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OPTICAL DETECTION OF SINGLE MOLECULES

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ABSTRACT

Recent advances in ultrasensitive instrumentation have allowed for the detection, identification, and dynamic studies of single molecules in the condensed phase. This measurement capability provides a new set of tools for scientists to address important current problems and to explore new frontiers in many scientific disciplines, such as chemistry, molecular biology, molecular medicine, and nanostructured materials. This review focuses on the methodologies and biological applications of single-molecule detection based on laser-induced fluorescence.

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PERSPECTIVES

Most experiments represent measurements of ensemble averages by which a vast multitude of duplicate systems are probed and average responses are recorded. As such, rare events often escape notice because they are buried in the average properties of the system under study. With advances in instrumentation we are able to detect individual particles and study their time evolution. Such studies permit exploration of a single particle interacting with its nanoenvironment and of how that interaction changes in time.

Perhaps the first such example is the celebrated Milliken oil-drop experiment in which the charge-to-mass ratio of an individual electron was determined (85). Since then, impressive strides have been made that allow the detection of isolated single atoms in traps, single molecules in low-temperature molecular crystals, and, recently, the more complicated but also more fascinating world of single molecules in room-temperature solutions and surfaces—a world that includes biological systems of interest.

Significant advances in instrumentation involve the class of scanning probe microscopies, of which the scanning tunneling microscope (STM) and the atomic force microscope (AFM) are the best known (20, 21, 54, 108). In these microscopies, a probe tip is brought into intimate contact with the molecule, and the presence of the molecule manifests itself as a tunnel current or a deflection in the tip-height as the tip is scanned across a relatively flat surface upon which the molecule is attached. Other variations exist, such as the use of an ultramicroelectrode probe. Single molecules in solution are detected by repeated oxidation and reduction as the molecule diffuses back and forth between the two electrodes that constitute a circuit, resulting in a measurable current flow (43). Alternatively, in some special cases, the presence of the electroactive molecule in solution can be sensed by the electrogenerated chemiluminescence at the microelectrode (32). Yet another electrochemical alternative is one in which the single molecule in solution is sensed by a membrane-bound receptor that opens an ion channel, so that the presence of the single molecule is amplified by the charges that flow through the membrane (93, 102, 113).

Scanning-probe microscopies hold much promise for molecules bound to a surface, but for molecules embedded in a medium, optical methods involving sensitive detection of emission or absorption appear in general to be more useful (11, 14, 86, 144). The key concept is that a single molecule can be repetitively cycled between its ground and excited electronic states with a laser beam at a wavelength resonant with this transition, which yields a large number of photons for detection. Optical methods probe chemical chromophores and are well suited for noninvasive studies of single molecules in low-temperature solids, in room-temperature liquids, and on surfaces.

Optical methods in the far field cannot localize the position of the molecule to better than the diffraction limit, which is equal to half the wavelength of light. It is possible, however, to combine probe microscopies with optical detection. One highly successful example is near-field scanning optical microscopy, in which a tapered single-mode fiber tip is used to provide both optical and topographical information in the same sampling area (8, 17). Another interesting development is a hybrid instrument that combines far-field laser-induced fluorescence and atomic-force microscopy via an inverted optical microscope (42). In this integrated microscope, ultrasensitive optical measurement at the single-molecule level is used to provide chemical/molecular information and atomic force imaging to resolve individual molecules at subnanometer resolutions.

This review concerns the optical detection of single molecules. We review the fundamental principles of optical detection and present a detailed description of the specific methods that have been developed for that purpose during the past decade. We then discuss emerging biological applications of single-molecule detection as well as some possible future directions.

PRINCIPLES

Optical detection of single molecules has been achieved by both frequency-modulated absorption and laser-induced fluorescence. Because of the low background and high signal-to-noise ratios, laser-induced fluorescence has become the most widely used method. In the condensed phase, fluorescence emission from a single molecule usually occurs in a four-step cycle: (*a*) electronic transition from the ground-electronic state to an excited-electronic state, the rate of which is a linear function of excitation power; (*b*) internal relaxation in the excited-electronic state; (*c*) radiative or nonradiative decay from the excited state to the ground state as determined by the excited-state lifetime; and (*d*) internal relaxation in the ground state. Vibrational and rotational relaxations generally occur on the picosecond timescale for small molecules in the condensed phase, whereas the excited-state lifetime and the absorption time are in the subnano to nanosecond range. Consequently, the fluorescence cycle is primarily determined by the absorption and emission steps. At low excitation powers, the absorption time is expected to be the dominant factor and the observed fluorescence intensity increases approximately linearly with laser power. At intermediate power levels, the absorption time becomes comparable to the excited-state lifetime, saturation occurs, and the signal is weakly dependent on laser power. At high power levels, the absorption time becomes much shorter than the excited-state lifetime, and the signal is determined by the molecule's intrinsic excited-state lifetime and is thus essentially independent of laser power. For common fluorescent dyes, the optical excitation-emission cycle rates are

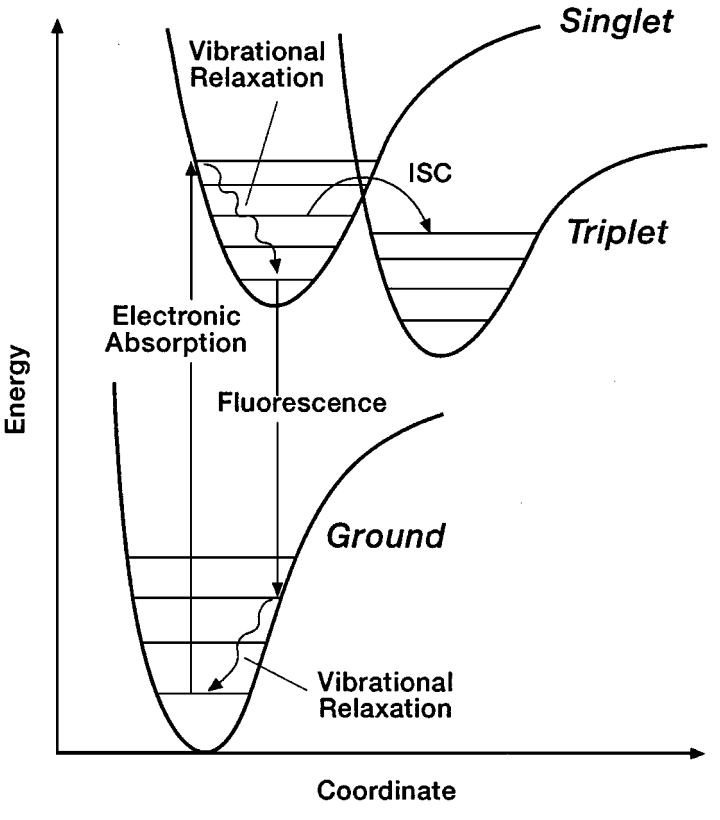

Figure 1 Fluorescence cycle of a single molecule (from S Nie, DT Chiu, and RN Zare, unpublished drawing). ISC: intersystem crossing from the excited singlet state to the triplet state.

approximately 10^7–10^8 per second with a 1.0 mW laser beam focused to the diffraction limit.

Fluorescence competes with several other deactivation channels and photochemical reactions that can lead to photodestruction of the molecule. Depending on the fluorophore and the solvent system and the intensity and wavelength of the radiation, several types of photoinduced reactions can occur, such as photooxidation, photoionization, photodissociation, and photoisomerization. Both photochemistry and intersystem crossing (ISC) from the excited-singlet state to the long-lived triplet states influence the behavior of the fluorophore. The maximum average number of fluorescent photons emitted is simply given by the fluorescence quantum yield divided by the photodestruction quantum

yield. For the typical dye molecule, rhodamine, dissolved in ethanol, the average number of fluorescent photons has been determined (125) to be 1.7×10^6, which is a number that can be readily detected.

Active chemical species such as singlet oxygen and hydroxyl radical often play an important role in photodestruction, but many photoinduced quenching and degradation reactions involve oxygen in its ground triplet state. A standard practice for reducing photodestruction is to add reducing agents such as mercaptoethanol or to add a mixture of catalase and glucose oxidase in order to remove dissolved oxygen (56). Under favorable conditions, individual molecules can emit an average of 10^5–10^6 fluorescence photons before photobleaching. Current ultrasensitive instrumentation using single-photon–counting avalanche photodiodes allows approximately 5 photons to be detected for every 100 photons emitted, which represents a 5% overall detection efficiency. Thus, 5000 to 50,000 photons can be observed from a typical well-behaved single fluorophore. This number is sufficient not only for single-molecule detection, but it is also sufficient for spectroscopic identification and real-time monitoring over an extended period of time. This estimate is strictly for molecules that contain a single fluorophore such as fluorescein, rhodamine, or cyanine. For biological macromolecules such as proteins and nucleic acids, each molecule can be labeled with many copies of a fluorescent tag to amplify the signal intensity. These concepts form the basis for the methods and applications of single-molecule fluorescence measurements discussed in this review.

The key challenge is to reduce the background interference, which may arise from Raman scattering, Rayleigh scattering, and impurity fluorescence. To put this need in perspective, a tiny volume of buffer solution on the order of one femtoliter or less ($<10^{-15}$ liter) still contains approximately $1–3 \times 10^{10}$ solvent molecules, $0.5–1 \times 10^8$ electrolyte molecules, and a large number of impurity molecules. This background interference has gradually been overcome by the use of high-performance optical filters, ultrapure solvents, and the reduction of illuminated-sample volume through the use of laser excitation in the confocal, near-field, and evanescent configurations. These special excitation geometries direct the laser beam to probe a small volume or a thin sample layer. The concept of improving the fluorescence-to-background signal-to-noise ratio by reducing the probe volume may be regarded as a leitmotiv that appears in each instrumental variation on the detection of single molecules by optical means.

Another experimental challenge has been to ascertain whether the observed signals arise from single molecules or from molecular aggregates and artifacts. The following criteria are commonly used as a practical test: (*a*) The frequency or number of detected fluorescence signals should depend linearly

on concentration, but the signal intensities should remain the same; (*b*) photobleaching should occur in an all-or-none fashion; (*c*) the recorded optical spectra may vary from molecule to molecule or from time to time because of environmental perturbations; (*d*) the signal dependence on excitation intensity should show saturation as expected for a single molecule; (*e*) the observed number of fluorescence photons should not exceed that limited by the fluorescence cycle of a single molecule; and (*f*) the time correlation of the fluorescence signal should exhibit antibunching (13).

METHODS

A number of optical methods have been developed to study single molecules. They differ in sampling conditions and means of delivering excitation energy, but all of them share the need to isolate single molecules for detection. One approach is to isolate individual molecules spectroscopically in low-temperature solids because matrix perturbations cause each molecule to have a slightly different absorption frequency. A more broadly useful approach is to isolate molecules on a surface or in dilute solution, i.e. individual molecules are spatially separated from each other in the area or volume probed by a laser beam. Specific methods embodying these principles are discussed below.

Spectral Isolation of Single Molecules in Low-Temperature Solids

Optical detection and spectroscopy of single-chromophore molecules were first achieved in solid matrices at very low temperatures (66, 88, 99). Guest molecules (for example pentacene) are embedded in a host material (for example *p*-terphenyl) at low concentrations (10^{-7}–10^{-9} M). Because of strains and imperfections in the host environment, the optical absorption lines of the embedded molecules are inhomogeneously broadened. These broadened lines consist of a superposition of the homogeneous lines of individual guest molecules, which still have narrow Lorentzian profiles but are spread over a range of absorption frequencies in a Gaussian distribution. In the absence of phonon-electronic coupling, a laser beam of narrow line width can be tuned to resonance with only one of the guest molecules. Such spectral selection can isolate 1 out of 10,000 molecules in the probe volume, which allows the measurement of a single absorber without ensemble averaging. This method forms the basis for studying the nanoenvironments in low-temperature solid materials (87, 100).

In the model system of pentacene doped into crystalline *p*-terphenyl, fascinating physical phenomena such as sudden changes of absorption frequencies (spectral diffusion) (4) and photon antibunching (13) were first observed.

Recently, this approach has been extended to polymeric and Shpol'skii matrices (12, 89, 101) and has been employed for wavelength-resolved vibronic spectroscopy of single molecules (45, 92). Double resonance methods even permit the magnetic resonance of a single molecular spin to be observed (69, 70, 71, 143). Far-field two-dimensional detection (50) and near-field optical spectroscopy (90) have also been achieved for single molecules in low-temperature solids.

The requirements for successful single-molecule measurements in low-temperature solid hosts are quite restrictive, including high peak-absorption cross sections, weak or absent bottlenecks in the optical pumping cycle, high fluorescence quantum yield, a strong zero-phonon lowest-electronic transition, and weak or absent hole-burning. This approach has been limited to solids with temperatures less than 15 K, which excludes the study of many interesting biological systems, but it has provided much insight into the photophysics of single molecules.

Single Molecules in Liquid Streams

The method for detecting and identifying single molecules in liquid streams works in much the same way as a flow cytometer counts and discriminates biological cells. A dilute liquid sample flows through a focused laser beam in a capillary tube. Fluorescence signals are collected at 90 or 180 degrees with respect to the direction of the laser beam before passing through spatial and spectral filters to the detection system. Sudden bursts of fluorescence photons correspond to single molecules passing across the laser beam. Dovichi et al (36) first suggested the use of laser-induced fluorescence for single-molecule detection in such a manner. Keller, Mathies and coworkers (94, 106) subsequently demonstrated single-molecule detection with the flowing stream technique for the multi-chromophore molecule B-phycoerythrin (equivalent to $\sim$25 rhodamine 6G molecules in fluorescence). Using time-gated fluorescence, the detection of individual single-chromophore molecules was first achieved with pulsed-laser excitation (121). The time-gated fluorescence technique has recently been extended to study mixture-dye solutions (123), to measure the lifetime of single rhodamine 101 molecules (129, 141), and to detect single near-infrared (IR) dyes (74, 124). Several groups (26, 27, 79) have recently achieved detection of single molecules in electrophoresis capillaries.

This method is well suited for counting and sorting of single molecules in liquid solution. Potential applications include rapid DNA sequencing, optical sizing of DNA fragments, rare-event detection, and ultrasensitive screening of combinatorial chemical libraries. Signal-to-noise issues are always important for accurate and reliable detection of single molecules, and a conflict currently exists between sample volume throughput and detection efficiency. In a simple

50-μm flow cell, only 4% of the molecules will actually flow through a 10-μm laser beam, which means that 96% of the molecules will not be detected. The detection efficiency can be significantly improved with a submicrometer-sized detection zone, but the volume throughput will become too low for practical use. A compromise may involve the use of a slightly defocused laser beam to completely cover the cross-section of a 2 to 5-μm detection zone in a 50- to 100-μm capillary. Recent research has shown that small inner diameter (i.d.) silica capillaries can be shaped to create a micrometer-sized detection zone by using a CO_2 laser micropipette puller (79). Such tapered capillaries could provide a unique means for high-efficiency detection and manipulation of single molecules at room temperature. Other issues important in the future will include spectroscopy-based sorting and chemical or biochemical amplification of the sorted target molecules.

Single Molecules in Microdroplets

The detection of single molecules in microdroplets was pioneered by Ramsey and coworkers (9, 10). The main concepts are to confine a single molecule in a picoliter-sized droplet and to use an electrodynamic trap for levitating the droplet so that the molecule can be interrogated by a laser beam for an extended period of time. The cavity electrodynamic effects in microdroplets may be exploited to the advantage of single-molecule detection. Indeed, it has been shown that both the spontaneous emission rate and the integrated fluorescence intensity for rhodamine 6G are significantly enhanced in glycerol microdroplets (10), yielding a signal-to-noise ratio of 10–40 for single rhodamine 6G molecules (9). The time required for levitation, fluorescence measurement, and droplet-size analysis is relative long (several minutes), however, which makes this approach impractical for single-molecule counting and sorting applications. Perhaps one way to overcome this problem is to produce a stream of free-falling droplets that are rapidly detected by a high-intensity laser beam (11). In some aspects, this approach resembles high-speed single-molecule detection in the continuous-flow liquid stream discussed above. The ultimate limit of analysis speed is determined by the time required for a single molecule to emit enough photons for detection under saturating fluorescence conditions. For fluorescence dyes with nanosecond–excited-state lifetimes, a single molecule will need to stay in a laser beam for approximately 10 to 50 μs to generate $\sim$20 photon counts (at 5% overall photon detection efficiency). Thus, the maximum possible detection speed is approximately 20,000–100,000 molecules per second. Paradoxically, the very aspects of cavity electrodynamics interactions that enhance fluorescence detection for single molecules in microdroplets may prove to be a disadvantage in those experiments that wish to characterize the behavior of single molecules in solution. Also using microdoplets, Ishikawa

et al (63) developed a method for parallel, two-dimensional detection of single molecules. In this approach, microdroplets of a dilute dye solution are sprayed on a mirror-like silicon surface, and a conventional microscope equipped with a photon-counting camera is used to image single molecules in the dispersed droplets after solvent evaporation. This method potentially allows rapid analysis of a large number of molecules, but it may not be suitable for sorting "hit" molecules as described above.

Near-Field Scanning Optical Microscopy

Near-field scanning optical microscopy (NSOM) has been developed primarily to break the optical diffraction limit, which restricts the spatial resolution of conventional optical measurements to approximately half of the light wavelength ($\lambda/2$). In NSOM, subdiffraction spatial resolutions on the order of 50–100 nm (5–10 times better than the diffraction limit for visible light) are achieved by bringing a sample to within 5–10 nm (the optical near-field) of a subwavelength-sized optical aperture so that photons from the aperture do not have enough distance to experience diffraction (8, 17). The resolution is only limited by the size of the aperture, which is often a pulled micropipette or a tapered single-mode fiber. The sides of the tapered near-field probes are coated with approximately 100 nm of aluminum to prevent light leakage, with an exposed aperture of approximately 50–100 nm at the tip. A feedback mechanism based on shear-force is used to regulate the tip-sample distance with a vertical resolution of $\sim$1 nm. The optical signals are detected in the far field by using an objective lens either in the transmission or collection mode. The high resolution and sensitivity of NSOM has been used to image fluorescently labeled actin filaments in fixed cells (18), to study light harvesting complexes in photosynthetic membranes (39), to detect single fluorescent allophycocyanin proteins (38), and to image DNA molecules labeled by intercalation dyes (1).

Recent advances in NSOM have allowed for the imaging (3, 19, 59, 81, 145), dynamics (145), spectroscopy (130), and resonance-energy transfer (51) of single molecules, as well as for the determination of each fluorophore's dipole orientation (Figure 2). This capability results from a high photon flux delivered by the tapered single-mode fiber, efficient background rejection owing to the small illumination volume, and excitation by a strong evanescent wave component near the tip end. At the present, however, NSOM suffers from several limitations, such as low power throughput (approximately 1–50 nW for a 50 to 100-nm tip) (57), poor reproducibility in tip preparation (132), and sample perturbation by the coated fiber probe (3, 145). The use of bent optical fiber tips with contact- or tapping-mode force-feedback may improve the performance of NSOM for imaging biological materials (91, 120). If the primary goal is to study single molecules, and if subdiffraction spatial resolution

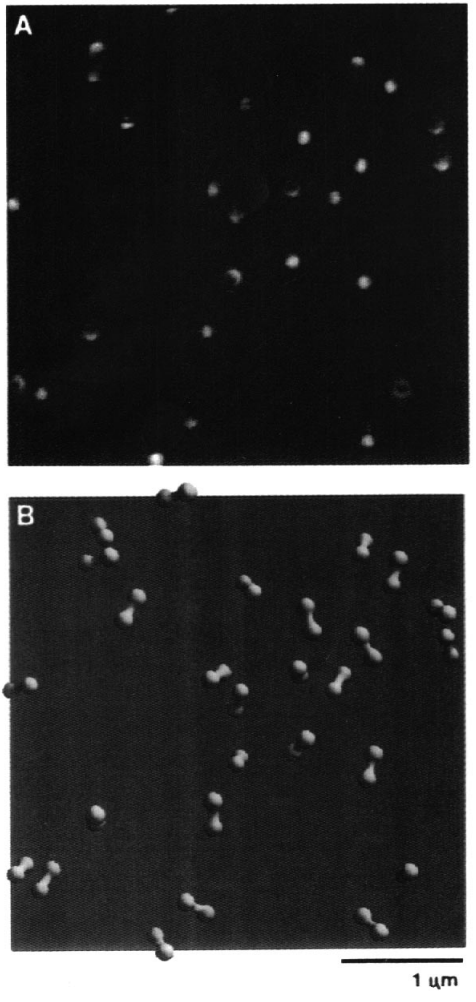

Figure 2 Near-field fluorescence images of single molecules and determination of each molecule's dipole orientation. (See figures 4*a,b* in Reference 16.) (Reprinted with permission from *Science* 262:1422–25. Copyright 1993 American Association for the Advancement of Science)

is not essential, then far-field confocal and evanescent-wave methods appear to be better choices because of their unlimited excitation throughput, their experimental simplicity, and their absence of tip perturbations. The benefits of near-field microscopy are its improved spatial resolution and the ability to correlate spectroscopic information with topographic data.

The theoretical aspects of near-field optics center on Maxwell's equations for a small aperture in a conducting screen, which were originally solved by

Bethe (15) and refined by Bouwkamp (23). Recently, more accurate descriptions of the electromagnetic field around an aluminum-coated NSOM tip have been made from simulations with the multiple-multipole method (97) and the finite-difference time-domain method (29, 67). These methods use a numerical approach for solving Maxwell's equations for the specific geometry of an NSOM fiber probe. Experimentally, what is needed in NSOM is to develop brighter and smaller probes. Since Betzig and coworkers first developed adiabatically tapered single-mode optical fiber tips for improved light throughput, no major breakthroughs have been made. Kopelman and coworkers (72) are currently developing "supertips" based on excitonic crystals and molecular antennas. A related approach is perhaps to attach nanometer-sized polymeric light-emitting diodes at the tip of a microfabricated atomic force microscope (AFM) cantilever (49).

Far-Field Confocal Microscopy

In confocal microscopy (104), a laser beam is brought to its diffraction-limited focus inside a sample using an oil-immersion, high-numerical–aperture (NA) objective (NA $\sim$ 1.3). A small pinhole (50–100 μm in diameter) is placed at the image plane to reject light from out-of-focus regions. This arrangement defines a small volume of 0.5–1.0 femtoliters in the sample. This tiny probe volume, which is approximately cylindrical in shape, is $\sim$0.5 μm in diameter (the diffraction limit for visible light) and 2 μm in height (limited by spherical aberration). The fluorescence signal that passes through the pinhole encounters appropriate optical filters before striking a photon-counting system. At a concentration of 3.3×10^{-9} M, an average of only one target molecule (of typical nanometer dimensions) resides in the probe volume. The actual number of target molecules fluctuates in time. Assuming a Poisson distribution (44), the probability of finding zero molecules at this concentration is 0.368, that of finding one molecule is 0.368, that of finding two molecules is 0.184, and that of finding more than two molecules is 0.078. In more dilute solutions, the detection events are increasingly dominated by single-molecule events.

Rigler and his coworkers were the first to exploit the use of confocal fluorescence microscopy for the detection of single molecules (111, 41, 140, 84, 139, 40). They recorded the temporal correlation among the fluctuating light signals. Specifically, they measured the autocorrelation function in which the product of the intensity recorded at time t times that recorded at time $t + \delta t$ is integrated over the finite time interval Δt, normalized by the intensity and time of acquisition. If Δt is sufficiently large, the autocorrelation function is the average of the fluctuating amplitudes. In the limit of short integration times and with the assumption that the fluorescing target molecules are distributed randomly in the sample (Poisson statistics), the time-dependent part of the autocorrelation function is equal to the inverse number of molecules within the probe volume.

By this method, Eigen & Rigler (41) estimate that they are able to monitor concentrations down to 10^{-15} M.

Using this technique of fluorescence correlation spectroscopy, the hybridization dynamics of fluorescently tagged DNAs with their complements have been investigated (40), the conformational transitions of single molecules have been observed under conditions relevant for biological systems (68), and the interaction between tetramethylrhodamine-labeled alpha-bungarotoxin and detergent-solubilized nicotinic acetylcholine receptor has been characterized (109). In addition, fluorescence-correlation spectroscopy under confocal conditions has been used to examine singlet-triplet intersystem crossings in fluorophores (140, 139).

With improvements in the photon-counting system, it is possible to follow single-molecule fluorescence directly in real time. This approach has been applied by Nie et al (95, 96) to study dye molecules in solution as well as fluorescently labeled proteins and DNA fragments. With a low background level and a high excitation throughput (milliwatts), single rhodamine molecules have been detected with a signal-to-noise ratio of ~10–20 in 1 ms, approaching the theoretical limit set by fluorescence saturation (Figure 3). Real-time measurements at a speed of 500,000 data points per second yield single-molecule fluorescence records that show not only the actual transit time of a particular molecule, but they also contain characteristically long (~50 μs) and short (~4 μs) dark gaps (96). Random-walk simulations of single fluorescent molecules provide evidence that these long and short dark periods are mainly caused by boundary recrossing motions of a single molecule at the probe-volume periphery and by intersystem crossing into and out of the dark triplet state, respectively.

As the laser excitation power is increased, short-time deviations from Poisson statistics appear (28). They are caused by an "optical tweezer" effect in which the fluorescent molecule undergoes biased diffusion with a propensity to return to where the electric field is the highest, i.e. to the focus. This behavior results from the electric-dipole moment induced by the electric field in the molecule being irradiated, which causes an attractive interaction. At still higher laser powers, it is possible to trap and manipulate individual macromolecules, such as DNA.

Although its resolution is diffraction-limited, the far-field confocal approach has several important advantages: It provides an unlimited laser throughput (mW/W), a three-dimensional sectioning capability, and noninvasive detection. These features, together with high sensitivity and experimental simplicity, have made confocal fluorescence detection a powerful method. Single molecules have even been detected in solution by confocal two-photon fluorescence, which has a much lower absorption efficiency but a higher axial spatial resolution (83). Recently, compact diode lasers have also been used as light sources in confocal single-molecule detection (116). In the scanning mode, Macklin

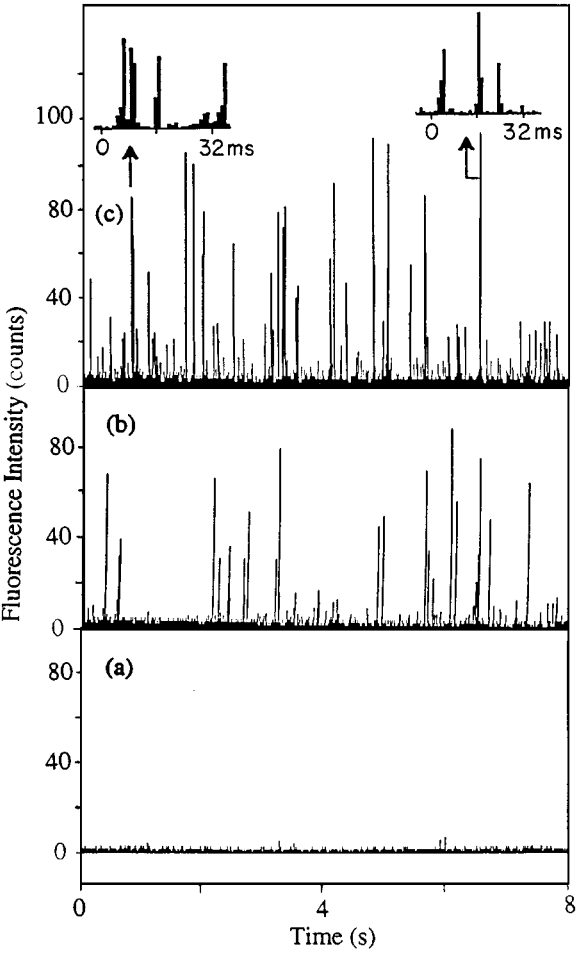

Figure 3 Real-time detection of single molecules in solution by confocal fluorescence microscopy. (See Reference 95.) (Reprinted with permission from *Science* 266:1018–21. Copyright 1994 American Association for the Advancement of Science)

et al (80) have employed confocal microscopy for imaging and time-resolved microscopy of single molecules at a polymer-air/liquid interface, and Lu & Xie (77) have extended this technique to study single-molecule dynamics at room temperature. Furthermore, this far-field approach appears well suited for probing fluorescence–resonance-energy transfer between a single donor and a single acceptor molecule (51).

Because a confocal laser beam probes a single point at a time, scanning is required to survey a large area of interest. Consequently, this method is well

suited for single-point spectroscopy and kinetic monitoring (on the millisecond timescale) but, it is intrinsically time-consuming if a large area needs to be searched. As discussed below, a confocal laser beam and wide-field optical illumination may be combined so that single molecules can be quickly located in a large area and the intense confocal beam can be positioned to that spot to initiate (or control) chemical reactions at the single-molecule level. Recent research has combined a confocal laser beam and wide-field illumination to make photochemical scissions on single chromosomal DNA molecules at any desired site.

Wide-Field Epi-Illumination

Wide-field epi-illumination is perhaps the most straightforward way to image single molecules at the diffraction limit. Although the background interference was a concern in the beginning, simple modifications and accessories can be made (or added) to an ordinary epifluorescence microscope to achieve single-molecule detection sensitivity. The optical excitation system generally consists of a laser source, defocusing optics, a high-performance (high-reflection and low fluorescence) dichroic beamsplitter, and an oil-immersion, low autofluorescence objective (NA $= \sim$1.3). Highly sensitive detection is achieved by using a cooled, back-thinned charge-coupled device (CCD) camera or an intensified CCD (ICCD). The imaged area is approximately 100 μm by 100 μm, depending on the illumination field and the CCD active area. With a slow-scan, high-resolution CCD, the sample molecules need to be immobilized because diffusion during the relatively long signal integration time (1–10 s) causes image blurring. Fluorescent dyes or fluorescently labeled biomolecules can be immobilized on a glass surface by covalent attachment, spontaneous nonspecific adsorption, or solvent evaporation.

Several groups have employed this method to study single molecules on dry surfaces and in aqueous media (e.g., biological buffers). Funatsu et al (46) have obtained epifluorescence images of single myosin molecules that are labeled with one or two copies of a fluorescent tag. The large protein molecules that are used appear to adsorb spontaneously on the glass slide surface. Also, Sase et al (115) reported the observation of individual tetramethylrhodamine fluorophores attached to actin filaments sliding on a surface coated with heavy meromyosins in dealing with molecular motors. For molecules that are not fixed on a surface but that can move slowly in a lipid membrane, Schmidt et al (118, 119) have measured the random two-dimensional motion of single fluorescence-labeled lipid molecules.

Using fluorescence photomicroscopy and digital video microscopy, the detection and tracking of individual low-density lipoproteins, each tagged with an average of approximately 36 fluorescent labels, have been demonstrated by

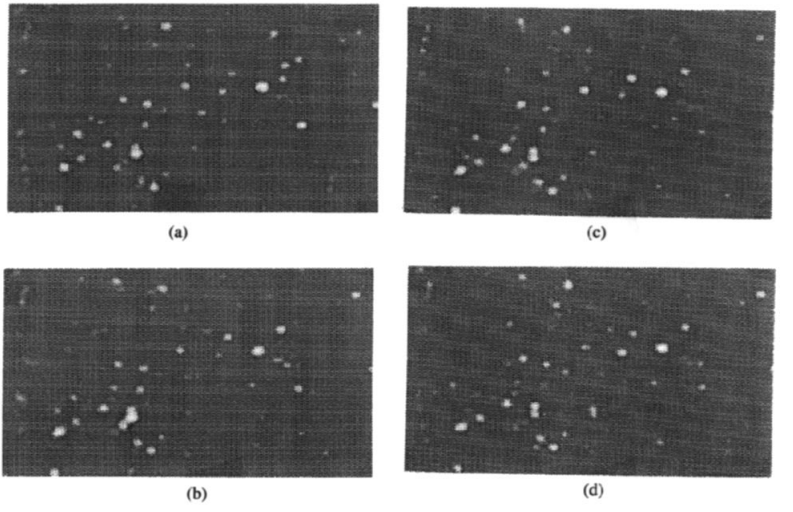

Figure 4 Consecutive video frames (*a* to *d*) of single phycoerythrin molecules obtained with an intensified charge-coupled device (CCD) camera. The video data acquisition system consists of a video–rate-intensified CCD camera, a high-speed (48 MHz) frame-grabber (Matrox), video-acquisition software (Microsoft), and digital image-processing software (Matrox). Digital movies of single molecules are acquired and stored as compressed image files on a high-capacity hard disk. Image areas shown: approximately $10 \times 20\ \mu m^2$. (From S Nie, unpublished data.)

Webb and coworkers (6, 7, 48). In a similar manner Georgiou et al (47) have monitored single influenza viruses labeled with approximately 100 fluorescent dye molecules.

With an intensified video-rate CCD camera, single molecules can be studied in real time at 30 video frames per second. Such digital movies of single molecules may be later analyzed frame by frame (33 ms each) on a computer. Figure 4 depicts a series of four consecutive video frames of individual B-phycoerythrin molecules. This capability will be important to real-time measurement of single-molecule dynamics and reactions, but the use of an image intensifier (either lens or fiber coupled) causes a blurring effect, and the recorded single-molecule images are noticeably less resolved than are those obtained with a cooled, back-thinned CCD camera.

So far, laser sources have been employed for wide-field imaging of single molecules; however, uniform illumination is difficult to achieve because of the defocusing optics used and the coherent optical interferences. For certain applications, high-power mercury lamps (100–200 W) commonly used in epifluorescence microscopy may replace lasers for single-molecule imaging. Laser-monochromatic excitation is essential to spectroscopy but not for

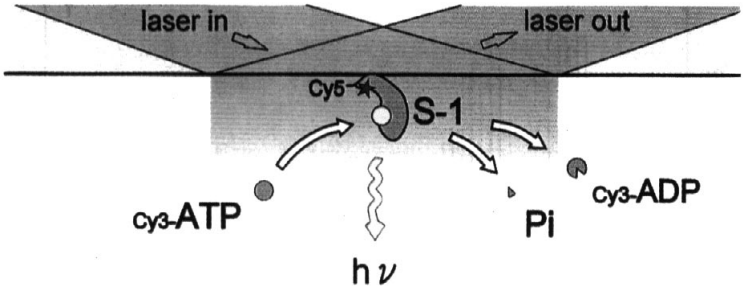

Figure 5 Visualization of individual ATP turnovers by single myosin molecules, by evanescent-wave laser excitation. (See figure 4*a*–4*c* in Reference 46.) (Reprinted with permission from *Nature* 374:555–59. Copyright 1995 MacMillan Magazines Ltd.)

imaging. A lamp-based light source is cheaper and should provide more uniform illumination at many wavelengths.

Evanescent Wave Excitation

Evanescent wave excitation is normally achieved by total internal reflection at the glass-liquid/air interface (138). At this interface the optical electromagnetic field does not abruptly drop to zero but decays exponentially into the liquid phase (or air). Molecules in a thin layer of ∼300 nm immediately next to this interface can still be excited by the rapidly decaying optical field (or evanescent wave).

The overall apparatus for evanescent-wave single-molecule imaging is similar to that for epifluorescence imaging except that the excitation beam is directed to the sample from the other side of the objective. In both techniques, the sensitivity that allows single-molecule detection arises from the small sample volume probed. Although the field of view may be wide, the sample thickness is extremely thin.

Hirschfeld (60) appears to be the first to have used evanescent wave excitation to detect single molecules, i.e. proteins labeled with 80–100 fluorescent tags. At the single-fluorophore level, this method has allowed the observation of fluorescently labeled myosin and kinesin molecules (46, 133) as well as individual ATP turnover reactions (Figure 5). Moerner and coworkers recently employed this technique for three-dimensional imaging of single molecules that are confined in nanometer-sized pores of polyacrylamide gels (35). Laser excitation in the evanescent field has also been used to study single molecules freely moving in solution at room temperature. With an intensified CCD camera, Yeung and coworkers have recently detected single molecules on the millisecond time scale as they rapidly move into and out of the evanescent field (149).

The evanescent method has certain advantages over epi-illumination. First, the background signal is generally lower because only a thin layer of ~300 nm is probed. Second, the dynamics of surface molecular events such as binding can be studied at the single-molecule level because fluorescence signals are detected only when a molecule moves into the evanescent field. Third, a confocal laser beam of the same or different wavelength can be brought to the sample via a high NA objective, which allows simultaneous wide-field imaging and confocal single-point measurement. One application of this dual wide-field and confocal approach is to perform spectroscopic measurements on a particular molecule or nanoparticle. Another application is to monitor chemical events that can be initiated (or controlled) by a high-intensity confocal laser beam.

Integrated Optical and AFM Studies

Although optical methods are sufficiently sensitive to detect single chromophore molecules, it is highly unlikely that they will be able to localize a single molecule to a spot comparable to its dimensions. The best spatial resolution achieved in NSOM is approximately 15 nm (16), still far from resolving individual molecules that are often smaller than 1 nm in size. Also, the light throughput in NSOM becomes vanishingly low when the aperture size is reduced to improve the spatial resolution. The ultimate measurement in single-molecule studies will need not only to detect a single chromophore but also to resolve the shape and size of a single molecule. At the present time, a number of criteria are used to ascertain whether the observed optical signals correspond to single molecules or molecular aggregates. When the size and shape of a single molecule can be determined simultaneously, it will be immediately clear whether optical detection of single molecules has been achieved.

One strategy toward meeting this goal is to integrate ultrasensitive fluorescence detection and atomic-force microscopy by using an inverted optical microscope. With lateral resolutions better than one nanometer and vertical resolutions in the Angstrom range, AFM is well suited for resolving individual molecules immobilized on glass or mica surfaces. It has been used to image a variety of small molecules and biomolecules (such as proteins and nucleic acids) in air and solution (24a, 55, 151). However, AFM normally does not provide chemical or molecular information, and it is difficult to differentiate target molecules from contaminants. In the integrated microscope, molecular information is obtained by optical imaging and spectroscopy, and AFM provides nanometer-resolved topographic information on the same molecule. Recent effort has led to the construction of such an integrated microscope (42). Preliminary results on double-stranded DNA molecules show correlated fluorescence images of intercalation dyes and AFM topography of double-stranded DNA at nanometer resolutions.

PROSPECTS

With the caveat that the most exciting and important insights are likely to be unforeseen, the rewards for potential success appear particularly high in the following areas of single-molecule detection. First, the instrumentation developed for the detection of a single molecule should have a wide range of applications in ultrasensitive diagnostics. Second, molecular dynamics and reactions may be examined at the single-molecule level to reveal rare conformational states or events or the time evolution of structures that are not detected by conventional, population-averaged measurements. Third, intrinsic single-molecule problems exist that can best be solved by single-molecule measurements, such as mechanical-force generation by molecular motors (myosin and kinesin molecules), the distribution of genes on chromosomal DNA, and the kinetics of polymerase enzymes moving on a DNA template during active replication or transcription. In the following, we discuss in more detail the emerging application areas of single-molecule detection.

Chemical Analysis and Microinstrumentation

Although ultrasensitive detection at the single-molecule level will benefit many research areas in chemical analysis such as microcolumn separations and immunoassays, a unique application appears to be the counting and sorting of target molecules in a complex molecular mixture of extremely low concentration ($<10^{-12}$ M). In contrast to bulk measurements, no standard samples will be needed and unwanted molecules can be discarded one at a time to eliminate interferences. The main challenges will be (*a*) to improve the detection efficiency so that all molecules in a sample will be counted, (*b*) to sort and enrich molecules with unique properties, and (*c*) to amplify the sorted materials for further characterization. Such a fluorescence-activated molecule sorter may resemble a flow cytometer, but the sorting speed is likely to be lower. As noted above, this limitation arises because a minimum number of photons is needed to overcome the statistical shot noise and a single molecule can emit fluorescence photons no faster than the lifetime-limited rate. An important potential application is to detect and separate rare "hit" molecules in a large chemical library composed of structurally similar polypeptides and oligonucleotides (30). In this experiment, a low concentration of fluorescently labeled target molecules is added to a combinatorial library. Only the hit molecules with the highest binding affinity will bind to the target. Single-molecule sorting would then be used to identify and separate the hit molecules that have bound to the fluorescent target.

Chen & Dovichi (27) have recently shown that more than 50% of the molecules injected into a capillary can be detected, but the sampling error becomes

significant when the total number of injected molecules falls below 3000. This work employed a sheath cuvette to hydrodynamically focus the sample stream and electrokinetic injection to introduce small volumes of solution into the capillary. A second approach to analyzing extremely dilute samples is based on fluorescence-correlation analysis of a large number of single-molecule detection events (41, 110). In this method, a confocal laser beam detects single molecules over a certain period of time, and the data are analyzed statistically to obtain the concentration and molecular profile of the sample.

In addition to analysis of ultradilute solutions, single-molecule detection methods fit well with microinstrumentation because of the small sample volumes used. This research area in advanced instrumentation seeks to fabricate and integrate complex analytical instruments on a single silicon or glass substrate. Microfabricated devices will be faster and more efficient and will require only very small amounts of materials for analysis. Recently, significant progress has been made in integrating capillary electrophoresis (CE) on a glass chip (CE on a chip) (64, 142, 58) and in developing high-density oligonucleotide microarrays (DNA chips) for medical diagnostics (126, 148, 105, 37, 24). Ultrasensitive detection and imaging methods should have an important role to play in the future development of micro- and nanodevices.

DNA and Protein-DNA Interactions

Because each chromosome is a single DNA molecule (the average size of a human chromosome is more than 100 million base pairs), many problems exist, such as DNA mapping and sequencing, that can be solved by single-molecule measurements. Keller and coworkers (65) were the first to put forward a novel method for rapid DNA sequencing based on single-molecule detection (Figure 6). In this method, DNA fragments (up to a few thousand base pairs) are enzymatically synthesized by using the four types of nucleotides that are labeled with different fluorescent tags. A single fragment is then attached to a microbead or a micropipette tip, which is placed in the center of a flow buffer stream. Exonuclease enzymes (5′-cutting or 3′-cutting) contained in the flow buffer digest the DNA fragment one nucleotide at a time. The cleaved nucleotides flow through a laser beam and are detected and identified, one by one, by their fluorescent tags. This approach potentially offers a much higher sequencing speed and the advantage of directly sequencing much larger DNA fragments. In addition to accurate spectroscopic identification of single fluorophores at high speed, significant experimental challenges may include the biochemical synthesis of DNA with fluorescent tags at all or a substantial portion of the bases, the attachment of only one DNA molecule to a microbead, the speed and continuity of base cleavage (modified bases) by exonuclease enzymes, and the need to detect all cleaved nucleotides. Keller and others

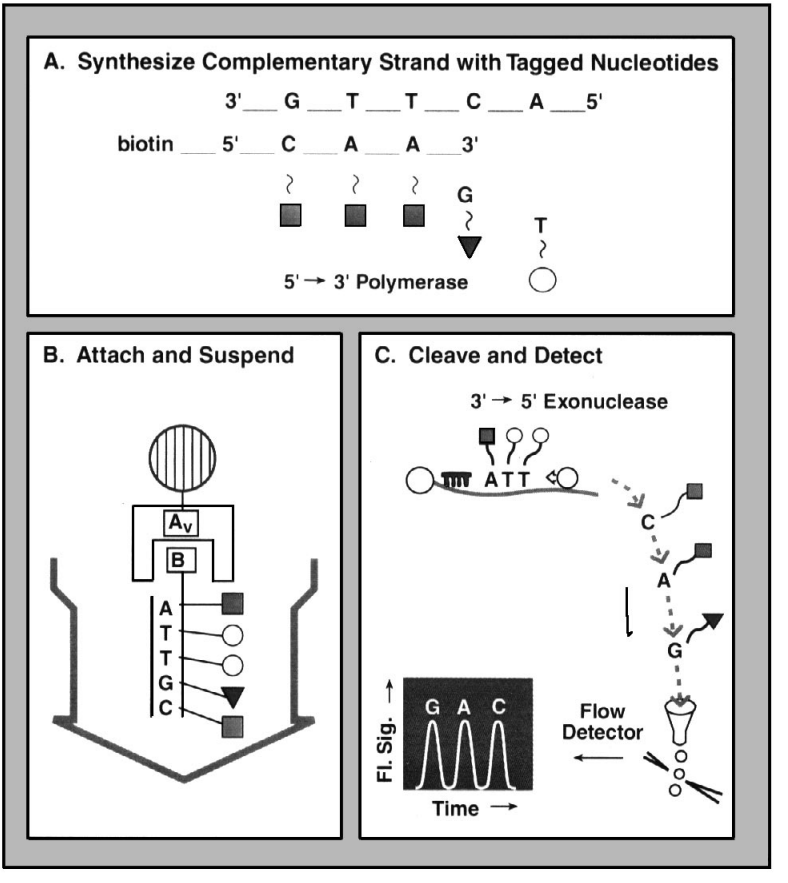

Figure 6 Method for rapid DNA sequencing based on single-molecule detection. (See figure 19 in Reference 67a.) (Reprinted with permission from *Applied Spectroscopy* 50:12A. 1996.)

(2, 117) have recently made significant progress in these difficult areas, and any major breakthrough will have an enormous potential payoff.

Although not representing strictly single-fluorophore detection, two single-molecule methods have been developed for rapid DNA fragment length analysis. In the first, DNA fragment lengths are determined by fluorescence intensities of single fragments that are stained by dye intercalation (25, 52). The DNA solution is introduced into a sheath flow cuvette, and individual DNA fragments are detected by a downstream laser beam. The smallest detectable fragment size is currently approximately 1000 base pairs. The second single-molecule approach also uses fluorescence intensities of intercalation dyes to determine

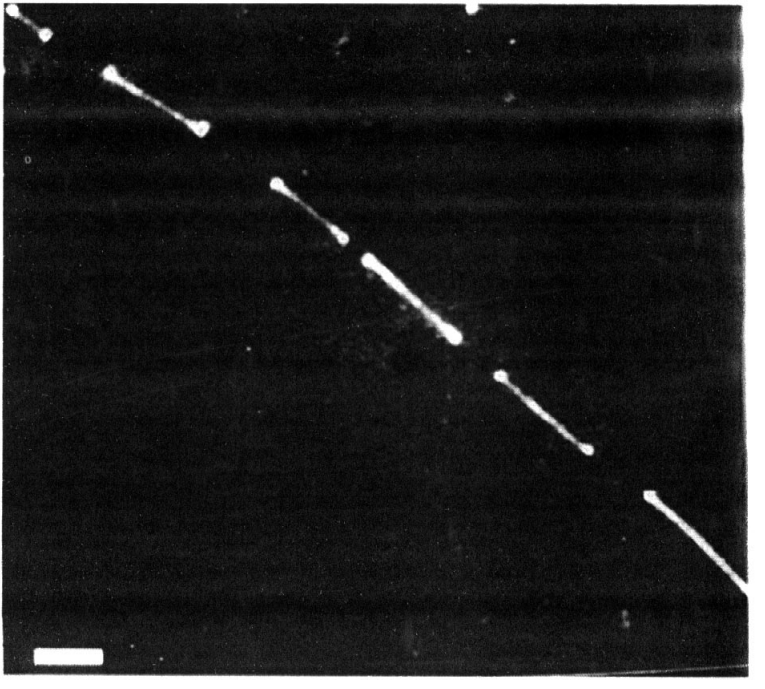

Figure 7 Optical mapping of single DNA molecules. (From Reference 114.) (Reprinted with permission from *Nature* 378:516. Copyright 1995 MacMillan Magazines Ltd.)

restriction-fragment lengths, but the DNA molecules are first stretched on a glass surface and then are cut with a restriction enzyme (137, 82). The cleaved sites relax to form visible gaps under the microscope, and the integrated fluorescence signals of individual fragments reflect the fragment lengths (Figure 7). An implicit assumption in both methods is that the intercalation dye has no sequence preference and is uniformly distributed along the DNA. This may not be always valid, because most of the intercalation dyes show some sequence preferences when an insufficient number of dye molecules are present to saturate the intercalation sites. An alternative way to determine fragment length is to use the gaps as distance markers. But these gaps are often several micrometers wide, which makes it difficult to determine accurately the original cutting site within a gap.

A number of methods have recently been developed to manipulate single DNA molecules (28, 107, 150, 31, 122, 128). These methods open many new opportunities for using single-molecule gene probes to target particular complementary genes or sequences on the human genome. One exciting research

area is likely the study of protein-DNA interactions at the single-molecule level. Real-time observation of the binding and movement of single-enzyme molecules on DNA may answer several important questions, such as how DNA-binding proteins find their specific sites in a sea of nonspecific sites and whether RNA polymerase molecules move in a continuous or an inchworm fashion during active transcription. These questions have been the subject of much interest and debate in biochemistry (53, 112, 135, 98).

The multi-enzyme assemblies involved in DNA replication and transcription exhibit even greater complexity. These molecular machines contain several protein subunits responsible for various functions such as ATPase activity, template strand separation, synthesis, and proofreading. An exciting breakthrough would be to observe directly the movement of a DNA replicase machine along a DNA template. Because DNA replication is much faster than transcription, collisions between DNA and RNA polymerases are inevitable when they use the same template DNA. Biochemical studies by Alberts and coworkers (75, 76) have provided much insight into the consequences of head-on and one-directional collisions. It would be fascinating indeed to observe a DNA polymerase and an RNA polymerase colliding on the same template.

Molecular Motors

Recent advances in in vitro motility assays have measured the force and motion of individual molecular motors (62, 131, 22, 127, 147). The new results on fluctuations of quantized motor force and velocity appear to support the conventional view that a motor molecule attaches to a polymer filament (e.g. actin), executes a power stroke, and then detaches. However, a key problem in the molecular mechanism of force generation still remains, i.e. how protein structural changes are related to mechanical-force generation. Optical detection of single fluorophores attached to motor proteins provides a powerful technique for addressing this problem. Fluorescence-polarization measurements at the single-molecule level will detect changes in the orientation of the light-chain region of the myosin head. Goldman and coworkers (61) have shown that the light-chain region of the myosin head tilts both during the imposed filament-sliding and during the subsequent quick-force recovery. It should thus be possible to measure the conformation changes of a single myosin molecule by polarized fluorescence and simultaneously measure the mechanical force of a single motor by optical trapping. Such correlated protein conformation and force studies can provide important insight into the dynamic process of protein structural changes and mechanical force generation.

Rapid progress is being made in this research area. At the single-fluorophore level, Funatsu et al (46) first reported the observation of individual fluorescently labeled myosin molecules and individual ATP-turnover reactions mediated by

the myosin ATPase activity. Vale and coworkers (133) showed that single kinesin molecules can be observed sliding along a microtubule. Sase et al (115) achieved real-time imaging of single fluorophores bound to actin filaments sliding on a meromyosin-coated surface. The present pace of research in this area is breathtaking.

Single-molecule methods using optical laser-trapping have also been developed to study the transcription of immobilized RNA polymerase molecules (150). The results reveal that a single *Escherichia coli* RNA polymerase molecule can generate a pulling force as large as 14 pN, which is enough to overcome the opposing force of transcription-induced DNA supercoiling ($\sim$6 pN).

Biomolecular Dynamics and Reactions

It is now experimentally feasible to observe single-molecule events, such as orientational motions, spectral shifts caused by the environment, and chemical changes, but the question that needs to be asked is: What new information can be obtained from such measurements? According to classical statistical mechanics, the population-averaged behavior of a large ensemble of molecules should be equal to the time-averaged behavior of a single molecule. For simple chemical systems, single-molecule studies would perhaps provide little or no new information. But for complex chemical or biochemical systems such as enzymes and nucleic acids, there could be rare, discrete conformational states that play an important role in function but that cannot be detected in population-averaged measurements. As an example, consider a growing cell culture that consists of a large population of individual cells. These cells are going through the various steps of the cell cycle such as interphase, metaphase, and mitosis. If only a few cells rapidly undergo mitosis at any given time, this phenomenon can best be revealed by measurement at the single-cell level and not by population-averaged analysis. From a more rigorous theoretical point of view, Wang & Wolynes (136) recently showed that single-molecule measurements in complex reaction systems can provide unique dynamical information such as non-Poissonian statistics and reaction intermittency, which are not readily obtainable from ensemble-averaged experiments.

Single-molecule dynamic studies have also revealed surprising phenomena such as sudden spectral changes (spectral diffusion) that occur at the single-molecule level, both in low-temperature solids (4) and at room temperature (77). The spectral changes are believed to arise from environmental perturbations, thermally activated or photoinduced changes of molecular conformations and orientations. For single-molecule dynamics at shorter time scales (nanosecond to picosecond), statistical methods such as photon correlation will be needed because a single molecule cannot emit enough photons for detection in such a short time period. At the present time, most single-molecule studies have been

carried out on the 0.01 to 100-s time scale. Nonetheless, many biological problems involving the kinetics of structural change appear to be within reach of current measurement capabilities at the single-molecule level.

An interesting research area is to examine the reaction activities of individual enzyme molecules, which may reveal whether enzymes can exist in different conformational states. If these conformations are stable and do not rapidly interconvert at room temperature, single-enzyme molecules in each conformational state should have a different catalytic rate. Rothman (112a) appears to be the first to have demonstrated that the activity of a single-enzyme molecule can be measured by detecting the accumulated product in a microdroplet. In this experiment, a diluted solution of galactosidase is dispersed into micrometer-sized liquid droplets so that each droplet contains mostly zero- or one-enzyme molecules. With a fluorogenic substrate, a single-enzyme molecule generates a large number of fluorescent-product molecules after a long period (hours) of incubation. The product molecules are all trapped in a single microdroplet and can be measured by fluorescence microscopy. Recently, Xue & Yeung (146) have employed a capillary tube to confine single molecules of lactate dehydrogenase in well-separated locations along the capillary. Because diffusion is a macroscopically inefficient process, each enzyme molecule generates a separate product zone after an inoculation period of 20 min to 1 h. Then, an electrokinetic or mechanical force is used to flush out all the product zones for detection. By using the same approach, Dovichi and coworkers (33) have examined the death of an enzyme by measuring the activity of individual alkaline phosphatase molecules with and without heat treatment.

These time-averaged measurements may be extended to study conformational changes of a single-enzyme molecule in real time. A large number of enzymes undergo structural changes associated with substrate (ligand) binding, bond cleavage (catalysis), and product release. For example, many restriction enzymes are able to search and bind at a specific DNA site but cannot cut the DNA in the absence of Mg^{2+}. The binding of Mg^{2+} ions is believed to induce a significant conformational change that leads to cleavage of the phosphodiester bond (134, 73). Such bond cleavages occur on the millisecond to second time scales, and single molecule measurements could reveal structural changes from substrate-binding to cleavage and product release. Such detailed information is difficult or impossible to obtain by other methods.

Another possibility is to apply single-molecule detection to study key intermediates involved in protein folding. This approach might solve the timing problem encountered in macroscopic measurements, which requires that all protein molecules fold in a synchronized fashion (103, 5). In this experiment, fluorophores would be attached to the protein at certain amino acid residues, and structural information might be obtained from fluorescence-polarization

measurements (which determines the orientation of the fluorophore dipole versus the laser polarization), fluorescence-resonance–energy transfer (which measures the donor-acceptor distance and orientation), and environmental-fluorescence quenching. Real-time monitoring of biological processes may turn out to be one of the most significant payoffs from this newly emerging capability to detect single molecules in biologically relevant environments.

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