97 Multiphoton Excitation Fluorescence Microscopy

INTRODUCTION

Although eminently useful for most localization applications, the technique of fluorescence microscopy suffers from serious limitations; notably, murky images of thick specimens and phototoxicity. The murkiness arises from photons originating from structures above and below the plane of focus, and the phototoxicity derives from nonproductive excitation of the fluorophore.

A high numerical aperture objective lens provides an in-focus image of a thin (submicron) plane within the specimen. However, the information from this optical section is often obscured by out-of-focus photons from fluorescent structures above and below the plane of focus. As discussed in the previous chapter, confocal imaging (Brakenhoff 1985; White et al. 1987; Pawley 1995) and computer deconvolution (Agard et al. 1989) are two techniques that have been developed to combat this problem.

These techniques can be effective in virtually eliminating out-of-focus photons; however, they do nothing to alleviate the other major problem, phototoxicity. When a fluorophore is excited, there is a probability that, instead of decaying to a singlet state and emitting a fluorescence photon, intersystem crossing will occur to a triplet state. These relatively long-lived states are very reactive and can damage living cells and bleach the fluorophore. One of the most significant damage mechanisms is the generation of highly reactive singlet oxygens from triplet states (Widengren and Rigler, 1996). When a specimen is being observed in a fluorescence microscope, fluorophores are excited throughout the bulk of the sample, even though only one focal plane is being observed at any time. Most of the phototoxic load in a live specimen therefore comes from regions away from the thin focal plane being observed.

Multiphoton Imaging: Principles of Operation

The basic principle of multiphoton excitation had its origins in the theoretical work of Maria Göppert-Mayer (1931). She proposed that, given a sufficiently high photon density, two or more photons could cooperatively excite an electronic transition that would normally require a single photon with twice as much (or more) energy. This means that in these situations, long-wavelength excitation radiation can be used to excite fluorescence of a shorter wavelength. The practical demonstration of this effect had to await the development of lasers in the 1960s when two-photon fluorescence excitation was demonstrated by Kaiser and Garrett (1961) and three-photon excitation by Singh and Bradley (1964). Multiphoton spectroscopy is now an active, if somewhat obscure, field.

In a groundbreaking paper, Denk et al. (1990) demonstrated that two-photon excitation could be used as a technique for optical sectioning in fluorescence microscopy. They used a modified laser-scanning confocal microscope with an ultrafast, pulsed laser source for fluorochrome excitation. The excitation wavelength was

beam intensity falls off as the square of the distance from the plane of focus, and as a consequence, the excitation rate of the fluorophore decreases as the fourth power of the distance from the focal plane. In other words, the local beam intensity decrease is proportional to the diameter of the cone of excitation light squared as it expands above and below the plane of focus and, as a result, the excitation rate of the fluorophore decreases by the fourth power of that diameter. Thus, excitation of the fluorophore is confined to a relatively thin optical section. The emitted photons originate only from the one optical section, so there is no longer a need to descan and reimage the spot in order to employ a discriminating aperture (pinhole). The emission signal can be collected directly from the microscope lens by a photon detector such as a photomultiplier tube (PMT) placed near the back aperture of the lens. In this way, the imaging pathway of a multiphoton microscope is similar to that of a scanning electron microscope.

As discussed above, the multiphoton process is peak-power-dependent. In order to deliver high-intensity light required for multiphoton imaging without the deleterious effects of saturation, light is introduced to the sample in ultrashort pulses. The average fluorophore has a fluorescent lifetime of a few nanoseconds. To avoid saturation, it is desirable to allow the fluorophore sufficient time between excitation events to return to its ground state in order to be able to absorb another photon packet. Mode-locked lasers have made it possible to generate pulses of femtosecond duration with repetition rates of 80 to 120 MHz. The short pulsewidth provides the highest instantaneous peak power for a given mean power, whereas the 100 MHz repetition rates maximize the signal that may be obtained from the fluorophore.

Pulses of light traveling through glass and dielectric coatings of a microscope are spread due to group velocity dispersion (GVD; blue wavelengths are slowed down, relative to red wavelengths, while passing through optical components). While the pulse's total energy remains unchanged, the peak intensity is lowered. It is possible to compensate for the pulse broadening by "pre-chirping" the beam with a series of prisms or gratings (Fork et al. 1984). The gratings effectively give the blue light a head start so that all the different wavelengths simultaneously arrive at the sample and the narrow pulse is preserved. Pulses of too short a duration should also be avoided.

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Advantages of Multiphoton Imaging

In confocal imaging, for each point of irradiation there is a conical volume expanding above and below the plane of focus that constitutes the excitation volume. Both photobleaching and phototoxic by-products are generated within this volume wherever excitation occurs. It is therefore possible to photobleach fluorophore in a plane before it has been imaged, which is problematic when attempting to collect three-dimensional data sets. In addition, the overall concentration of phototoxic by-products of excitation, such as singlet oxygens and free radicals, is in excess of what might be expected from imaging a single plane. Multiphoton excitation is an elegant imaging solution for generating images of individual optical sections without generating interfering information from other planes of focus. Since excitation is restricted to the volume close to the geometric focus of the light, photobleaching and photodamage are also restricted. The reduced volume of photobleaching means that a three-dimensional data set may be collected for the same integrated excitation dose as a single focal plane in a confocal microscope. It is likely that lowering the

overall rate of phototoxic by-product generation is a major factor in increased viability of samples viewed by multiphoton excitation. It is also possible that the intrinsic scavenging mechanisms of living samples, such as superoxide dismutase and catalase, can cope with the lower level of production of phototoxic compounds of multiphoton excitation so that there is little or no buildup that would cause photodamage. Phototoxic damage may occur when these mechanisms become swamped.

Conventional fluorescence excitation results from a fluorophore absorbing light energy of one wavelength, achieving an excited state, and then releasing energy in the form of light at a less energetic, longer wavelength. To detect the emitted fluorescent signal, it is necessary to use imaging filters that separate this signal from the excitation light. The shift in wavelength between light of excitation and emission (Stokes shift) is often only a few tens of nanometers, and there is generally considerable overlap between the emission and excitation spectra. Consequently, the discriminating filters disregard much of the light emitted. In contrast, multiphoton imaging can collect all the emission from an excitation event because the wavelength separation between the excitation wavelength and the spectrum of emitted light from a fluorophore is very large.

Multiphoton excitation has been particularly successful at imaging deep within thick, heavily stained, light-scattering samples (e.g., brain, skin, cheese). Useful images have been obtained at two or more times the depth attainable by conventional confocal microscopy (Fig. 97.2). There are several possible reasons for this: (1) The excitation beam in single photon imaging may be attenuated by fluorophore absorption above the plane of focus. Highly fluorescent structures in planes above the one being imaged could significantly absorb the excitation beam, thereby blocking the excitation beam from reaching fluorophore below. In the deeper sections, discrete regions under the highly fluorescent structures would then be shadowed and remain undetected. (2) The excitation beam may be scattered by the specimen, thereby reducing the amount of excitation light that reaches the focal plane. The shorter wavelengths of light such as those used for confocal imaging are scattered more strongly than the longer wavelengths used for multiphoton imaging, resulting in a loss of excitation intensity at the point of focus, especially at levels deep within a sample. (3) The visible wavelength photons emitted from the fluorophore may also be scattered by the specimen. The photons from a spot excited by conventional confocal imaging that suffer scattering do not contribute to the final image because they become indistinguishable from emitted light from planes that are out of focus and are prevented from reaching the detector by the confocal pinhole. Multiphoton excitation does not generate fluorescence out of the plane of focus, so there is no need to use a confocal pinhole. Therefore, all photons emanating from the point of excitation, scattered or not, that are incident on the face of the detector can contribute to the multiphoton image.

FIGURE 97.2
Demonstration of increased depth of sectioning with multiple photon excitation imaging. A hamster brain slice stained with FM 4-64 was imaged by confocal microscopy (*left*) and by multiple photon excitation imaging (*right*). The images shown were taken at 20 μm intervals. The confocal images become murky and the structures lose definition at depths of 20–30 μm, whereas definition of structure in the multiple photon images remains at depths of at least 70 μm. (Photo provided by J. White and V. Centonze, University of Wisconsin.)

chosen to be around twice the wavelength of the absorption peak of the fluorophore. There was sufficient peak power in the pulses such that, within the focal volume of the objective lens, there was enough photon density to produce sufficient two-photon events for imaging. The use of short pulses (around 100 femtosec) allows high peak powers to be generated so as to attain the very high photon densities needed for the nonlinear effects to be apparent. The relatively low duty-cycle (1:100,000) keeps the mean power levels low to avoid specimen damage by heating.

Three-dimensionally localized fluorescence results from multiphoton excitation because the fluorophore is excited only in the focal volume where the photon density of the excitation light is highest (Fig. 97.1). Two photons must be absorbed simultaneously to elicit an excitation event; therefore, the excitation rate is proportional to the square of the local beam intensity (Kaiser and Garrett 1961). The local

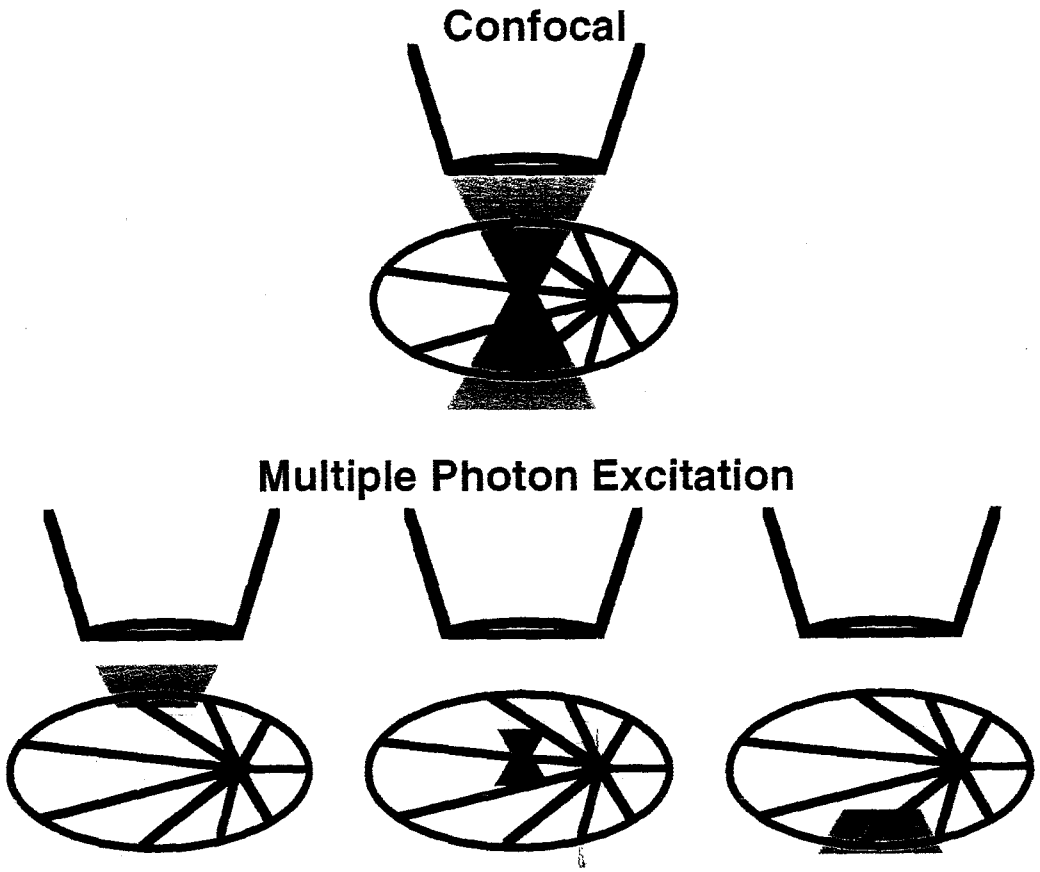

FIGURE 97.1
Principles of multiple photon excitation imaging. Upper panel depicts illumination used in confocal imaging: all fluorescent structures in the beam path are excited; light emanating from above and below the plane of focus must be eliminated by the confocal blocking aperture. Lower three panels depict passage of a short pulse of infrared light through a sample. Only at the point of focus is the photon density sufficient to elicit multiphoton excitation. Fluorescence continues to be emitted for the duration of the excited-state lifetime of the fluorophore after the passage of the short-pulse excitation.

12

Near-Field Imaging in Biological and Biomedical Applications

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12.1 Introduction

Optical microscopy is a standard technique in biology and medicine because it is convenient and easy to use and allows rapid assessment of problems at an early stage. Ability to view the sample is very important; all techniques other than optical microscopy must devise "tricks" to visualize sample information content. Samples ranging from tissue to single cells like bacteria can be investigated in a wide variety of environments, temperatures, and pressures. Sample preparation is straightforward and depends primarily on what information is desired. Because investigation is typically noninvasive, samples are not destroyed and can be reused in other applications.

From the beginning, a major challenge has been the need to improve spatial resolution. Since the late 19th century instrument resolution has come close to the so-called diffraction limit.[1] This is a fundamental restriction that limits the resolution of any optical instrument based on diffraction. Essentially, the diffraction limit uses the greatest possible collection angle and the shortest possible wavelength. Simply put, blue light is better than red light and allows a spatial resolution of 300 nm and smaller. Using an electron microscope increases the resolution; the energy (wavelength) is very high (short) and a resolution down to 1 nm can be achieved. Elaborate sample preparation is a drawback; experiments require an extensive vacuum system. This method is not suitable for environments with great variations and high energy used often destroys the sample.

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Another problem often encountered in biological samples is transparent specimens that show only weak contrasts. In the 20th century, contrast was improved by using the phase information of the image or special illumination schemes. One of the most important achievements was the introduction of fluorescent labeling of the specimen, which provided a means of distinguishing distinct cell compartments.

In 1928 the Irish scientist E. Synge proposed a totally different approach to imaging that scanned very small apertures across a surface.[2] It was almost 50 years before this approach was experimentally tested in the microwave region by Ash and Nichols.[3] A decade later two research groups independently demonstrated use of the technique for optical frequencies.[4,5]

Near-field optical microscopy uses different instrumentation based on scanning probe microscopies, such as scanning tunneling microscopy (STM) or atomic force microscopy (AFM).[6,7] STM/AFM and NSOM are similar in two ways. First, image formation is based on step-by-step recording. Second, the electronic feedback mechanism that moves the subwavelength aperture across the sample is essentially the same. The acronyms used for the technique, NSOM for near-field scanning optical microscopy and SNOM for scanning near-field microscopy, refer to exactly the same technique. These feedback mechanisms have a very useful side effect unique to near-field microscopy. Because the tip–sample distance remains constant, the feedback signal directly yields a topographic image of the sample. Optical and topographical images are therefore measured simultaneously and can be directly correlated. In the last 10 years improvements have resulted in better stability and handling. As a result, near-field microscopy has become a powerful tool for a large variety of applications in physics, chemistry, and biology.[8–12]

Despite the differences in image formation mechanism, the basics of standard (far-field) microscopy and near-field optical microscopy are very similar. In both techniques a change in refractive index or in absorption can be identified. Consequently, many of the established techniques in microscopy can be adapted to the near-field optical technique. Fluorescence labeling has been especially successful in biological applications and can be used to increase spatial resolution to less than 50 nm.

This chapter provides a brief introduction to near-field optics (NFO); however, the main focus is on state-of-the-art applications for investigating biological samples. Many of the current investigations focus on model systems under precisely controlled conditions; however, the first investigations have already been made on living cells. Recently, new techniques related to the original idea of NSOM have been used successfully to investigate problems in life science. Some examples will be introduced here.

12.2 Near-Field Optical Microscopy

12.2.1 Basic Principles of Near-Field Optical Microscopy

A detailed review of the theory and physics of near-field imaging is not appropriate here. The reader is referred to several excellent publications and reviews.[13–17] The focus here is on basic principles and the instrumentation required to conduct a near-field optical experiment.

Image formation in a classic (far-field) microscope is achieved by specimen illumination using a monochromatic plane wave. The object scatters the light in a characteristic way. The light is then collected by transmission or reflection and focused on a detector. The lens is several wavelengths away from the object, in the optical far-field. High spatial frequencies correspond to the fine details of the specimen and generate Fourier components of the field that decay exponentially along the normal object.[18] Such frequencies cannot be collected by the lens. This effect is the well-known Abbé limit of diffraction, $\Delta x = 0.61 \cdot \lambda/NA$,[1,19] where Δx corresponds to the smallest resolvable distance between two points, λ is the wavelength of the light, and NA is the numerical aperture of the microscope objective or the lens. If a confocal setup is used instead of wide-field illumination, the resolution increases slightly;[20] multiphoton techniques can also improve the resolution.

In contrast to far-field microscopy in which the light source is confined by a lens, in near-field optical microscopy the light source is confined by a metal aperture. Within a short distance beyond the screen, the size of the illuminated spot is limited only by the dimensions of the aperture. This area

is the so-called optical near-field. If such a small light source is scanned above a surface and the distance between aperture and sample is in this near-field region, all scattering or absorption phenomena must originate from that small illumination spot. Consequently, aperture size and distance determine the resolution.

12.2.2 Instrumentation

12.2.2.1 General Considerations

The key development for practical application of near-field optics was the fabrication of a functional subwavelength aperture. Instead of a hole in a planar metal screen, an aperture was formed at the apex of a pointed glass tip by coating it with a metal.[4,5,21–24] This design ensured an easy way to approach the sample and keep it in close proximity to the surface, while still satisfying the optical requirement for the aperture size. Figure 12.1 shows some of the most important setups using aperture tips. Figure 12.1A shows the most common setup. The sample is illuminated through the aperture and the light is collected by reflection or transmission by standard optical techniques (see, for example, References 4, 5, 11, 21–23, 12, and 25). Figure 12.1B shows an example in which the light is also collected back through the aperture in the illumination–collection mode.[24] Figure 12.1C shows a more specialized arrangement that uses a different illumination by evanescent waves.[26–29] Figure 12.1D illustrates a relatively new development

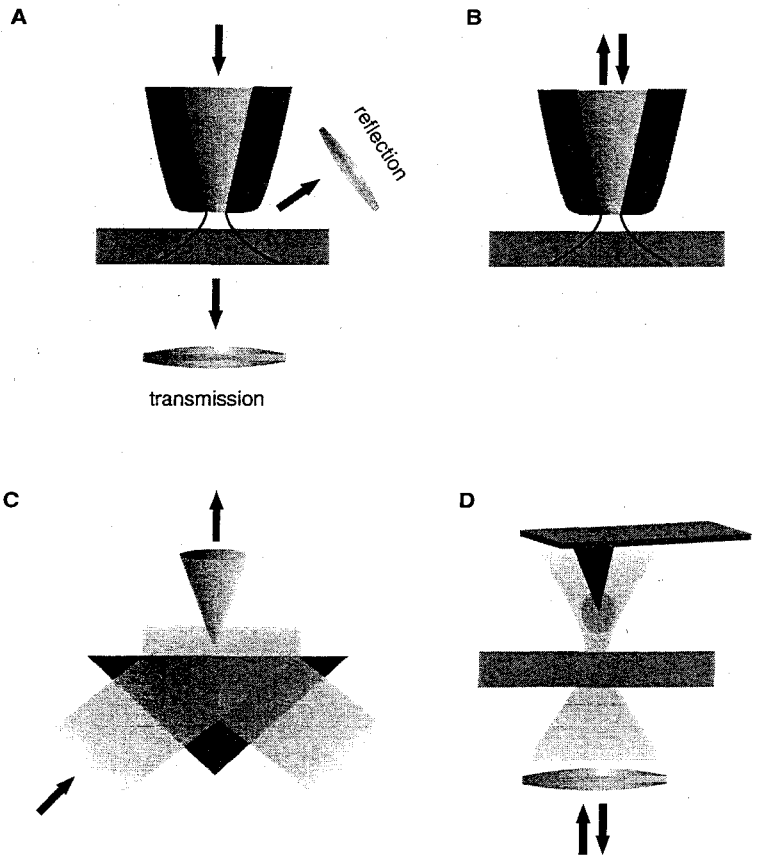

FIGURE 12.1 Different modes for near-field scanning optical microscopy: (A) illumination through the aperture, detection reflection or transmission via standard optics; (B) illumination–collection mode, both via the near-field aperture; (C) photon scanning tunneling microscope (PSTM); (D) apertureless or probe-enhanced techniques. The illuminated tip enhances the field and serves as a subwavelength light source.

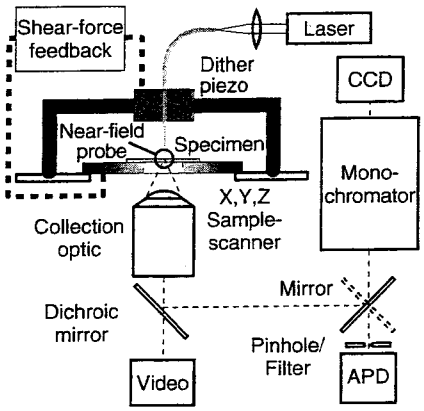

FIGURE 12.2 Standard experimental setup for transmission of NSOM. For specific analytic requirements different detection schemes can be applied.

without an aperture. Here, the light source is generated at the tip apex and can therefore be even smaller than any aperture.[30–37] This technique will be discussed in more detail in Section 12.4.2.

The most commonly used setup is the design depicted in Figure 12.1A. The entire instrument is built around the core shown. It can be described as a microscope in which the standard illumination source has been replaced by a near-field probe. Figure 12.2 sketches the major parts of a modern NSOM. Laser light is coupled into the back end of the fiber probe, which is held above the sample at a distance of 5 to 15 nm by a shear-force feedback arrangement.

This is a noncontact AFM mode where the tip is vibrated at its resonance frequency.[38,39] As soon as the tip "feels" the surface this vibration is damped. The damping serves as the feedback signal for the tip–sample distance control and accordingly allows topographical mapping synchronously to the optical signal acquisition. The light is collected in transmission by an appropriate objective and, depending on the experimental requirements, simply imaged onto a detector. If spectral information is also desired, the collected light is dispersed by a spectrograph and then imaged onto a multichannel detector. To maintain the alignment of near-field probe and collection optics the sample is scanned underneath the tip; hence, the image is built up, point by point.

12.2.2.2 Near-Field Optical Probes

The fabrication of near-field optical probes is a crucial prerequisite for the experiment. Aperture size, efficiency of light transmission, and the damage threshold are considerabions in probe fabrication. Because large taper angles result in a higher transmission,[40] the goal is to produce probes with a large angle that are still able to approach the sample surface and yield a useful topographical image. The straightforward approach would be a microfabrication similar to the process used to manufacture AFM cantilevers. However, because commercial microfabricated probes still are not readily available, many groups prepare these tips themselves. Recent progress made in the production of such tips may lead to commercial manufacture of these probes.[41–47]

Fortunately, two rather simple methods are used to prepare the tips in virtually every lab. The most frequently used technique is the melt-drawn, or "heating and pulling," method. Glass fiber is heated locally using a laser or a filament and the fiber is then pulled apart. The resulting tip shapes depend largely on the temperature and the timing of the procedure.[48,49]

The second method, based on chemical etching of glass fibers, is often called "Turner's method."[50,51] The tip formation occurs at the meniscus between hydrofluoric acid and an organic overlayer. Tips generated in this way generally show considerably larger angles and therefore the transmission is higher than for melt-drawn tips.[52,53] However, the parameters are difficult to control and the quality is less reproducible. Recently, a variation of the standard etching scheme was proposed in which the taper is

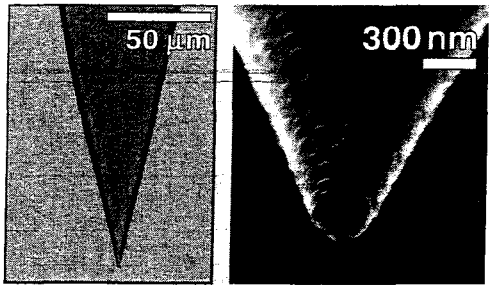

FIGURE 12.3 Optical (left) and scanning electron microscope image (right) of a "tube-etched" near-field probe.

formed inside the polymer cladding of the glass fibers.[54,55] This so-called "tube-etching" preserves the advantages of the etching; however, it is much more reproducible compared to the original Turner etching.

The metal coating needed to form the aperture is relatively easy to achieve. The glass fiber tip is rotated along its main axis while the metal is evaporated. The arrangement is such that the metal vapor cannot reach the very end of the tip, but only the sides of the taper. Using this kind of shading, the aperture size can be selected by varying the angle between the rotating tip and the evaporation source. Figure 12.3 shows an example of a tube-etched near-field probe and a zoom into the aperture region.

Finally, the near-field tip must be mounted into the scanning probe head. Here the dithering necessary for the feedback loop will be applied by piezoactuators or quartz tuning forks.[38,39,56,57]

12.3 Biological Applications of Near-Field Optical Microscopy

12.3.1 Practical Considerations

The main reason for using a near-field microscope in biological applications is the need for spatial resolution beyond the limits of a typical microscope. Another advantage is the synchronous sampling of the sample morphology and its optical characteristics. If one of these two considerations cannot be addressed using another, simpler method, then NSOM is the researcher's only choice. NSOM is only 20 years old and still not as developed as standard optical microscopy; hence, the technique is not yet used routinely. The scanning probe part of the instrument, the delicate probes, and the very long acquisition times are aspects still unfamiliar to scientists accustomed to confocal microscopes.

These problems are especially obvious when the method is applied to biological problems, where the samples and their environments differ considerably from those of surface science. Soft samples and scanning in liquids are still challenges peculiar to scanning probe microscopy. As the following discussion of biological and medical applications shows, model systems often must be considered before investigation can commence. Finally, we show that NSOM is now used to investigate living cells.

12.3.2 Investigation of Cell Material

12.3.2.1 Near-Field Fluorescence Microscopy

12.3.2.1.1 Stained Cell Tissue

Betzig and co-workers were among the first to investigate biological tissue using an NSOM.[58] Thin tissue sections from the hippocampus region of monkey brain were investigated in the standard transmission mode (see Figure 12.4.) From the toluidine blue stained section, different features can be clearly identified in Figure 12.4: pyramidal cell dendrites (D), nucleus (N), glial cell (Gc), and myelinated axon (M). As indicated by the scale bar, the spatial resolution is clearly better than that of a conventional microscope. An electron microscope would yield an even better resolution, but sample preparation for an NSOM is much simpler and the measurement can be done more quickly with an NSOM.

FIGURE 12.4 Illumination mode NSOM image taken from a thin-tissue section of the hippocampus region of the monkey brain. (From Betzig, E. and Trautman, J.K., *Science*, 257, 189, 1992. Copyright 2002, American Association for the Advancement of Science. Reprinted with permission.)

12.3.2.1.2 Actin Filaments

Similar experiments attempt to distinguish single actin fibers in the cyto skeleton.[59,60] Single filaments are too big for confocal microscopy and they cannot be readily accessed by force microscopy because they are below the cell membrane. Figure 12.5 illustrates one way to approach that problem. Here MDCK cells (Madin-Darby canine kidney) were grown on a silica slide using glutaraldehyde and lysed so that the interior of the cell walls became accessible.[61] The actin filaments were stained selectively with rhodamine labeled phalloidin. Figure 12.5A shows the NSOM fluorescence image and Figure 12.5B shows the topography image. In contrast to the fluorescence image, the topography image shows no clear structure inside the cell. This rules out topographic coupling, which often obscures near-field images and may give rise to incorrect or misleading results.[62] A line plot across the fluorescent image visualizes the spatial resolution of the measurement. In this case the so-called 10:90 step resolution is around 100 nm.

Recent experiments by Doyle et al. on fixed or living Glial cells involve a novel feedback scheme that relies on the detected fluorescence.[60] By measuring the signal at different distances from the specimen, Doyle et al. distinguished between the pure near-field and the ubiquitous far-field contributions.[62] Such experiments would then allow measurement of at least small distances through the plasma membrane. The achieved spatial resolution that has been extracted from the data is already on the order of 50 nm. Although no further conclusions have been drawn from the data, these experiments provide a useful methodology for soft samples such as cells.

12.3.2.1.3 NSOM inside Cells

Correlation of topographic and optical information is usually considered a unique advantage of NSOM. In the case of cell investigation, it can also be considered a great disadvantage because the interior of the cells cannot be accessed. Recently, Lei and co-workers[63] approached the mitochondrial membrane of a living breast carcinoma cell by penetrating the outer cell membrane. The cells were treated with JC-1, a dye that changes conformation — and hence its emission characteristics — when it is aggregated. This high concentration state occurs particularly in the mitochondrial membrane and thus distinguishes this membrane from other cell compartments.

To access the mitochondria, the surface topography of the cell was first determined, using the shear-force feedback in the aqueous environment of the setup. Next, the plasma membrane of the cell was penetrated by the NSOM probe and three fluorescence spectra were recorded at different distances from the mitochondrium. Figure 12.6 schematically shows this penetration of the cell membrane and the corresponding near-field fluorescence spectra, respectively. When the probe is close to the mitochondrial membrane a clear change in the two emission peaks can be detected. The relative peak height change between positions 1 and 2 is negligible and the overall intensity changes are due to concentration

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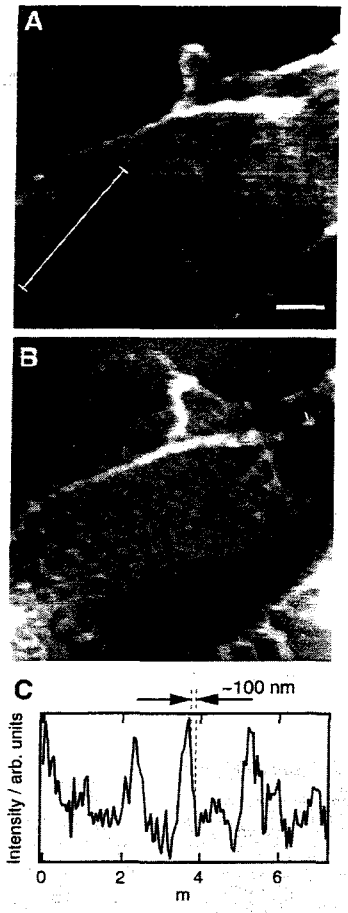

FIGURE 12.5 (A) Reflection NSOM image of labeled actin filaments in Madin-Darby canine kidney (MDCK) cells; (B) corresponding topography image; (C) line scan through the section marked in (A).

fluctuations. However, the ratio between the two emission peaks at position 3 changed significantly, which indicates that the probe is very close to the mitochondrial membrane.

The main emphasis here is on the height resolution, which is not generally considered in normal NSOM. Lateral scanning inside the cell is not possible with this technique because the tip would drag the entire cell. Therefore, high spatial resolution imaging in the x and y directions is not feasible.

12.3.2.1.4 In Vitro Chemical Imaging of Tobacco Mosaic Virus

An intriguing advantage of near-field optical microscopy is the ability to visualize virus particles that cannot be investigated at all by standard microscopy techniques. Keller and co-workers were the first to apply NSOM to virus samples.[64] They used a special force feedback setup, in which the sample is placed on an AFM cantilever underneath the actual near-field aperture.[65] Their specific arrangement allows a more sensitive feedback for the distance control between sample and near-field probe. This is important because of the previously discussed difficulties of measuring directly in liquids.

Figure 12.7 shows the results of this investigation. Figures 12.7A and 12.7B show the topography and near-field optical image, respectively. An interesting labeling technique was used to determine the direction of the virus. The tobacco mosaic virus (TMV) was labeled with a metatope monoclonal antibody (M) that binds only to the 5′ end of the virus. A biotin labeled polyclonal antibody (P) was attached to the monoclonal antibody. Finally, an avidin labeled latex bead (~30 nm diameter) was bound to the remaining biotin sites. The composition can be seen from Figure 12.7D. The presence of bovine serum albumin (BSA) prevents aggregation of the virus and is needed to obtain single virus particles. This kind of label

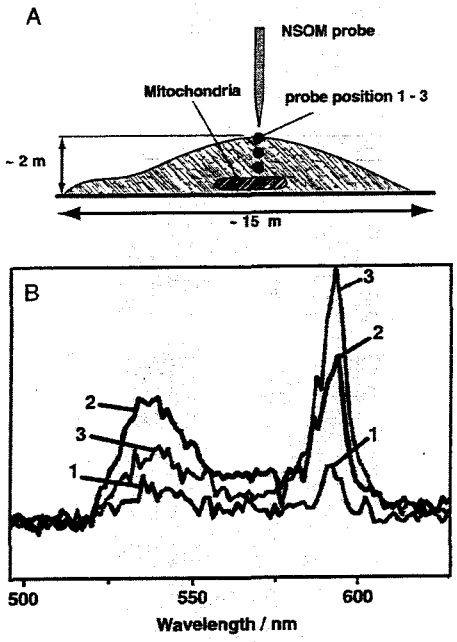

FIGURE 12.6 (A) Experimental setup for an inside cell near-field fluorescence experiment. (B) Fluorescence spectra taken at different distances from the mitochondrium. (Adapted from Lei, F.H. et al., *Appl. Phys. Lett.*, 29, 2489, 2001.)

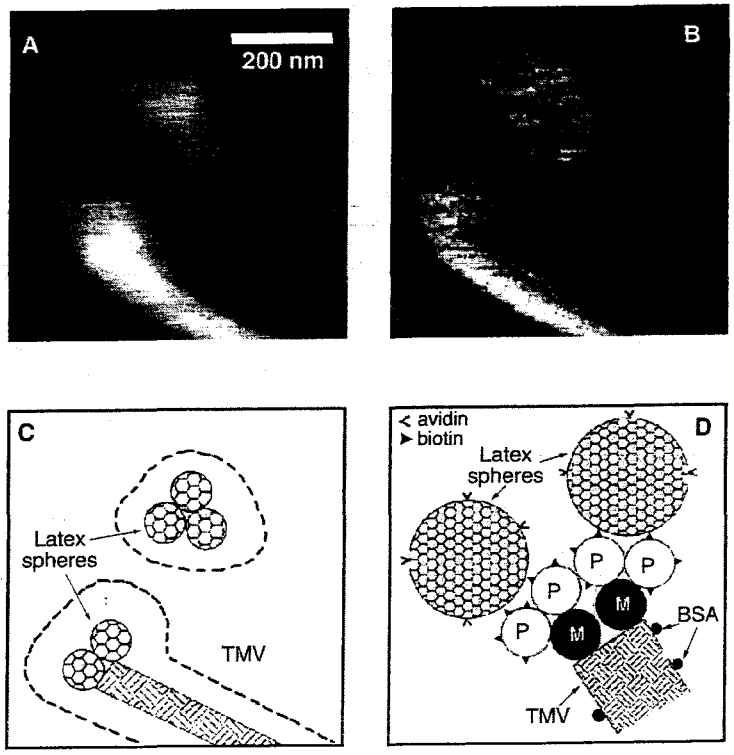

FIGURE 12.7 AFM and NSOM images of tobacco mosaic virus (TMV): (A) topography; (B) NSOM image; (C) interpretation of the optical image; (D) schematic diagram of the labeling procedure (P = polyclonal antibody, M = monoclonal antibody, BSA = bovine serum albumin). (From Keller, T.H. et al., *Biophys. J.*, 74, 2076, 1998. Copyright 2002, Biophysical Society. With permission.)

can be detected by either topographic or optical measurements, as shown in Figure 12.7. The sketch in Figure 12.7C shows the results from the raw images more clearly. Because a near-field optical image is always a convolution between the object size and the aperture dimensions, it is possible to extract the object features if the size and shape of the near-field source are known. This is also valid for the topographic image.

A remarkable fact that can be deduced from the images is that the optical resolution in this experiment was better than the topographic resolution. In principle, even without the extensive labeling, the features of the virus could be easily detected with a resolution of ~60 nm; however, only the label provides direct information about the virus.

A major drawback of this method is the elaborate sample preparation on an AFM cantilever. Nevertheless, this method offers a means to control environmental conditions in a way not possible with other methods.

12.3.2.1.5 Single Green Fluorescing Proteins

The detection of intact single molecules is the ultimate goal of microscopy. In 1993, Betzig and Chichester[66] were the first to observe individual fluorescing molecules at room temperature, using a near-field optical microscope, thus establishing single-molecule detection as a new branch of science.[67–72] Although, in principle, single-molecule sensitivity can be achieved readily by standard microscopy techniques and appropriate dilution, near-field optical techniques offer some distinct advantages:[73] the small detection volume, which allows localization to a few nm; the sensitivity toward the molecular orientation in all three dimensions; and, perhaps most important of all, the correlation to surface morphology resulting from the simultaneous detection of topography and optical signal.

As an example, the near-field fluorescence and the corresponding topography images of single green fluorescing proteins (GFP) are shown in Figure 12.8.[73] This molecule has attracted considerable attention because it can be produced by many proteins without changing the biological function. An extensive exogenous staining procedure is obsolete in many cases.[74,75] The individual proteins were adsorbed on freshly cleaned hydrophilic glass.

The image shows a 2 × 2-μm scan of two distinct sample areas, each with corresponding fluorescence and topographic information. The most obvious feature is the irregular shape of the spots in the fluorescence image. This is a direct indication of single-molecule behavior, the so-called on/off switching observed very early by researchers.[76] The origin of the photodynamic is not yet fully understood.[77,78] The fluorescent events can be correlated with topographical features that, with a height of 2 to 5 nm and a width of ~10 nm, correspond well to the known size of 4 nm of the barrel-shaped GFP.

Interestingly, virtually all the fluorescent spots also can be found in the topography, whereas not all the morphological features fluoresce. Subsequent measurements revealed that most of the features are indeed proteins; however, they light up only from time to time. This indicates that only a few molecules are "active" at a given time and that most of the time the GFP is in a so-called dark state.[79] The structure

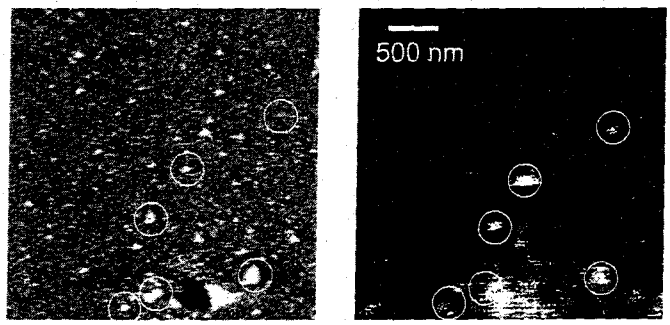

FIGURE 12.8 NSOM and topography images (2 × 2 μm) of individual S65T GFPs. Right: Fluorescence image showing abrupt changes in the emission of isolated spots (circles). Left: The corresponding topography shows individual GFPs 2 to 4 nm high. (Courtesy of Niek van Hulst, University of Twente, the Netherlands.)

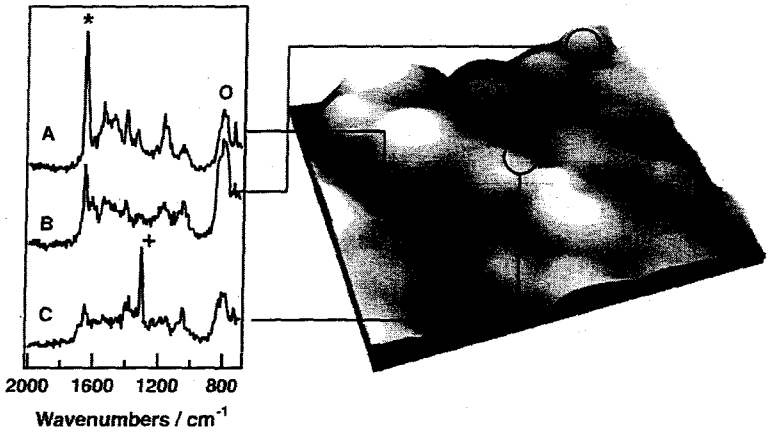

FIGURE 12.9 Shear-force topography of a SERS substrate covered with brilliant cresyl blue labeled DNA fragments. The Raman spectra on the left correspond to the specified local positions on the substrate.

and biological nature of this dark state are still under investigation; nevertheless, these factor affect the sensitivity of the single-molecule detection because the off-time can last for several seconds and the probability of detecting a single GFP molecule is only 5%.

12.3.2.2 Near-Field Raman Spectroscopy of Labeled DNA

Raman spectroscopy has the potential to identify any material by its characteristic molecular vibration without the need for further labeling and, hence, in many cases without any further sample preparation. Raman microscopy is fast becoming a standard technique to characterize surfaces directly.[80] The major disadvantage of standard Raman spectroscopy is the poor scattering cross section. Its efficiency is about ten orders of magnitude lower than fluorescence spectroscopy. This makes routine investigations impossible for many analytical problems. In biology, nevertheless, many structural problems of proteins have been solved by using resonance Raman scattering.[81] Here, the efficiency is much better because the molecules or certain fragments of the molecule are resonantly excited.[82] This results in an enhancement of several orders of magnitude.

Even greater signal enhancements can be gained by the implementation of surface-enhanced Raman scattering (SERS).[83–86] Using certain rough metal substrates, enhancement factors up to 10^{10} and even higher have been reported, resulting in single-molecule sensitivity comparable to fluorescence, as discussed previously.[87,88] Kneipp and co-workers reported on the detection of single DNA base molecules using SERS.[88] At present, the still lower light emission of SERS compared with fluorescence restricts the use of near-field optical techniques. To utilize the enhancement it is helpful to label the substance of interest with an especially active SERS compound. This may seem like a more difficult way of performing fluorescence labeling, but the advantage here is the narrowness of the emission lines in Raman scattering compared to those in fluorescence. This narrowness allows the simultaneous detection of several labels with just one excitation line.

This kind of labeling is shown in Figure 12.9, which shows the rough topography of a SERS substrate covered with brilliant cresyl blue (BCB) stained DNA fragments.[89,90] Near-field Raman spectra were recorded simultaneously and some example spectra are shown. The change in the spectral pattern indicates a change in sample composition without an elaborate band assignment. The central part of the right substrate shown in Figure 12.9 differs clearly from the outer regions. The signal-to-noise ratio is not sufficient for further investigation. Because this was an atypical result, all but two prominent bands can be used for analysis. The only conclusion that can be drawn from the data is that the results are definitely not due to the dye label, and therefore a change in the sample composition occurred. The nature of this change remains, however, unclear.

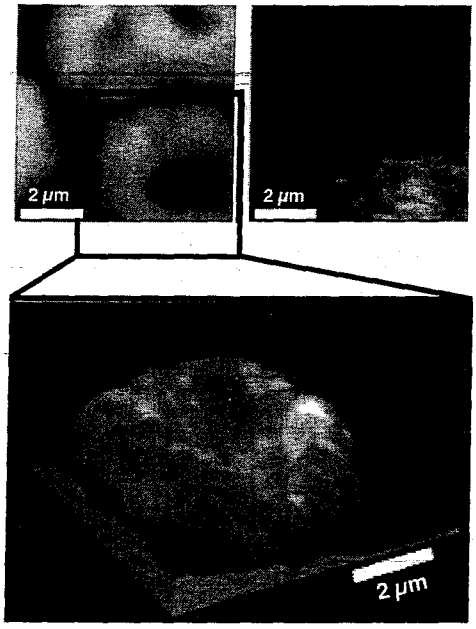

FIGURE 12.10 Fluorescence and topography images of malaria-infected cells.

The combination of SERS and near-field optical techniques is promising because of the ease of distinguishing many samples simultaneously. However, there are still drawbacks, the most important of which is the long acquisition time of about 1 min per position. Therefore, the specific investigation of selected regions of interest appears to be more promising than a complete imaging.

12.3.3 Model Cell Membranes

An important issue in biology is the probing of cell membranes. Typical questions concern the organization and orientation of the membrane or the location of specific molecules such as proteins. Because of their softness, many membranes tend to fluctuate, and this requires a very sensitive feedback scheme. Consequently, many researchers choose Langmuir-Blodgett mono- and bilayers that are easier to control and serve as a model system.[91–93] The capabilities of NSOM to gain information on phase changes or changes resulting from additives such as cholesterol or small peptides have been demonstrated by several research groups.[94–99] One very interesting approach is the detection of the three-dimensional dipole orientation of single fluorophores to determine the structure and phase composition of a membrane.[66,100] Depending on environmental conditions, these orientations change; in combination with simultaneous topographic measurements, the correlation between structure and morphology can be deduced.

Some groups have begun to look at cell membranes directly.[101–104] Figure 12.10, for example, shows the fluorescence and topography images of malaria-infected cells.[105] Here the mapping of malarial protein in the erythrocyte membrane is shown. A blood sample was reacted with antibodies against a specific malaria protein (*Plasmodium falciparum* histidine-rich protein) and then stained with tetramethylrhodamine. The topography image clearly shows the red blood cells, but cannot distinguish between infected and healthy cells. The fluorescence image to the upper right clearly shows that only the cell in the lower right is infected. In the superposition of fluorescence and topography images, the distribution of the antibodies on the erythrocyte membrane can be visualized quite clearly. The investigators claim the resolution to be around 100 nm — well below the far-field control experiment. A further near-field dual-color experiment (not shown) proved that different malarial proteins interact differently with certain blood proteins. The high resolution of the near-field experiment is necessary to distinguish the local origins of the labeled proteins.