Optical Detection and Manipulation of Single Molecules in Room-Temperature Solutions

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Abstract: With the tight focus of a gaussian laser beam, single molecules in solution at room temperature can be trapped and detected by observation of fluorescent photons. The focus defines an ultrasmall probe volume on the order of 1 femtoliter, and the electric field gradient associated with this focus enables an individual molecule to be trapped and manipulated.

Keywords: single-molecule detection - single-molecule manipulation - laser-induced fluorescence - optical trapping - confocal fluorescence microscopy

Introduction

Single-molecule detection and single-molecule manipulation have generated intense interest in recent years. Analytically, they have far reaching implications in chemistry, biology, molecular medicine, and nanotechnology. Applications include the screening and sorting of a combinatorial chemistry library, the sequencing of a single long strand of DNA without amplification, the mapping of genes on a single chromosome, and the real-time observation of conformational changes. Fundamental investigations into the behavior and dynamics of single molecules offer exciting possibilities for gaining insight into events that are otherwise "buried" under statistical averaging. Recent experiments on single enzymes, for example, have revealed very different catalytic rates for individual enzyme molecules. The rate variation may be caused by differences in local environment or in conformational states of the enzyme.

A number of schemes exist to detect a single molecule, including the use of the family of scanning probe microscopes to detect and image single molecules immobilized on flat surfaces. Perhaps the best known of this family are the scanning tunneling microscope and the atomic force microscope. For molecules embedded in a solid medium, frequency-modulated optical absorption and fluorescence excitation have been used to investigate the spectral properties of single molecules in low-temperature crystalline solids. In these experiments, sudden spectral changes were observed that may be ascribed to local environmental fluctuations or to orientational changes of the probed molecule. With confocal fluorescence microscopy, the dynamical behavior of single molecules in room-temperature solution was also studied.

The capability of manipulating a single molecule opens even more exciting possibilities. Whereas the ability to detect a single molecule permits interesting and important observations to be made, the ability to manipulate a single molecule allows more than passive observation, so that the system is directly influenced and controlled. Various methods have been proposed and developed to accomplish this feat. Electric traps based on the quadrupole configuration were proposed and implemented by Rigler and co-workers for the screening and sorting of biological molecules. Optical traps based on the single-beam gradient force were first demonstrated by Ashkin and co-workers, and have since found numerous applications in biology. This brief review emphasizes one of the available diverse techniques, the optical detection and manipulation of single molecules in room-temperature solution under conditions relevant to biological processes.

Discussion

Optical Detection of Single Molecules: The key to the optical detection of a single molecule in each instrumental variation is the reduction of background interference, which may be caused by Rayleigh scattering, Raman scattering, or false signals arising from impurities. To put this consideration into perspective, at a concentration of \(3.3 \times 10^{-9}\) M, a single target molecule will reside, on average, in a small volume of \(10^{-15}\) liters. But this small probe volume still contains about \(10^{10}\) solvent molecules, \(10^{4}\) electrolytes, and many impurity molecules. This problem of background interference can be overcome by defining an ultrasmall probe volume, thus spatially isolating the molecule to be studied. Advances in microscopy have solved this technical difficulty by reducing the probed volume through near-field,

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evanescent, confocal, and two-photon configurations. With a confocal fluorescence microscope, for example, this tiny probe volume is created latitudinally (with a diameter of 0.5 μm) by the tight focusing capability of a high numerical aperture oil-immersion objective and longitudinally (with a height of 2 μm) by a pinhole placed in the primary image plane of the microscope. In this way, a probe volume (shown in Figure 1) of less than 1 femtoliter is defined.

![Figure 1: The conical drawing is the 1/e² boundary of the tightly focused laser beam. The dark, wiggly line represents the diffusional path of a single Rhodamine 6G molecule in a 1 ms period. When the molecule enters the probe volume defined by the focused laser beam, it is repeatedly cycled between its ground and first excited electronic state. This process results in the emission of the detected photons. Source: ref. [16].](image)

Once background interference is minimized, the presence of the molecule must be registered through a detectable signal, which usually involves some intrinsic amplification system. In the patch-clamp technique, for example, the binding of a single ligand molecule to its receptor results in the receptor-mediated opening of an ion channel so that the presence of the ligand molecule is amplified by the charges that flow through the membrane. In the optical detection of single molecules with laser-induced fluorescence, the amplification involves cycling the molecule between its ground electronic state and first excited electronic state. Each time a molecule completes this cycle, a photon can be emitted depending on the quantum yield of the molecule. This fluorescence cycle is depicted in Figure 2. It consists of four steps: 1) excitation from the ground electronic state to the first excited electronic state by the laser beam whose wavelength is resonant with this transition, 2) relaxation to the lowest lying levels of the excited electronic state, 3) radiative or nonradiative decay to the ground electronic state, and 4) relaxation to the lowest lying levels of the ground electronic state. Relaxation is typically fast in the condensed phase (in the picosecond range), and the rate of the fluorescence cycle is limited by the excited-state lifetime of the molecule, which is typically in the range of a few nanoseconds. For common fluorescent dye molecules, this fluorescence cycle can occur at a rate of $10^{10}$ to $10^{12}$ per second under favorable conditions. With an optimal geometric setup, optics, and detector, an overall photon detection efficiency of 5% may be realized.

![Figure 2: Fluorescence absorption and emission cycle of a single molecule. This fluorescence cycle is occasionally interrupted by intersystem crossing (ISC) from the singlet to the triplet state. Source: ref. [5].](image)

Nonetheless, detecting more than 50000 photons from a good chromophore in solution is difficult, because the molecule is photochemically destroyed at some point during the fluorescence cycle. This photobleaching process is not fully understood, but it is generally believed to involve dissolved oxygen and other radicals present in solution. For example, photobleaching of the YOYO dye causes YOYO-intercalated DNA ($\approx 2000$ YOYO per DNA) to fluoresce less strongly and to photofragment as bleaching progresses. Photobleaching makes observation of the dye-stained DNA difficult for more than a few seconds. With a mixture of glucose, glucose oxidase, catalase, and mercaptoethanol that enzymatically scavenges oxygen and other radicals, however, YOYO-intercalated DNA is stable for almost one hour.

Another interruption of this fluorescence cycle is the crossover from the singlet state to the triplet state in the first excited electronic level of the fluorescent molecule (Figure 2). Although the probability for this spin-forbidden transition is very low, once the crossover occurs, the cycle is interrupted for microseconds, the typical lifetime of the triplet state in solution. This interruption is relatively long, because the lifetime of the triplet state is about three orders of magnitude longer than that of the singlet state. The phenomenon of macroscopic fluctuations in the fluorescence signal caused by singlet–triplet intersystem crossing, which are characteristic of a single-molecule quantum system, is one of the earlier interesting observations made on the photophysical properties of a single molecule.

One of the key challenges in single-molecule detection is the need to ascertain that the observed signal indeed arises from a single molecule. This was first confirmed for single-molecule detection in low-temperature crystalline solids. The most convincing proof came from the time correlation of the fluorescence signal, which shows antibunching. Antibunching simply means that for a single molecule, the probability for two photons being emitted at the same time is zero. Other qualitative criteria exist, including the fact that 1) the frequency of the observed signal should scale proportionally with the concentration, 2) the intensity of the signal should vary in an expected way with a different buffer or environment, 3) the observed
signal intensity should be less than the cycle rate between the ground electronic and excited electronic states, and 4) photo-bleaching should happen in an "all-or-none" fashion.\[^{15}\]

**Applications of Single-Molecule Detection:** Single-molecule detection schemes have been proposed by Keller and co-workers\[^{23, 24}\] for the rapid sequencing of a single large fragment of DNA molecule. These techniques are based on the fluorescent labeling of each nucleotide followed by enzymatic cleavage of individual bases in a flowing stream. A detection window is placed downstream for subsequent identification of the fluorescently tagged base. In this way, a large piece of DNA can be sequenced without amplification so that the laborious procedures of subcloning and mapping can be omitted. In addition, similar techniques have been applied to the rapid sizing of single DNA fragments, which may find useful applications in molecular biology and medicine.\[^{25, 26}\]

Using fluorescence correlation spectroscopy coupled with electric manipulation of biomolecules, Rigler and co-workers\[^{16}\] have screened and isolated rare and specifically marked compounds among large numbers of alternatives. This procedure might find use in screening a combinatorial library or in identifying rare types in molecular evolution. Similar techniques have also been applied to monitor the hybridization between complementary DNAs,\[^{27}\] the binding of a single ligand molecule to its receptor,\[^{28}\] and the conformational changes of a single molecule.\[^{29}\]

The real-time dynamical behaviors of single fluorescent molecules and dye-tagged biomolecules in solution were studied by Zare and co-workers with fluorescence confocal microscopy.\[^{15, 16, 20}\] Interesting diffusional behavior was observed, such as probe volume boundary recrossing motions, and photophysical behavior of a single molecule as a function of exciting intensity. Because this technique does not involve statistical averaging, it should find useful applications in following the real-time dynamics of single-molecule reactions.

Experiments performed on single-molecule systems should offer insight into many physical processes. Single-molecule reaction dynamics in fluctuating environments, for example, has been the subject of theoretical study by Wang and Wolynes.\[^{31}\] By examining the statistics of an individual reacting molecule, this report provided insight into reaction kinetics that was not easily understood when a large number of reacting molecules were monitored simultaneously. In short, by peering into events hidden by statistical averaging, which in this case was the occurrence of intermittency in the barrier crossing of single molecules, complex population behavior can be readily inferred.

Another attraction in single-molecule chemistry is the ability to study dynamic or kinetic events without the need for synchronization. Much effort has been spent to synchronize reactions with the shortest possible time distribution, such as the use of fast-mixing and temperature-jump techniques. In addition to the costly or tedious nature of such experiments, unwanted perturbations introduced by these synchronization methods often complicate data analysis. Single-molecule experiments inherently do not require synchronization, because only one molecule is investigated at a time. This advantage may be exploited to study a variety of biological processes. One example is the intercalation of single dye molecules into double-stranded DNA. The action of various chemicals, including mutagenic polycyclic aromatic hydrocarbons and antibiotics, originates from intercalation.\[^{12}\] Gaining a better understanding of this process is therefore important from both the environmental and medical perspectives. However, the kinetics of intercalation is still poorly understood, and its study has been impeded, in part, by the difficulty of synchronizing the intercalation process without introducing confusing interferences.\[^{23}\] Many of these difficulties could be overcome by monitoring and identifying key intermediates in the intercalation of single dye molecules into DNA with polarization, lifetime, or energy-transfer measurements.

Another example is conformational kinetics, for which protein folding is the most celebrated example. By labeling two amino acid residues with two different dyes, structural information on protein-folding intermediates might be inferred by measuring energy-transfer efficiencies between the dye molecules. By monitoring the polarization of the two chromophores, orientational information might also be obtained. And again, the difficulty associated with synchronizing the folding process is effectively overcome. One obvious challenge in single-molecule chemistry is sensitivity. The amount of details that can be obtained depends critically on the number of photons that can be collected. With improved collection optics and detectors, single-molecule experiments should find increasing applications and offer many exciting insights.

**Optical Manipulation of Single Molecules:** Optical traps make use of radiation pressure, the force that is exerted by light on matter through scattering, absorption, emission, or refraction.\[^{8, 34 - 37}\] This force may be regarded as the transfer of momentum from photons to the object being irradiated. Perhaps the most common and versatile optical trap used for the manipulation of micro- and nanoparticles in solution is the single-beam gradient force trap based on the spatial gradient in light intensity when a laser beam is brought to its diffraction-limited focus. Figure 3 illustrates the geometric setup of this

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*Figure 3. Geometric illustration of the single-beam gradient trap: (a) A gaussian laser beam is focused by a high numerical aperture objective. A particle irradiated by this laser beam will seek out the region of the highest light intensity, which is caused by the gaussian profile in the transverse direction (b) and by the presence of the focal point in the longitudinal direction (c). \( F \) represents force, \( \nabla I \) is the gradient of the intensity, and \( M_\theta \) is the beam waist.*
trap. A gaussian laser beam (TEM\(_{00}\)) is sharply focused by a high numerical aperture objective. This focus creates a light intensity gradient in the transverse direction by the gaussian profile of the laser, and in the longitudinal direction by the presence of the focal point. The electric field \(E\) of the laser beam induces a dipole moment \(p\) in the molecule, and this induced dipole oscillates in phase with the driving electric field, provided the wavelength of the laser is to the red of the main absorption feature of the molecule. Because this interaction energy is attractive, of the form \(-p \cdot E < 0\), the molecule will seek out the region where the radiation intensity is highest. For a trapping potential that is sufficiently deep to overcome Stokes drag (force exerted on the particle when translated in an aqueous medium), the trapped molecule can be moved at will in solution. If the laser beam is polarized, the induced dipole moment will align itself with the driving electric field. If the polarization then rotates in space, the induced dipole moment will rotate with the polarization, which results in the rotation of the molecule that is trapped. In this way, single particles or molecules can be trapped, translated, and rotated with great dexterity.

To avoid optical damage to the object being trapped, the appropriate laser wavelength must be selected. For biomolecules, using near-infrared lasers is best, because absorption by biological samples and water is minimal at this wavelength. The destructive nature of the shorter wavelength UV lasers can be taken advantage of as an optical "scalpel" or "scissors" to dissect biomolecules. Visible wavelength is best suited for the excitation of fluorescent dyes for the purposes of detection or imaging. With a correct combination of laser wavelengths, therefore, manipulation and microdissection can be carried out for the study of biological systems at the single macromolecule level.

**Applications of Single-Molecule Manipulation:** Since the first demonstration by Ashkin and co-workers\(^{50}\) of the single-beam gradient trap, many intriguing biological observations have been reported. Single bacteria and viruses, for example, were transported in solution with ease and with no apparent damage.\(^{38}\) The manipulation capability of optical traps was then exploited to study the tubelike motion and relaxation of single DNA molecules.\(^{39, 40}\) In these experiments, a single piece of DNA is first attached to a polystyrene bead, which acts as a "handle" for manipulating the attached DNA. Using this technique, the reptation model of de Gennes, Edwards, and Doi, which describes the interactions between entangled polymer chains, was directly tested. These experiments performed on single DNA molecules go beyond passive observation of the Brownian dynamics of polymers by exercising direct control on the nanoscopic level of single chains of DNA.

Optical traps have also been used as force-measuring devices. By combining optical trapping with interferometric feedback, it is possible to measure with exquisite accuracy the force needed to maintain the trap against an applied force. With this technique, the force generated by single kinesin molecules was measured to be 1.9 piconewtons.\(^{41, 42}\) The force applied during transcription by RNA polymerase was also monitored and estimated to be 14 piconewtons by Block and co-workers.\(^{43}\) This force exerted by RNA polymerase is substantially larger than those generated by the cytoskeleton motors kinesin and myosin.

Because polymerases usually are not thought of as mechanoenzymes, it is rather surprising that the observed energy conversion efficiency into biologically useful force is comparable to or exceeds that of the typical mechanoenzymes.

The elasticity of single double-stranded DNA was investigated by Cluzel et al.\(^{44}\) and Bustamante and co-workers.\(^{45}\) By applying force to both ends of the DNA through polystyrene bead "handles", they found that DNA molecules existed in a super-stretched form (S-DNA). The deformation of DNA molecules is involved in many biological processes, such as the binding of RecA to double-stranded DNA. These experiments may have important implications in the energetics and dynamics of these processes.

In all of the above experiments, a single DNA molecule is manipulated through "handles" that were biochemically attached to its ends. Recently, Chiu and Zare\(^{30}\) have demonstrated the direct trapping and manipulation of a single molecule of DNA, as shown in Figure 4. This feat is accomplished by first causing the DNA molecule to take on a coiled-compact structure, which results from lowering the pH (from pH 8.0 to pH 5.75) of the solution. This supercoiled DNA can then be readily trapped and moved at will in solution. This ability to directly manipulate DNA opens the exciting possibility of exploiting the optical trap as a reaction-initiation device. A single coiled DNA molecule is first optically trapped, and the pH is then raised by introducing base. When the trap is subsequently turned off, the DNA should "unfold" in this higher pH environment. In this way, the optical trap effectively initiates the "unfolding" of a single DNA molecule. Coupled with a second
wavelength for monitoring purposes, the dynamics of DNA conformational changes may be probed at the single-molecule level.

**Conclusion**

Although single-molecule detection and manipulation is still in its infancy, these techniques are already offering insights and interesting observations into the world of nanostructures. With advances in instrumentation, an ever-widening spectrum of questions arising from biology and chemistry to material sciences become addressable. By these means, we enter a new world in which we can study and control chemistry in solution, molecule by molecule.

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