect the photoluminescence (PL) from the sample. The PL, after passing through the dichroic mirror, a notch filter and a bandpass filter is focused onto the confocal aperture. Light that passes through the aperture is detected. A complete image can be obtained by either raster scanning the sample or by raster scanning the laser beam and confocal aperture synchronously.

With that basic overview, lets consider the role of each element with respect to the goals of (1) maximizing the collection of PL signal and minimizing the (2) generation and (3) collection of other light. In terms of the first goal, three elements are crucial – the objective lens, the final focussing lens and the detector. The objective lens must efficiently collect and image light from the single molecule. Using a high numerical aperture (NA = 1.4), oil immersion lens, ensures high collection efficiency. Infinity focus type objectives allow for additional filters to be placed in the beam path without affecting the ability of the final lens to provide a tight focus. To accompany the infinity focus objective lens, a tube lens is generally used to image the PL at the confocal aperture

plane. Due to the low signal expected, it is generally necessary to have a low noise, high quantum efficiency detector operating in photon-counting mode (generally either a photo-multiplier tube or an Avalanche Photo-Diode).

In terms of the 2nd goal, minimizing the generation of background light, again three elements are crucial – the laser, the objective lens and the sample – to minimizing the excitation volume. In order for the objective lens to obtain a minimal spot size in the focus plane it is necessary to ensure that the incoming beam is TEM_{00}, not a combination of higher order modes. This can be done either by using a single lateral mode laser or by employing a spatial filter before a multimode source. A high-NA objective lens allows the spot size in the focal plane to be minimized. As a rule of thumb, the minimum spot size obtainable by an objective lens is defined by:

$$\omega_o = \frac{1.22\lambda}{2 \times NA} \quad (1)$$

where λ is the wavelength of the excitation light. Thus for 488nm excitation, on can limit the lateral spot size to ~220 nm by using a NA = 1.4 objective. Well, the spot size is minimized in the focal plane, it is rapidly diverging outside the focal plane. Thus to minimize the excitation volume it is necessary to have the molecule of interest embedded in a thin film. (An alternative technique to minimizing the generation of out-of-plane photons is to employ two-photon absorption making use of pico- or preferably femto-second lasers.[29]

Finally, one seeks to minimize the collection of unwanted background light. This is done both spatially and spectrally. The spatial reduction of unwanted background light is accomplished by an aperture placed in front of the detector – from which this technique derives its name. This aperture allows only light emitted from the excitation volume to pass into the detector (Scattered and out of focus light is thus rejected). The spectral reduction of background is accomplished in two ways. First the combination of the narrow band filter (in the excitation path) and the holographic notch filter following the dichroic mirror, enables scattered and reflected excitation light to be rejected before it reaches the detector. This is key as the excitation intensity is many orders of magnitude greater than the single molecule signal. Finally a bandpass filter designed to pass specifically the wavelength of interest allows the rejection of other light based on its spectral characteristics.

Using these design principles, we built a low-cost sample-scanning confocal microscope (Fig. 3) for the observation of single polymers of DOO-PPV, a polymer who absorbs in the green and whose PL is centered at ~550 nm. The vari-

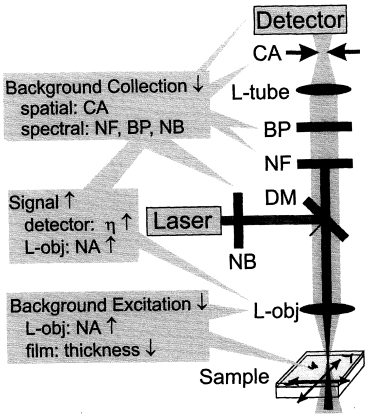

Fig. 2. Principles and schematic of a confocal microscope designed for single molecule detection. Excitation light from the laser first passes through a Narrow Band filter (NB) before being reflected towards the sample by a dichroic mirror (DM). An objective lens (L-obj) focuses the light onto the sample. Photoluminescence is collected by L-obj, and after passing through DM, a holographic notch filter (NF) and a bandpass filter (BP) are focused by another lens (L-tube) onto the detector. An aperture (CA) *confocal* to the focus on the sample aids to reject scattered background light. Molecules are found by raster scanning either by scanning the sample or by scanning the laser and confocal aperture synchronously.

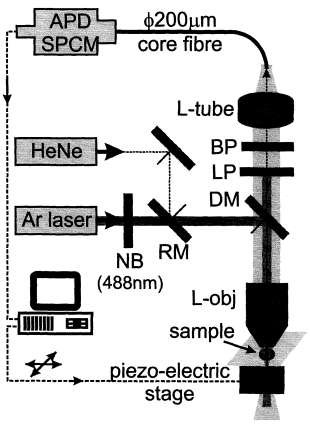

Fig. 3. Schematic diagram of the confocal laser setup used for imaging single polymers. Excitation is provided by a single mode Argon-ion laser. The confocal aperture is provided by the optical fiber. For alignment, a HeNe laser is directed to follow the Ar-ion laser beam path. After alignment, a single mirror (RM) is removed from the beam path. A computer controls data acquisition and scanning (see text for details).

ous optics were placed in/on a standard Nikon (Ellipse 660) optical microscope. The stage was controlled. Laser excitation from the TEM_{00} mode of a linearly polarized Ar-Ion laser (488 nm), after attenuation and passing through a narrow band filter (Omega Optical 488NB3) was reflected down to the sample by a dichroic beam splitter (Omega Optical 525DRLP). The size of the beam was chosen to slightly underfill the back of the objective (Nikon 100X oil immersion, N.A. = 1.3, infinity focus) to minimize self-fluorescence from the objective. The fluorescence signal was collected through the same objective. After passing through the dichroic beam splitter, a long-pass filter (Omega Optical 530EFLP) and a band-pass filter (CVI-F70-550-3), the fluorescence was focused onto a ϕ200 μm optical fiber that acted as the confocal aperture. Although a holographic notch filter is recommended to block reflected and scattered excitation radiation, we found that the combination of the dichroic mirror, long-pass and band-pass filters proved to be sufficient and represented a considerable cost savings (total cost ~US$450). Upon exiting the fiber, the light was refocused onto an APD (EG&G SPCM-AQ-131) operating in single photon counting mode (dark-count ~15cps, η_{QE}~65% in the region of interest). The TTL output of this detector was fed into a computer via a pulse counter card (National Instruments PCI-6602 in a CA1000 configurable connector accessory enclosure with CB-68 LPR I/O connect Type SH6868EP 68 Pin). The same computer was also used to control the motion of the piezoelectric stage (Physik Instrumente P-730) in the X-Y plane. Data acquisition and control software was written in LabView (National Instruments). A highly attenuated TEM_{00} HeNe laser (λ = 633 nm) was used to aid in focusing the excitation beam on the thin film and aligning the confocal aperture (optical fiber) as it emits in a region in which (1) the polymer in question is non-absorbing, and (2) the various filters are transparent. It is important to note that not only should the two laser beams be collinear, but their divergence and beam size also *must* be approximately equal in order for the alignment to be successful.

Once the sample is in place and the system is aligned, a fast low irradiance scan was made to determine not only the density but also the relative positions of the individual polymers. For those samples in which molecules are well separated, individual molecules were systematically positioned at the focal point of the objective lens and their fluorescent time decays recorded. Fig. 4 shows a typical photoluminescence image obtained by raster scanning the excitation laser beam. The individual bright spot corresponds to one single molecule. We control the density to around 1 molecule per 10 μm^2. Fig. 5 represents a typical fluorescent time decay for DOO-PPV in which we have extracted polymers of a specific molecular weight (average molecular weight of 8.2 K Daltons (M_n) and a polydispersity (δ), M_w/M_n of 2.23) using the technique of "sorting by solubility control".[15,17] This corresponds to persistence length polymer of ~24 monomers as determined from the "number average" molecular weight (M_n). (It should be noted that for single molecule experiments this is more appropriate than the standard "mean-average" molecular weight $(M_nM_w)^{1/2}$) As can be seen from the figure, emission occurs at a number of discrete intensities. Changes in intensity are always abrupt and not gradual. The histogram (Fig. 4 inset) illustrates that although emission was observed

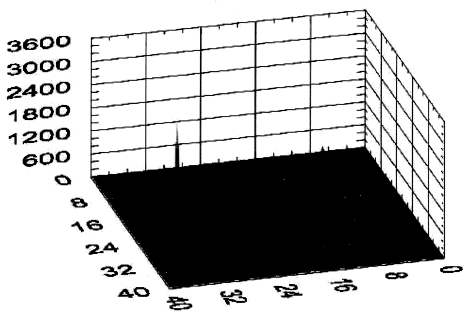

Fig. 4. A typical confocal fluorescent image of a well-dispersed single polymer samples. The image was obtained by raster scanning the sample stage. The x-y axis indicates the scan area, 40 μm by 40 μm. The vertical axis is the photon counts.

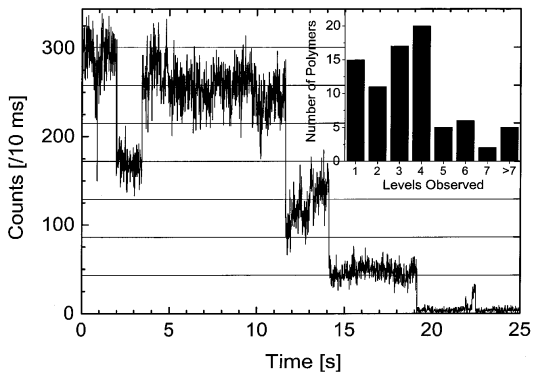

Fig. 5. Photoluminesence time trace of a single DOO-PPV polymer ~24 monomers long under irradiance excitation of 1600W/cm2 with linearly polarized light. Emission is observed at a number of discrete intensities. The inset summarizes the number of discrete fluorescence levels observed for some 91 molecules and shows that in general emission occurs at 3-4 discrete intensities.

at only one level for a few polymers (similar to the case for small dye molecules[31]), for the majority of polymers emission is observed at four discrete levels. This is consistent with a view of the polymer as being composed of a number of independent chromophores, each having a conjugation length of ~ 6 monomers.

Despite the low signal, spectroscopic information can also be obtained from single molecule experiments. In our lab, we have taken spectroscopic information both with long (seconds) and short integration times (10 ms). When we recorded data with long integration times, we simply, once a single molecule had been located (via the fast, low power scan), blocked the laser, redirected the fiber to a spectrograph and liquid N_2 cooled CCD system (Acton Research 0.25 M and Princeton Instruments) and then recorded the spectrum. For these experiments, the band-pass filter (BP) was removed. Fig. 6 shows the PL spectrum for two single DOO-PPV polymers superimposed on fluorescence spectrum in chloroform solution. While the ensemble average of the spectrum of individual polymers is equivalent to that in solution, the spectrum individual polymers vary greatly from one to another. It is also interesting to note that the spectrum for these persistent length polymers are clearly single peaked – in contrast to that the two peaks observed for long chain polymers,[33] again suggesting that the mechanics of energy migration are different in short and long-chain conjugated polymers of PPV.

On the other extreme is the desire to obtain spectral information on a short time scale. Here one is faced with the problem of low S/N ratios which makes it impossible to take a spectrum using a conventional spectrograph. However, one clearly desires to know whether or not the spectra is constant with constant intensity emission and whether changes in emission are accompanied by any spectral change. As illustrated in Fig. 7, one way of doing this is to use a beam splitter to split the PL spectrum into two approximately equal portions[32] for detection by two APDs (APD_{red} and APD_{blue}). By simultaneously recording the fluorescent transient and then calculating the normalized spectral shift coefficient, *S*, defined as:

$$S = \frac{APD_{red}\text{-}APD_{blue}}{APD_{red} + APD_{blue}} = \frac{R\text{-}1}{R + 1}; \quad where \ R = \frac{APD_{blue}}{APD_{red}} \qquad (2)$$

spectral changes can be observed with high S/N ratio. (If the photoluminescence (PL) spectrum does not change with time, *S* will be constant with time. A gradual change will appear as a slanted line and a sudden spectral jump by a discontinuity.) In our investigation of spectral changes during emission, a polarization insensitive beamsplitter with λ_{center} = 555 nm was used to split the PL into approximately two equal portions. The results of this work are published elsewhere.[30]

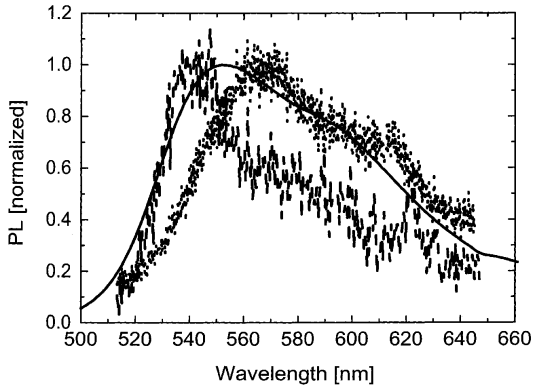

Fig. 6. PL emission spectra (in dot … and dash ---) from two single DOO-PPV polymers compared with that obtained for polymers in a dilute solution of chloroform (solid —). The spectral width for both molecules is narrower than that in solution with each molecule exhibits significantly different spectral behavior.[16]

Wide-field Technique

While confocal microscopy is the most common method of performing single molecule detection on immobi-

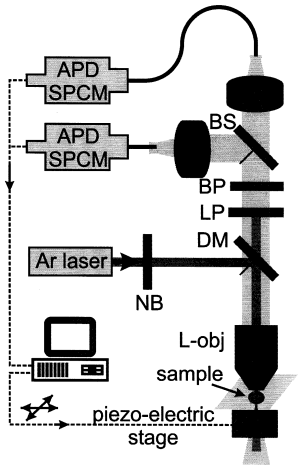

Fig. 7. Schematic diagram of the confocal laser setup used for obtaining spectral data with short integration time from a single polymer. As before a HeNe laser (not shown is used for alignment). In this case an additional beam splitter (BS) is used to split the PL into approximately two equal portions based on its spectrum.

lized molecules, wide-field single molecule detection has found application in areas in which one is interested in tracking *mobile* molecules due to its ~100 μm field of view. As such, it has found its primary application in biology having being used to observe a single kinesin sliding along a microtubule[23] and individual actin filaments sliding over heavy meromyosin.[24] While a full discussion of this technique is beyond the scope of this paper, we would just like to mention that these techniques require that a highly efficient fluorophore (i.e. Green Fluorescent Protein (GFP)) be attached to, or incorporated into, the molecule of interest.[25] Compared to the confocal technique, S/N is significantly lower as background light can no longer be spatially rejected, requiring the use of considerably more expensive optics. In addition, the APD must be replaced with a CCD (higher cost, lower speed). Integration times must be increased making it difficult to look at short time fluctuations. Applications of spectroscopic techniques also become less straightforward, though still possible. While background can be greatly reduced by coupling excitation beam in total internal reflection configuration,[23,25] the time resolution of wide-field imaging is generally restricted by the imaging CCD detector to be around 100 ms (10 frames/second). That said, in many ways, confocal and wide-field techniques are complimentary. The wide-field technique is useful when one is interested in following the *two*-dimensional trajectory of single molecules over distances of tens of micrometers (i.e. biology). The confocal technique, on the other hand, is more appropriate for studying the photo-physics and photochemistry at high time resolution of the light-emitting molecule itself.

CONCLUSIONS

Summarizing, in this paper we have introduced three optical methods for single molecule detection: (1) near field optical (NOM) microscopy, (2) confocal microscopy, and (3) wide-field microscopy. The three methods are complementary to each other. Confocal method stands out due to its relative low cost (a complete system including optical table, detectors and lasers can be assembled at a cost ~ US$60,000), relative ease of use, and high S/N ratio. The near field technique (capable of ~15 nm resolution compared to about ~300 nm for confocal method)[26,27] finds application where its relatively high resolution is required – for example to observe the relative position of two different photoluminescence sites on a single large molecule.[28] The 3rd technique, although possible to use with single molecules, is most appropriate for long-range *two*-dimensional diffusion studies where immobilizing the molecule is not an option.

We believe that in the future, besides providing fundamental insights into the physics and chemistry of single molecules, single molecule detection should also find increasing application in biology. A critical problem in molecular and cellular biology is obtaining the highest possible spatial resolution images of biological structures. The information obtained from such images ranges from mapping of the genome on chromosomal length DNA molecules to obtaining images of surface components on cells. Traditionally, X-ray diffraction and Nuclear Magnetic Resonance (NMR) have been used to determine protein structure. The main advantage of the X-ray diffraction method is high-resolution. However, it is difficult to grow protein crystals, and not all proteins can be prepared in such a crystallized form. In fact, since this technique was developed about 40 years ago, only a few hundred of protein structures have been determined by this method. While the structure obtained by NMR is not as accurate as that determined by x-ray diffraction, this technique allows for much simpler methods of sample preparation. Its key disadvantage is that it is limited in application to low molecular weight proteins. With large protein molecules, the complicated, overlapping resonant spectra makes positive identification of different peaks impossible. However, most important of all, these two techniques can only measure structure in an equilibrium state. If the protein is in non-equilibrium condition, such as an unfolded or partially folded state, neither method can obtain high quality images. A new method is needed to probe protein structures under non-equilibrium conditions. We suggest that single molecule spectroscopy will find widespread application in dynamical studies of DNA and protein under non-equilibrium conditions.[34,35]

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