MINIREVIEW

The Laser-Scanning Confocal Microscope in Biomedical Research (43321B)

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The laser-scanning confocal microscope (LSCM) produces improved light microscope images of both fixed and living cells and tissues. Moreover, the serial optical-sectioning power of the LSCM has made three dimensional reconstruction of light microscope images a practical option. The different confocal microscopes that have resulted in the current generation of the LSCM and the applications of the LSCM for biomedical research are briefly reviewed; further details can be found elsewhere (1–3).

Historical Perspective

Marvin Minsky's Microscope. The confocal microscope was invented in 1955 by Marvin Minsky specifically for studying neural networks in the living brain (4, 5). All modern confocal microscopes are based on Minsky's original idea, which was patented in 1957. Basically, illumination and detection are confined to a single diffraction-limited point in the specimen. The point is scanned across the specimen and light from the specimen is built into an image of a precise optical section of the specimen. The method of image formation in a confocal microscope is fundamentally different from that in a conventional wide field microscope, in which the entire specimen is bathed in light, usually from a mercury or xenon source, and the final image quality can be degraded by light scattered from out-of-focus structures.

In Minsky's original confocal microscope, the point source of light is produced by a pinhole placed in front of a zirconium arc source. The point of light is focused by an objective lens onto the specimen, and light that passes through the specimen is focused by a second objective lens onto a second pinhole, which has the same focus as the first pinhole, i.e., it is confocal with the first pinhole. Light that passes through the second pinhole is detected by a low noise photomultiplier. This second pinhole prevents any of the light from above or below the plane of focus from striking the photomultiplier. This is the key to the confocal approach, namely, the elimination of out-of-focus glare in the specimen by spatial filtering using a point source of light for excitation and a pinhole confocal with the excitation pinhole in front of the detector. Minsky also described a reflected light version of his microscope that uses a single objective lens and a dichromatic mirror arrangement. The reflected light arrangement, rather than the transmitted-light arrangement, is the basic configuration of most modern confocal systems.

In order to build an image, the confocal spot of light must be scanned across the specimen in some way. In Minsky's original microscope, the beam was stationary and the specimen was moved on a vibrating stage. This optical arrangement had the advantage of always scanning on the optical axis, which eliminates any lens defects. However, for biological specimens, movement of the specimen can cause wobble and distortion, which results in a loss of resolution in the image. Moreover, it is impossible to perform micromanipulations, such as microinjection, when the specimen is moving. Stage-scanning confocal microscopes have evolved into instruments used mainly in materials science and, to a limited extent, in biomedical research applications (6–8). The stage-scanning confocal microscope has recently been developed into a gel scanner for detecting fluorescently labeled DNA at a high spatial resolution (9).

A real image is not formed in Minsky's microscope; rather, the output from the photodetector is a stream

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of pulses related to the pattern of scanning of the specimen. This "data stream" from the photodetector can be translated into an image of the region of interest. In Minsky's original design, the image was built up on the screen of a military-surplus, long-persistence oscilloscope. In modern confocal microscopes, the image is either built up in a computer framestore, or by a sensitive video camera, and then displayed on a high-resolution video monitor and recorded on modern hard copy devices. Thirty-five years later, the video display system can still be the limiting factor in reproducing the resolution achieved in the microscope.

An alternative to moving the specimen is to scan the beam across the specimen. There are two fundamentally different methods of beam scanning: multiple-beam scanning or single-beam scanning. The flying spot microscope (10) and the scanning mirror microscope (11) were nonconfocal forerunners of beam-scanning confocal microscopes.

Multiple-Beam-Scanning Microscopes. The pioneer of multiple-beam technology was Mohimir Petran, who, like Marvin Minsky, was interested in imaging living, growing nerve cells in the intact brain (12). His ingenious design, the tandem-scanning reflected-light microscope, incorporates a bright white light source and a modified Nipkow disc to scan the specimen with hundreds of points of light. The disk spins rapidly and acts as the point source and the spatial filter that prevents out-of-focus light from reaching the photodetector. The disk is designed with apertures that are matched on the excitation and emission side of the light path. The photodetector in this case can be the human eye or a sensitive video camera mounted on the microscope (13).

The tandem-scanning reflected-light microscope has several advantages for biomedical research. The image is visible in the microscope itself, so the user can view the image as in a conventional microscope. The microscope scans in almost real time, which means that fast events such as blood flow can be observed (14). Moreover, the true colors of the specimen are imaged (15, 16), and it is relatively easy to select different wavelengths of light, including ultraviolet and near infrared excitation, simply by optically filtering for multiparameter fluorescent imaging (17). Fluorescence microscopy can be a practical option using the tandem-scanning reflected-light microscope, since the Nipkow disc and the efficiency of light through the microscope have been improved (18).

Petran's idea of using a modified Nipkow disc as a spatial filter has been incorporated by Xiao and Kino (19) in the real-time scanning optical microscope. In this microscope, light passes through one side of the disc only. This reduces the number of mirror elements in the light path and reduces light losses in the microscope. A third type of multiple-beam scanning microscope, now commercially available, is based on the design of Lichtman *et al.* (20).

Single-Beam Scanning Microscopes. The first practical single-beam scanning microscope for confocal epifluorescence microscopy was designed by John White and Brad Amos (21) in Cambridge, England with the specific aim of imaging changes in cytoarchitecture of blastomeres during early embryogenesis of fluorescently labeled nematode embryos. The first two cycles of division were visualized relatively easily using conventional epifluorescence microscopy. However, images of later stages became uninterpretable because of glare from out-of-focus structures. When he investigated the confocal microscopes available at the time, White found that his options were limited. The technology consisted of the stage-scanning instruments, which tended to be slow to produce images (approximately 10 sec for one full-frame image), and the multiple-beam microscopes, which were difficult to align and produced fluorescence images that were extremely dim. White and his colleagues designed a LSCM for epifluorescence microscopy that has evolved into an instrument which has been applied to many different biomedical problems. This microscope, together with several other LSCM (22, 23), is now available to biomedical researchers through several commercial vendors.

Basic Design Features of the LSCM

A typical LSCM has a laser light source, which replaces the zirconium arc-pinhole arrangement in Minsky's original design. The laser beam is directed into the scanning head, which can be mounted on either an upright or an inverted fluorescence microscope. An advantage of using an add-on unit to a conventional epifluorescence microscope stand is that the region of interest in the specimen can first be located by eye using conventional optics and then the user can switch to the confocal mode of operation. This is an essential feature for any confocal microscope, since unlike in a conventional epifluorescence microscope in which out-of-focus structures are visible as a bright background glow and can be used as an aid to focusing, no such visible cues are available in a confocal microscope. The specimen is either in focus, in which case structures are visible, or out of focus, in which case absolutely no structure is seen. Consequently, the user could spend much time finding a specimen when using confocal mode alone.

Scanning of the laser beam across the specimen is achieved by two mirrors: one scans in the X direction and the second mirror in the Y direction. The mirrors are driven by galvanometers under control from the host computer. Light that returns from the specimen is reflected back through the scanning system (since the mirrors are moved by a negligible amount in the time taken for the light to return from the specimen). A

pinhole is placed in front of the photomultiplier tube (PMT) so that light out of the plane of focus is prevented from reaching the PMT. The design of White *et al.* uses an infinity-focused light path, which means that the optical path is folded within the scan head by mirrors. This arrangement allows for a larger confocal aperture, the diameter of which can be adjusted.

The microcomputer controls the scanning mirrors and correlates their position with the data stream from the PMT in order to build the image in the framestore. The rate of image production varies with the type of microscope, and specifically in terms of the scanning rate of the mirrors and the speed of the computer. The rate of scanning is generally in the range of one full frame (512 × 746 pixels) per second. Faster scanning LSCM at video rates are now available in which scanning is achieved with an acoustico-optical modulator (24) or an image dissector tube (25).

Advantages of LSCM for Biomedical Research

There are two major advantages to using confocal microscopy in preference to conventional light microscopy for biomedical research. The first is that the amount of glare from the specimen is reduced by the spatial filtering of the pinhole. In general, by reducing the diameter of the pinhole, the amount of specimen sampled is reduced, giving a thinner optical section. There is an optimal pinhole size for every objective lens (26). The second major advantage of the confocal microscope is that image resolution is improved by a factor of two if the input and output apertures are both imaged as a diffraction-limited spot.

These factors vary with the objective lens and with the biological specimen. Generally, it is better to use a planapochromat objective lens with the highest possible numerical aperture and no phase rings (27). Some specimens, (for example, the flattened leading edge of a fibroblast growing in tissue culture and stained with a fluorescent dye), are not improved dramatically by confocal microscopy, whereas thicker specimens with significant three-dimensional structure (for example, fluorescently labeled microtubules within multicellular embryos) can only be visualized using confocal microscopy.

In day-to-day operation, the limitations imposed by the biological specimen usually force a trade-off between achieving theoretical limits of resolution and an image with acceptable information. The theoretical limits are measured using ideal test specimens, whereas certain compromises are made when gaining information from biological specimens. Thus, for biological applications at least, it is essential to incorporate as much flexibility as possible in the imaging system. As an example, an adjustable pinhole not only allows optimal confocal conditions to be met using ideal specimens and a wide variety of objective lenses, but also allows "not quite confocal" images to be obtained from relatively dim and difficult specimens.

The LSCM, therefore, has found additional biomedical applications to critical confocal imaging. Some of these features are made possible by the ability to control the scanning mirrors by using the microcomputer. For example, the magnification can be changed by optically zooming using the same objective lens with no loss in image resolution. This is achieved by decreasing the area scanned by the beam and, thus, imparting a wide range of magnifications to a single objective lens (usually between one and eight times). Furthermore, the scanning mirrors can be controlled to scan a spot or a shape onto the specimen for photobleaching or photoactivation experiments.

Confocal Fluorescence Microscopy

Single Labels. The LSCM was designed primarily for epifluorescence microscopy of fixed specimens. Most of the commercially available LSCM employ an Argon laser, which has two major lines at 488 nm and 514 nm. The 488 nm line is well matched to the excitation spectrum of fluorescein and related dyes, and the 514-nm line is suboptimal for rhodamine and better suited to Texas Red. The image of any fluorescent specimen that is stained with the appropriate fluorochrome whose excitation spectrum is matched to the major wavelengths produced by the laser is improved using the LSCM. The most dramatic improvements in image quality are seen in thick and brightly labeled fluorescent specimens, which includes eggs, embryos, tissue sections, and whole tissues where there is a lot of glare from out-of-focus structures (28). Indeed, in some relatively thick and brightly labeled samples, no image is possible by conventional epifluorescence microscopy because so much out-of-focus glare contributes to the image, whereas a crisp optical section is produced in the LSCM.

White *et al.* (29) have compared images from the same region of a range of fixed specimens examined by conventional epifluorescence microscopy and LSCM. These examples illustrate the power of the LSCM in improving epifluorescence microscopy of biological samples. Most of the examples have significant three-dimensional structure and, therefore, a significant contribution from out-of-focus glare. The following list of examples from the paper illustrate the different types of specimens that are well suited to investigation with the LSCM. The specimens include cell types with a significant 3-D structure, for example, the microtubules in interphase and mitotic HeLa cells, microtubules in the two-cell-stage sea urchin embryo, and the endoplasmic reticulum in plasmocytoma cells. In addition, the banded chromosome patterns in chromomycin-stained preparations of salivary glands from Drosophila

larvae and whole mounts of various embryos were more clearly imaged with the LSCM.

Many studies continue to use the optical sectioning power of the LSCM to produce improved images of fixed specimens labeled with single fluorochromes. These studies are too numerous to include here, but representative examples include studies of spindle rotations in nematode embryos (30), of prolactin sites on lymphoma cells (31), of replication sites in nuclei (32), of nuclear staining in yeast (33), of fluorescently labeled endoplasmic reticulum (34), of neurons in tissue (35), and of nuclei in HeLa cells (36).

Samples are typically overstained for LSCM as compared with staining for conventional epifluorescence microscopy. This is because the total fluorescence within the specimen is undersampled with the LSCM as compared with the conventional epifluorescence microscope. In addition, various antifade agents are generally used to combat photobleaching of the fluorophore by the laser beam (37, 38). Photobleaching in the more recently available LSCM is generally not a problem, especially when the instrument is operated optimally and digital image processing is used to optimize the gray levels in the image. Photobleaching rates are dependent upon the specific properties of the fluorophore used, the quality of the specimen, and the amount of laser power used for imaging (39).

Multiple Label Immunofluorescence. Double labeling can be achieved by using the 514-nm line to excite two fluorochromes; for example, fluorescein can be excited, albeit rather inefficiently, at 514 nm (usual max. 488 nm) and Texas Red at 596 nm. The light emitted from the two fluorochromes is subsequently split by filters and directed to two separate photomultipliers. A great advantage of simultaneously collecting images in the same framestore is that they are in register and can be merged to map the distribution of two fluorescent labels in a tissue. One of the images can be colored green for fluorescein and the second image can be colored red for Texas Red; the regions of overlap will then appear yellow. The double-labeled specimens collected in this way are prone to bleed through from one channel to the other and require careful specimen preparation to avoid this. This can be rectified in a variety of ways: by digitally subtracting the bleed-through of one image from the other or by using newly available dyes whose excitation and emission spectra are better suited to the laser (40). Several successful double-label studies using this method include the localization of human growth hormone to a subset of cytoplasmic vesicles in PC12 cells (41) and the determination of gene expression in the development of Drosophila wing (42).

An improved method for double labeling is to use two different excitation wavelengths that are sufficiently separated for double labeling. This can be achieved by using two lasers (43), with a single laser, and changing filter blocks and digitally realigning the images (44), or by using a Krypton-Argon laser, which gives good separation of fluorescein and rhodamine (45). Moreover, the Krypton-Argon laser has a third major line at 647 nm (red), so three different fluorochromes can be imaged simultaneously with a single laser. In fact, new dyes that are excited around the 647-nm line of the Krypton-Argon laser are now available (46–48). Imaging at these longer wavelengths should give improved viability of living samples and allow deeper penetration into samples.

Transmitted Light Imaging

A nonconfocal transmitted light image can also be obtained with the LSCM. This is extremely useful for image display purposes where a fluorescent image can be merged with a transmitted light image. In many cases, the fluorescent image alone is difficult to interpret out of the context of the transmitted light image for reference. The transmitted light image produced by the LSCM can be bright field, phase contrast, differential interference contrast (DIC), or dark field. This image can be merged with the fluorescent image. The transmitted light detector collects light that passes through the specimen and then pipes it up to the second PMT by means of a fiber optic. The double-label fluorescence-transmitted light technique has been used in phagocytosis research to study fluorescent microspheres ingested by murine macrophages (49). By optically sectioning the fluorescent image and superimposing the transmitted light DIC image, it is possible to determine the number of fluorescent beads phagocytosed by individual macrophages versus the number of beads on the surfaces of the macrophages. This technique is especially useful in studies of cells that tend to form three-dimensional clumps.

A confocal transmitted light image is possible using Minsky's original confocal design, although it is difficult to match precisely the two objective lenses. A confocal, transmitted light, phase contrast system has recently been described (50). Optical sections can be obtained using critical transmitted light microscopy with DIC optics. Indeed, 3-D reconstructions have been produced from specimens that do not scatter much light by DIC, such as Golgi-stained neurons, for example (51).

Confocal Reflected Light Microscopy

The LSCM produces improved images of specimens that are unstained but reflect enough light to produce contrast in the image. When viewed with a conventional light microscope, many of these specimens scatter so much light back into the objective lens that structural details are obscured. The LSCM eliminates much of this scattered light and produces extremely clear images. For example, 5-nm gold beads

have been detected, but not resolved, using reflected light LSCM (52). One advantage of using immunogold labels instead of immunofluorescence is that, unlike fluorescent dyes, gold is not subject to photobleaching by the laser beam. For example, movements of immunogold particles on the surface of cells have been tracked for several hours, whereas fluorescently labeled specimens would photobleach in a similar time (53).

Reflected light confocal microscopy improves the detection of peroxidase-labeling on the surface of tissue culture cells (54) and can be used as an alternative to interference reflection microscopy for observing cell substratum adhesions of cells growing in tissue culture (55). Reflected light LSCM has been applied to unstained living tissues. The layers of cells in the living rabbit cornea have been imaged using reflected light LSCM in conjunction with a long working distance, water-immersion objective lens (56). The lens was placed directly into the medium bathing the eye. Under these conditions, it was possible to image the epithelium, stroma, and endothelium of the cornea, and image into the lens of the eye with no distortion of the eye itself. If the light efficiency of the LSCM can be improved to eliminate photodamage to the cells of the eye, then this type of imaging may be applied to the detection of early pathological changes (57).

When using the reflected light mode of the LSCM, a specular reflection can appear in the image as a series of diffraction rings. This is caused by a reflection from the eyepiece in the microscope and can be eliminated by inserting two polarizing filters and a quarter waveplate in the optical path (58), or by scanning slightly off the optical axis when the reflection detracts from the information in the image.

In situ Hybridization

In situ hybridization is a powerful technique for detecting the position of nucleic acids and nucleic acid sequences within cells and tissues. It has a specificity and spatial distribution equivalent to fluorescent antibody techniques, especially when the LSCM is the chosen imaging system. The application of *in situ* hybridization using LSCM has been reviewed recently (59). Specific chromosome domains or gene loci within nuclei, i.e., specific sites on isolated chromosomes using either single or double labeling, have been detected (60, 61). It is possible to label several sites along a chromosome using several different fluorochromes. This technique has been applied to rapid screening of chromosomal and nuclear abnormalities in certain inherited diseases, such as Down's syndrome and Burkitt's lymphoma (62). Chromosome 21 defects in Down's syndrome have been detected; the normal chromosomes show a two-spot pattern, whereas chromosomes from Down's syndrome patients show a three-spot pattern.

Reflected light LSCM improves visualization of silver grains in autoradiographs of specimens prepared by *in situ* hybridization (63). The out-of-focus image of the background silver grains present in the emulsion is eliminated from the in-focus image of the radiolabeled probe associated with the cells by optically sectioning with the LSCM. The images produced by the LSCM provide a significant increase in the sensitivity of detecting positively labeled cells and tissues prepared by *in situ* hybridization. The method has been utilized in samples of human immunodeficiency virus-infected human peripheral blood cells (64), tissue sections of human placenta (65), and skin (66). Here, the reflected light confocal image can be merged with the transmitted light (bright field or dark field) image so that infected cells are clearly distinguished from uninfected cells in a mixed population of cells in tissue sections or whole mount preparations.

Three-Dimensional Reconstruction

Serial optical sections in the Z plane can be collected at user-determined increments by driving the fine focus control of the microscope with a stepper motor. A stereo pair can then be constructed by digital offset methods or by digital merging to give a two-color stereo (67). Alternatively, a 3-D image from the accumulated series of optical sections can be generated (68). The series of optical sections produced by the LSCM are suitable for 3-D reconstruction using programs written for the host microcomputer or more sophisticated computer graphics programs that run on large, high-capacity workstations. The computer graphics systems currently used for 3-D reconstruction of confocal data sets have emerged from applications in the film industry for the production of computer-animated films and in medical imaging for the reconstruction of CAT scan data as an aid in clinical diagnosis and the planning of surgical strategies. There are two basic methods currently applicable to the display of confocal data sets. These are volume rendering (69) and geometric surface rendering (70).

Data sets collected using the LSCM are well-suited to computer reconstruction, since each of the optical sections is in register with its neighbors when collected with the microscope. This is a tremendous advantage to physically sectioning the specimen, e.g., frozen sections, since registration is lost using these methods and can only be regained by using lengthy registration algorithms or by placing fiduciary marks in the specimen. The 3-D data sets can be transferred directly into a 3-D reconstruction program that runs on a graphics workstation and, after a relatively trivial file reorganization, they can be processed into a 3-D image that can be rotated or dissected on the screen of the graphics computer in a relatively short time (71).

Confocal Microscopy of Living Tissues

Confocal microscopy is essential for imaging vitally stained tissues, since it avoids damaging the specimen by physically cutting sections of it. In fact, Minsky's original dream of observing living and growing neurons *in situ* is rapidly being approached by currently available confocal microscopes in conjunction with newly developed vital fluorescent dyes. Using the high sensitivity of the latest LSCM, it is possible to visualize both structural and ionic changes in living cells and tissues by taking time-lapse series at a single level of focus or time-lapse Z series. The LSCM can be incorporated into a multiparameter imaging system so that confocal and conventional images are combined (72).

A major prerequisite for successful live-cell imaging is to reduce the number of photons that interact with the specimen and then to use all photons leaving the specimen to produce an image. This is because living cells, and especially fluorescently labeled living cells, are prone to photodamage (73, 74). The early LSCM was extremely light inefficient, whereas recent developments have improved the light budget so that live-cell imaging is now a practical option (75). These improvements include scanning mirrors with better light efficiency (99%) and improved methods of digitizing images, including a fast photon-counting mode.

Several different approaches for observing living cells and tissues using the LSCM have been developed. These include static imaging of unfixed material either with reflected light or using epifluorescence. Recent examples of static imaging of fluorescently labeled living tissue include tracing the pathway of labeled peptides through the cornea (76), visualization of the endoplasmic reticulum in plants stained with the vital dye DiOC6 (77), living rat heart stained with acridine orange (78), and cell membranes stained with lucifer yellow (79).

Time lapse can be used for imaging changes in structure. Examples include time-lapse observations of single optical sections or Z series with subsequent graphic reconstructions of each time point, sometimes referred to as 4-D imaging. Programs are now available that automatically collect optical sections over time and store them on a large hard disc or optical disc recorder. These programs also allow the series to be played back over time or allow the user to select individual optical sections to be played back over time. This can be useful in following a particular structure that moves in the Z plane over time. An example of time lapse using the LSCM is the recording of Golgi dynamics in cultured rat hippocampal astrocytes labeled with the vital dye *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-ceramide. This study showed both tubulovesicular processes and small submicron particles emerging from the trans-Golgi and migrating along the microtubules (80). Furthermore, laser photobleaching experiments showed that tubulovesicular processes can provide direct pathways for the diffusion of membrane lipids between joined trans-Golgi elements.

Confocal Ion Imaging

The following are the advantages of using confocal microscopy for imaging ions such as calcium: (i) the narrow depth of field allows better visualization of intracellular details by eliminating out-of-focus signals; (ii) there is improved lateral spatial resolution; and (iii) measurement errors due to path-length variation are minimal because a small and relatively constant volume of the cell is sampled through the focal plane. Fast events can be imaged or measured using a smaller region of the specimen or a point in the specimen.

Various fluorescent dyes are now available that change their fluorescence intensity relative to the concentration of free ions, especially calcium ions (81). The first dyes available were excited at relatively short wavelengths (for example, FURA-2), whereas most commercially available LSCM are not supplied with a UV laser, but rather with an Argon laser with lines at 488-nm and 514 nm, and utilize mirrors that are not efficiently reflecting at UV wavelengths. However, excitation of the shorter wavelength dyes has been achieved using two-photon laser-scanning fluorescence microscopy, although the laser tends to be prohibitively expensive for routine work (82).

Various ion-sensitive dyes are now available that are excited at longer wavelengths and better matched to the Argon laser. The dyes include FLUO-3, RHOD-2, calcium green, calcium orange, calcium crimson, and FURA-Red for calcium, and SNAFL, SNARF, and BCECF for pH measurements (83). FLUO-3 has proved to be an ideal dye for measuring calcium using the LSCM. Calcium gradients in voltage clamped bull frog neurons were observed with localized calcium signals in the nuclei after electrical stimulation (84). Using FURA-2 and conventional epifluorescence microscopy, the calcium wave during fertilization of the sea urchin embryo was observed to spread rapidly across the cell (85). Using FLUO-3 and LSCM, the relative rise in free calcium was found to be higher in the cortex than in the center of the egg, fertilization caused the egg nucleus to undergo a dramatic and transient increase in free calcium, and the egg contracted directly after fertilization (86). The rise in nuclear fluorescence was not visualized in previous work using conventional epifluorescence microscopy because the nuclear fluorescence was obscured by cytoplasmic fluorescence.

Waves of intracellular free calcium have been observed to pass at velocities of 10–20 μm/sec through networks of astrocytes within slices of neonatal rat hippocampus (87). The waves occur spontaneously or more frequently when neurons are selectively stimu-

lated using an electric current. Changes in calcium and pH in maize coleoptiles stained with FLUO-3 or BCECF and imaging with the LSCM have been correlated with increases in cell elongation stimulated with light and gravity (88, 89).

The effects of photobleaching can be corrected by obtaining the ratio of two wavelengths. This has been achieved using an Argon and helium cadmium laser combination (90). The pH in individual principal cells in the isolated cortical collecting tubule of the rabbit kidney was measured by obtaining the ratio of the 488-nm excitation from the Argon laser to the 422-nm excitation from the helium cadmium laser in order to counter the severe photobleaching of BCECF.

Future Applications of the LSCM in Biomedical Research

The biomedical potential of the LSCM, and of confocal microscopy in general, has yet to be fully realized. Several applications have been highlighted in this brief review. These applications include single- and multiple-label immunofluorescence, reflected light imaging, *in situ* hybridization, 3-D reconstruction, and live cell imaging. The current generation of instruments is already used in novel ways that have the potential for development into clinical instruments. Two examples are a confocal ophthalmoscope and the confocal screening system for *in situ* hybridization. However, for routine work in a pathology laboratory, it will be necessary to develop an instrument that is relatively inexpensive, easy to use, and perhaps incorporates a real-color imaging system for the interpretation of pathological specimens, since pathologists have been trained for many years to diagnose diseases with specimens stained with colored dyes. Thus, the confocal image could be merged with the more familiar bright-field real-color image for more accurate diagnosis. Perhaps the optical section will one day replace the frozen or the electron microscope thin section in the pathology laboratory.

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