Optical Detection and Spectroscopy of Single Molecules

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The optical study of single molecules opens new paths in molecular spectroscopy, solid state physics and quantum optics. And recent experiments at room temperature hold great promise for chemistry and biology as well

"Through these phenomena an insight is afforded into the very laboratory of nature itself, and we are thus enabled to view the interior structure and molecular arrangement of bodies."
Humphrey Lloyd, in his lectures on light, 1846

What a dream for a physicist, a chemist or a molecular biologist to be able to observe and even to handle matter atom by atom or molecule by molecule. Thanks to near-field techniques such as the scanning tunnelling microscope (STM) or the atomic force microscope (AFM), science is on its way to make this dream come true. These 'contact' microscopy techniques were developed approximately fifteen years ago and they gave rise to a real revolution in our approach to nanoscale physics. Their basic principle is the exploration of the surface of an object with a sensor, usually a very small tip, which interacts with the surface through short range interactions. However, as we shall see hereafter, individual molecules can also be studied by optical methods, which have much longer characteristic interaction distances.

Optical methods have specific advantages over close-range (contact) methods. One is the possibility to probe matter in a very sensitive, and usually non-invasive, way; another is the possibility to probe molecules located inside the studied object. However, what makes these optical techniques especially attractive is the existence of a large variety of spectroscopic methods which have been developed since the invention of the laser. A few examples of these are the scattering of light, high resolution frequency-resolved spectroscopy, and ultra-short pulse spectroscopy. Hitherto, all these techniques were applied to bulk objects.

Now we may ask, what more is there to learn from spectroscopy done on individual molecules or on nano-volumes? In fundamental physics, restricting the study to the observation of a single quantum system will make it possible to observe specific quantum effects of the interaction between light and matter, collectively called quantum optics. These could be monitored in new ways. In solid-state physics, the study of the electronic transition of a single molecule or of a single quantum dot will give information about the dynamics, and indirectly also about the structure, of the local micro-environment, a few tens of nanometers deep – the typical distance of the interactions influencing these electronic states. These local probes will also be useful to the engineer wishing to assemble and study nanometric devices, or else to the physical chemist looking at interfaces and at chemical reactions in heterogeneous environments, where up to now he had access only to average responses.

However, the most far-reaching effects of this possibility to study single systems in heterogeneous ensembles at the nanometer scale will be found in biology. A few of the fascinating possibilities opened up by single-molecule detection techniques are the tagging of a molecular probe inside a single protein – for instance a pore in a membrane, so as to observe its behaviour in real time, or the identification of one single photosynthesis reaction centre or of a single photoreceptor. The ultimate detection sensitivity of the single molecule will greatly reduce detection time (from a day to a minute) of rare compounds for DNA sequencing or for immunological tests since it will suppress the long and tedious intermediary step of molecular multiplication.

Optical detection of individual molecules in dense samples is very recent. At the end of the 80s, a few groups, stimulated by the success of the scanning tunnelling microscope, attempted the optical detection of single molecules. In 1989, W.E. Moerner, then at IBM, after having observed statistical absorption fluctuations in small samples, published the first detection of single molecules by a technique of modulated absorption[1]. Unfortunately, the signal-to-noise ratio in this experiment was rather poor. In 1990[2], our group showed the great superiority of the fluorescence excitation technique for detecting single molecules. Within just a few years, this technique brought forth a whole array of results, some of which will be summarised in this article. Independently, single molecules were observed as early as 1990 in liquid solutions, also by detecting their fluorescence. In 1993, E. Betzig, then at Bell Labs, published the first room temperature detection of single molecules in a polymer, using near-field excitation[3]. In 1994, a similar result was obtained using an optical confocal microscope[4]. This last method, which is very easy to use but whose spatial resolution is limited by diffraction, is now developing rapidly.

The optical detection of individual molecules, which just ten years ago seemed inconceivable, has now become a routine technique. New results and new tech-

Further reading
The essence of the work in this paper is the detection of single atoms and molecules in dense samples. For most quantum opticians, *the* book to read that describes detection in *dilute* samples is Laser Photoionisation Spectroscopy (published by Academic Press in 1987) by **Vladilen Letokhov** – as the problem of solitary atom and molecule detection by laser-induced fluorescence in dilute samples was solved in the mid 70s by scientists of the former USSR.

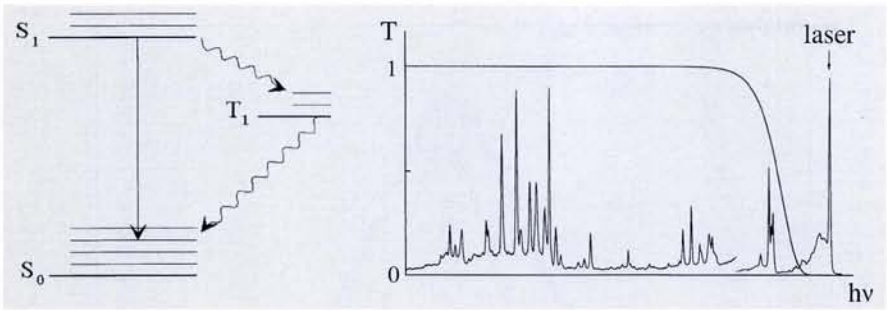

Fig 1 Energy level diagram of an organic molecule showing the three levels mentioned in the text: two singlet levels (S_0 and S_1) and one triplet level (T_1). Each of these levels carries a progression of vibrational levels. The diagram shows how a filter eliminates the laser photons while transmitting most of the molecular fluorescence

niques keep cropping up in this rapidly expanding field. The present article will mention a few recent results, but mostly it will describe the state of our own field: low temperature studies.

Experimental methods

Individual absorbing molecules dispersed within a transparent matrix are detected by using a laser to excite their fluorescence. This method is extremely sensitive since optical photons can be counted individually. It also results in a drastic reduction of the continuous background on which the molecular signals appear because the fluorescence photons are easy to separate efficiently from the exciting photons (see *fig* 1). If a molecule can emit a large number of photons without disappearing ('burning out') it will be easy to observe with a good signal-to-noise ratio even with the overall detection efficiencies of usual setups, *ie* varying between 10^{-2} and 10^{-4}.

In order to isolate the response of a single molecule, the first thing to do is to operate a spatial selection (one could also use microscopic samples, but since these are difficult to make and difficult to handle this method has not, to our knowledge, been explored thus far). This is mostly brought about by the exciting laser itself. The laser beam is focused onto a thin sample. Whatever the setup, the fluorescence photons, which are emitted in all directions, must be collected as efficiently as possible[2]. In the confocal setup, the same optical part leads the exciting beam onto the sample and collects the emitted fluorescence[4] (see *fig* 2). In the case of near-field excitation, the laser beam can be sent onto the sample by way of a pulled optical fibre while the fluorescence is collected by an ordinary microscope objective[3] (see *fig* 2).

For low-temperature experiments, a fourth dimension, the frequency dimension, can be used to separate molecules from one another. Indeed, in some specific solute-matrix systems, each electronic transition is associated with a single, very narrow line, called the zero-phonon line (that is, the line without any phonon creation), which is analogous to the Mössbauer line in gamma spectrometry. This narrow line is usually not observed as such because of the inhomogeneous broadening due to the disorder of real macroscopic samples, whether they be glass or even crystals, but it will emerge when the number of molecules is small enough (see *fig* 3). The individual frequencies of the molecules are distributed along the frequency axis. Each single molecule can therefore be selected by tuning a monochromatic laser onto its own frequency. In this frequency dimension, the 'width' of the laser is much 'narrower' than the molecular 'width', while of course in the spatial dimensions, the contrary is true. Since the ratio of inhomogeneous width versus homogeneous width can reach values up to 10^5 to 10^6, spectral selection is very efficient. Consequently, at cryogenic temperatures the excited volume can be much larger than at room temperature where spectral selection does not work. For instance, the exciting beam can be sent onto the sample by way of a simple non-pulled monomode optical fibre, such as was the case in our initial experimental setup[2] (see *fig* 2).

At low temperatures

Experiments at high spectral resolution in condensed matter require cryogenic cooling. Of course, low-resolution experiments could also be carried out at such low temperatures, but they are much easier at room temperature. The high-resolution experiments we are about to describe have been done on a limited number of systems because of the restrictive requirements demanded by this kind of spectroscopy.

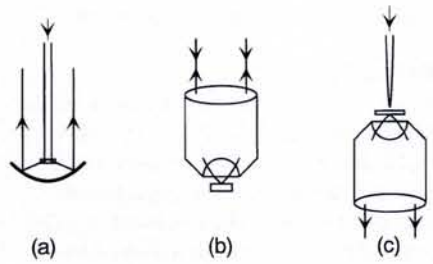

Fig 2 These diagrams show a few of the optical setups used up to now to operate spatial selection and to collect the fluorescence. a) spatial selection of a few microns by a monomode non-pulled optical fibre and collection by a parabolic mirror. b) Confocal setup with a microscope objective. c) Near field excitation with a pulled optical fibre and collection by a microscope objective

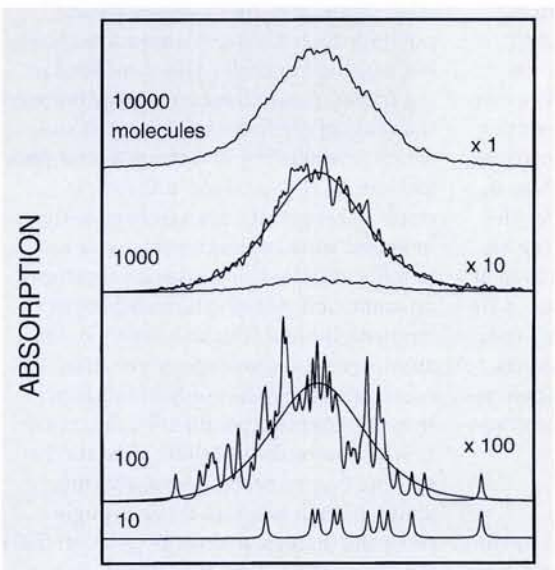

Fig 3 Illustration of the principle of spectral selection at low temperatures: a bulk sample contains a large number of molecules and has a broad spectrum (top). When the number of molecules is drastically decreased, the homogeneous lines no longer overlap and are clearly resolved (bottom)

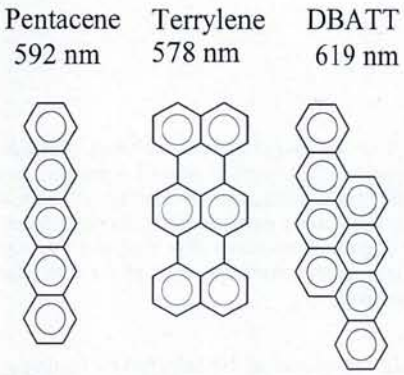

Fig 4 Above Schematic chemical structures (with only the carbon skeleton shown) and main absorption wavelengths of a few of the molecules used in our experiments. DBATT stands for dibenzanthanthrene

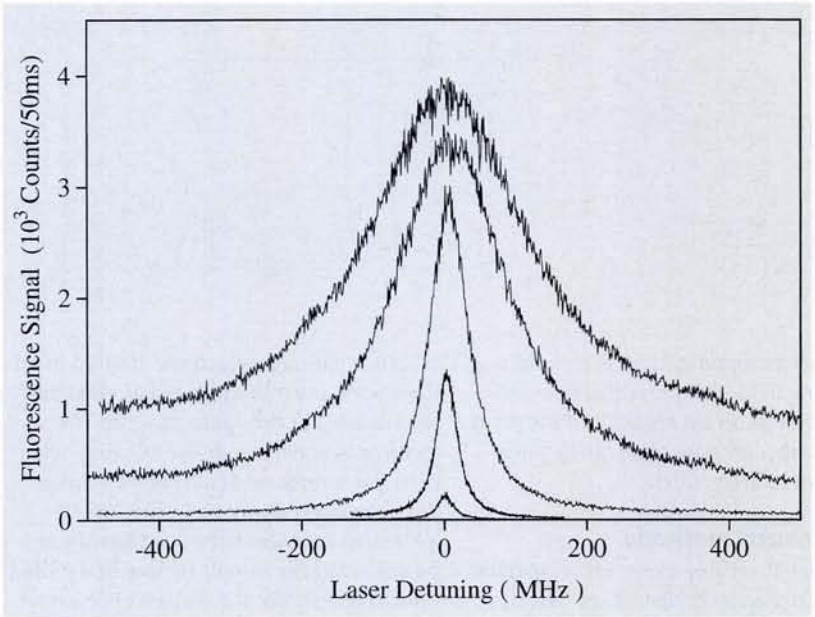

Fig 5 Right An example of a single molecule line as a function of the excitation intensity (shown here is dibenzanthanthrene in a naphthalene crystal). The optical saturation of the response at strong intensity is clearly seen

For a solute-matrix system to be well adapted to frequency-resolved single molecule spectroscopy, it must have the following characteristics: the optical transition must be intense, with a strong zero-phonon line, which implies that the excitation does not modify the shape of the molecule too much. The matrix itself must be stable in order to make long acquisition times possible. And, last, the photophysical properties of the molecule involving the triplet state must be favourable so as to have sufficiently high saturation intensities. The molecules which up to now have given the most satisfying results are polycyclic aromatic hydrocarbons (see *fig* 4). These molecules are known to have narrow zero-phonon lines and an intense fluorescence. Several types of matrices have been used: high quality molecular crystals (such as para-terphenyl or naphthalene), or more disordered matrices (such as Shpol'skii matrices, *ie* rapidly cooled linear alkane crystals), or even polymers (semi-crystalline as well as completely amorphous polymers). When the molecules are included in stable matrices, they can be studied for themselves, or they can be used to study the light-molecule interaction. In more disordered systems, complex dynamics usually persist even at the lowest temperatures. In this case, a single molecule can play the part of a probe exploring the movements of the matrix. We shall examine these different applications of single molecule spectroscopy successively.

Molecular Physics

A single molecule can be explored by the various techniques of molecular spectroscopy. *Figure* 5 shows how the homogeneous excitation line of one aromatic molecule changes with the intensity of the exciting laser. At high laser powers, the line shows saturation and broadening. From this study we can deduce the homogeneous line-width, directly related to the life-time of the phase of the optical oscillation, as well as the saturation intensity. As the temperature drops to zero, the homogeneous linewidth tends to the natural width (the inverse of the lifetime of the excited state) of the molecular state since all dynamical effects disappear. This has been confirmed by lifetime measurements on single molecules. The saturation intensity itself does not give any information about the saturation mechanism. Saturation can be due to the two-electronic-level system in which the transition takes place, or it can be due to optical pumping towards a third, long-lived level. For organic molecules, this third level is the triplet manifold whose energy is lower than that of the first excited singlet state which is reached by absorbing a laser photon (see *fig* 1). A passage in the triplet states interrupts the fluorescence of the molecule since optical resonance is no longer possible. This causes an associated phenomenon, namely the bunching of emitted photons, which shows up in the auto-correlation function of the fluorescence intensity[5]. The study of this function provides two parameters: the transition rate towards the triplet state and its lifetime (see *fig* 6). Paradoxically, this study is much easier to do on a single molecule than on a whole population. This is because the molecule provides its own trigger-signal so that a time-resolved response can be obtained while using a continuous excitation. The correlation technique was also used to demonstrate the existence of photon anti-bunching due to the dynamics of the two-electronic-level system, first in an atomic beam[6], later on a single molecule[7]. The emission of a photon means that the molecule has just reached its ground state so that it cannot immediately emit a second photon. The fluorescence photons are therefore emitted one by one, like drops out of an eye-dropper, instead of being emitted randomly in time as is the case for a classical source. Incidentally, this anti-bunching effect shows that the radiation emitted by a single system is fundamentally quantum in nature. Another interesting experiment is to induce transitions between the levels of the triplet manifold. This can be – and has been – done by applying the oscillating magnetic field of a micro-wave to the magnetic moment of the electrons. The observed fluorescence response is the optical signature of the paramagnetic resonance of a single electronic spin. This kind of experiment has recently been extended to the study of the nuclear magnetic resonance of a single proton[8] by the group of Ch. von Borczyskowski at the University of Chemnitz, in Germany.

Matrix Dynamics

A molecule may also be used to probe the movement of its neighbours, that is, to probe the dynamics of its environment on a nanometer scale. These experiments have been done on the one hand in a disordered crystal presenting domains with

different symmetries, and on the other in a completely disordered, or glasslike, polymer environment. Unlike a crystal, a glass still presents a very rich dynamical behaviour, even at extremely low temperatures. This is due to the out-of-equilibrium nature of the glassy state into which the liquid was frozen when cooled. Namely, this state is quasi-degenerate with many other states so that tunnel transitions between these may take place, even at the lowest temperatures. Here we must stress that these transitions, which are still not well-known, probably involve the combined movement of several atoms represented by a pseudo-particle of large effective mass. However, since the observation time is much longer than the oscillation time of these particles, which is of the order of the picosecond, such tunnelling events can be observed, notwithstanding their very low probability. In fact, because of the dispersion of the values of barrier heights within glasses, the range of relaxation times is very wide, which is a well-established observational fact.

Traditionally, the different specific properties of glasses at low temperatures, thermal as well as optical, are modelled by a collection of two-level-systems (TLS) which represent the tunnelling possibili-

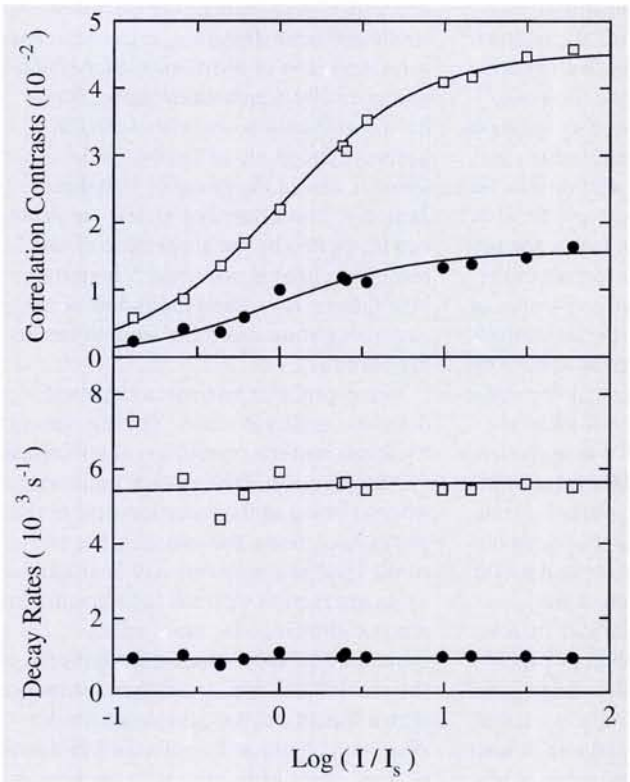

Fig 6 Contrasts and decay rates of the two components in the autocorrelation function of the fluorescence intensity of a single molecule (here for dibenzanthanthrene in hexadecane). The experimental data are fitted with a four-level model including two triplet sublevels

ties. Each TLS represents the rearrangement of the atoms between two metastable configurations. Thanks to single molecule spectroscopy, it has been possible to study single TLSs in polyethylene and in polyisobutylene. A first experiment consists in characterising the intensity fluctuations, caused by fluctuations of the molecular resonance frequency induced by jumps of a nearby TLS, for a given excitation frequency (see *fig* 7).The correlation function shown on *fig* 8 reveals several specific TLSs[9].

Another possibility is to follow the resonance frequency of the molecule in the spectrum by recording a large number of successive spectra. These spectral trajectories, of which *fig* 9 is an example, have the advantage of indicating directly the different frequencies occupied by a molecule and showing each jump of a neighbouring TLS. The price to pay for this extra information is the limited acquisition time of each spectrum, since the new spectral position must be searched for and found. Because the time-resolution of this method cannot be much shorter than a second, it can only be applied to study slow processes. It was used to determine the dynamics of domain walls in a ferroelastic para-terphenyl crystal and to identify the characteristic jumping times of a wall[10].

Non-linear Optics

A single molecule is also an isolated quantum system whose interaction with the radiative field is liable to reveal some effects predicted by quantum optics. This is the case, for instance, in the experiments showing the anti-bunching of emitted fluorescence photons discussed earlier, and which were done in the early days of single molecule detection, at low temperatures, and later in solutions. Other experiments involving non-linear optics have been carried out more recently in our group, in the group of Th. Basché in Munich, and in the group of U. Wild in Zürich. A non-centrosymmetric molecule can be excited by the absorption of two infra-red photons, after which it emits blue fluorescence photons, and these are easy to separate spectrally from the exciting photons[11].

Pump-probe experiments can also be done on single molecules. The energies of the levels and the transitions of a material system (atom, molecule...) are modified when coupled to the radiation field of the pump laser beam. For instance, the electronic level of a molecule will be modified by its interaction with the light beam. This effect is similar to the Stark effect observed in a static electric field. When the pump beam is practically in resonance with a transition, the alternating Stark effect will of course be enhanced, and may become observable, even with the relatively weak field of a light beam.

Not only is there a shift of the molecular resonance ; the pump-molecule system also acquires a new resonance whose frequency is symmetrical to the molecular resonance with respect to the pump frequency. This resonance is the hyper-Raman line, characteristic of a three-photon process in which two pump photons are absorbed and one photon is added to the probe beam by stimulated emission. The molecule is left in the excited state. This phenomenon is associated with an amplification of the probe beam – which has to be cut off for detection, rendering any direct observation of this amplification impossible. Its existence has, however, been demonstrated indirectly on single molecules by the enhancement of their fluorescence. If the probe beam is strong enough, multiphoton effects arise. These can be explained simply by the movement of the molecular Bloch vector, the optical analog of the magnetisation vector of a spin in magnetic resonance. The Bloch vector is driven by a modulated laser field consisting of two intense interfering laser beams, the probe beam and the pump beam. Because of saturation, the Bloch vector stays practically transverse during most of the beat, except when pump and probe beams interfere destructively. The sudden drop of the field intensity causes a transient movement of the Bloch vector which interferes at the next intensity drop. These interferences, which are similar to those of an anharmonic oscillator subject to a periodic kick, can be seen in *fig* 10 and are in excellent agreement with calculations based on the Bloch equations[12]. This shows that such a complex system as a large molecule in condensed matter is very faithfully described by the simple

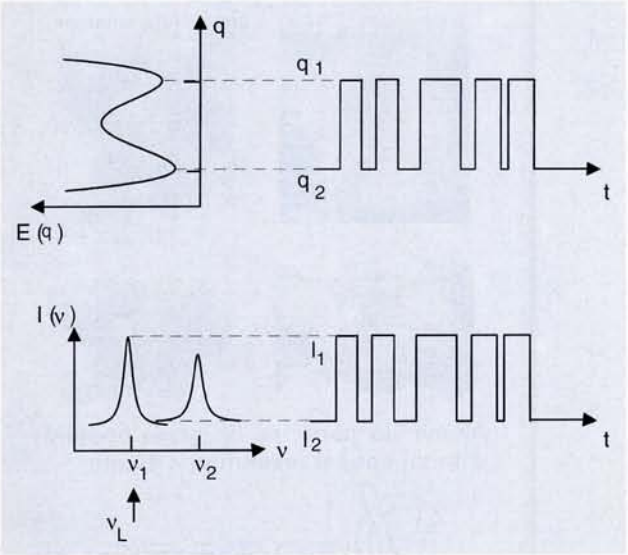

Fig 7 Diagram showing a two-level system in a glass as a function of a generalized coordinate q representing the combined movement of several atoms. The two accessible positions q_1, q_2 are separated by a potential barrier which is crossed by tunnelling at low temperatures. The lower diagram shows how a single molecule fluorescence intensity I, excited by a laser at a fixed frequency ν_L, is modulated in time by spectral shifts as the TLS jumps back and forth

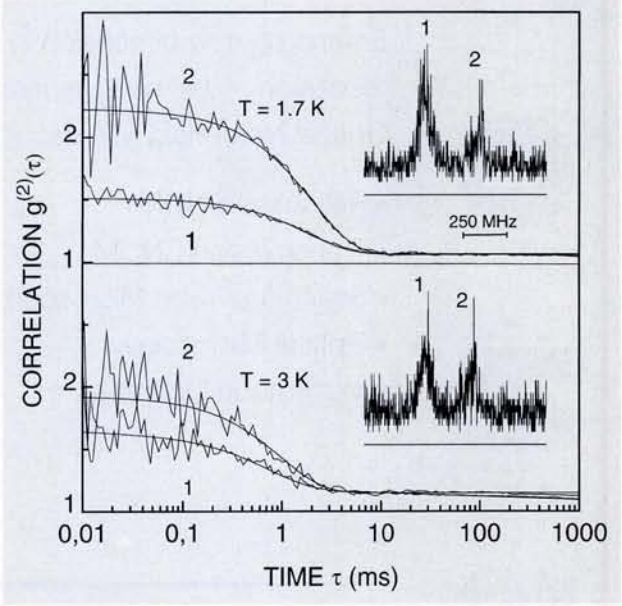

Fig 8 This figure shows the effect of jumps between the two levels of figure 7 on the correlation function of the fluorescence of a single molecule coupled to a single TLS (here terrylene in polyethylene). It can be seen that the jump rates increase when the temperature goes up

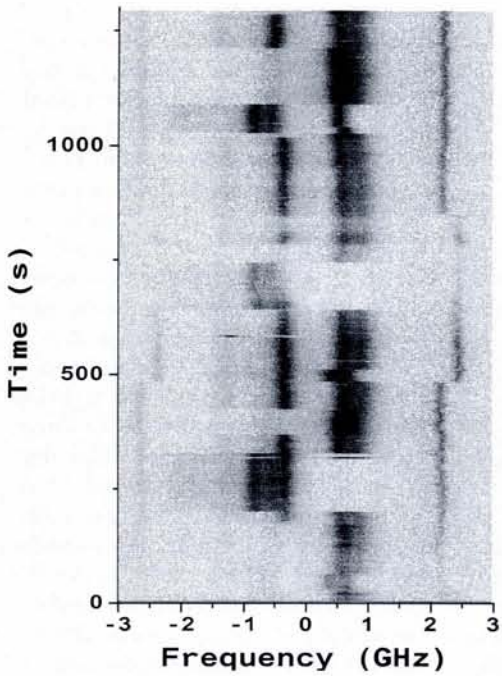

Fig 10 Sub-harmonic structures in the excitation spectrum of a single molecule in a resonant pump beam, while the probe beam, of equal intensity, scans a small range around the transition frequency. This is shown for several values of the intensity (left-hand side). The oscillating structures are due to multi-photon processes which are well reproduced by calculations based on Bloch's equations (right-hand side)

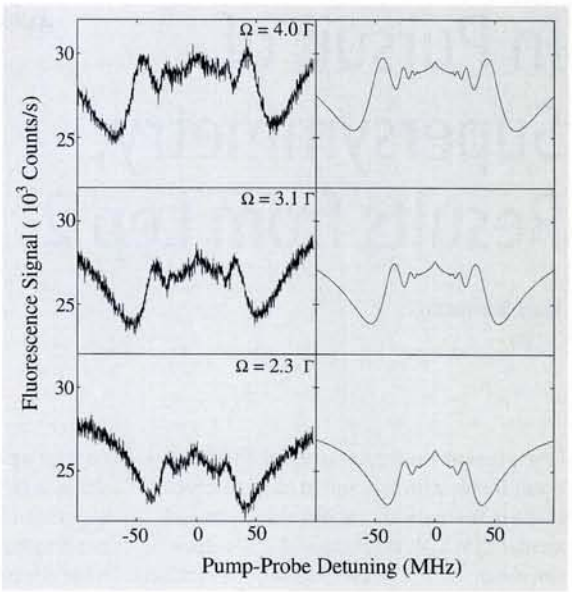

Fig 9 Evolution of the excitation spectrum of a single molecule (terrylene in polyisobutylene) as a function of time. The intensity is colour-coded with shades of grey. Besides jumps between two frequencies corresponding to a molecule-TLS coupling, this spectral trajectory also reveals more complex behaviours which are now being analysed.

model of a two-level system in a laser field.

Even though the absorption cross-section of a dye molecule loses several orders of magnitude when the molecule is heated from helium temperatures to room temperature, room temperature observations of single dye molecules have become quite common now. The benefit of these observations to physical chemistry, to the study of surfaces and to biology in general is obvious, and many groups, especially in the United States, have started single molecule experiments in the past two years.

Remarkable Results

Among a few remarkable results, let us mention the effect of interfaces on spontaneous emission[13], the observation of frequency and orientation jumps of molecules included in a polymer[14], the study of translational diffusion in a gel[15], where it is slower than in a liquid solution, and the visualisation of the energy transfer between donor and acceptor part of the same molecule[16]. Very recently[17], single molecules have been detected by surface enhanced Raman scattering (SERS), an effect involving special amplification arising at the tips of irregularities on a rough metal surface, or on minute metallic particles. These experiments form a remarkable tool for the study of SERS, which is not well known in its details (for instance, they showed that the SERS effect arises from an enormous amplification of the response of just a very few molecules). But above all, since they are based on the Raman effect rather than on fluorescence, they provide fairly narrow and characteristic lines, rather like a fingerprint of the single molecule.

It is seven years since single molecules were first studied by optical spectroscopic methods. In this short period of time, spectacular effects have been discovered, some of which do not exist in bulk systems. The many advantages of studying molecules one by one have as yet not been fully explored. Only single molecule methods can do away with all the drawbacks of averaging, only the study of single systems will give access to the distributions of microscopic parameters and to the statistical correlations between these parameters. Moreover, single molecules can be used as truly local, nanoscopic probes. Perhaps the greatest advantage of studying a single system lies in its automatic synchronisation. This property has been used in our experiments on quantum jumps and on spectral diffusion. For biological applications, it will be absolutely essential to obtain self triggering single systems, because it is practically impossible to synchronise a large assembly of systems without perturbing them, as was brilliantly demonstrated by the patch-clamp experiments. We may certainly expect a profusion of spectacular results in the coming years, for instance if it were found that time-resolved spectroscopic methods could be used with single molecules. Let us hope that European scientists, who did a good part of the initial single molecule experiments, will also contribute some of the many possible developments in this domain.

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