Single Molecule Detection of Macromolecules

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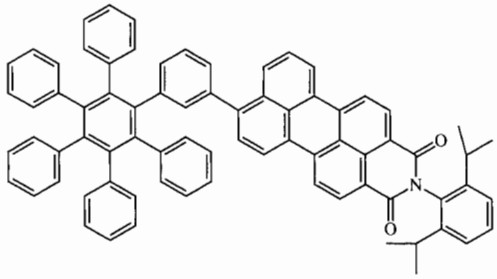
SUMMARY: The detection of single molecules is first exemplified for a model compound and then applied to a macromolecular system of a dendrimer with multiple chromophores at the rim.

Introduction

Single molecule detection [1] is the ultimate in chemical analysis and has applications in a broad range of scientific domains. The advantage is that one can obtain values for the properties of one molecule that are no longer affected by the statistical averaging inherent in ensemble measurements. In this contribution the methods used in single molecule spectroscopy will be discussed and illustrated with a model system of a peryleneimide derivative **1.**



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The use of this technique in the study of macromolecules, in particular dendritic structures decorated with such chromophores at the rim, will be discussed.

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Instrumentation

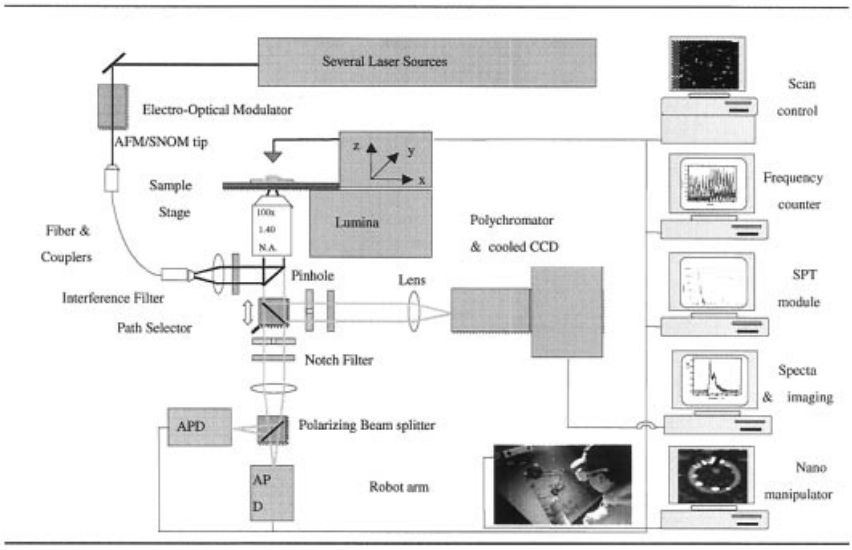

Fig. 1: Plate scanning confocal fluorescence microscope.

Single molecule spectroscopy at room temperature is performed in polymer matrices in which the compound under investigation is dissolved in very high dilution (10^{-9} molar) and a thin film is formed on a glass plate by spin coating the polymer solution. The plate is then placed in a confocal piezo driven plate scanning fluorescence microscope (Figure 1). Light is coupled in through an optical fiber in the microscope. The fluorescence of single molecules was detected using a confocal microscope (Diaphot 200, Nikon) with an oil immersion lens (NA 1.4) equipped with an avalanche photodiode (APD) in single photon counting mode (SPCM AQ15, EG&G) as the detector. Suitable filters were placed in the detection path to suppress remaining excitation light. The fluorescence intensity transients were measured with a dwell time of 5 or 10 ms. Excitation sources were an Argon ion laser for 488 nm (Spectra Physics Stabilite 2017) and a Helium Neon (HeNe) laser (Melles Griot 05-LGR-193) for 543 nm. The fluorescence spectra were measured with a liquid nitrogen-cooled, back-illuminated CCD camera (LN/CCD-512SB, Princeton Instruments) coupled to a 150 mm polychromator (SpectraPro 150, Acton Research Cooperation), using 5, 8 or 10 seconds integration time. The recorded spectra were corrected for the background, the response of the CCD-camera and the

optics used. Determination of the peak position of each spectrum was done by calculating the first and second derivative. The resulting accuracy was about ± 1 nm. For the time resolved measurements, the signals from the APD were collected in a Time-Correlated Single Photon Counting (TCSPC) PC Card (SPC 430, Picoquant GmbH) together with the trigger signal to record the fluorescence decays of the single molecule in steps of 1 to 10 seconds. The decays were fitted with the least square (LS) method. The quality of the fits was judged by the values of the reduced χ^2 (<1.2) and Z_χ^2 as well as by the residuals and the autocorrelation function. For the combined measurements (transients, spectra and decays) the fluorescence signal of the single molecule under investigation was split with a hybrid beam splitter cube (Newport 05BC17MB.1), guiding 50 % of the light to the CCD camera and 50% to the APD. Polarization measurements were performed by splitting the signal with a polarizing beam splitter cube (Newport 05FC16PB.3) and detecting s and p polarized components of the fluorescence light with two independent detectors. Modulation of the excitation was obtained by passing linear polarized laser light through a λ/2 plate rotating with a stable frequency.

Imaging of a Single Molecule

Raster scanning of the sample leads, upon detection by one APD computer-linked to the co-ordinates of the scanner, to a fluorescence image as presented in figure 2.

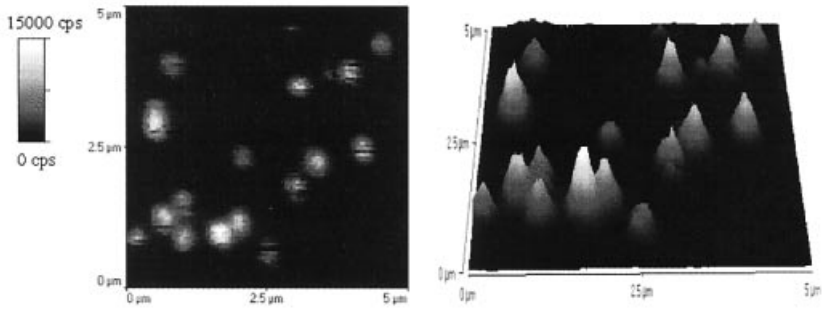

Fig. 2: 2D and 3D fluorescence images of **1** in a polymer film obtained by raster scanning.

The spot size of one molecule exceeds its dimensions but is determined by the diffraction limit in this measurement and equals 300 nm. The dark stripes seen in the

images are due to the fact that the molecule visualised in the given spot while it is being scanned goes into an "off state", that is a state in which it does not absorb light anymore and hence does not emit. For a system containing one chromophore this could be the triplet state. This can be seen in the transient trace of a given molecule obtained by focussing on one molecule and measuring the intensity of fluorescence as a function of time binned over a chosen time interval (Figure 3). From the second trace in Figure 3 an off time of several milliseconds is visible. If a large number of individual molecules of **1** are analysed it is found that for excitation at 488 nm with 350W/cm^2 one or more off times are observed for **1** in about 35% of the molecules. Taking into account the efficiency of the detection system and the excitation power, an intersystem crossing efficiency of about 10^{-7} can be calculated. Furthermore, the average survival time for a molecule before irreversible bleaching does occur is about 70 seconds. In this time about 10^5 photons are detected from one molecule.

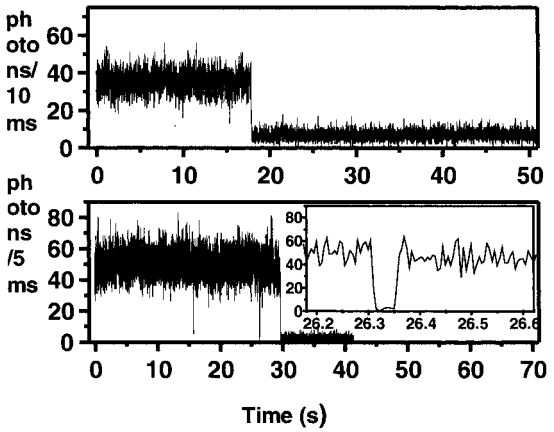

Fig. 3: Transient traces of **1**.Top trace : no off time is observed. Bottom trace : a few off times are observed and in the insert a small time domain is blown up to show the off time.

Using polarised excitation, two detectors can be used to detect both polarised emissions simultaneously. Such traces, presented in Figure 4, show that no change of polarisation occurs, as can be seen by the insert P, the polarisation in the top trace. In the bottom trace the polarisation does vary during detection, indicating that the transition dipole of the molecule and hence the molecule itself has undergone some reorientation in the

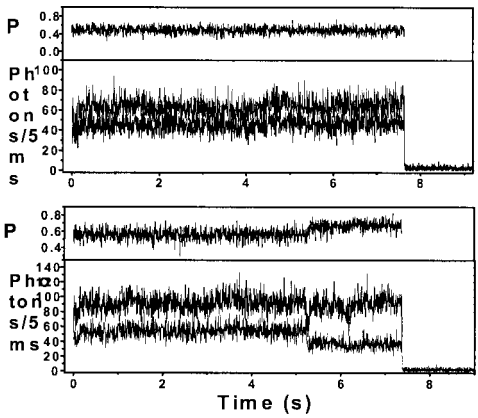

Fig. 4: Two polarised transients of **1**

polymer matrix. This can be used to study mobility at the molecular level or to investigate blending at the molecular level. It is also possible to modulate the linearly polarised excitation light and again detect both polarised emissions. If the system really contains only one chromophore and hence one molecule the modulation of the emission should be in phase with the excitation and the trace should become zero when the transition dipole is perpendicular to the excitation (Figure 5). This allows us to state unequivocally that a single chromophore is being investigated.

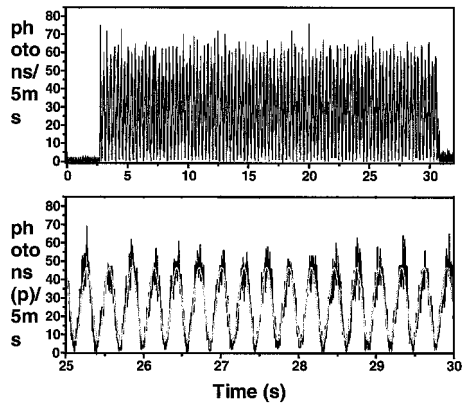

Fig. 5: Transient of **1** obtained with modulated linearly polarised light. Bottom part is a blow up of a shorter time region.

Spectral Properties

Focussing on one spot, the detection can be done with a CCD camera and the wavelengths can be spread by a polychromator. This allows determination of the emission spectrum of the emitting entity and is, in view of the high dilution of molecules in a polymer film, a way to test if the emission is due to the molecule or an impurity in the polymer matrix.

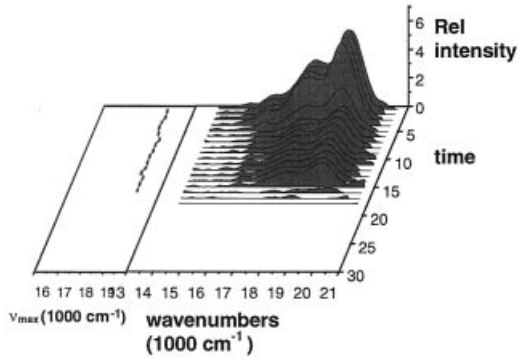

Fig. 6: A spectral run of **1**.

In Figure 6 a spectral run of 1, i.e. a series of spectra as a function of time of one given molecule, is presented. The obtained emission spectrum is very similar to the one obtained for an ensemble of **1** in solution.

Time Resolved Fluorescence

Not only the spectral characteristics of an excited single molecule can be measured but also its fluorescence decay. Since only a limited number of photons can be detected, exact and dependable methodologies have to be developed and evaluated. A comparison between the in single photon timing analysis standard used "Least square" method and the " Maximum Likelihood Estimation" [2] shows that the former is not for decays that contain less than 600 photons. The latter method can be applied at any time. Figure 7 shows simultaneously detected transients and decays of **1.** As can be seen from the figure the decay of **1** over the time euqals the transient was recorded 4.2 nanoseconds and varies only slightly.

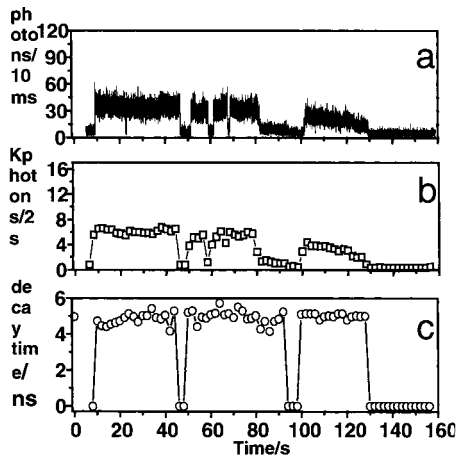

Fig. 7: a) Transient of **1** b) Reconstruction of the transient from simultaneously recorded decay traces c) Decay times as a function of time detected with a bin time of 2 s.

Detection of Single Macromolecules

Within the research group a special class of macromolecules, namely dendritic structures, are being investigated. Dendrimers have been shown to possess unusual physical and chemical properties, which differ significantly from that of linear polymers. New applications, based on these structure-controlled architectures, have made dendrimers the object of wide interdisciplinary interest. Fluorescent chromophores can also be attached to the external surface of the dendrimer. Thus, dendrimer synthesis serves as a way to obtain a well defined number of chromophores in a confined volume element. Not only the number of chromophores can be easily controlled, but also the interactions among the chromophores can be governed by changing the structure of the branches to which the chromophores are attached or by attaching the branches to different cores. The chromophores in each branch of the dendrimer readily allow us to probe interactions of the branches, conformational distortions as well as excitation energy transfer or electron transfer among the chromophores. One of the interesting questions that might be addressed with SMS of multichromophoric dendritic systems is whether there is a transition from single molecule behavior (discrete on/off jumps in fluorescence intensity of one chromophore) to ensemble behavior (exponential bleaching of the fluorescence intensity). The system discussed in this contribution,

although containing eight chromophores, still behaves as a single emitting quantum system, showing collective on/off behavior similar to the on/off behavior reported for single molecules containing one chromophore [3].

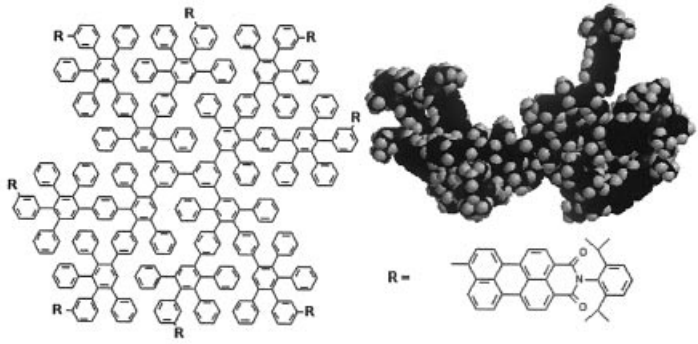

Fig. 8: Representation of the dendritic macromolecule **2** containing 8 chromophores.

Fluorescence intensity traces, polarized fluorescence intensity traces, spectral runs, decay runs and combinations thereof were recorded for a large number of individual molecules. These data were compared with similar sets of data obtained for the model compound **1**. Excitation delocalisation as well as emission from a dimer-like entity, that might be already present in the ground state, could be observed at the single molecule level. Combination of decay runs and spectral runs could clearly link the long decay time of 9 ns with this dimer-like entity. This clearly establishes single molecule spectroscopy, besides being an interesting and emerging field of spectroscopic investigations, as an important investigative tool in the whole of spectroscopic techniques.

One of the most interesting findings of this study is the presence of collective or cooperative effects in the dendritic system.

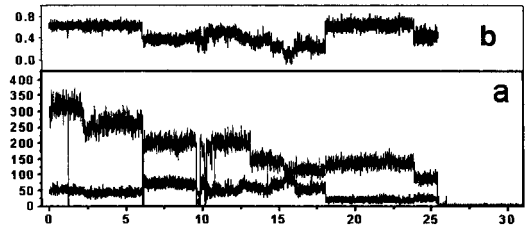

Fig. 9: a) P (top) and s (bottom) polarized transients of 2 b) polarization as a function of time.

Figure 9 clearly shows collective on/off behavior and changes in the polarization of the emitting chromophore as a function of collection time.

Collective or cooperative effects are known to occur in strongly coupled systems such as J-aggregates, antenna systems of bacteria and conjugated polymers. These effects are related to the processes of energy transfer and exciton coupling. The observation of collective on/off jumps of all eight non-conjugated only weakly interacting chromophores is rather unexpected. One possible mechanism leading to this behavior is singlet triplet energy transfer in the multichromophoric system. All the previously presented data suggest structural nonhomogeneity at the single molecule level leading to complex temporal behavior, in part accentuated by the stepwise photobleaching of the multichromophoric system. A schematic presentation of this mechanism is given in Figure10.

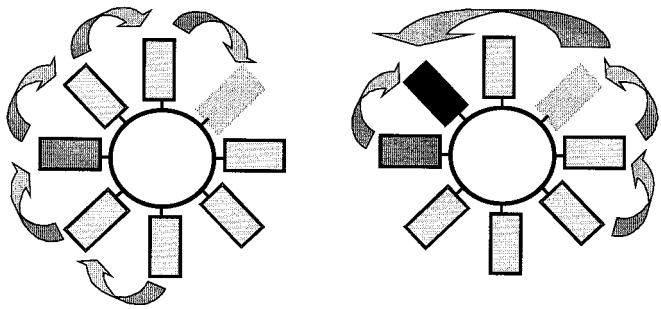

Fig. 10: Left : a scheme representing Forster excitation transfer to the energetically lowest lying chromophore (light box) and right : trapping of the excited singlet by a triplet excited state (dark box).

It can be envisioned that this and similar studies will deepen the understanding of fundamental photophysical processes occurring in all kinds of multichromophoric systems including biosystems.

Acknowledgments

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