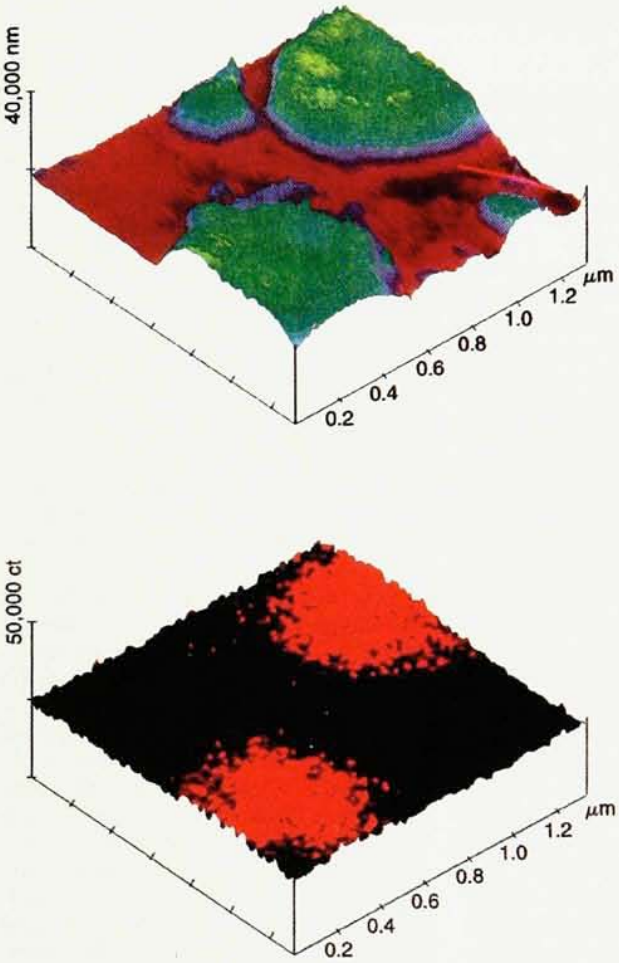
NEAR-FIELD OPTICAL MICROSCOPES TAKE A CLOSE LOOK AT INDIVIDUAL MOLECULES

Researchers have recently looked at individual dye molecules with a resolution of about 50 nanometers, about six times finer than the diffraction limit for the 600-nm light they are using.[1] They achieved these unprecedented optical resolutions and the sensitivity needed for single-molecule detection with near-field scanning optical microscopy. This technique surpasses the diffraction limit by placing a subwavelength-sized optical element within tens of nanometers of the sample: Traveling over such short distances, light has no opportunity to diffract and take on its asymptotic far-field characteristics. And unlike other high-resolution imaging techniques, such as electron microscopy and scanning tunneling microscopy, the technique allows the study of fluorescence in molecules' natural biochemical environments, such as the biological membranes responsible for photosynthesis.[2,3] (See the figure at right.)

The pioneers of single-molecule detection with near-field microscopy are Eric Betzig and Robert Chichester[1] (AT&T Bell Labs), who used an innovative fiber optic scanner tip devised by Betzig, Jay Trautman and coworkers[4] in 1991. In addition to determining the positions of individual molecules to within 5–15 nm (or $\lambda/120$ to $\lambda/40$) Betzig and Chichester deduced the orientation of each molecule's optically active dipole. (See the figure on page 18.) The technique could have numerous applications, including the study of how molecules diffuse across cell membranes, the monitoring of protein conformational changes and the study of biochemical reactions at the molecular level in their native environments.

Trautman (also at Bell Labs) has gone beyond Betzig and Chichester's work, using near-field microscopy to study the spectra of individual fluorescing molecules,[5] while separate groups at Los Alamos National Laboratory[6] and the Pacific Northwest Laboratory[2,3] have done time-resolved studies.

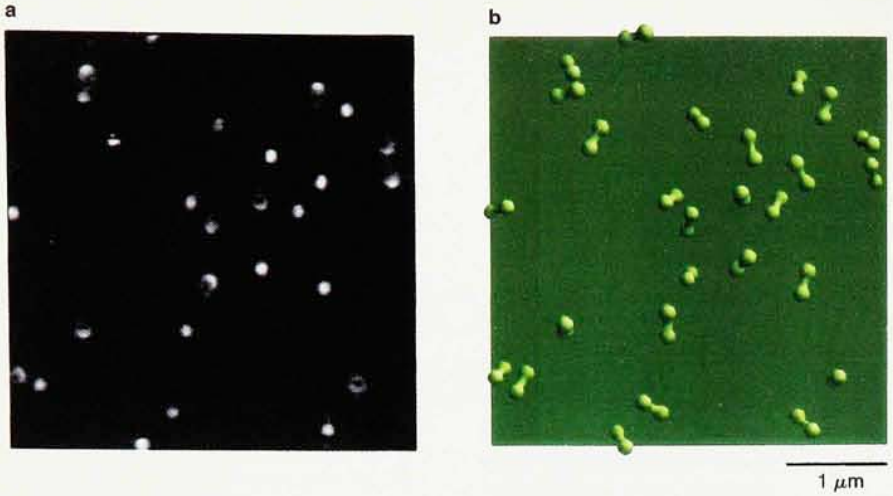


Fragments of photosynthetic membrane on a mica surface studied simultaneously with a shear-force scan (top) and a near-field optical scan (bottom) using the same tip. The shear-force scan shows four fragments (green), each about 7 nm thick, consistent with their being single bilayer membranes. The fluorescence image, with a resolution of 90 nm (about $\lambda/7$), suggests that only the two larger fragments (red) contain light-harvesting complex II, a group of proteins to which the fluorescing chlorophyll *a* molecules are bound. (From ref. 2; © 1994 American Chemical Society.)

The ultimate close-up

The $\lambda/2$ diffraction limit applies to everything from astronomical telescopes to conventional optical microscopes and arises from the way that light propagates over distances greater than about a wavelength (the "far field"). If a feature smaller than $\lambda/2$ emits light, its details are lost after the light travels on the order of a wavelength. This can be understood from the perspective of Fourier analysis: A propagating electromagnetic wave can't carry information of much higher spatial frequency than $1/\lambda$—the higher spatial frequencies decay exponentially. With a far-field system the only way to improve the resolution is to reduce the wavelength, either by using higher-energy photons such as x rays or by switching to different imaging particles, such as electrons.

Nevertheless, as long ago as 1928 E. H. Synge proposed a near-field scanning optical system that gets around the diffraction limit by taking images that depend on the "near field," which closely mirrors the subwavelength features producing the light.[7,8] In the simplest system, one



Individual carbocyanine dye molecules scanned with a resolution of about 50 nm ($\lambda/12$). Eric Betzig and Robert Chichester used the actual spot shapes (left) to deduce the orientation of the optically active dipole in each molecule (as rendered at right). (From ref. 1.)

places a screen with a subwavelength-sized hole less than a wavelength above the object to be imaged. One then shines light through the hole and moves the screen to scan across the whole object. After it passes through or reflects off the object, the light can be collected by a conventional microscope. Features smaller than a wavelength can be resolved because the signal depends only on the subwavelength region near the aperture. An experimental system of this sort was demonstrated in 1972 by Eric Ash and coworkers,[9] who used 3-cm-wavelength microwaves and achieved resolutions of about $\lambda/60$.

An aperture at the tip of a cone is more convenient for scanning within tens of nanometers of a surface, which may be uneven. The first scanners of this type used glass pipettes pulled almost to a point or quartz rods etched to a point and coated on the outside with aluminum.[8] Various groups constructed apertures smaller than 20 nm across in this way.

A basic problem with all such probes is that much intensity is lost: Light passing through a region of subwavelength cross section decreases in intensity exponentially, because there are no propagating modes in such a region. (This is analogous to the exponential decay of a wavefunction tunneling through a classically forbidden region.) The pipette and quartz-tube probes suffer from further losses due to reflections and absorption into the probe walls over the entire tapering region. In 1991 Betzig, Trautman and coworkers developed an optical fiber probe that achieves intensities four orders of magnitude greater than those in previous designs.[4] They make such a probe by heating a single-mode optical fiber with a CO_2 laser and pulling it to an aperture as small as 20 nm across. The sides of the fiber are evaporatively coated on the outside with aluminum. If the taper is sufficiently gradual ("adiabatic"), the light remains bound to the core of the fiber until just a few microns from the tip, dramatically decreasing the losses and thus making single-molecule detection by near-field microscopy possible.

For apertures smaller than about 20 nm, leakage of the electromagnetic field into the aluminum cladding starts to play a significant role, limiting the ultimate resolution achievable to about 12 nm. In addition, as aperture size is reduced, the signal is tremendously attenuated, forcing a trade-off between sensitivity and resolution.

The distance between the tip and the sample is carefully controlled by doing a shear-force scan simultaneously with the optical scan, using the same tip for both. At each point of a shear-force scan, the tip is dithered laterally by a few nanometers at its resonant frequency (about 30 kHz). Frictional forces damp the oscillation when the tip is within tens of nanometers of the surface. Controlling the height of the tip to maintain a constant damping keeps the tip at a fixed distance from the sample. This technique was devised independently by Betzig and Mehdi Vaez-Iravani (Rochester Institute of Technology).[8]

Substantially different, apertureless near-field optical techniques have also been devised. Kumar Wickramasinghe (IBM, Yorktown Heights, New York) reported one such method at a NATO Advanced Study Institute in Schluchsee, Germany, in March.[10] In his device a conventional microscope focuses a spot of light through a microscope cover slip onto a 2-nm-radius, solid silicon tip. Light scatters back from the tip $\pi/2$ out of phase with light reflected from the rear face of the cover slip. The resulting small phase shift in the total reflected light is detected relative to a second spot of light focused away from the silicon tip.

By considering the smallest detectable phase shift and how rapidly the scattered intensity from the silicon tip drops off with decreasing tip size, Wickramasinghe predicts resolutions as small as 2 Å should be feasible with his technique. So far he reports seeing 30-Å features on the cover slip's surface, which he believes are small variations in the refractive index. He hopes to apply the technique to study very thin biological membranes. This apertureless design hasn't been used for studies of molecules.

Optics and the single molecule

Betzig and Chichester used their fiber-probe microscope to see single molecules.[1] (See the figure above.) In their mode of operation light passes through a 100-nm aperture to illuminate the sample, and a standard optical microscope on the other side of the sample detects the fluorescence. They have studied a number of different dye molecules, all of the type that biologists typically use as fluorescent probes. All have molecular weights in the range of a few hundred to a thousand and are about 10 Å across. The researchers prepare the samples by drying a dilute solution of the dye on a microscope slide coated with a thin (about 30 nm) polymer film.

Because the optically excited volume is about 1000 times smaller than in diffraction-limited systems, the background is reduced by a similar factor. The high sensitivity this allows is significant: It permits detection of molecules, such as carbocyanine, that have a single chromophore (an optically active group of atoms). Betzig points out that if one wishes to label, for example, specific cell features with a molecule and then study them with a near-field microscope,

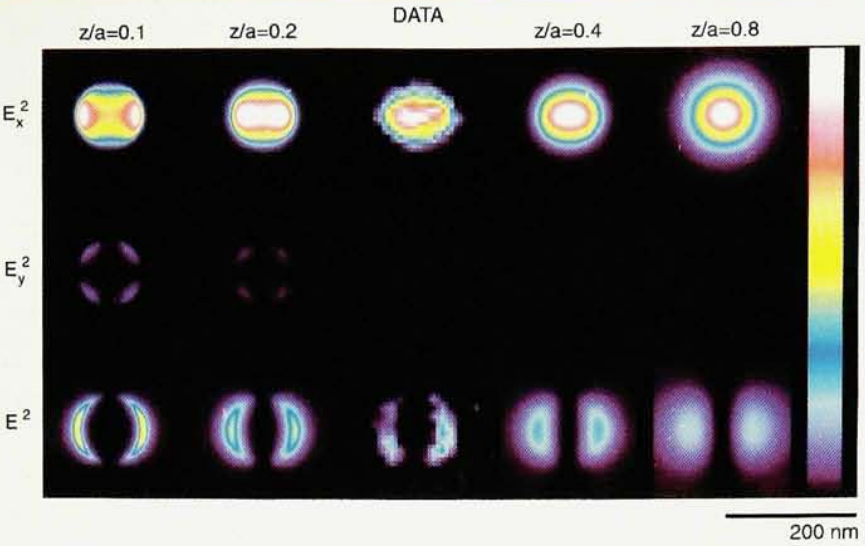
one doesn't want to be restricted to using "big Christmas tree ornaments" with many chromophores. "You don't want to use a bowling ball to label a flea," he quips.

The spots of light corresponding to individual molecules were resolved to about a twelfth of a wavelength, or 50 nm. Each molecule's position was determined to within about $\lambda/40$ in the plane of the scan and to within $\lambda/120$ along the axis of the probe. The spots have a variety of forms, including symmetric peaks, rings, arcs and double arcs. (See the left-hand part of the figure on page 18.) The particular shape and intensity depend on the orientation of the dipole $\mathbf{p}$ responsible for the transition and on the polarization of the light, since the absorption rate is proportional to $|\mathbf{p}\cdot\mathbf{E}|^2$. (The excitation light can be polarized, and the detected light can be analyzed with a linear polarizer.) The figure at right shows some examples. Another curiosity of the near field is the presence of a longitudinal mode in addition to the usual two transverse polarizations. By comparing predictions of a simple model with the observed intensity profiles for different polarizations, Betzig and Chichester deduce the dipole orientations rendered in the right-hand part of the figure on page 18.

Conversely, the intensity profile for each molecule provides information about the electric field distribution in the region of the microscope's aperture: In a sense one is doing a scan of the aperture region using an individual molecule as an ultrasmall near-field probe! The results of this "scan" closely agreed with the simplest model of an aperture's near field, which dates back to a 1944 paper by Hans Bethe, who assumed an infinitesimally thin, perfectly conducting sheet of infinite extent with an aperture much smaller than the light wavelength. Betzig says that "considering the poor conductivity of aluminum at optical frequencies and considering the tapered geometry of our probe, it's astonishing to see how well Bethe's simplified model worked. An often-claimed advantage of near-field optical microscopy over other techniques is that we can fall back on 300 years of contrast mechanisms in optical microscopy. Knowledge of the precise near-field geometry at a tip tells us how good that claim is."

Spectra and lifetimes

Trautman and his group at AT&T have studied[5] the spectra of individual dye molecules similar to those scanned by Betzig. They did the work as a test run of their instrument, which they hope to use to do spectroscopy of silicon clusters of about 40 Å diameter. (They are interested in learning how quantum confinement within the clusters affects which transitions are allowed.)



Electric field components (squared) predicted by a simple model at various distances z from a subwavelength aperture of diameter a. The data in the middle are the signals from two molecules deduced to be oriented nearly parallel to the x axis (top row) and the (longitudinal) z axis (bottom row), respectively. (From ref. 1.)

Their microscope was set up in essentially the same configuration as Betzig's. After scanning to locate individual molecules, they positioned their tip over a selected well-isolated molecule. Instead of directly measuring the intensity of the fluorescence from that molecule, they passed the emission through a spectrometer before detecting it with a CCD camera.

As expected, the molecules exhibited a variety of spectra as a result of differences in their local environments. Far-field experiments conducted at liquid helium temperatures have previously allowed spectral lines of individual molecules to be discerned in the wings of the overall distribution. In Trautman's near-field biomolecule work the spectral shifts are readily seen at room temperature, holding out the promise of spectroscopy of single molecules in their natural biochemical environments. In addition there was variation in the shapes of individual spectra, which relates to how electronic excitations couple to molecular vibrations, and some individual-molecule spectra changed over time.

Trautman and his coworkers also observed that for certain molecules the process of photobleaching was greatly inhibited. Photobleaching occurs when a molecule undergoes an irreversible structural change that renders it unable to fluoresce. The biomolecules studied by Trautman typically photobleach after about 10^6 cycles of excitation and photon emission, but he noticed that there were occasional molecules that lasted for more like 10^9 transitions. In his present setup, with the biomolecules embedded in a random polymer film, it is impossible to determine the specific local environment that inhibits photobleaching.

At the Pacific Northwest Laboratory, Sunney Xie, Robert Dunn and coworkers are using a fiber-tip microscope to study the thylakoid membranes, in which photosynthetic reactions take place in plants.[2,3] Embedded within these membranes are complexes of proteins such as light-harvesting complex II and photosystem I, which collect sunlight and transform its energy through a sequence of reactions. (See the article by Graham R. Fleming and Rienk van Grondelle in PHYSICS TODAY, February, page 48.) The structure of LHC II was recently solved, but little is known about its orientation and distribution in the membrane, which are equally important for completely understanding photosynthetic processes. Previous studies of the fluorescence properties of these proteins involved washing the molecules out of the membrane with a detergent, which can damage the proteins and in any case removes them from their usual environment. The spatial distributions of molecules such as LHC II have been studied with electron microscopy, but this involves freeze-fracturing the sample, making a metal replica, washing it with acid and so on, and provides no spectroscopic information.

Xie's group took membrane samples prepared from a mutant strain of green

algae by Laurens Mets (of the University of Chicago's department of molecular genetics) and spread them on a mica surface. The researchers then did a simultaneous shear-force scan and near-field optical scan. The former provided a topographic image of the 7-nm-thick membrane fragments, while the latter revealed which regions were fluorescing. (See the figure on page 17.) Xie and his group have also performed separate scans of allophycocyanin and sulforhodamine 101 molecules dispersed on glass, with sensitivity and resolution sufficient to detect individual molecules. (Allophycocyanin is a six-chromophore light-harvesting protein that occurs in red algae, and sulforhodamine 101 is a single-chromophore laser dye.) After doing a scan they positioned their microscope tip over a bright region of the membrane or over a single sulforhodamine 101 molecule and took time-correlated photon counts, which revealed the fluorescence lifetime of that region or molecule. The field of fluorescence-lifetime imaging is very active, but most work uses confocal microscopy, which is diffraction limited.

Ultimately Xie's goal is to map the photosynthetic membrane spectroscopically and to study the many reactions involved in photosynthesis at the single-molecule level, including electron transfer, proton transfer, energy transfer and protein conformational changes.

His group has also made a movie of a 2-micron-square field of sulforhodamine 101 molecules fluorescing after being hit by a pulse of laser light. (Strictly speaking, the movie shows the temporal probability distribution of the fluorescence, since it is mapped out with a series of laser pulses.) Hollywood epics are not under challenge, however: The movie's 64 frames represent 6 nanoseconds of action.

In addition, Xie's group has studied the effect of the microscope tip itself on fluorescence. When the edge of the tip was close to a fluorescing molecule the aluminum in the tip quenched the fluorescence, reducing the lifetime in one experiment from about 3 nsec to about 1 nsec. When the center of the tip was over the molecule, however, the lifetime was close to the bulk lifetime (measured for a collection of molecules using far-field light), suggesting that the tip is not greatly influencing the results in that case.

Patrick Ambrose and collaborators at Los Alamos have also done time-resolved studies of single-chromophore molecules.[6] They saw individual molecules' fluorescence blinking out due to photobleaching and they also observed the effect of the tip on fluorescence lifetimes. In their experiments, using the dye rhodamine 6G on silica, they saw both lengthening and shortening of the fluorescence lifetime according to whether the molecule was centered under the tip or was near the edge of the tip, respectively. Ambrose attributes this effect to phenomena observed in the 1960s with molecular layers close to metal surfaces: As a molecule approaches the surface, radiation reflected from the metal suppresses or enhances the spontaneous emission rate, depending on the distance from the metal. Within 50 nm of the metal, direct energy transfer from the molecule to the metal can occur, quenching the fluorescence.

In the immediate future a rapidly growing body of researchers will likely be using fiber-probe scanners based on Betzig's design for increasingly detailed studies of small, delicate systems such as photosynthetic membranes and silicon clusters. When asked about his own future plans, Betzig only hints suggestively about the prospects for a near-field optical microscope with a molecule-sized tip. He and Trautman took out a general patent on such a concept a couple of years ago.

—GRAHAM P. COLLINS

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Reaching for the Top

The uninitiated would have had difficulty explaining the excited crowd of physicists filling Fermilab's auditorium on 26 April—especially since the presenters were careful to disclaim an actual discovery. Yet even though skepticism remained the watchword, the excitement of even the most ardent critics was palpable. The multinational 440-member Collider Detector Facility group at Fermilab's Tevatron may have glimpsed the top quark—the long-sought partner of the bottom quark—at 174 ± 17 GeV. This would make the t quark the heaviest fundamental particle yet seen. Indeed, because the observed mass is so close to the mass at which electroweak unification becomes manifest, many speculate that the top quark may hold the secrets to the primordial breaking of vacuum symmetry that is thought to have given masses to elementary particles.

CDF spokesmen William Carithers and Melvyn Shochet discussed the t-quark candidates distilled from 10^{12} 1.8-GeV $p\bar{p}$ interactions that took place during the ten-month run beginning August 1992. Three analyses looking for different final states of t quarks decaying into a W boson and a b quark found a total of 12 candidates. Three of those events were seen independently by two of the analyses, adding to their credibility. Depending on the background estimates used the statistical significance of the observation lay between 2.8 and 3.5 standard deviations. The t-quark hypothesis is also supported by most subsequent analyses of the top sample. On the other hand, spokesmen for CDF and the other Fermilab collider experiment, D0 (which reports no significant signal, despite similar sensitivity), pointed out that limited statistics, inconsistencies in some backgrounds and event complexity (many events produce more than 100 charged particles) suggest caution. The CDF analyses are so complex that the paper submitted to *Physical Review D* on 22 April was 153 pages long. As CDF physicist, Paul Tipton said, "If we'd had twice the signal or half the signal, it would have been a much shorter paper." The CDF and D0 teams hope that the 1994–95 data run will help them make an intriguing observation believable. —RAY LADBURY