Methods in Cell Biology

Prepared under the Auspices of the American Society for Cell Biology

VOLUME 63

Cytometry
Third Edition, Part A

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ACADEMIC PRESS
A Harcourt Science and Technology Company
San Diego San Francisco New York Boston London Sydney Tokyo

CHAPTER 4

Principles of Confocal Microscopy

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I. Brief History of Microscope Development

Microscopy techniques have taken over 200 years to mature into technologies capable of the measurements now possible using confocal microscopy. Prior to 1800, production microscopes using simple lens systems were of higher resolution than compound microscopes despite the chromatic and spherical aberrations present in the double convex lens design. Microscopy did not really prosper until W. H. Wollaston made a significant improvement to the simple lens in 1812.

METHODS IN CELL BIOLOGY, VOL. 63

0091-679X/01 $35.00

Soon after, Brewster improved on this design in 1820, and in 1827 Giovanni Battista Amici introduced the first matched achromatic microscope. Key features of the Amici design were the recognition of the importance of coverglass thickness and the development of the concept of "water immersion." Then came Carl Zeiss and Ernst Abbé, who introduced oil-immersion systems by developing oils that matched the refractive index of glass. By 1886, Dr. Otto Schott formulated glass lenses that color-corrected objectives and produced the first "apochromatic" objectives. Just after the turn of the twentieth century, Köhler illumination revolutionized brightfield microscopy. This discovery has been considered one of the most significant developments in microscopy prior to the electronic age.

Later developments such as the use of phase-contrast illumination, Nomarski illumination, and epi-illumination have each had significant impact on cell biology. In more recent years, the advent of confocal microscopy has changed the way cell biologists prepare and examine material, because we now have more options. Using this technology, the biologist can pinpoint the location of labeled molecules (e.g., a growth factor) in relatively thick specimens. This allows him to identify the organelle or location for the synthesis of the molecule. It is also possible to reconstruct the three-dimensional structure of many cells, organs, and even small organisms or animals quite accurately. Such information has given us a great deal of insight into the structure and function of many biological systems. This chapter discusses the basic principles of confocal microscopy. Detailed texts on the subject include Pawley (1995), Matsumoto (1993), Paddock (1999), and Sheppard and Shotton (1997).

II. Development of Confocal Microscopy

Marvin Minsky, then at Harvard University, filed the first patent for a confocal microscopy in 1957 (Minsky, 1988). Many scientists contributed to the enhancement and practical application of the technology, including Brackenhoff (Brackenhoff *et al.*, 1979, 1985), Wijnaendts van Resandt (Wijnaendts van Resandt *et al.*, 1985), Carlsson (Carlsson *et al.*, 1985), Amos (Amos *et al.*, 1987; Amos, 1988), and White (White *et al.*, 1987) among others. A confocal microscope achieves crisp images of structures even within thick tissue specimens by a process known as optical sectioning. The image source is primarily the photon emission from fluorescent molecules within—or attached to structures within—the object being sectioned. An alternative to fluorescence emission, reflectance, is discussed later. A point source of laser light illuminates the back focal plane of the microscope objective and is subsequently focused to a diffraction-limited spot within the specimen. Within this spot fluorescent molecules are excited and emit light in all directions. However, the emitted light is refocused in the objective image plane and any out-of-focus light is essentially removed from the image by passing the light through a pinhole aperture, so only a thin optical section of the specimen is formed. The effective removal of out-of-focus light from the emission creates an essentially background-free image—as opposed to the traditional fluorescent

microscope that includes all of this out-of-focus light. The comparison between traditional fluorescence microscopy and confocal microscopy is illustrated in Fig. 1. To create "depth" of the optical section, the diameter of the pinhole is reduced, thereby decreasing the light collection from the specimen but reducing the "thickness" of the optical sections. Although this effectively decreases the light collection for the images obtained, it allows a greater definition of the structure of the sample because more sections are collected over the sample thickness.

The resolution of a point light source is defined by the circular Airy diffraction pattern formed on the image plane. This pattern consists of a central bright region enclosed by an outer dark ring. The radius r_{Airy} of this central bright region is defined as $r_{Airy} = 0.61\lambda/NA$, where λ is the wavelength of the excitation source and NA is the numerical aperture of the objective lens. To increase the signal-to-noise (background) ratio and decrease the background light, it is necessary to decrease the pinhole to a size slightly less than r_{Airy}; a correct adjustment can decrease the background light by a factor of 10^3 over conventional fluorescence microscopy. Thus, achieving the correct pinhole diameter is crucial for achieving maximum resolution in a thick specimen. This becomes a trade-off, however, between optimizing axial resolution (optimum = 0.7 r_{Airy}) and lateral resolution (optimum = 0.3 r_{Airy}).

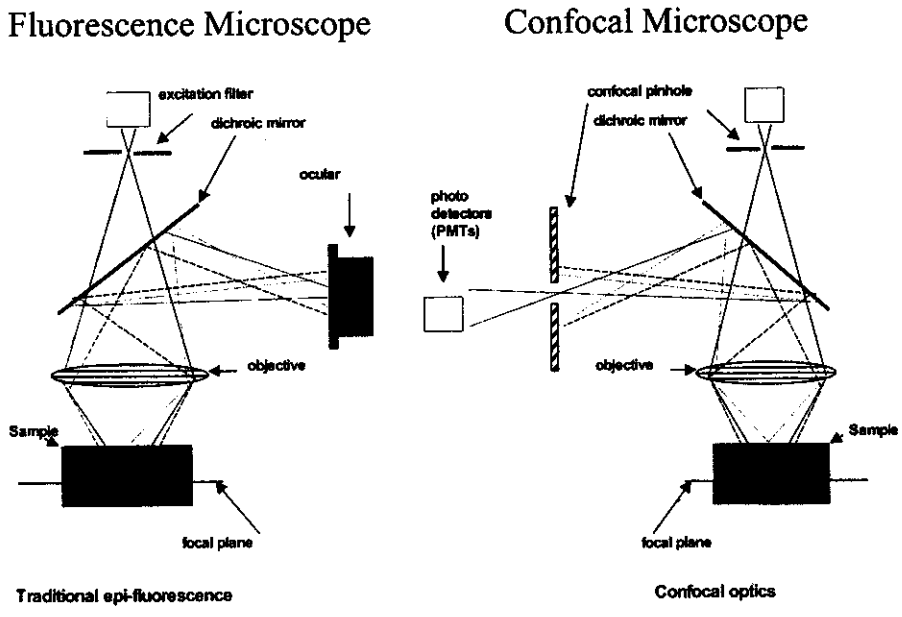

Fig. 1 The light pathway in conventional fluorescence microscopy versus in confocal microscopy. It should be noted that the principle of confocality is that the emission signal recorded will be exclusive for each focal plane within the specimen that is imaged. This feature is essentially the role of the confocal iris as described in the text.

Although the image collection optics removes the background light and creates a nice clean section, it is important to realize that the entire image is still being bathed in excitation light. By the time a thick section has been imaged a number of times, the impact of substantial photobleaching must be considered.

III. Image Formation in Confocal Microscopy

There are several methods for achieving a confocal image. The most common method scans the point source of light (a laser beam) over the image using a pair of galvanometer mirrors. One galvanometer scans in the x direction and the other in the y direction. The emitted fluorescence traverses the reverse pathway, is separated from the excitation source by a beam-splitting dichroic mirror, and is reflected to a photomultiplier tube amplifying the signal. After passing through an analog-to-digital converter (ADC), the signal is displayed as a sequential raster scan of the image. Depending on the desired measurements within the imaging requirement, it is possible to collect very small scan ranges from 50 × 50 points (or even smaller) up to rather large scanning areas with as many as 4096 × 4096 points. Most current systems utilize 16-bit ADCs, allowing an effective image of 1024 × 1024 points or more with at least 256 gray levels. Some confocal microscopes can collect high-speed images at video rates (30 frames/sec), whereas others achieve faster scanning by slit scanning. Just because an instrument can collect more points (and thus often claims higher resolution) does not necessarily mean it is useful. For example, a 512 × 512 image might require 0.3 sec to collect, and a 1024 × 1024 image perhaps 1.5 sec. A single scan of a 4096 × 4096 image might take 15 sec. To collect a relatively small number of sections (50) with signal averaging of three scans per image would take nearly 40 min, an impractical time constraint with most biological specimens. Regardless, the perfect image could take several runs to acquire, and so the "high-resolution" mode is less practical for three-dimensional imaging than the commercial literature might suggest.

Frequently, practical operation of the confocal microscope will be 512 × 512 image collection using the fastest possible point scanning available on the instrument. Once the imaging area is selected, the top and bottom (in the z-axis) of the image sections are identified; if desired, the image collection parameters can be changed at this point to obtain higher resolution. Electronic magnification is one of the most useful components of the confocal collection system and is universally available on all microscopes. The principle of electronic magnification is that the imaging area is reduced, but the number of pixels in the collection area remains constant. This effectively magnifies the image. However, it is generally not feasible to magnify the image beyond the point exceeding the Nyquist criterion (2.3 f), since beyond this is considered empty magnification—although there are cases where "super-resolution" is possible (Plasek and Reischig, 1998). An important point to consider is that the power delivered to the specimen

increases with the square of the magnification. Therefore, a zoom factor of 2 places four times the laser power onto the object. This could cause serious bleaching or physically heat the specimen beyond a reasonable level. An example of electronic magnification is shown in Fig. 2. The primary advantage is that one can view a larger sample field and zoom in to areas of particular interest using the zoom feature.

Investigators have demonstrated two-photon excitation, in which a fluorophore simultaneously absorbs two photons each having half the energy—and twice the wavelength—normally required to raise the molecule to its excited state. A significant advantage of this system is that only the fluorophore molecules in the focal plane are excited, as this is the only area with sufficient light intensity. The higher wavelengths mean that considerably less background noise is collected and the efficiency of imaging thick specimens is significantly increased. Those probes requiring ultraviolet (UV) excitation can be excited by means of three-photon excitation (Sako *et al.*, 1997), which may have the advantage of causing less tissue damage (particularly when imaging live cells); this is still subject to verification, since little to no supportable data have been published on this matter. What is clear, is that two-photon microscopy has decided advantages in imaging to a greater depth. Although resolution in most thin (<70 μm) tissues

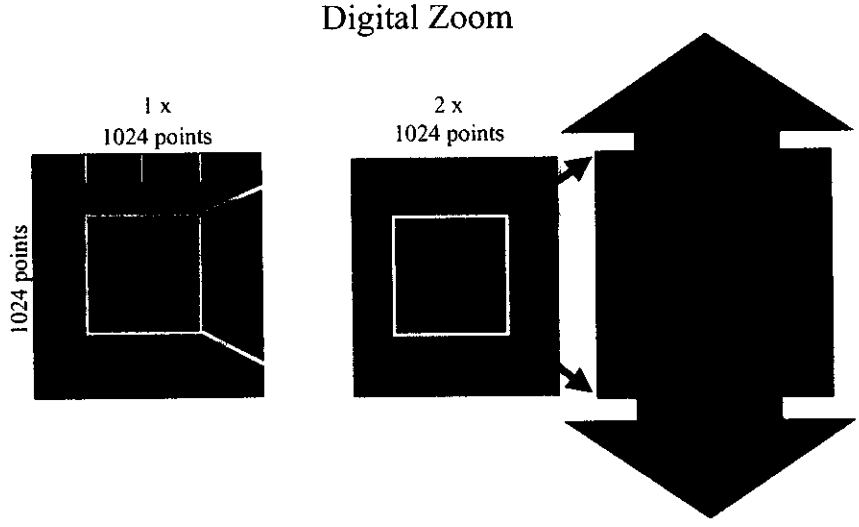

Fig. 2 The principle of electronic zoom is based on the notion that by reducing the area of the scan, but not reducing the number of points within that scan, the image will be electronically magnified. This is shown in the cartoon above. The first box represents a 1024 × 1024 matrix. If a box half this size is now imaged using 1024 × 1024 points, the resultant image will be magnified by a factor of 2. If this is repeated the resultant image will be magnified by a factor of 4. The impact is that the area of the object to be image is reduced as demonstrated by the arrowlike object in the cartoon.

is actually worse than in conventional confocal microscopy, it is actually improved in thick tissues, where conventional confocal microscopy is unable to image well at all.

A. Benefits of Confocal Microscopy

A now-familiar tool in the research laboratory, confocal microscopy has a number of significant advantages over conventional fluorescence microscopy. Among these are:

Increased effective resolution: A point source of light imaged through a pinhole provides increased resolution.

Reduced blurring of the image from light scattering: Because out-of-focus light is excluded from the image plane, collected images are sharper than those obtained with regular fluorescent microscopes. Photons emitted from points outside the image plane are rejected.

Improved signal-to-noise ratio: Compared to fluorescence microscopy, the background light is decreased, allowing for significantly improved signal-to-noise.

z-axis scanning: A series of optical sections can be obtained at regular distances by progressively moving the objective through the specimen from bottom to top.

Depth perception in z-sectioned images: Reconstruction techniques are used to reconstruct an image of the fluorescence emission of the specimen through the entire depth of the specimen.

Electronic magnification adjustment: By reducing the scanned area of the excitation source, but retaining the effective resolution, it is possible to magnify the image electronically. This has a number advantages over conventional microscopy.

B. Excitation Sources

The most common light sources for confocal microscopes are lasers. The acronym LASER stands for light amplification by stimulated emission of radiation. Most lasers on conventional confocal microscopes are continuous wave lasers (CW) and are either gas, dye, or solid-state lasers. Argon ion (Ar) lasers are the most popular gas lasers, followed by either krypton ion (Kr) or a mixture of argon and krypton (Kr-Ar). Helium–neon (He-Ne) and helium–cadmium (He-Cd) are also used in confocal microscopy. The He-Cd laser can provide UV lines at 325 or 441 nm, although use of the 325-nm line is very difficult in most microscopes because of loss of signal transmission at wavelengths below 350 nm. The most common source of UV excitation for the confocal microscope is the argon ion laser, which can emit 350- to 363-nm UV light. Frequently the UV

excitation source for UV–vis confocal systems is the Coherent "Enterprise." The power necessary to excite fluorescent molecules at a specific wavelength can be calculated. For instance, consider a standard system with 1 mW of power at 488 nm focused via a 1.25 NA objective to a Gaussian spot whose radius at $1/e^2$ intensity is 0.25 μm. The peak intensity at the center will be 10^{-3} $W/[\pi(0.25 \times 10^{-4}\ cm)^2] = 5.1 \times 10^5\ W/cm^2$ or 1.25×10^{24} photons/(cm^2 sec^1) (Sheppard and Shotton, 1997). If fluorescein isothiocyanate (FITC) were the fluorochrome used in such a system, 63% of its molecules would be in an excited state and 37% in the ground state at any one point in time. This photon flux would be sufficient to obtain efficient excitation of this probe. For optimal confocal microscopy, the power delivered to the fluorescent probe must be sufficient to saturate the fluorescent molecules in the specimen.

Most confocal microscopes are designed around conventional microscopes, with the modification of the light source, which can be one of several lasers. An example of the layout of a typical confocal microscope is shown in Fig. 3. Here the instrument is divided into three essential components: (A) the light sources, (B) the optical components for manipulating the signals, and (C) the microscope itself. For most cell biology studies, arc lamps are not adequate sources of illumination for confocal microscopy. When using multiple laser beams, it is vital to expand the laser beams using a beam-expander telescope so that the back focal aperture of the objective is always completely filled. The beam widths from several different lasers must also be matched if simultaneous excitation is required. The most important feature in selecting the laser line is the absorption maximum of the fluorescent probe.

C. Line Scanners

One of the limiting factors that must be addressed in confocal microscopy is photobleaching of the fluorophore because of the intense illumination. An alternative to a moving-spot microscope is one in which the laser spot is "extruded" to form a line of light which is then used to scan the sample. Such scanning is naturally considerably faster than a moving-spot scan, so these systems can produce very rapid kinetic image sets. The principal reason for using a line scanner is to obtain rapid successive images of a fluorescence emission. Lower-intensity, even illumination can be applied at high rates by scanning a "line" of laser light instead of a point of light across the specimen. In general, these instruments are referred to as "slit-scanners" because they utilize a slit aperture, which can either scan or remain stationary. Quite different from most point scanners, line scanners commonly use a sensitive video camera—either silicon intensified target (SIT), intensified silicon intensified target (ISIT), or cooled charge coupled device (CCD)—to capture the fluorescence signal. An example of the light path of one such slit-scanner is shown in Fig. 4. Figure 5 is provided to demonstrate how this system might actually image cells in culture. In this figure, the cells are scanned by the light source using an inverted microscope.

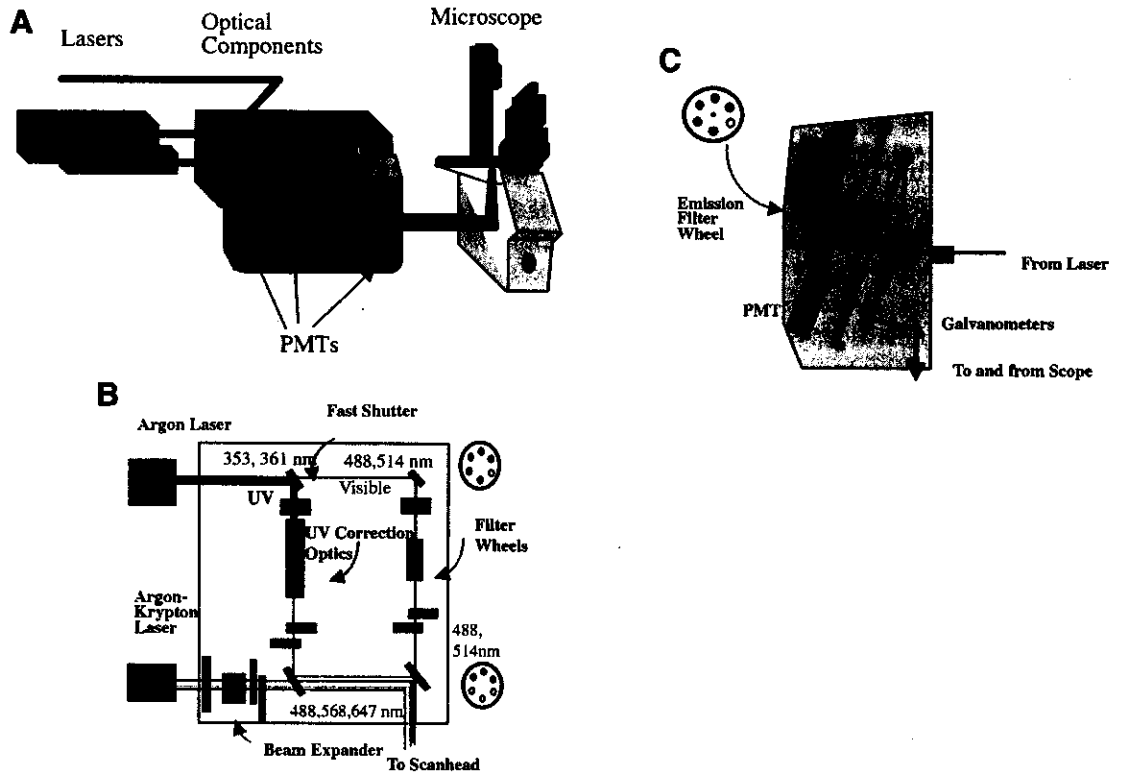

Fig. 3 The light paths of a confocal microscope with multiple lasers and using an inverted microscope. This shows three components: (A) the light sources, (B) the optical components that manipulate the signal, and (C) the microscope system. Shown are several laser lines that can be used together or independently to excite an object. Resultant signals are collected in the detector region where several PMTs reside. A computer system controls the system and creates the images. (See color plates).

The figure also demonstrates how the point source is converted to a line source for such scanning.

IV. Useful Fluorescent Probes for Confocal Microscopy

The essential requirement for a fluorescent molecule is an appropriate excitation source. Because most lasers can be successfully used in confocal systems, the number of fluorescent probes available for use in confocal microscopy is very broad. A series of tables is provided detailing the properties of fluorescent

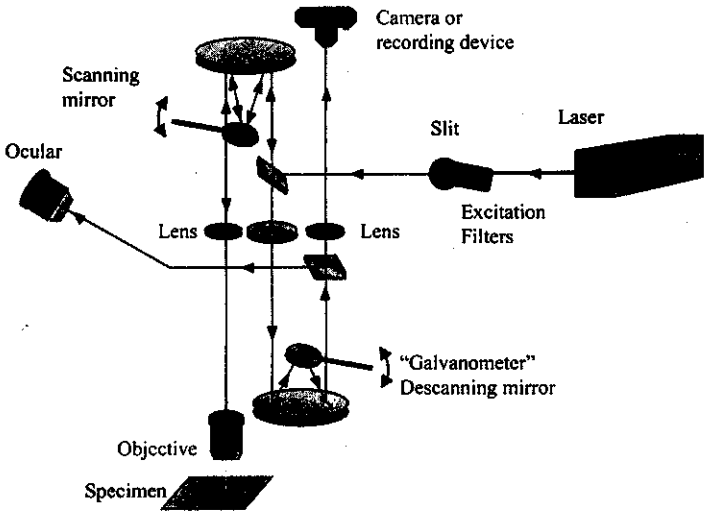

Fig. 4 The line scanning confocal microscope showing the laser light source, scanning and descanning mirrors, and the light path in the system. This system was originally designed for the BioRad DVC 250 line scanning confocal microscope (Hercules, CA). In the line scanning microscope, the confocal image is visible to the viewer at the ocular. Normally, a CCD camera is attached to the ocular to record the image.

probes for proteins (Table I), for intracellular organelles (Table II), for nucleic acids (Table III), for ions (Table IV), and for measuring intracellular changes in oxidation state (Table V). The excitation properties of each probe depend on its chemical composition. Ideal fluorescent probes will have high quantum yield, large Stokes shift, and nonreactivity with the molecules to which they are bound. It is vital to match the absorption maximum of each probe to the appropriate laser excitation line. For fluorochrome combinations, it is desirable to have fluorochromes with similar absorption peaks but significantly different emission peaks, enabling use of a single excitation source. It is common in confocal microscopy to use two, three, or even four distinct fluorescent molecules simultaneously, although it is usually necessary to image each probe independently and combine the images postcollection.

A. Fluorochrome Photobleaching

Photobleaching is defined as the irreversible destruction of an excited fluorophore by light. Uneven bleaching throughout the thickness of a specimen will bias the detection of fluorescence, causing a significant problem in confocal microscopy. Methods for countering photobleaching include shorter scan times, high magnification, high-NA objectives, and wide emission filters, as well as reduced excitation intensity. A number of "antifade" reagents are available (see

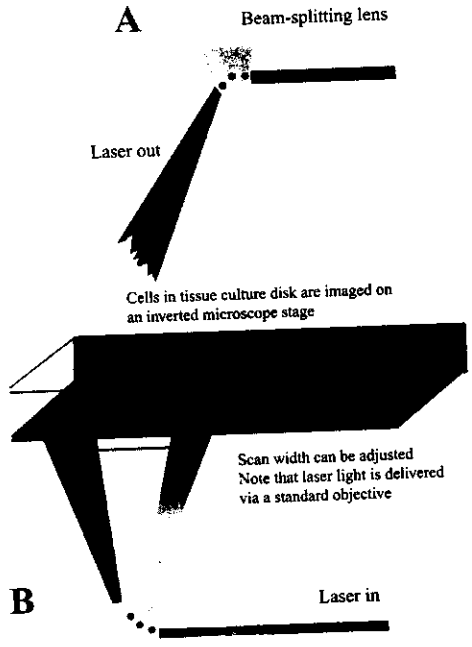

Fig. 5 The light source for a line scanning confocal microscope is a laser; however, instead of a point source of light, a line must be delivered to the specimen. This can be achieved by stretching the point into a line by means of a prism as shown in the diagram. As shown in (A), the line source is scanned (B) across the specimen. This allows very fast scanning of the specimen since the scanning requires a change in the y direction (not both x and y as in spot scanning).

Table I
Probes for Proteins

Probe	Excitation (nm)	Emission (nm)
FITC	488	525
PE	488	575
APC	630	650
PerCP	488	680
Cascade Blue	360	450
Coumarin-phalloidin	350	450
Texas Red	610	630
Tetramethylrhodamine-amines	550	575
CY3 (indotrimethinecyanines)	540	575
CY5 (indopentamethinecyanines)	640	670

Table II
Organelle-Specific Stains

Probe	Specificity	Excitation (nm)	Emission (nm)
BODIPY	Golgi	505	511
NBD	Golgi	488	525
DPH	Lipid	350	420
TMA-DPH	Lipid	350	420
Rhodamine 123	Mitochondria	488	525
DiO	Lipid	488	500
DiI-Cn-(5)	Lipid	550	565
DiO-Cn-(3)	Lipid	488	500

later); unfortunately, many are not compatible with viable cells. In the absence of an antifade reagent, FITC in particular is very susceptible to photobleaching.

B. Antifade Reagents

Many quenchers act by reducing oxygen concentration to prevent formation of excited species of oxygen. Antioxidants such as propyl gallate, hydroquinone, and *p*-phenylenediamine can be used for fixed specimens but are not useful for live-cell studies. Quenching fluorescence in live cells is possible using either systems with reduced O_2 concentration or singlet-oxygen quenchers such as carotenoids (50 m*M* crocetin or etretinate in cell cultures), ascorbate, imidazole, histidine, cysteamine, reduced glutathione, uric acid, or trolox (vitamin E analog). Photobleaching can be calculated for a particular fluorochrome to determine the maximum scan time possible for that molecule. For example, the most commonly used fluorescent probe, FITC, bleaches with a quantum efficiency Q_b of 3×10^{-5}. A standard laser intensity would pump 4.4×10^{23} photons cm^{-2} sec^{-1} and FITC would be bleached with a rate constant of 4.2×10^3 sec^{-1}. After 240 μsec of irradiation, only 37% of the molecules would remain. In a single plane, 16 scans would cause 6–50% bleaching (Sheppard and Shotton, 1997).

Table III
DNA Dyes

Probe	Excitation (nm)	Emission (nm)
Hoechst 33342	350	460
DAPI	350	470
PI	530	620
Acridine orange	500	520
TOTO-1	514	530
TOTO-3	640	660
Thiazole orange (vis)	510	525

Table IV
Probes for Ionic Fluxes

Molecule	Probe	Excitation (nm)	Emission (nm)
Calcium	Indo-1	351	405, >460
Magnesium	Mag-Indo-1	351	405, >460
Calcium	Fluo-3	488	525
Calcium	Fura-2	363	>500
Calcium	Calcium green	488	515
P1 A	Acyl pyrene	351	405, >460

V. Applications of Confocal Microscopy

A. Cell Biology

The applications in cell biology are expanding on a daily basis, in no small part owing to a new generation of simple-to-use confocal microscopes that have been designed to remove the technical difficulties previously associated with operating these instruments. Currently one of the more frequent applications is cell tracking using green fluorescent protein (GFP), a naturally occurring protein from the jellyfish *Aequorea victoria* which fluoresces when excited by UV or blue light (Moerner *et al.*, 1999; Jordan *et al.*, 1999; Sullivan and Shelby, 1999). A fluorescent protein such as GFP can be transfected into cells so that subsequent replication of the organism carries with it the fluorescent reporter molecule, providing a valuable tool for tracking the presence of that protein in developing tissue or differentiated cells. This is particularly useful for identifying regulatory genes in developmental biology, and for identifying the biological impact of alterations to normal growth and development processes. In almost any application, multiple fluorescent wavelengths can be detected simultaneously. If a UV–vis confocal microscope is available, Hoechst 33342 (460 nm), FITC (525 nm),

Table V
Probes for Oxidative States

Probe	Oxidant	Excitation (nm)	Emission (nm)
DCFH-DA[a]	(H_2O_2)	488	525
HE[b]	(O_2^-)	488	590
DHR 123[c]	(H_2O_2)	488	525

[a] DCFH-DA, dichlorofluorescin diacetate.
[b] HE, hydroethidine.
[c] DHR-123, dihydrorhodamine 123.

and Texas Red (630 nm) can be simultaneously collected to create a three-color image, providing excellent information regarding the location of the labeled molecules and the structures they identify, and the relationships between them.

B. Microscopy of Living Cells

Evaluation of live cells using confocal microscopy presents some difficult challenges. One is the need to maintain a stable position while imaging a live cell. For example, a viable respiring cell may be constantly changing shape, preventing a finely resolved three-dimensional image reconstruction. Fluorescent probes must be found which are not toxic to the cell. For example, it is possible to evaluate cells attached to an extracellular matrix. In such a situation, the cells can be accurately identified and enumerated, and their relative locations within the matrix determined as well. This requires not just excellent instrumentation, but also an array of analytical tools for image analysis. Confocal microscopy is an effective tool for qualitative and quantitative assessment and for creating three-dimensional image reconstructions of live cells. In most cases it is possible to image several fluorescent markers within a single cell system, making powerful use of the correlative tools of confocal microscopy. As shown in Fig. 6, another example of three-dimensional imaging of live cells demonstrates how the three

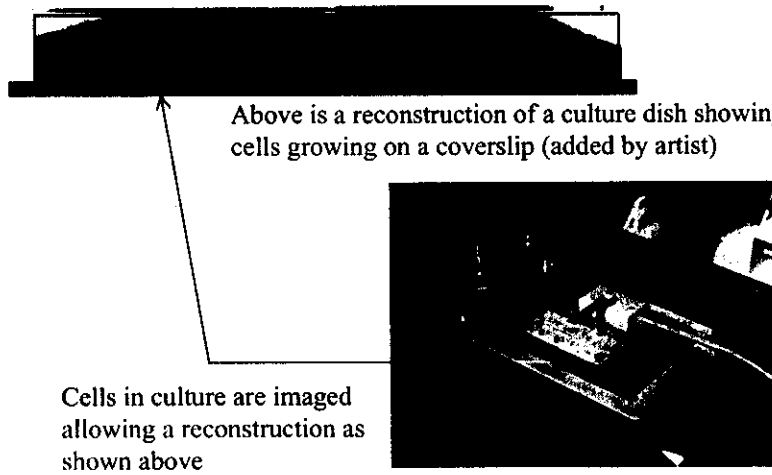

Fig. 6 Endothelial cells growing on a coverslip–tissue culture chamber (shown in the photograph) were imaged using a confocal microscope mounted on an inverted scope. Approximately 60 sections were collected at 0.1-μm z-axis steps. The images were then analyzed using VoxelView (a three-dimensional software package, Vital Images, Inc., Plymouth, MN), and the cell image was reconstructed from an electronic slice through the cells. The cells appear to be sitting on the coverslip (which was added to the figure to demonstrate its position). (See color plates.)

dimensionality of the image can provide powerful information. In this case endothelial cells were grown on glass in a tissue-culture dish. Thirty image sections were taken 0.2 μm apart; the image plane presented shows an x–z plane with the cells attached to the coverglass. The coverglass is represented by a cartoon insertion to show where the cells would be actually attached.

C. Calcium Imaging

Confocal microscopy can be used for evaluation of physiological processes within cells. Examples are changes in cellular pH, changes in free Ca^{2+} ions, and changes in membrane potential and oxidative processes within cells. One of the most successful methods for evaluating these phenomena is emission ratioing in real time. Usually the molecules under study are excited at one wavelength but emit at two wavelengths depending on the change in properties of the molecule. Changes in cellular pH can be identified using SemiNaphtho-Rhodafluor (SNARF-1) (Edwards *et al.*, 1998) or 2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) (Yip and Kurtz, 1995; Stephano and Gould, 1997), which is excited at 488 nm and emits at 525 and 590 nm. The ratio of 590/525 signals reflects the intracellular pH. Calcium changes can be detected using Indo-1, which can be excited at 350 nm (Niggli *et al.*, 1994; Sako *et al.*, 1997). Indo-1 can bind Ca^{2+}, and the fluorescence of the bound molecule is preferentially at the lower emission wavelength; the ratio of emission signals at 400/525 nm reflects the concentration of Ca^{2+} in the cell. Rapid changes in Ca^{2+} can be detected by kinetic imaging—taking a series of images at both emission wavelengths in quick succession. An example is shown in Fig. 7.

D. Cell Adhesion Studies

One early example of the power of confocal microscopy was the study of chondrocytes essentially *in vivo* (Errington *et al.*, 1997). In addition, studies of osteoblastic cell adhesion have been performed using confocal microscopy. Investigators in those studies were interested in the cell attachment and release mechanisms of human osteoblasts to orthopedic devices used for bone or joint replacement (Shah *et al.*, 1999).

E. Colocalization Studies

One of the routine uses for confocal microscopy is the colocalization of or distribution of molecules produced within living organisms. For example, studies of the distribution of HMG-1 protein, a high-mobility group protein which interacts *in vitro* with the minor groove of AT-rich B-DNA, have demonstrated that it is found exclusively in the nucleus (Amirand *et al.*, 1998). Other examples of colocalization have been shown in studies of the TR6 protein produced by equine herpes virus. Confocal microscopy was able to determine that the IR6 protein

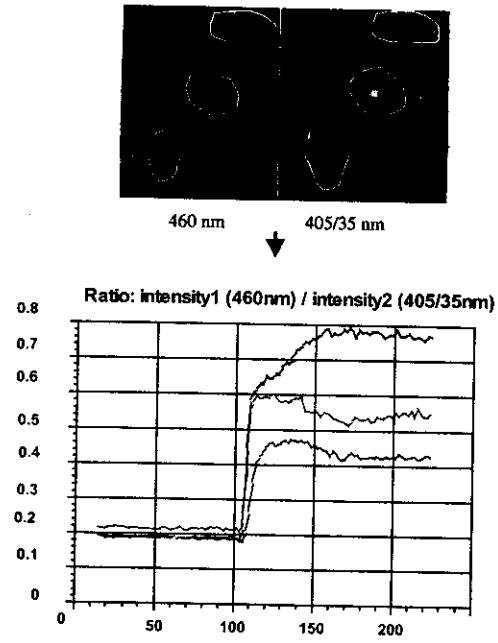

Fig. 7 Changes in the fluorescence of cells loaded with a calcium-sensitive dye were measured using a confocal microscope system with calcium ratioing software. The same region in each wavelength was measured, and the relative change was recorded and exported to a spreadsheet for analysis. The ratio of fluorescence signals can be plotted on a cell-by-cell basis as shown in the graph.

of wild-type RacL11 virus colocalizes with nuclear lamins very late in infection, whereas the mutant IR6 protein encoded by the RacM24 strain did not colocalize with the lamin proteins (Osterrieder *et al.*, 1998).

Similarly, colocalization studies using confocal microscopy have determined that gene 1 products associated with murine hepatitis virus (MHV) are directly associated with the viral RNA synthesis. Confocal microscopy revealed that all the viral proteins detected by these antisera colocalized with newly synthesized viral RNA in the cytoplasm, particularly in the perinuclear region of infected cells. Several cysteine and serine protease inhibitors—E64d, leupeptin, and zinc chloride—inhibited viral RNA synthesis without affecting the localization of viral proteins, suggesting that the processing of the MHV gene 1 polyprotein is tightly associated with viral RNA synthesis. Dual labeling with antibodies specific for cytoplasmic membrane structures showed that RNA and MHV gene 1 products colocalized with the Golgi apparatus in HeLa cells. However, in murine 17CL-1 cells, the viral proteins and viral RNA did not colocalize with the Golgi apparatus but, instead, partially colocalized with the endoplasmic reticulum (Shi *et al.*, 1999). It is fair to say that despite the many alternative technologies available, only confocal microscopy, via its unique ability to create accurate

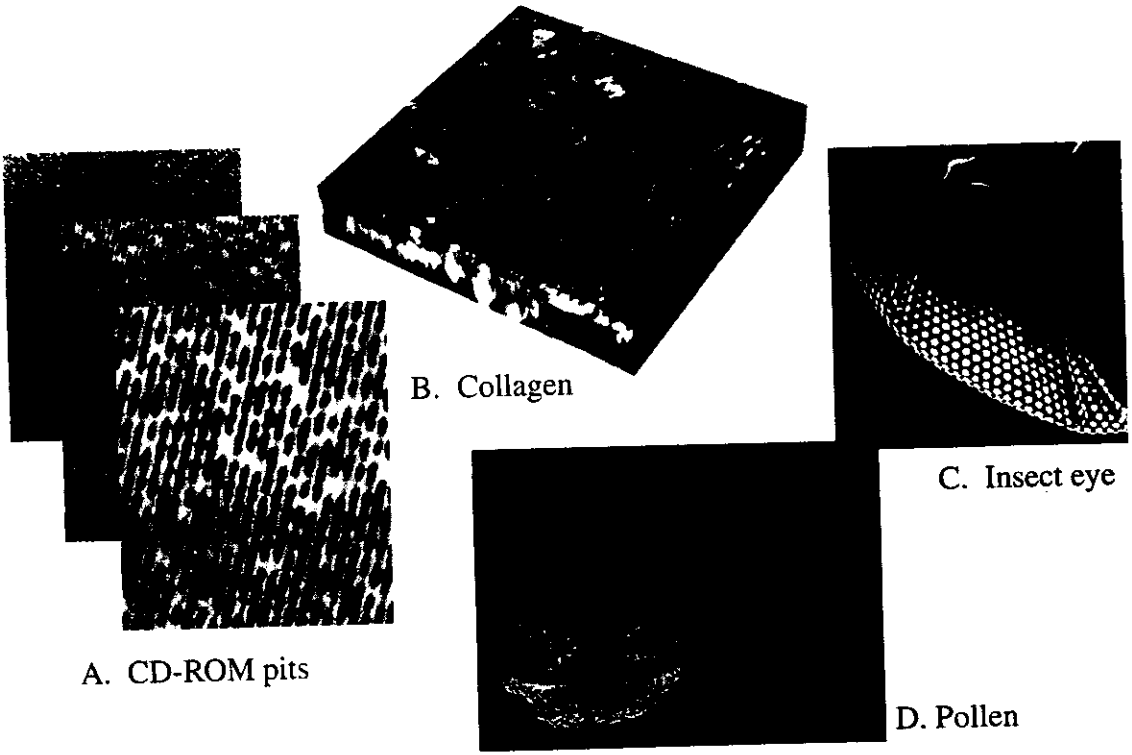

Fig. 8 A variety of confocal images demonstrating (A) reflected light imaging—the pits from a CD-ROM: (B) collagen autofluorescence—an image reconstructed from approximately 150 sections; (C) a reconstructed image of the eye of an insect; and (D) a three-dimensional representation of pollen grains from a pine tree.

three-dimensional structural representations of cells and their organelles, was able to demonstrate the location of the viral RNA.

F. Fluorescence Recovery after Photobleaching

Fluorescence recovery after photobleaching (FRAP) is a measure of the dynamics of the chemical changes in a fluorescent molecule within an object such as a cell. A small area of the cell is bleached by exposure to an intense laser beam, and the recovery of fluorescent species in the bleached area is measured. The recovery time (t) can be calculated from the equation $t = W^2/4D$, where W is the diameter of the bleached spot and D is the diffusion coefficient of the fluorescent molecule under study. An alternative technique can measure interactions between cells: one of two attached cells is bleached and the recovery of the pair as a whole is monitored. To perform FRAP experiments satisfactorily,

it is necessary to be able to park the laser beam over a particular cell, or part of a cell, and effectively bleach the fluorescence of this component of the cells. A number of studies designed to explore multistep signal-transducing events can be performed using FRAP apparatus, e.g., tracking G-protein segments to localized regions of the cell (Kwon *et al.*, 1994). FRAP has also been used to determine macromolecular diffusion of biological polymers (Gribbon and Hardingham, 1998).

VI. Conclusions

Confocal microscopy has reached the point that instruments are now effective and inexpensive compared to the early 1990s when commercial technologies were introduced. More complex systems such as UV microscopes are still relatively rare; however, these may eventually be superseded by the multiphoton microscopes now becoming almost turnkey in operation. For routine one-, two-, or three-color fluorescence microscopy where three dimensionality is important, conventional confocal microscopes surpass multiphoton resolution and will be around for many more years. A new breed of low-cost instruments is now available, making this technology a simple tool requiring no significant technical or engineering knowledge. New fluorescent dyes are also available, increasing the applications for confocal microscopy. Finally, new applications are being developed that utilize reflectance (Fig. 8) (Brightman *et al.*, 2000) or autofluorescence (see Chapter 27 of this volume), which while previously considered a problem can be used a tool for extracting three-dimensional information from within thick tissue.

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