Chapter 7

Optical Sectioning (Depth Discrimination) with Different Scanning Techniques: The Beginnings of Confocal Microscopy

This chapter describes a variety of confocal microscopes, based on several types of scanning techniques, that were invented by different individuals in several countries around the world. Marvin Minsky invented a confocal microscope in which the specimen was mechanically scanned with respect to the illumination light. Petràn invented a confocal microscope based on the rotating Nipkow disk for scanning and descanning the light with respect to the specimen. Guoqing Xiao and Gordon Kino used a similar rotating Nipkow-type disk, but only used one side of the disk for scanning and descanning the light on and from the specimen. Svishchev used a two-sided mirror for the same process. Finally, laser scanning confocal microscopes are described.

In many cases, the motivation was a research problem that was not accessible with existing types of optical microscopes. Many of these problems were in the domain of *in vivo* microscopy. For example, Petràn investigated the living brain cortex and the live retina, and Svishchev studied the brain cortex in a living animal. These diverse inventors had a common requirement: an optical microscope that could image live, unstained, thick, highly scattering specimens. There is one striking exception. Minsky was attempting to use the light microscope to observe thick, fixed, Golgi-stained brain slices.

Instead of the low-contrast, blurred images from these specimens, these researchers dreamed of a microscope with depth discrimination that could be used to observe such specimens as the live brain cortex and living retina. Many of the early inventions of various types of confocal microscopes were driven by the limitations of existing optical microscopes.

7.1 The Confocal Microscope: The Problem and Its Solution

The lack of depth discrimination or optical sectioning capability is the major limitation of the conventional (nonconfocal) fluorescence microscope. In the past, the common solution was to use very thin specimens such as cells in tissue culture monolayers or thin smears of cells for pathology. Nevertheless, this limitation precluded the use of the light microscope for thick, highly scattering specimens, e.g., *in vivo* human skin, live embryos, intravital microscopy of organs, brain imaging, and studies of hard tissues such as teeth and bone. Similar problems occurred when such specimens were observed with reflected light in an optical microscope.

Wide-field fluorescence (nonconfocal) microscopy, when used with very thin sections under appropriate conditions, is still a very useful technique. When applied to thick, highly scattering specimens such as skin, the eye, brain slices or whole embryos, the inherent limitations become apparent. The lack of axial (depth) discrimination greatly degrades image quality. This is because of scattered and fluorescent light in the regions above and below the focal plane that are imaged together with the light from the focal plane.

Confocal microscopy offers a solution to this problem. The rejection of light from out-of-focus planes and the smaller depth of field result in images with high fidelity and high contrast. The confocal microscope also has the important ability to acquire optical sections from thick specimens. The solutions offered by a confocal microscope include enhanced axial and transverse resolution, enhanced contrast, and depth discrimination.

This chapter discusses inventions of various confocal microscopes. These differ from standard light microscopes in a critical manner. In the standard light microscope, the image blurs with defocus along the optical axis. In the confocal light microscope, the image becomes black with defocus; there is depth discrimination.

Another computational solution (not confocal microscopy) to the problem of depth discrimination exists: **deconvolution techniques**. Wide-field fluorescence microscopy can be used to acquire a stack of blurred images through the full thickness of the specimen. A measure of the actual axial resolution of the wide-field microscope can be made by imaging subresolution fluorescent particles such as submicron fluorescent beads under the same conditions as were used to acquire the stack of blurred images through the thick specimen. Various computer algorithms can deconvolve the blurred images and restore the image. These deconvolution techniques are not only of use with wide-field fluorescence microscopy, but may also help improve images taken with other types of light microscopy, e.g., confocal or multiphoton excitation microscopy.

The confocal microscope provides *en face* images of the specimen; hence, the plane of the image is orthogonal to the specimen thickness. For example, the confocal microscope, when applied to the skin surface of the arm, acquires images parallel to the skin surface; i.e., first the surface layer of cells, then the deeper cell layers. This is very different from the typical sections obtained in histopathology in which the tissue is cut along the thickness. In histopathology, a section of skin is removed, fixed and stained. For microscopic observation, the excised specimen is imaged in a plane perpendicular to the skin surface. Therefore, the microscopic image shows cells from the skin surface to the deeper cellular layers in a single image.

In contrast to the conventional light microscope, which images all of the points in the specimen in parallel, a confocal optical microscope optimizes illumination and detection for only a single spot on the specimen. In order to form a two-dimensional image with a confocal microscope, it is necessary to scan the illumination spot over the area of the specimen or to scan the specimen.

The next section describes and compares several generic types of confocal microscopes to explain their basic principles. That discussion is followed by the pre-

sentation of three designs that were based on modifications of the earlier instruments. Again, the reader is urged to read the original papers and patents.

7.2 Stage-Scanning Confocal Microscope Invented by Marvin Minsky

Marvin Minsky is credited with the invention and experimental realization of a stage-scanning confocal microscope. Minsky was motivated by his need to study the structure of fixed, Golgi-stained, thick brain slices. Minsky clearly stated the advantages of stage or specimen scanning in his 1961 patent on the confocal microscope. This idea decoupled the magnifications of the objective from the resolution. The magnification could be electronically varied by changing the number of pixels that form the scanned image. That implied that a single microscope objective with a fixed magnification could be used to form images of various magnifications. His patent also clearly showed the folded mode of modern confocal microscopes. Minsky described, but did not construct, a confocal microscope based on an **epitaxial** design, where the same microscope objective is used for both illumination and detection.

It is both instructive and of historical interest to follow the thinking of Minsky on his invention. First, he correctly stated the problem: How to make a microscope in which scattered light from a given point in the specimen is uniquely defined by a given illuminated point on the specimen. He realized that each focal point on the specimen would also have contributions from other points in wide-field microscopy.

He also realized that a second microscope objective could be used to illuminate one point of the specimen. That second objective replaced the usual condenser; it imaged a point source of light (obtained by using a pinhole aperture in front of a lamp filament). Now the illumination objective focused all the light from the point source (the pinhole aperture) onto a single point on the specimen.

Third, he noted that even with the second microscope objective illuminating a single point of the specimen with the image of a point source of light, the problem of scattered light from above and below the focal plane still existed. However, he noted that these out-of-focal-plane light rays could be eliminated by placing a second pinhole aperture in the image plane beyond the exit side of the microscope objective lens. This arrangement describes the principle of a confocal microscope.

The Minsky solution had elegant symmetry (see Fig. 7.1). There are two microscope objectives, one on each side of the specimen, and two pinhole apertures, one on the illumination side and one on the image side; therefore, both pinholes are located in conjugate planes. A point source of light illuminates a point on the specimen. The light scattered from that point is detected; hence, stray light from out-of-focus planes located above and below the focal plane is excluded (see Figs. 7.2 and 7.3). The word confocal denotes that fact: the images of these pinholes are cofocused or "focused together."

Minsky noted three other points. First, how can one build up an image from a series of single spots? The previous work on flying-spot microscopes solved that problem: the specimen could be moved in a raster scan pattern through the optical

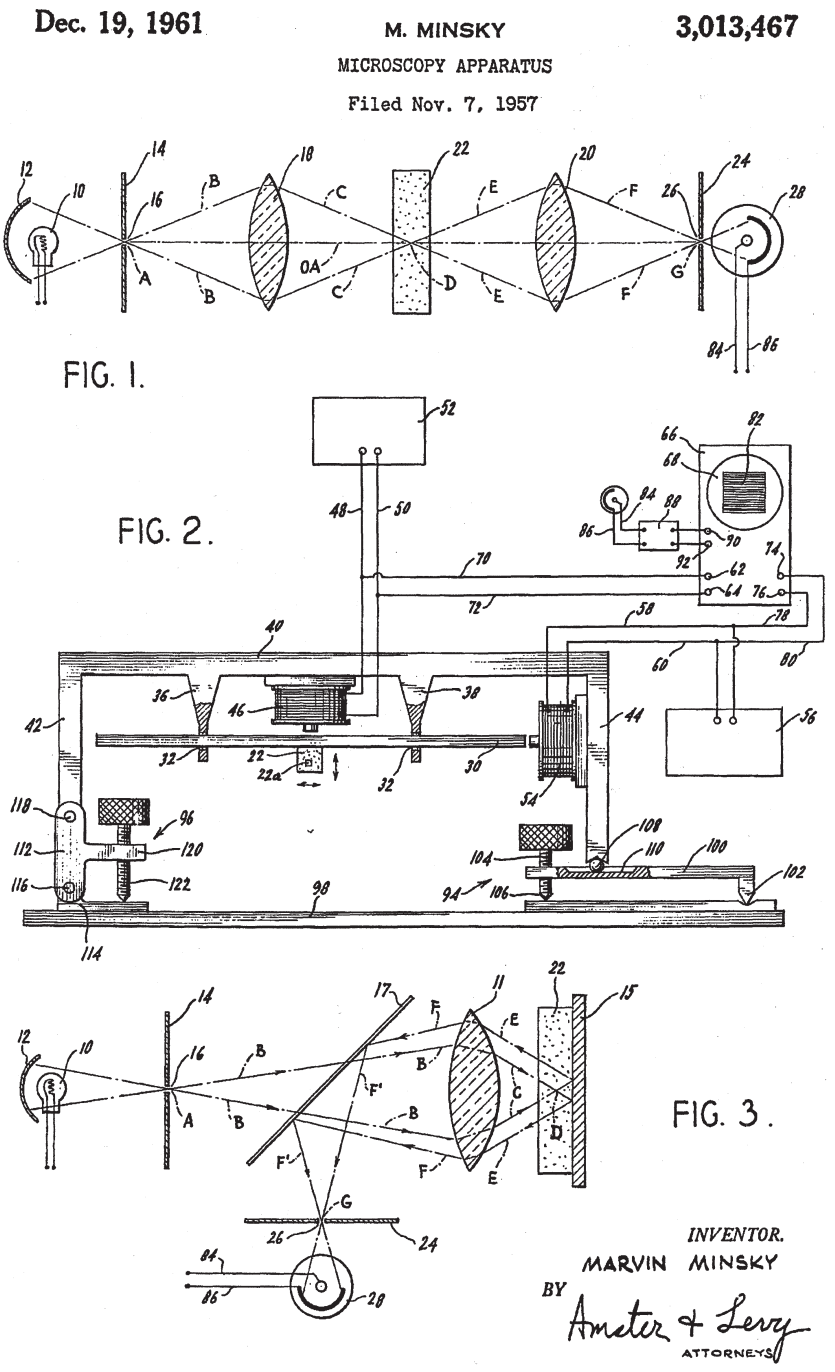

Figure 7.1 Drawing of the confocal microscope invented by Marvin Minsky, from his 1961 U.S. patent, in which Fig. 1 shows the transmission mode confocal microscope; Fig. 2 shows the stage-scanning system; and Fig. 3 shows the reflection mode confocal microscope with a single microscope objective and a beamsplitter.

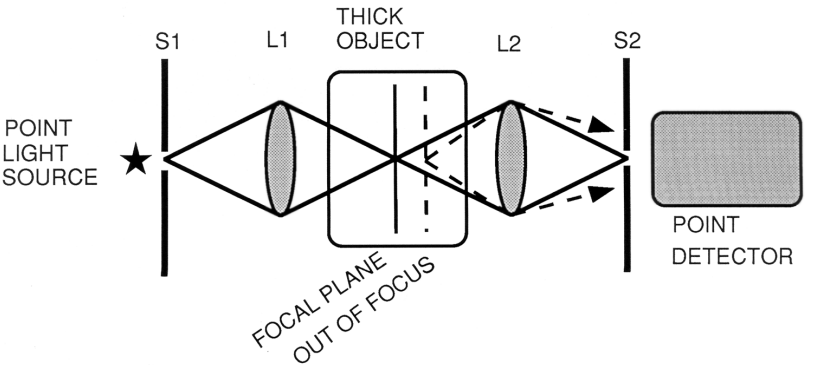

Figure 7.2 Schematic diagram of a transmission microscope illustrating the principles of a confocal microscope and depth discrimination, where S1 and S2 are confocal apertures located in conjugate planes, and L1 and L2 are focusing lenses for illumination and detection, respectively. The drawing is modified from Fig. 1 in the Minsky patent shown in Fig. 7.1.

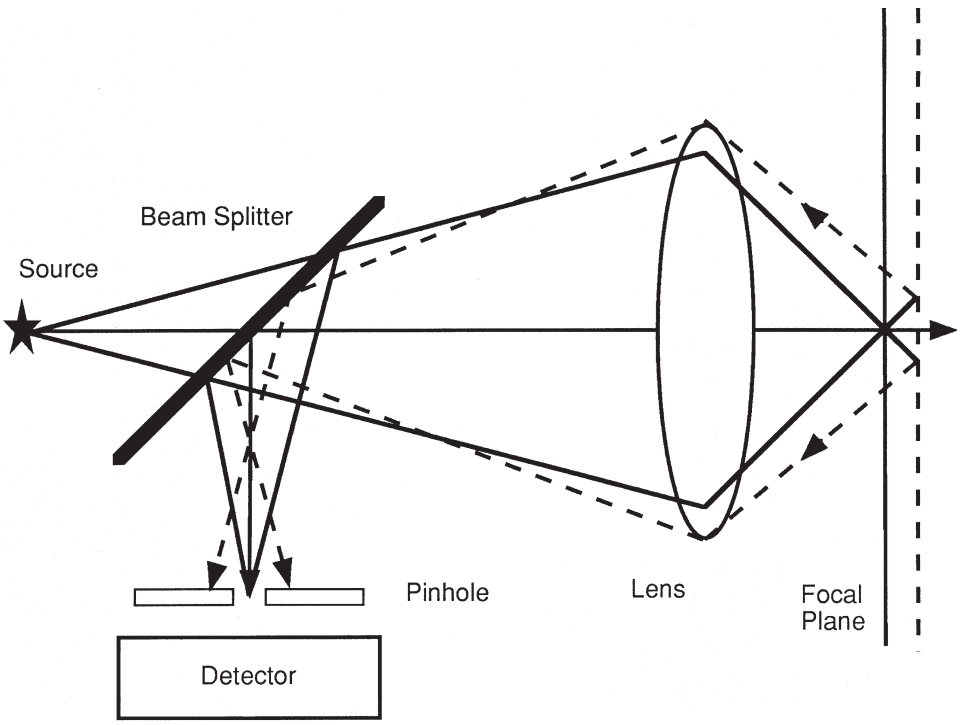

Figure 7.3 Schematic diagram showing the depth discrimination capability of a confocal microscope and how it discriminates against reflected light. The dashed vertical line to the right of the focal plane represents an out-of-focus plane.

axis of a stationary microscope, and the image would be built up, spot by spot, to make the complete image. The detector could be an integrating device such as photographic film or an electronic CRT display.

Second, the first design that we discussed operates in the transmission mode; he also described a reflected-mode confocal microscope, which is similar to the modern confocal microscope. A single microscope objective was used both to illuminate a single spot on the specimen and to image the collected light from that point. It was used together with a pinhole aperture on only one side of the specimen. A half-silvered mirror was used to separate the illumination rays from the imaging rays. Minsky stated that the diffraction patterns of both pinhole apertures are multiplied coherently with an increase of both axial and transverse resolution.

Third, in order to obtain a three-dimensional image, which was his original goal, an image is obtained on the CRT display, and then the specimen is translated a small distance (microns) along the optical axis of the microscope and the next image is built up. This process is repeated until a stack of images is formed, and the stack or optical section could then be formed into a three-dimensional image using techniques of stacked sheets of plastic containing a single optical section. These techniques were known in the fields of x-ray crystallography and light microscopy where they were used to reconstruct thick tissues and embryos.

The 1955 invention of the Minsky confocal microscope was a breakthrough, but one major limitation was the slow image acquisition time, since the image was slowly built up spot by spot, and the stack of images required moving the specimen incrementally along the optical axis. Minsky used an arc lamp for the light source. The detector was a low-noise photomultiplier tube. The display was a long-persistence radar scope. The acquisition time for one image was 10 seconds.

Two major technological advances were not available at the time of Minsky's invention: the laser was not yet invented, and the desktop computer was not yet available. These two inventions had a great impact on the popularity of modern confocal microscopy. Not until 1983, when Cox and Sheppard published their seminal paper, "Scanning optical microscope incorporating a digital framestore and microcomputer," did the microcomputer became part of the confocal microscope (Cox and Sheppard, 1983).

In 1971, P. Davidovits and M. D. Egger published, "Scanning laser microscope for biological investigations," which combined a 5-mW He-Ne continuous wave laser with a confocal microscope (Davidovits and Egger, 1971). Another unique feature of their confocal microscope was that the objective scanned over the specimen to form the image. Their paper also pointed out the problem of using coherent light in wide-field microscopy, in which interference effects severely degrade the image. They stated that another advantage of point scanning is that coherent interference does not occur.

Nevertheless, Minsky spelled out all of the key principles of confocal microscopy and constructed a working confocal microscope! Many of his ideas are implemented in the basic designs of modern confocal microscopes. Therefore, it is valuable to discuss his ideas further.

Minsky noted that two types of scanning were available: scanning the specimen or scanning the beam of light. Minsky correctly noted that beam scanning is fast; nevertheless, it is easier to keep the optics fixed, and to scan the specimen. He also noted that the use of beamsplitters always results in a reduction in image brightness. Minsky's confocal microscope used 45× microscope objectives in air. It could resolve points closer than 1 μm.

Minsky pointed out an important advantage of specimen or stage scanning. The microscope only used the central part (paraxial optics) of the microscope objectives; thus no off-axis or lateral optical aberrations exist that need correction. Chromatic and spherical aberrations still required correction. Minsky also stressed the advantage of combining stage or specimen scanning with paraxial optics. In addition, with stage scanning, if the microscope objective is used on-axis, then field curvature of the object is unimportant.

7.3 Mojmir Petràn, Milan Hadravsky, and Coworkers Invent the Tandem-Scanning Light Microscope

While the Minsky confocal microscope illustrated many technical developments that would appear decades later in modern commercial designs, it did not have an impact on the life science community at the time. A very different result followed the invention of the tandem-scanning confocal microscope; the life sciences community became deeply interested in this new invention.

The modern development of the real-time tandem-scanning confocal microscope is credited to the 1965 invention by Petràn and Hadravsky. In 1964, Petràn, who was a qualified medical doctor, visited Dr. Robert Galambos' laboratory at Yale University. They discussed the need for a microscope that could study live, unfixed, unstained neurons in the brain. During this visit, the tandem-scanning reflected light microscope was conceptually developed. A year later at Charles University in Plzeň, Czechoslovakia, Petràn and Hadravsky constructed the first prototype of a tandem-scanning confocal microscope based on a Nipkow disk. **Tandem scanning**, or double scanning, is defined as simultaneous scanning in both illumination and detection (see Fig. 7.4).

Petràn and Hadravsky were interested in intravital optical microscopic imaging neurons in live brain tissue. At Charles University, Petràn used his tandem-scanning microscope to investigate the live retina and the live brain. He and his students combined optical *in vivo* imaging and electrophysiological techniques. It is of interest that Minsky had a similar motivation in the design of his confocal microscope; however, he designed his microscope to study the three-dimensional organization of fixed, Golgi-stained, thick brain slices.

In addition to intravital imaging of brain tissue, Petràn and Hadravsky were interested in microscopic imaging of the structure of other living tissues such as epithelia, capillaries, nerves, muscles and glands *in vivo*. This was the driving force for the development of their Nipkow disk confocal microscope. Petràn later brought his tandem-scanning confocal microscope to the U.S. and collaborated

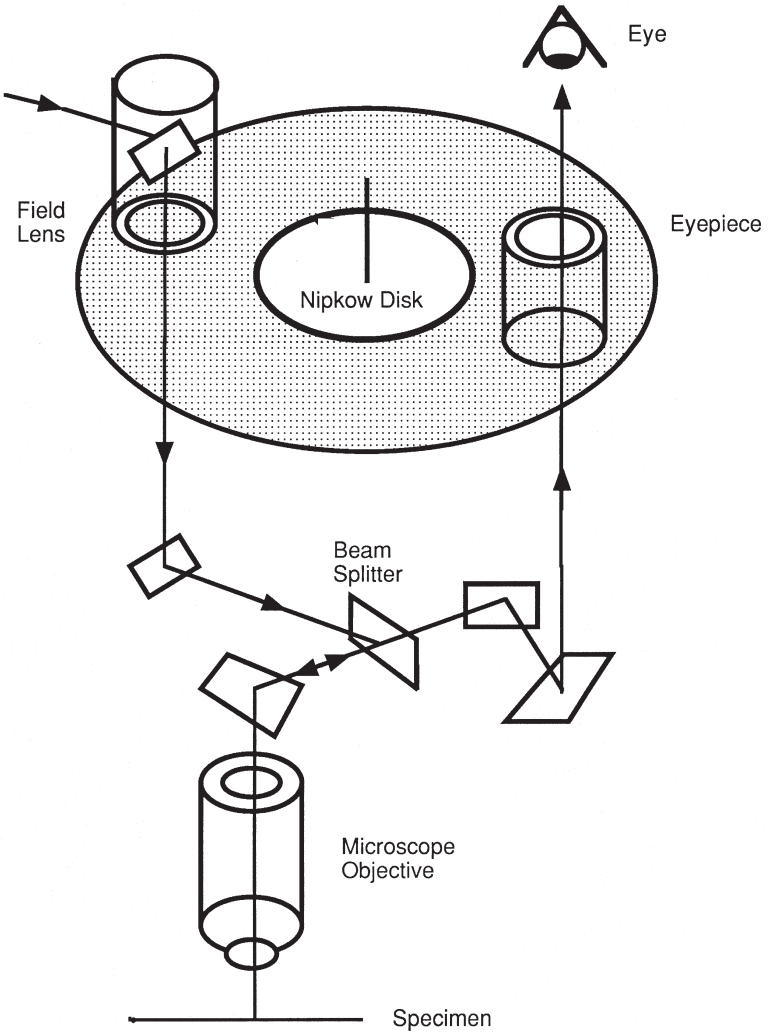

Figure 7.4 Schematic diagram of the real-time, direct-view, tandem-scanning Nipkow disk confocal microscope. The light source is a mercury arc or a tungsten filament lamp.

with Egger and Galambos at Yale University on experiments with live animals. Their 1967 paper was published in *Science* and included a composite hand drawing of the three-dimensional structure of a ganglion. In 1968 Petràn, Hadravsky, Egger, and Galambos published a paper on the tandem-scanning reflected-light microscope in the *Journal of the Optical Society of America*. That paper states that Galambos was the principal investigator of a NASA grant on the microscope, and that Petràn and Hadravsky were research associates at Yale University.

At that time, the small computers with three-dimensional volume rendering software that we have today did not exist. Therefore, there was not much interest in this technological development for the next 20 years. It is interesting to follow their formulation of the problem and then to appreciate their unique solution, which modified a very old invention.

A real-time tandem-scanning confocal microscope, in which the image could be observed with the naked eye, was developed by Petràn and Hadravsky in the mid-1960s. They acknowledged the contribution of Nipkow, who in 1884 invented the disk that provided real-time, tandem point illumination and detection. Petràn and Hadravsky invented their confocal microscope while living in Czechoslovakia, which had severe restrictions on travel, communication, instruments, and equipment. Therefore, great credit is due Alan Boyde, who aided them in the development and publication of their work. This shows the importance of free communication and free travel for the development of science and technology.

In order to understand their contribution, it is helpful to first formulate and state the problem: how to design a confocal light microscope that was simple, inexpensive to construct with materials then available, would use either the sun (it was first tested on a mountain) or an arc lamp as the source and a Nipkow disk for mechanical scanning, operate in real-time, and result in a real-color image.

Early on, the inventors decided against a transmission-light confocal microscope. A reflected-light microscope has several advantages. First, specimens such as whole animals, tissues, or organs could not be observed with a transmission microscope without sectioning; therefore, intravital microscopes would be excluded. Second, transmission confocal microscopes required two identical flat-field microscope objectives, which are difficult to obtain. Third, transmission light microscopy offers a high background of illumination. For these reasons, they decided to construct a tandem-scanning confocal microscope that operated in the reflection mode. Their patent, however, also describes a confocal microscope based on a spinning Nipkow disk constructed in the transmission mode.

The spinning Nipkow disk is the key component. The design concept was to have simultaneous point illumination of the specimen and detection of light from the same point. In order to form a two-dimensional image, a scanning device was required to simultaneously scan the image of both the illumination aperture and the cofocused image of the detection point over the specimen. A point-scanning confocal microscope suffers from the fact that the image is formed point by point. Petràn required a confocal microscope that would work in real-time. The use of a rotating Nipkow disk provided a mechanical device to permit the parallel illumination of many points on the disk; in effect, many confocal microscopes that work in parallel. At each pinhole on the illumination side of the disk, the light is focused by the objective to a diffraction-limited spot on the specimen. The light reflected from the sample is passed through a conjugate pinhole in the disk and can be observed in the eyepiece. When the Nipkow disk is rotated, a real-time image of the specimen can be observed.

The principle of the tandem-scanning confocal microscope is as follows (see Fig. 7.4). In the hypothetical case of only two pinholes on a stationary disk, the illu-

mination light passes a pinhole on a stationary Nipkow disk and is focused by the microscope objective onto a spot on the specimen. The scattered and reflected light from that illuminated spot is collected by the aperture of the microscope objective. A very thin beamsplitter separates the illumination light from the reflected light. The reflected light is focused on a second pinhole, which is located in a position conjugate to the first pinhole; the images of both pinholes are cofocused on the specimen. The first illumination pinhole and the second imaging pinhole are located on a diameter of the disk at conjugate points. Only the light from the focal point in the specimen is focused on the second pinhole, and can therefore pass through the pinhole and form an image in the ocular. Light that is from above and below the focal plane of the objective is defocused and does not pass through the second pinhole. That is the origin of the axial discrimination in the tandem-scanning confocal microscope.

The idea of Petràn and Hadravsky was to pass the illumination light through one set of pinholes on one side of the Nipkow disk, and to pass the light from the specimen through a conjugate set of pinholes on the opposite side (see Fig. 7.4). This arrangement provided a solution to the problem of reflected light from the top surface of the Nipkow disk. The design of the tandem-scanning confocal microscope requires that the distribution of apertures have a center of symmetry, which results in identical aperture patterns in both the illumination and image fields.

In the actual microscope, the Nipkow disk contains many spiral arrangements of holes. Each aperture is in the range of 30 to 80 μm in order to avoid cross talk. About 100 pinholes at a time are illuminated on one side of the Nipkow disk, and the same number of conjugate holes pass the reflected light from the specimen. When the disk is stationary, the observer sees many spots of light from the specimen; when it rotates, the real-time image of the specimen is observed in the eyepiece. In addition to the microscope objective, beamsplitter, and Nipkow disk, a number of mirrors and lenses are contained in the microscope.

The first designs used several mirrors in which reflecting surfaces were perpendicular to the optical axis of the microscope. In order to reduce reflections from the microscope itself, it was necessary to use polarizers. Later designs of the direct-view tandem-scanning confocal microscope used prisms for beam inversion, and reduced the reflections from surfaces in the optical path. For example, the 1967 *Science* paper by Egger and Petràn describe a Nipkow disk confocal microscope with a polarizer, analyzer, and a quarter-wave plate to reduce reflections from the optical surfaces within the microscope. Prisms were used inside the microscope. For some of the experiments, the authors used the sun as a light source.

Petràn and Hadravsky decided to use multiple-aperture (multibeam) scanning since that would reduce the frame time to scan the field as compared to single-point scanning. The Nipkow disk contains several sets of pinholes (30–80 μm in diameter) arranged in several sets of Archimedes spirals. Each pinhole on one side of the disk has an equivalent and conjugate pinhole on the other side. The illumination light passes through a set of pinholes and is imaged by the microscope objective to form a diffraction-limited spot on the specimen. The reflected light from the specimen passes through a conjugate set of pinholes on the other side and can be ob-

served in the eyepiece. Both the illumination and the reflected light are scanned in parallel over the specimen to generate the two-dimensional image of the focal plane by spinning the Nipkow disk. This microscope is called a **tandem-scanning reflected light microscope** since both (double or tandem) conjugate pinholes, located on opposite sides of the disk diameter, operate together or in tandem.

The user will notice that there is a large loss of signal in the confocal microscope designs that incorporate a Nipkow disk; since the ratio of the areas of the holes to the area of the disk is usually only about 1–2%, only a small fraction of the illumination reaches the specimen. This loss of signal is even more apparent when the Nipkow disk-based confocal microscope is used to image specimen fluorescence. Typically, the intensity of a fluorescent specimen is much lower than that from a highly reflecting specimen (i.e., semiconductors, hard tissue, mineral) and the fluorescent images are extremely weak. Therefore, the illumination must be very bright (a xenon or mercury arc lamp is usually required). Historically, to test the first instrument and in further work at Yale University, the sun with a heliostat on the roof of the laboratory was used as the light source.

These tandem-scanning confocal microscopes based on a Nipkow disk are best suited for reflected light confocal imaging. But, even in the reflected light mode, confocal microscopes based on a Nipkow disk containing pinholes have a very poor light throughput. In order to minimize cross talk between adjacent pinholes on the Nipkow disk, it is usually designed so that the separation between adjacent pinholes is about 10 times the pinhole diameter.

Various designs of the tandem-scanning Nipkow disk-based confocal microscope have been made. It is possible to make the Nipkow disk with several bands of apertures of varying sizes that are placed in the beam path. Real-time, direct-view scanning Nipkow disk confocal microscopes use round holes in the spinning disk; however, other designs have used square and rectangular holes. Another design used a spinning disk with slit apertures. In fact, in 1969 Egger, Gezari, Davidovits, Hadravsky, and Petràn designed and constructed a confocal microscope based on a rotating disk with slit apertures.

A tandem-scanning Nipkow disk-based confocal microscope is a poor choice for weakly reflecting specimens such as living cells, tissues, and organs. It is also not suitable for imaging weak autofluorescence or weakly stained fluorescent specimens. The low intensity of light that reaches the detector results in an image with marginal quality. However, for strongly reflecting objects such as hard tissue, composites, and microelectronics, the use of this type of confocal microscope is reasonable.

The advantages of the Nipkow disk-type confocal microscope are that it allows for real-time viewing, true specimen color, and direct observation. The microscope can also be used with white light and a microscope objective specifically selected because of its large chromatic aberrations. When the profile of a surface is to be imaged, the chromatic aberrations in the objective will separate the focal planes of light in the specimen according to the wavelength (color) of the light, and the resulting image will resemble a topographical map of the surface with different

heights encoded into different colors. Finally, the clever design of tandem scanning presents a simple mechanical solution that incorporates point scanning to form the image, and the use of sets of conjugate apertures to strongly discriminate against light from above and below the focal plane provides axial discrimination.

In conclusion, the real-time direct-view scanning confocal microscope based on a spinning Nipkow disk is an elegant solution to the inadequacies of previous wide-field light microscopes. With bright or highly reflecting specimens, the images are seen in real color and with excellent contrast. The microscope design is simple and it can be manufactured at low cost.

The limitations of this type of microscope are evident when the specimen has weak fluorescence. The size of the disk apertures is fixed; nevertheless, several sets of apertures with varying sizes could be located on the Nipkow disk. Also, the mirrors of the tandem-scanning confocal microscope are difficult to align and maintain correctly. Mechanical vibration that causes the disk to wobble while rotating can degrade image quality and brightness. Also, the large number of optical surfaces, in which each contributes to the loss of light throughput to the detector, reduces the image brightness.

Petràn also suggested that the use of an image-intensified video camera sensitive in the infrared would yield an additional advantage as a detector. The use of infrared light as the illumination would permit increasing the penetration depth within the specimen because of the reduction of scattering at the longer wavelengths (compared to visible light) as the light penetrates the tissue, and also as the reflected light passes through the tissue toward the microscope objective. In Part III, we shall again see the utility of illuminating the specimen with infrared light and the concomitant increase in penetration depth.

The next two sections provide innovative solutions to the problems associated with the tandem-scanning confocal microscope: the one-sided disk and the Nipkow disk confocal microscope with a microlens array.

7.4 Guoqing Xiao and Gordon Kino Invent the One-Sided Confocal Scanning Light Microscope

The two-sided, or tandem-scanning, Nipkow disk confocal microscope is optimally suited for the observation of reflecting specimens; it solves the problem of stray light reflected from the top surface of the disk. However, it is difficult to adjust and align the microscope due to the number of internal mirrors or prisms and to wobble.

The idea of using only one side of the Nipkow disk for a confocal microscope was first suggested by Egger and Petràn. With this instrument, there are no mirrors below the Nipkow disk that must be aligned. In spite of this great advantage, they subsequently decided against it because of the serious problem of eliminating the light that was reflected from the top surface of the disk.

Other groups also explored the one-sided Nipkow disk confocal microscope. In 1975 Albert Frosch and Hans Erdmann Korth were granted a patent that they filed

in 1974 for a "Method of increasing the depth of focus and or the resolution of light microscopes by illuminating and imaging through a diaphragm with pinhole apertures." Their invention was based on a **one-sided Nipkow disk** and they described a method to eliminate light from the disk, part of which involves tilting the Nipkow disk with respect to the optical axis of the microscope.

Xiao, Corle, and Kino, working at Stanford University, invented a real-time, one-sided, Nipkow disk-based confocal microscope (see Fig. 7.5), for which they

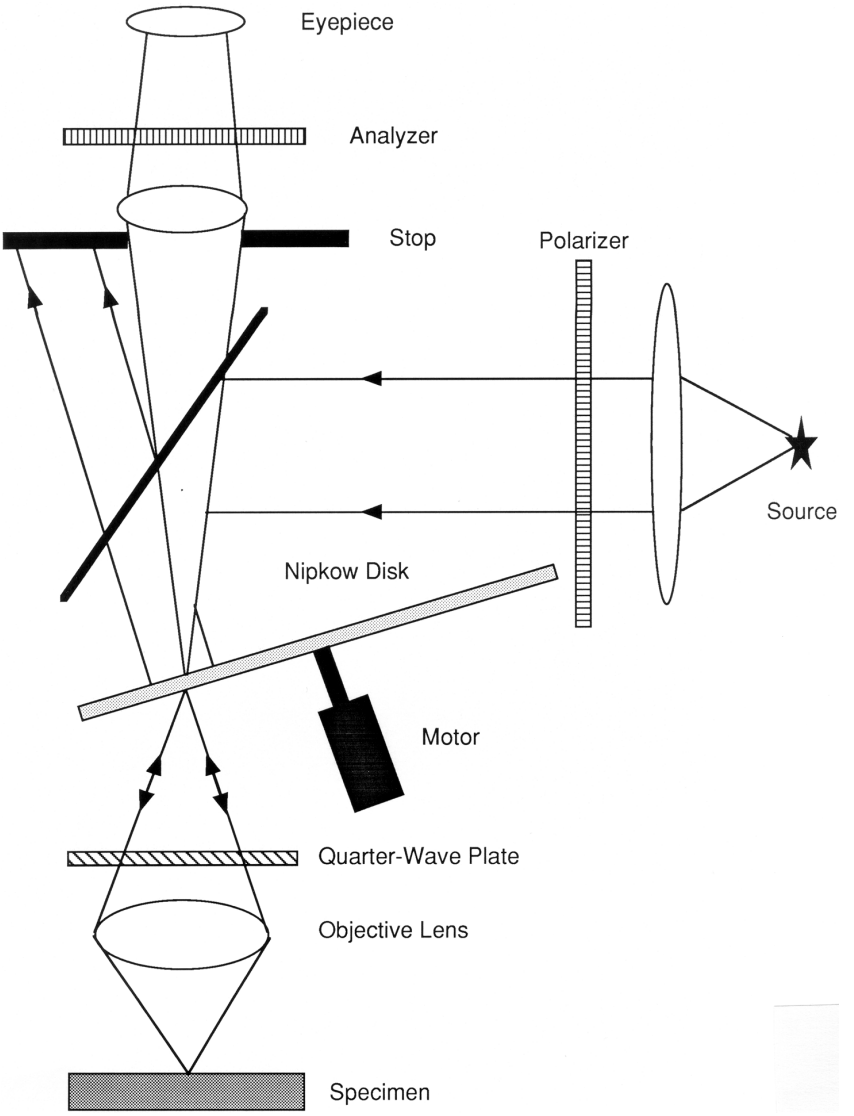

Figure 7.5 Schematic diagram of a real-time, single-sided, Nipkow disk confocal microscope. The actual Nipkow disk is about 10 cm in diameter and contains 200,000 pinholes, which are typically 20 μm in diameter.

received a patent in 1990. This design has several advantages over the tandem-scanning confocal microscope: it is less sensitive to vibration, has a simplified optical design, and is easier to align. Still another advantage is that while the disk is rotating, it can be translated horizontally, so different bands containing different pinhole sizes and/or shapes will be placed in the light path. This is a technique to change the pinhole or slit size without removing the disk and replacing it with another. This feature can be useful in the observation of specimens that have regions of differing reflectivities.

The driving force for their invention was the need to improve the metrology of semiconductor devices using simple optical confocal microscopes. Their confocal microscope used a rotating Nipkow disk in which the illumination and the reflected light passed through the same holes.

To reduce the reflected light from the surface of the Nipkow disk, three techniques were implemented. The disk was tilted approximately 5 deg. from the optical axis, so that the light reflected from its surface was reflected into a beam stop. The surface of the disk was blackened to reduce surface reflections. A polarizer was placed between the light source and the disk; hence, the disk was illuminated with polarized light. A quarter-wave plate was placed between the Nipkow disk and the microscope objective, and an analyzer between the Nipkow disk and the detector. The combination of polarizer, quarter-wave plate, and analyzer effectively separates the light from the specimen and that reflected from the disk surface. This optical arrangement sharply discriminates light reflected from the surface of the disk; similarly, it slightly reduces the light reflected from the object that reaches the detector.

A disadvantage of the one-sided design is that since the illumination and reflected light follow the same optical path, it is not easy to correct for chromatic aberrations in the microscope. This design, as with the tandem-scanning Nipkow disk-based microscope, still has the disadvantage of the low disk transmission, which also makes the microscope a poor choice for weakly reflecting specimens. Neither the one-sided nor the tandem-scanning Nipkow disk confocal microscope are suitable for use with weakly fluorescent specimens.

7.5 Effect of Pinhole Size and Spacing on the Performance of Nipkow Disk Confocal Microscopes

I have discussed the arrangements of the pinholes in the Nipkow disk and their various shapes. What about their size and spacing? How does the size and spacing of the pinholes affect their performance?

In previous discussions I introduced the words “point source of light” and “pinhole aperture.” An example of a point source of light is a star as observed by a telescope. The operational definition of these terms is that the geometrical image of the aperture is much smaller than its Airy pattern. Another way to frame the concept is as follows: if the geometrical size of a pinhole is less than the point spread function

of the lens, then it can be considered a point pinhole. Sometimes in the literature on confocal microscope we find the words "point" and "spot" are interchanged. A point is a geometrical object with no dimensions or extent.

In a Minsky-type of confocal microscope, the images of both the source and the detector aperture are co-focused on the specimen. The tandem-scanning and the one-sided confocal scanning microscope were invented in response to the long image acquisition time associated with the Minsky type of microscope. The Nipkow disk confocal microscopes reduced the image acquisition time and thereby permitted real-time imaging by using multiple apertures—and therefore multiple beams—in parallel.

The designer of a Nipkow disk confocal microscope has several choices in the construction of the aperture disk. First is the consideration of the optimal diameter of the disk apertures. Second, there is pinhole spacing or the distance between adjacent apertures. Third is the aperture shape. Fourth, the aperture-disk designer must select the pattern of the apertures.

All these design parameters depend on the nature of the object to be observed. Are the objects highly reflective semiconductor devices or are they weakly reflecting objects? Are they highly or weakly fluorescent? Microscope performance depends on the specimen and on the criteria most important to the observer: for integrated circuits, the user is often interested in measuring the profiles of stepped surfaces; for biological applications, the user is more often interested in distinguishing two neighboring point reflectors or fluorescent points.

When selecting the optimal pinhole size, the basic rule is that the aperture size should be adequate for the resolving power of the microscope, i.e., smaller than the central intensity spot of the Airy disk on the eyepiece side of the objective. For example, the aperture size is about 20–30 μm when used with a 100×/1.3 oil-immersion microscope objective with light at a wavelength of 550 nm. The aperture size should be selected for a given microscope objective; however, if it is selected for a high-NA objective, then the effect of using a low-NA objective is not critical.

For a given wavelength of illumination and an aberration-free lens with a defined NA, a point of light will be imaged as the Airy diffraction pattern. The geometrical image of the source aperture should be smaller than the main intensity peak of the Airy pattern. When the apertures are too small, there is a loss of signal because of the loss of light intensity at the object. As the aperture size decreases, the ratio of the aperture area to the disk area decreases, and therefore illumination to the object is decreased. If the size of the apertures is too large, a loss of resolution occurs in both axial and transverse directions; also, the illumination may not fill the pupil of the microscope objective, which results in a loss of NA and a consequent loss of resolution.

Pinhole spacing is the second important design parameter. In the ideal Nipkow disk confocal microscope are many sets of conjugate points, one on the illumination side and one on the detection side of the disk. The images of these conjugate apertures are co-focused on the object. Cross talk, which occurs when light passes through apertures other than the conjugate apertures on the image side of the disk,

is undesirable. This stray light from out-of-focus planes within the object, as well as from reflections in various parts of the microscope, will severely degrade the image contrast. When the Nipkow disk confocal microscope is used in the fluorescence mode, filters can separate the illumination and the fluorescence light based on their wavelength differences. When the pinhole spacing is too small, another effect occurs for the case of coherent light as the illumination source, e.g., laser sources: the appearance of speckle in the image resulting from the interference of light from adjacent points in the object.

7.6 Akira Ichihara and Coworkers at Yokogawa Institute Corporation Invent a Microlens Nipkow Disk Confocal Microscope

The fundamental limitation of the real-time, direct-view, Nipkow disk tandem-scanning confocal microscope is that the very small area of the disk that is covered with holes (typically 1–2%) results in an enormous light loss and low illumination efficiency. Therefore, it is difficult to use this microscope with weakly fluorescent specimens. This problem has been partially solved by a group of researchers of the Yokogawa Institute Corporation in Tokyo, Japan.

In the Yokogawa confocal microscope, a laser illuminates the upper spinning disk, which contains about 20,000 microlenses over the pinholes on the disk. The lower disk contains another 20,000 pinholes arranged in the same pattern as the microlenses on the upper disk. The key point is that the lower pinhole disk is localized in the focal plane of the microlens disk (see Fig. 7.6). The improved performance of this microlens confocal microscope is because of the enhanced "fractional area of the apertures," which results in increased illumination efficiency. Both disks rotate on a common axis. The light transmitted by each pinhole is focused by the microscope objective to a spot on the specimen. The reflected light from the specimen returns on the same path through the microscope objective and pinhole, and is reflected by a beamsplitter through a relay lens to a two-dimensional detector. The microscope uses the full NA of the objective. Approximately 1000 illumination beams of light are focused on the specimen at one time. This results in a brighter signal (because of improved illumination) and faster image acquisition.

Figure 7.6 shows the principle of the microlens confocal microscope. With the presence of the microlenses, the pinholes pass 40% of the light incident on the upper disk. The design achieves high light throughput and therefore high sensitivity even in the presence of weakly reflecting specimens. The small pinholes in the Nipkow disk achieve high resolution in the transverse and axial axes. Another advantage is the high frame rate: 1 frame/ms, though it is usually operated at video rates.

This clever microscope design has no optical relays between the pinhole and the objective lens. This is a great advantage for minimizing optical aberrations and distortions present in other designs. It also dramatically reduces the light loss so prevalent in other tandem-scanning Nipkow disk confocal microscopes. For a mi-

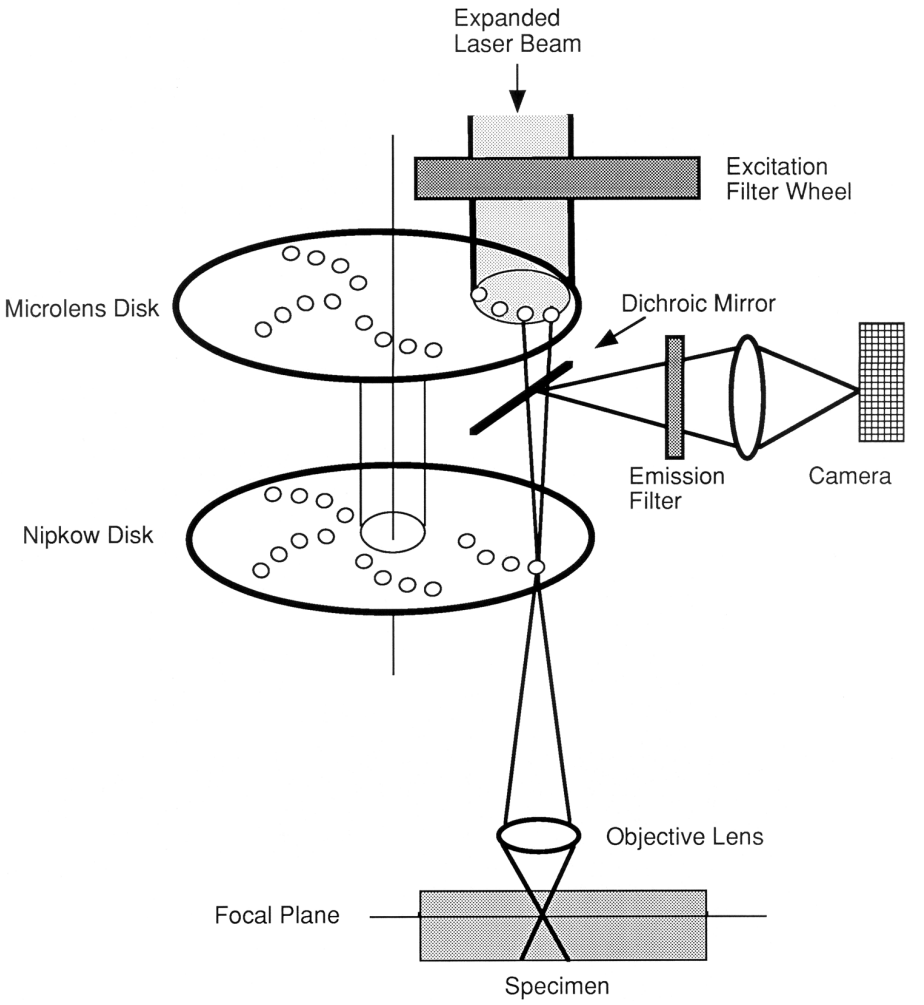

Figure 7.6 Schematic drawing of the microlens Nipkow disk confocal microscope.

croscope objective having an NA of 0.9 and a laser with a wavelength of 488 nm, the measured resolution on the optical axis is 0.6 μm (FWHM). Similar to the Kino design, a minimal effect of disk wobble on the image occurs. This type of confocal microscope is an alternative to laser-scanning confocal microscopes for studies on live cells and may have potential benefits for long-term cell biology studies. Now we leave the Nipkow disk confocal microscopes and discuss an alternative design based on conjugate slits.

7.7 Svishchev Invents an Oscillating Mirror Scanning-Slit Confocal Microscope

G. M. Svishchev, who worked at the Optical Laboratory, Institute of Biophysics of the Soviet Ministry of Public Health, Moscow, and was driven by the need to investigate neural tissue *in vivo*, invented a scanning-slit confocal microscope. Note that Minsky's confocal microscope was invented to study the three-dimensional structure of fixed, stained, thick brain slices. Petràn and his coworkers were also motivated to develop a new type of light microscope that could investigate the three-dimensional structure of unfixed, unstained, living brain slices. It is striking to note that different inventors, working independently in different parts of the world to achieve a common goal, invented three types of confocal light microscopes.

The key development in the Svishchev invention was a scanning system based on an oscillating two-sided mirror (see Fig. 7.7). This design is both simple and elegant; consequently, it eliminates the need for precision-controlled galvanometer mirrors for scanning and descanning. A simple two-sided mirror mounted on an oscillating rod performs the synchronized scanning, descanning, and rescanning for viewing. First published in a Russian journal in 1967 (and translated into English in 1969 and 1971), this clever design was subsequently redeveloped and reinvented in various designs of confocal microscopes in Europe and America.

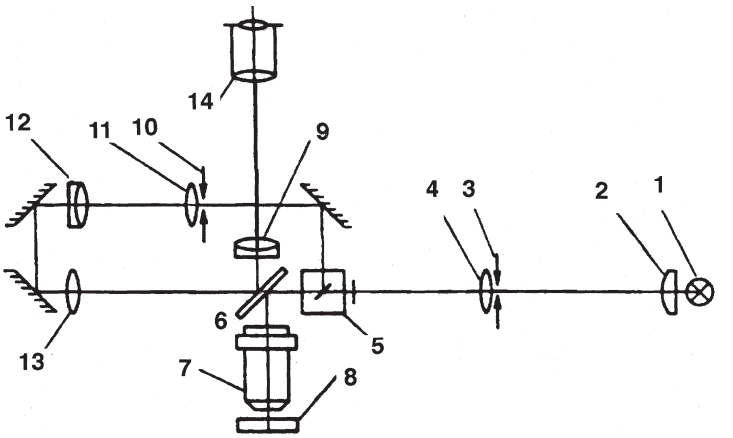

Figure 7.7 Schematic diagram of the Svishchev two-sided, oscillating mirror, scanning-slit confocal microscope. The light source, 1, is projected by a condenser lens, 2, onto the first slit, 3; the light passes through a prism cube, 5; an image of the first slit is scanned over the back focal plane of the microscope objective, 7, by the two-sided oscillating mirror, 6, which descans the reflected light from the focal plane in the specimen. The second slit, 10, is conjugate with the first slit (confocal) and excludes the light that is not in the specimen's focal plane, 8. The two-sided oscillating mirror, 6, performs three functions: it scans the image of the slit 3 over the back focal plane of the objective, descans the beam from the object, and rescans the beam for observation in the ocular, 14, or the film plane. Parts labeled 4, 9, 11, 12, and 13 are lenses.

Svishchev's confocal microscope provided for the effective removal of the light scattered by all the sample layers except the focal plane (Svishchev, 1969, 1971). This design produced a significant increase in the contrast of the image observed. Svishchev used an 85×, NA 1.0, water-immersion microscope objective. The use of a high-magnification, high-NA water-immersion microscope objective was subsequently incorporated into the instruments of Masters and Thaer. Svishchev published high-contrast photographs of the fine structure of cells and tissues in reflected light without the use of stains or ultraviolet light.

Furthermore, in a vision of future developments, Svishchev suggested the use of point apertures instead of slits together with continuous-wave lasers and solid-state detectors for improved imaging of thick, transparent, light-scattering objects. These proposals in his paper predated the development of laser-scanning confocal microscopes.

An alternative to point scanning, as exemplified in the designs of Nipkow disk confocal microscopes, is to use an illumination slit that is scanned over the back focal plane of the microscope objective. Since many points on the axis of the slit are scanned in parallel, the scanning time is markedly decreased; it can operate at video rate. Also, scanning-slit confocal microscopes have superior light throughput compared with point-scanning Nipkow disk systems. The disadvantages are that the microscope is truly confocal only in the axis perpendicular to the slit width, and it provides lower transverse and axial resolution than a pinhole-based confocal microscope. This comparison is for the same illumination and reflected light wavelength and the same microscope objective in each case. Even so, for confocal imaging of weakly reflecting living biological specimens, the trade-off between lower resolution and higher light throughput is acceptable.

Several arrangements have been developed to provide scanning of the illumination slit over the specimen and the synchronous descanning of the reflected light from the object. The simplest is the Svishchev design of a two-sided mirror mounted on a single oscillating shaft, which is the technique used in several modern designs of real-time confocal microscopes with bilateral scanning.

Scanning-slit confocal microscopes have several other advantages. The slit width can be adjusted, which allows the user to vary the thickness of the optical section as well as control the amount of light that reaches the sample and of reflected light that reaches the detector. This is important for samples that are very transparent, which can be imaged with a very narrow slit width; more opaque samples require a larger slit width.

As an example: the basal epithelial cells of a normal *in vivo* human cornea cannot be observed with a tandem-scanning confocal microscope. However, corneal basal epithelial cells can be observed *in vivo* when examined with a real-time slit-scanning confocal microscope. The reason is that although the tandem-scanning confocal microscope has higher axial and transverse resolution, the very low light throughput of the disk does not transmit enough reflected light from the specimen to form an image on the detector (in a single video frame) with sufficient signal to noise and, therefore, contrast to show an image of the cells. The reason is the low illumination efficiency of the Nipkow disk.

Now we have completed our discussion of Nipkow disk confocal microscopes, innovative modifications such as one-sided Nipkow disk confocal microscopes, Nipkow disk confocal microscopes with microlens arrays, and scanning-slit confocal microscopes. Today the most common type is a laser-scanning confocal microscope. Its origin was in the Minsky confocal microscope and patent, as well as many clever inventions in confocal microscopy since the time of his invention. In the next section we discuss several of these.

7.8 Laser-Scanning Confocal Microscope Designs

In this section, the design of the modern laser-scanning confocal microscope is presented. Two terms are often used interchangeably: laser-scanning confocal microscope (LSCM) and confocal scanning laser microscope (CSLM); this text will primarily use the former term. The previous discussion introduced many features and components, while this section provides some of the missing details and presents the LSCM as a complete instrument. Figure 7.8 shows the design of the LSCM.

Minsky's patent pointed out the key design principles for a confocal microscope. A careful reading of these patents is instructive to understand the various solutions to similar problems. Many of the ideas cited in these patents were eventually incorporated into commercial confocal microscopes.

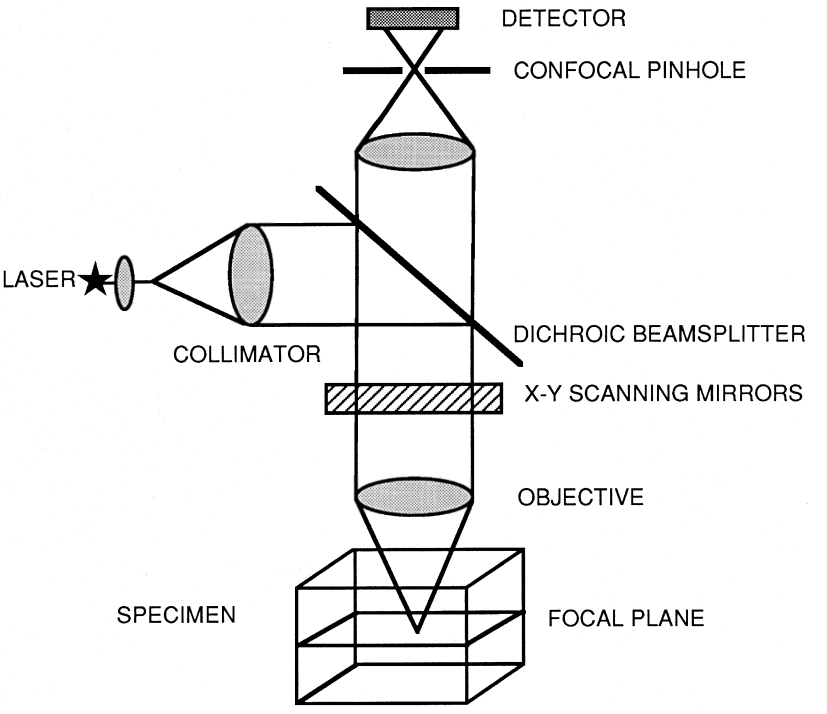

Figure 7.8 Schematic diagram of a confocal fluorescence microscope.

The design specifications of a commercial confocal microscope are constrained by many factors, but chief among them is intellectual property. Who owns the patents? Sometimes new designs were manufactured only to bypass the patents of others. Manufacturing costs and microscope maintenance is another consideration. The market is eventually heard, but change comes slowly.

Today, the user of a fluorescent confocal microscope has a choice of several excellent instruments. Many high-quality, corrected microscope objectives are available for a variety of purposes. Laser light sources are available to cover a range of wavelengths. The scanning systems and intermediate optics as well as the detectors are of high quality. These new confocal microscopes usually have several channels (wavelengths), a graphical user interface to operate and control the microscope, and software is available to process, analyze, and archive the images.

In the last few years, commercial laser-scanning microscopes have offered a variety of useful and improved features. New confocal microscopes are available with very compact scanning systems. They can be attached to a standard fluorescence microscope to convert it to a confocal microscope. A variety of air-cooled, low-noise compact lasers with a range of wavelengths can be purchased. Lifetime imaging can be added to these confocal microscopes by the use of add-on components. The graphical interface has been improved and a keyboard and mouse have replaced rotary dials.

While the original **analog-to-digital converters**, the electronic chips that convert the analog output of the photomultiplier detector to a digitized output, had a dynamic range of 8 bits, the new systems use 12, 14, or 16 bits. That results in a great improvement in the dynamic range of the system. A larger dynamic range permits more intensity steps between the lowest and highest light level in the image.

At first, the standard fluorescent microscope objectives were the only ones available. More recently, new series of microscope objectives have been specially designed for use with the fluorescent confocal microscope; the use of wider threads permits new microscope objectives with a high NA and a low magnification, which result in a wide field of view.

Confocal microscopes are also designed for clinical diagnostics. The goal of many of these is that the axial resolution will permit optical biopsy, that is, the diagnostic evaluation of tissue without the need for excision. There are confocal microscopes designed to image the skin *in vivo*; some use laser and others slit scanning.

In the field of ophthalmology, there are a variety of clinical instruments. Scanning-slit confocal microscopes that use noncoherent halogen lamp light sources are preferable for examining the cornea. Older designs are based on the Nipkow disk tandem-scanning confocal microscope. In addition, LSCMs are designed to image the cornea. Laser-scanning ophthalmoscopes are extremely useful diagnostic confocal microscopes for retinal examination.

The original patent of Minsky contained the concepts that are implemented in the commercial LSCMs used for both laboratory investigations and in the scanning laser ophthalmoscope. The availability of the laser provided a new, bright light source that resulted in several new laser-scanning microscopes. In the last decades,

many technological innovations in beam-scanning confocal microscopes were developed. Wilson in Oxford, UK, and Sheppard in Sidney, Australia, developed various types of confocal microscopes. Brakenhoff demonstrated the importance of high-aperture immersion microscope objectives for optical sectioning.

The scanning-stage confocal microscope, which uses stage or specimen scanning, is another development that follows Minsky's original ideas. This type of confocal microscope uses the paraxial rays of the microscope objective, so the images are of excellent quality and contrast. The disadvantages are that the image acquisition time is slow (several seconds per frame) and the instrument is sensitive to vibration. For biological and clinical applications, the slow image acquisition time is undesirable; most users wish to view the image in real time as they vary the field of view and the position of the focal plane.

We now will review some interesting technological developments in components that preceded the modern LSCM. Full details are available in the original publications and patents. Not all of these technologies have become available in modern commercial instruments, although some of them have been implemented. Sometimes a company will approach the inventor and license the technology protected in a patent. Sometimes the company will market instruments based on the use of unlicensed intellectual property. In that case the patent owner may go to court; consequently, out-of-court settlements are not uncommon. In other cases, for example, with the patent covering the multiphoton excitation microscope, a company may go to court to challenge the validity of a particular patent. In the next paragraphs we briefly survey some of these key technical, patented developments.

In 1983, Werner Schmidt, Gerhard Müller, Klaus Weber and Volker Wilke, while working at Carl Zeiss-Stiftung in Oberkochen, Germany, invented a "Method and apparatus for light-induced scanning microscope display of specimen parameters and their distribution." One part of their invention contained all the components of a modern LSCM: a laser light source, point scanning of the diffraction-limited spot of light on the specimen, use of two orthogonal oscillating scanning mirrors that form the raster scan, and an aperture in front of the detector. Following the idea of Minsky, the Zeiss microscope used a folded design with a beamsplitter that operated in the reflected-light mode. What was new was the laser light source and the set of orthogonal scanning mirrors for laser beam scanning. The scanning microscope was designed to separate the illumination light from the fluorescence emitted by the specimen. It was also designed to simultaneously operate in two channels: e.g., Raman scattering and fluorescence, or scattered light and fluorescence. Their patent contained another important proposal: the light source could be an array of light sources (point sources in a linear or two-dimensional array), and the detector could be a linear array. The scanning and detection could be electronically synchronized by activating each single-point light source in the source array and the corresponding point detector in the detector array.

The next invention solved the problem of slow image acquisition that characterized the laser beam scanning confocal microscope based on two orthogonal, oscillating galvanometer mirrors (Draaijer, Houpt, 1988). Pieter M. Houpt and Arie

Draaijer in the Netherlands received a patent in 1989 for a "Confocal laser scanning microscope." Their goal was to design a laser scanning microscope with rapid line or frame scanning. They proposed to combine electronically a number of thin "optical sections" to form an image with an increased depth of focus. They proposed the use of an acousto-optical deflector together with a mirror galvanometer to increase the frame rate. (An **acousto-optical deflector** is a solid state device in which sound waves in a crystal form a diffraction grating that can be used to deflect the incident light. This phenomenon was predicted in 1921 by L. Brillouin for the case of a liquid traversed by ultrasonic waves and illuminated with white light. He predicted that diffraction would occur in analogy to a grating. In 1932 his prediction was experimentally confirmed by P. Debye and F. W. Sears, and independently by R. Lucas and P. Biquard.)

An acousto-optical deflector uses the first order of the diffracted light; hence, its wavelength and intensity are controlled by the frequency and amplitude of the ultrasonic wave in the crystal. The problem with acousto-optical deflectors is their wavelength dependence in both the deflection angle and efficiency. Therefore, longer fluorescent light cannot be passed back on the optical path through the acousto-optical deflector.

For the reflected-mode confocal microscope, the light is descanned from the specimen and the reflected light is passed through a pinhole aperture before detection. However, for work in fluorescence imaging, a dichroic mirror redirects the fluorescence light to a slit in front of the detector. Therefore, the fluorescence light does not re-enter the acousto-optical deflector. The fluorescence-mode microscope is only confocal in one dimension. The advantage is high image frame rates.

The use of galvanometer mirrors to speed image acquisition in confocal microscopes is embodied in the invention of Yoshiaki Horikawa, who worked at the Olympus Optical Company in Japan. His 1990 patent, "Scanning optical microscope," describes the invention: Light from a laser source is deflected by an acousto-optic deflector to scan the specimen at high speed; the light from the specimen does not pass back through the acousto-optical modulator, but is focused through an aperture to the detector. Since the diffraction in the acousto-optical light deflector is sensitive to wavelength, it cannot be used to descan the emitted fluorescence. If the device has a high diffraction efficiency for the laser light used for illumination, then the diffraction efficiency for the wavelength of the fluorescence is low. The result is a loss of fluorescence light. Another key part of the patent is that instead of a circular pinhole, a slit aperture is used in front of the detector. In summary, the light source is a laser beam that is scanned over the specimen. Scanning is provided by an acousto-optical device that scans the beam in the horizontal direction. An oscillating galvanometer mirror scans the laser beam in the vertical direction. The reflected light or the fluorescence light is taken out of the optical path by a beamsplitter, passes a slit aperture, and then is detected.

Another important advance was the 1987 publication by Kjell Carlsson and Nils Aslund, working at the Royal Institute of Technology in Sweden, of "Confocal imaging for 3-D digital microscopy," showing how the optical sectioning capabil-

ity of the confocal microscope can be combined with digital image processing to provide three-dimensional microscopy. The inventors obtained a patent and constructed a beam-scanning confocal microscope that could be attached to a Zeiss Universal microscope, the only modification being a stepping motor on the fine focusing screw. The light source is an argon laser. Laser beam scanning is performed by placing two oscillating mirrors on orthogonal axes above the eyepiece of the light microscope. The slow-scan mirror is driven by a stepping motor, and the fast-linescan mirror by a galvanometric scanner. The laser beam is raster scanned over the back focal plane of the microscope objective. The light from the specimen is collected by the microscope objective, retraces the optical path, and is separated from the illumination light by a beamsplitter (dichroic mirror). The light from the specimen passes an aperture and is detected. Various dichroic mirrors are mounted on a rotatory wheel, and various pinhole apertures are mounted on a second rotatory wheel in front of the detector. The entire microscope system is controlled by a microprocessor. The confocal microscope could display 1024 × 1024 pixels, although it was sensitive to misalignment from vibration.

John White, William Bradshaw Amos, and Michael Fordham, working at the Medical Research Council Laboratory of Molecular Biology in Cambridge, UK, invented another variation of the LSCM. Their motivation was to produce a confocal microscope that was stable, insensitive to vibration, and optimized for fluorescence microscopy of biological specimens, mainly cells and tissues in culture. In addition, they wanted a design that could be retrofitted to an existing standard fluorescence light microscope. It was their 1987 paper, "An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy," that convinced the biological research community of the great utility of the LSCM.

Their invention is discussed in detail in their 1991 patent, "Confocal imaging system." In a 1991 correction to the U.S. patent, they list references to the missing U.S. Patent Documents—the patents of the following inventors: Barnes; Weber, Davidovits et al.; Baer; and Divens et al. Clearly, the White, Amos, Fordham design is based on many innovations of prior inventors. The unique feature of their invention is the nature of the input and detector apertures. An afocal set of mirrors transfers the beam from the first to the second galvanometer mirror. By expanding the optical path to almost 1.5 m, yet folding the optical path to make the instrument more compact, the apertures are millimeters in diameter. The optics formed an image about 80 times the magnification of the microscope objective at a distance of ~1.5 m. The Airy pattern at the detector was also enlarged. Therefore, a variable iris aperture from 0.7 to 7 mm could be used in front of the detector. Depending on the light intensity from the specimen, the iris could be made larger to pass more signal, or smaller to afford enhanced optical sectioning. An iris diaphragm has several important advantages compared to a pinhole aperture: it is adjustable, inexpensive, easy to align, and the dust and dirt that readily spoil a very small pinhole are not a problem.

A number of mirrors are used to fold the beam inside the laser scan assembly. Since each optical surface contributes to a loss of signal, the sum of all the surfaces

from the folding mirrors, the two scanning mirrors, and the two concave mirrors of the afocal assembly result in considerable signal loss.

In the invention, the scanning elements are two plane mirrors oscillating on orthogonal axes. An afocal set of mirrors is another key component. These concave mirrors serve as a telescope to transfer the light from the plane mirror on the first galvanometer (y axis) to the plane mirror on the second galvanometer (x axis) and then to the eyepiece of the microscope. The scanning optics assembly is situated so that the exit pupil of the microscope eyepiece falls on the area of the plane mirror near its axis of oscillation. The use of spherical concave mirrors in the afocal assembly provides a system without chromatic aberrations, which is critical since the two- channel confocal microscope is used with at least two different fluorescent probes.

Other components of the scan head include a filter set adjacent to the laser source, and a second filter set that separates the light from the specimen into two channels, each with its own variable iris and its own photomultiplier tube.

Finally—and this is a key point in their patent—by adjusting the angle between the oscillating axis of the slow (frame) galvanometer mirror and the line of the input laser and returning beam, the scan lines on the specimen are linear.

7.9 Analytical Expression of Resolution in a Confocal Microscope

Lateral or **transverse resolution** is in the plane of the specimen or the x-y plane. **Axial resolution** is along the z-axis or the optical axis of the microscope. The lateral resolution of a confocal microscope is proportional to the NA of the microscope objective; however, axial resolution is more sensitive to the NA of the microscope objective. Therefore, to obtain the maximum axial resolution, and hence the best degree of optical sectioning, it is preferable to use microscope objectives with the largest NA. For an oil-immersion microscope objective with a NA of 1.4 and blue-light wavelength of 442 nm, the lateral resolution is 0.14 μm and the axial or depth resolution is 0.23 μm.

The theoretical analysis of the resolution in a confocal microscope expresses the resolution as a function of the wavelength of the light and the NA of the microscope objective. Another factor that affects the resolution is the contrast of the image. The contrast is determined by such factors as the number of photons detected per pixel, the noise contribution (Poisson statistics and instrument noise), the SNR, and the signal-to-background ratio. Resolution in a confocal microscope is linked to image contrast, and therefore to the number of detected photons.

We begin with the definition of a **resel**, which is the resolution unit transverse to the optical axis. The central bright portion of the diffraction pattern is the Airy disk, which has a defined radius of 1 resel. A real aberration-free microscope objective has a finite aperture, and therefore the resolution is diffraction-limited. If the lens has a NA that is given by $n \sin \Theta$, then a resel is defined as one-half of the diameter of the Airy disk, which is equivalent to the first dark fringe in the diffraction pattern.

The lateral resolution of a conventional and a confocal microscope are now compared following the analysis of Wilson (Wilson, 1990). We examine the case

of a conventional microscope with the pinhole removed, and a confocal microscope with the pinhole placed in front of a detector. In each case, the image of a single point specimen is viewed in reflected light.

The following equation is also the analytical expression for the Airy disk. The Airy pattern is the response for an aberration-free lens with a circular pupil. This Airy pattern, also called the Fraunhofer diffraction pattern of a circular aperture, is what is observed when a point source, e.g., a star, is imaged with a telescope; it has a bright central disk surrounded by concentric bright and dark rings. The intensity of the bright rings decreases rapidly with their radius.

The image intensity of a conventional microscope is given by Eq. (7.1), which was derived by Airy in 1835,

$$I_{conventional}(\nu) = \left[\frac{2J_1(\nu)}{\nu}\right]^2. \tag{7.1}$$

where $I_{conventional}$ is the intensity of light from the object, J_1 is a Bessel function of the first kind of order unity, ν is a coordinate related to the lateral distance, r, in the focal plane. Where n is the refractive index of the medium in the space between the objective and the specimen, r is the real radial coordinate in image space, and λ is the wavelength of the incident light, the NA of the objective is n sin Θ, the coordinate ν is perpendicular to the optical axis and is a normalized coordinate:

$$\nu = \frac{2\pi}{\lambda} rn \sin\Theta. \tag{7.2}$$

For the confocal microscope case, a pinhole is in front of the detector (a point detector); the image intensity of a point source of light in the focal plane is now given by

$$I_{confocal}(\nu) = \left[\frac{2J_1(\nu)}{\nu}\right]^4. \tag{7.3}$$

These equations are for a point object and will be different for plane objects, as well as in the case of fluorescence (incoherent light). The key result is that the lens images a point object and an intensity distribution called the Airy disk. When we compare a confocal (point detection) with a conventional microscope, in which both image a point object in reflected light, we find that the Airy disk in the confocal microscope is narrower and the sidelobes from the central intensity peak are reduced, which is the physical explanation of the increased resolution obtained with a confocal microscope.

We now consider the axial distribution of light from a point source of light that is imaged with an aberration-free lens with a circular aperture. The light distribution is given by Eq. (7.4) for a conventional microscope:

$$I_{conventional}(u) = \left[\frac{\sin(u/4)}{(u/4)}\right]^2. \tag{7.4}$$

The normalized axial coordinate, u, is given by Eq. (7.5), where z is the real axial coordinate in image space, and λ is the wavelength of the incident light:

$$u = \frac{2\pi}{\lambda} z(\mathrm{NA})^2. \tag{7.5}$$

For comparison, we present the result for the confocal microscope:

$$I_{confocal}(u) = \left[\frac{\sin(u/4)}{(u/4)}\right]^4. \tag{7.6}$$

For the case of a point object, these equations show that for a confocal microscope the central peak is sharpened as compared to the conventional microscope.

The axial resolution is higher for a plane object than for a point object in reflection, and can be measured from a plot of the variation in the axial image intensity of a point object on the optic axis as the object is displaced through the focal plane. Experimentally, it is observed in a confocal microscope that the depth of field, which is operationally defined at the half-maximum height or a plot of intensity against axial distance, is reduced relative to a nonconfocal microscope by a factor of 1.4.

7.10 Comparison of Different Confocal Microscope Designs: Which One Should You Purchase?

How does the direct view, real-time, tandem-scanning microscope compare with the LSCM? How does beam scanning compare with stage or specimen scanning? The answers to these questions are related to the more general question: Which confocal microscope is best for my needs? The answer depends on what the user wishes to observe. A confocal microscope used to observe *in vivo* human skin or the living eye in the ophthalmology clinic will require different features than one used to study computer chips or integrated circuits. Different types of confocal microscopes may be optimal for intravital studies of the nervous system, embryo development, or single-molecule fluorescence studies. In the case of a microscopy center with multiple users, the choice of a confocal microscope would involve a compromise of several features. Confocal microscopes suitable for general use are never optimal for a specific use.

In general, commercial confocal microscopes are usually purchased and used. While the cost is high, there are many benefits; for example, the systems are ready to operate and the time from installation to use in research is short. There are also commercial courses that some manufacturers provide to compress the learning curve. The commercial confocal microscopes usually provide computer software

to control the operation of the system, from changing lasers and filters to changing objectives.

As for which confocal microscope is best, the answer is simple: try before you buy. Either take your samples to a working confocal microscope in the area or arrange for the manufacturer to place a demonstration unit in your facility. What is critical is that you test your particular specimens over a period of time (weeks rather than days is preferred). Try all the modes and all the features that you think are critical to your research. That period of time is important to help you decide how the confocal microscope meets other requirements related to economic considerations, maintenance contracts, record of service for the user, number of independent channels required, possibility of conversion to a multiphoton excitation microscope in the future, upright or inverted configuration, and ease of modification, e.g., to add lifetime imaging. Most commercial systems have extensive specifications that are available. It is also important to speak with several individuals who use a particular commercial confocal microscope and hear about their experiences.

Another approach is to purchase the basic building blocks of a commercial confocal microscope and then modify the system to meet your specific needs. That option requires a broad knowledge of optics, mechanics, signal processing, computer interfacing, and system design. Still, the rewards can be great. It is possible to construct a confocal microscope with specifications unique to your requirements. Also, in the process of constructing your microscope, you experience the great learning process of doing it yourself. This approach can also be accomplished if skilled individuals are available to design the systems and machine and assemble the components. Today there are an abundance of precision mechanical, optical, and photonic components. Commercial software programs such as LabView® are useful for control functions, data acquisition, and manipulation. Also, there are a wide variety of computer software programs for image processing; for example, the free software package *ImageJ*, which is available for several computer platforms from the National Institutes of Health.

A third approach is to construct your own confocal microscope, by purchasing the laser light sources and the basic microscope, and adding commercial components to meet your requirements for scanning and detection. Some people design and construct custom circuit boards for control functions and data acquisition as well. There is a wonderful feeling of accomplishment when you design and construct your own confocal microscope, and it can be an educational experience for students and others who are involved in the process.

When determining what type of confocal microscope to buy, ask yourself what data acquisition rates are required for your studies. If you are only observing fixed, stained sections, then rapid image acquisition rates are unnecessary. If you are interested in observing rapid transient events, such as calcium spikes, or events in excitable tissue, then the kinetics of these events set higher requirements for image acquisition speeds. For reflected-light confocal microscopy of highly reflecting specimens such as hard tissue (bone, teeth) or semiconductor wafers, the real-time, direct-view tandem-scanning confocal microscope could be ideal. For other appli-

cations, a rapid beam scanning confocal microscope that uses acoustic-optical scanners may be ideal. Stage or specimen scanning confocal microscopes would not be suitable, as they are too slow.

Another important decision is whether to use stage scanning or beam scanning. Both types of confocal microscopes present different advantages and limitations. As previously stated, with stage or specimen scanning there are no off-axis aberrations to correct. In principle, the specimen could be quite large. If the specimen is stable, the stage could be programmed to scan the complete specimen even if the process required many hours. Beam scanning confocal microscopes scan the light beam over the back focal plane of the microscope objective. Therefore, many rays are not paraxial, and off-axis optical aberrations are a consideration. Image acquisition time in beam scanning is usually limited by the signal intensity at each spot or pixel in the scan. With a strong signal it is sufficient to have a short **dwell time** on each pixel to collect the light. With much weaker signals, i.e. low-level fluorescence, a longer dwell time on each pixel is usually required to obtain a sufficient signal –to noise ratio for good contrast and image quality. The disadvantage to increasing the dwell time is that the photobleaching rate increases and the viability of living specimens decreases. Alternatively, the aperture in front of the detector can be opened to increase the strength of the detected signal; but the wider aperture will adversely affect the optical sectioning capability of the confocal microscope.

It is important to match the microscope capabilities to the use of the instrument. Applications in medical research and biology generally use fluorescence techniques. In the transmission mode, it is possible to use DIC and other contrast methods such as fluorescence.

7.11 Limitations of the Confocal Microscope

What are the major limitations of tandem-scanning confocal microscopes and LSCMs? I have previously discussed the loss of light in the illumination side of a Nipkow disk-based confocal microscope. This loss of signal scales with the ratio of the aperture area to the disk area.

Another, more subtle loss of illumination occurs within the specimen itself. If the thickness of the specimen homogeneously scatters and/or absorbs the incident illumination light, then the intensity will be successively reduced layer by layer throughout the specimen. Alternatively, the specimen can have a highly absorbing or highly scattering layer on the surface or within its thickness. In each case, the illumination decreases at the lower layers of the specimen. Light from the illumination beam lost because of scattering or strong absorption cannot be regained and used to illuminate lower regions of the specimen. The observer will see a reduced signal intensity at the lower planes.

The main limitation is detection of the signal used to display the image. First, we ask, what limits the signal? On the illumination side, we can increase the brightness of the light. Typically, lasers are used as the excitation source. At first you may think that the brighter the light source, the stronger the induced fluorescence of the

specimen, and therefore the stronger the detected signal. Can we increase the source brightness without limit? The answer is no.

The laser sources typically used with confocal microscopes are extremely bright, and the diffraction-limited focused spot on the specimen is many times the brightness of the surface of the sun. If the specimen to be observed is living—for example, any specimen of live cells, live tissue such as brain slices, a developing embryo, or clinical *in vivo* microscopy of human eyes or skin—then there is a chance for light-induced damage to the specimen, which could be caused by thermal, mechanical, photochemical, photophysical, or any combination of these means. The goal of intravital microscopy is to minimize damage to the specimen induced by illumination. The same goals hold for human clinical microscopy, for which there are added safety and ethical considerations. Light-sensitive organs such as the eye are only permitted to be subjected to light levels that have been shown to be safe.

The second process that limits the useful intensity of the illumination light is the photophysics of the fluorescence process. In the absence of light, the fluorescent molecules (whether naturally occurring, such as NAD(P)H or serotonin, molecules labeled with fluorescent probes, or genetically expressed fluorescent molecules) are in their ground electronic state. Only when illuminated with the appropriate light are they excited to higher electronic states. When the fluorescent molecules return to the ground electronic state they emit light, which is what we call fluorescence. The process of fluorescence is not instantaneous; it has a finite duration, which is measured by the fluorescence lifetime (typically nanoseconds). As we increase the intensity (brightness) of the illumination light from zero to increasing intensities, we first observe an increase in the fluorescence intensity. Above a certain threshold, which is a property of a particular fluorescent molecule and its electronic structure and energy states, we observe that the fluorescence intensity saturates. It is the **fluorescence saturation** of a population of fluorescent molecules that places an upper limit on the intensity that we use to induce the fluorescence. The physical basis for the fluorescence saturation is that at high light intensities, all of the molecules are in the excited state, and there can be no further absorption of exciting light. Only when a molecule emits a photon and returns to the ground state can absorption recur.

Once the specimen is induced to emit fluorescent light, the goal is to collect and detect every photon from the focal plane. Since we are talking about both confocal microscopes with their conjugate apertures to discriminate against defocused light, we limit our arguments to the focal plane. Assume the fluorescent molecules are randomly distributed in the focal plane; that means the molecules have an equal probability of emitting a photon in the fluorescence process in any direction. If we further assume that these emitted photons all escape the specimen (although there could be multiple points of scattering along the escape path), then we see the immediate problem.

Since every photon contains information, every photon counts and should be collected, detected, and contribute to the displayed specimen image. When the intensity of the emitted fluorescence is high with respect to other losses in the micro-

scope and noise and quantum effects in the light detectors, the loss of many photons is not very important. However, when the intensity of the detected fluorescence is very low with respect to problems of detecting these few photons, then we are up against real limitations. In order to collect all these photons, the microscope objective should completely surround the specimen! As many photons will be emitted in the direction of the microscope objective as will be emitted in the opposite direction, and are therefore undetected.

Assume we use high-NA microscope objectives with the correct thickness of cover glass and immersion fluid for the objective; then only those emitted photons that enter the acceptance angle of the microscope objective (higher NAs collect more light) can be detected.

Between the microscope objective and the light detector, considerable light loss occurs in the confocal microscope. In general, each optical element, whether mirrors, filters, polarizers, or lenses, contributes to the loss of light. As the number of optical surfaces increases, the loss of light increases, which is assuming the use of state-of-the-art antireflection coatings on each surface. Therefore, the goal is to design confocal microscopes with a minimum number of surfaces that result in light losses.

So, the remaining light that comes from the specimen is to be detected. Note that in low-level fluorescent confocal microscopy, electronic detectors typically are used. With the direct-view tandem-scanning confocal microscope it is possible to use a two-dimensional imaging device such as a film or charge-coupled device (CCD) camera. LSCMs usually use a low-noise photomultiplier to detect the fluorescence light.

For extremely low levels of fluorescence at the face of the detectors, the quantized nature of light (discrete photons) and the sources of noise in the detector and the associated amplifiers place limitations on how few photons or how low the light intensity can be and still be detected.

When our eyes are correctly adapted, they can detect single photons over a range of several log units of intensity. Solid state light detectors are less efficient. At best, we may achieve 90% detection efficiency with CCD cameras, but typically the value of the quantum efficiency is less than that. The **quantum efficiency** is the percent of photons incident on the detector that generate a signal. For a photomultiplier the quantum efficiency, which depends on wavelength, may be in the range of 1% to 40%. Note also that the detection efficiency of solid state detectors also varies with wavelength. Usually, these devices are selected to have high quantum efficiency in the wavelength regions that match our experimental conditions.

An important consideration is the statistical distribution of detected photons. The quantized nature of photons in a light beam is the physical cause of the distribution. For a coherent light source this probability distribution can be described by the Poisson distribution, which is valid for an ideal laser source that emits monochromatic light; the light is coherent and single-mode. As an example, if 100 photons are generated in a beam of light, then the inaccuracy in detection is about ± 10 photons.

Our ideal laser is assumed to output photons in a random manner. If the mean value of the average intensity is n, then the noise associated with this average signal is given by the root mean square of n. A useful parameter is the SNR. Typically, the SNR is equal to the mean value squared, divided by the variance. For the Poisson distribution, the SNR is equal to the mean value of the signal. This has an important consequence: the SNR increases as the mean number of photons, or signal strength, increases.

Another set of problems limits the use of fluorescent confocal microscopes for intravital microscopy of thick, highly scattering specimens. Confocal microscopes, their light sources, their microscope objectives, and all the optical components are designed, optimized, and constructed for use with visible light. When these microscopes are adapted with ultraviolet light sources without extensive modification of the components, new problems arise in three classes: (1) the requirement to drastically modify confocal microscopes that were optimized for visible wavelengths; (2) ultraviolet damage to live cells, tissues, organs and embryos; and (3) the limited penetration depth of ultraviolet light due to enhanced light scattering in thick specimens.

First, many examples of fluorescence confocal microscopy exist in which the fluorescence is excited in the ultraviolet wavelength region. For example, studies of NAD(P)H fluorescence in cell metabolism, measurements of intracellular ion concentrations with ion-indicators that absorb in the ultraviolet, photoactivation or uncaging of trapped ions and molecules that are triggered with ultraviolet light, studies of cellular autofluorescence, and fluorescence of many biological molecules with absorption peaks in the ultraviolet. Several investigators modified commercial confocal microscopes by introducing lasers and other light sources with ultraviolet light; furthermore, they changed the microscope objectives, beamsplitters, filters, optical coatings, and other components to maximize the system for use with ultraviolet light. While these drastic modifications had partial success in individual instruments, other limitations arose.

It was demonstrated many years ago that ultraviolet light damages and is lethal to living cells, tissues, and organisms. The use of a confocal microscope with highly focused ultraviolet light is dangerous for *in vivo* human studies such as diagnostics in ophthalmology and dermatology. When used for fluorescent intravital microscopy over an extended period of time, the high-energy ultraviolet light is absorbed by critical cellular components and induces damage and eventual death. This precludes the use of ultraviolet confocal microscopy to study the development of embryos and long-term observation of cells in tissue culture.

Also, it is observed that the penetration depth of ultraviolet light into thick biological tissues is less than that with longer-wavelength visible light. That effect results from increased light scatter and increased absorption of the ultraviolet light in cells and tissues.

All these limitations are severe and place limits on biological studies that require the advantages of a fluorescent confocal microscope but with the added constraint of ultraviolet excitation of the fluorescent molecules of interest.

All confocal microscopes have limitations. The signal strength can be degraded by multiple sources: illumination efficiency of Nipkow disk-based confocal microscopes; absorption and scattering of the illumination light within the upper regions of the specimen, which reduce the illumination intensity at the lower regions of the specimen; reflecting surfaces within the microscope resulting from multiple optical components and surfaces; stray light reaching the detector; misalignment of the components; dirt and dust on the apertures and other optical surfaces; quantum efficiency of the detector; the quantized nature of light; the signal to noise ratio; the image contrast; and the type of object imaged.

The partial solution to these formidable problems came with the development of nonlinear microscopy, specifically multiphoton excitation microscopy. Part III is devoted to this important advance.

7.12 Summary

- The greatest advantage of the confocal microscope is the elimination of out-of-focus light (depth discrimination). Point illumination and conjugate point detection is the principle, with the images of both apertures cofocused in the specimen. Spatial filtering is used to eliminate the out-of-focus light. Optical sections of less than 1 μm thickness can be imaged within thick, scattering tissue.
- The optical sectioning (depth discrimination) capability of a confocal microscope is the basis of three-dimensional microscopy. With defocus, the image becomes darker and disappears. A conventional fluorescence microscope has no optical sectioning capability; with defocus, the signal is constant but fuzzier.
- Consideration of signal intensity and the related signal-to-noise ratio is of paramount importance for detection.
- Marvin Minsky is credited with the experimental realization of a stage scanning confocal microscope. He clearly stated the advantages of stage or specimen scanning in his 1961 patent on the confocal microscope. This idea decoupled the magnifications of the objective from the resolution. The magnification could be changed by changing the number of pixels in the image. His patent also clearly showed the folded (reflected) mode of modern confocal microscopes.
- Minsky pointed out an important advantage of specimen or stage scanning. The microscope only used the central part (paraxial optics) of the microscope objectives, thus there are no off-axis or lateral optical aberrations to correct. Chromatic and spherical aberrations still required correction.
- Minsky's confocal microscope used a 45× microscope objective in air. It could resolve points closer than 1 μm apart. Its disadvantage was the slow scan time: 1 frame per 10 seconds.
- A real-time tandem-scanning confocal microscope, in which the image could be observed with the naked eye, was developed by Petràn and Hadravsky. They

decided to use a multiple-aperture (multibeam scanning) device since it would reduce the frame time to scan the field as compared to single-point scanning.

- The advantages of the Nipkow disk-type confocal microscope include real-time viewing, true color observation of the specimen using color to map the depth of the features in the specimen when used with an objective with low correction of chromatic aberrations, and direct-view observation.
- The tandem-scanning Nipkow disk-based confocal microscope is a poor choice for weakly reflecting specimens such as living cells, tissues, and organs. Consequently, it is not suitable for imaging weak autofluorescence or weakly stained fluorescent specimens. This is because of the low illumination efficiency of the Nipkow disk.
- Xiao, Corle, and Kino invented a real-time, one-sided, Nipkow disk-based confocal microscope. This design has several advantages over the tandem-scanning confocal microscope: it is less sensitive to vibration of disk, has a simplified optical design, and is easier to align.
- In the Yokogawa Nipkow disk confocal microscope, a laser illuminates the upper spinning disk, which contains about 20,000 microlenses over the pinholes. The lower disk contains pinholes in the focal plane of the microlenses that are arranged in the same pattern as the microlenses on the upper disk. Both disks rotate on a common axis. There is a great increase in light throughput.
- The key development in the Svishchev invention of a confocal light microscope was a scanning system based on an oscillating two-sided mirror. The two-sided mirror scans and descans a slit of light on the specimen side and on the image side.
- In a confocal microscope, if the illumination light is confined to a diffraction-limited spot on the specimen by the microscope objective, and the detection is also confined to the same spot with a pinhole aperture placed in front of the detector, then the confocal microscope would strongly discriminate against light from above and below the focal plane. The detector pinhole aperture is in a plane conjugate with the plane containing the illumination spot. With this confocal arrangement, the intensity of a point source of light on the specimen falls off with the fourth power of distance from the focal plane.
- A modern LSCM will only perform optimally if the following conditions are met: the system should be mounted on an anti-vibration optical table; laser-cooling fans and other sources of vibration should not be placed on the optical table; the optical elements should be correctly aligned and free of dirt, oil, and scratches, especially the microscope objectives.