Principles and Practices of Laser Scanning Confocal Microscopy

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Abstract

The laser scanning confocal microscope (LSCM) is an essential tool for many biomedical imaging applications at the level of the light microscope. The basic principles of confocal microscopy and the evolution of the LSCM into today's sophisticated instruments are outlined. The major imaging modes of the LSCM are introduced including single optical sections, multiple wavelength images, three-dimensional reconstructions, and living cell and tissue sequences. Practical aspects of specimen preparation, image collection, and image presentation are included along with a primer on troubleshooting the LSCM for the novice.

Index Entries: Confocal microscopy; laser scanning; fluorescence light microscopy.

1. Introduction

The major application of confocal microscopy in the biomedical sciences is for imaging either fixed or living tissues that have been labeled with one or more fluorescent probes. When these samples are imaged using a conventional light microscope, fluorescence in the specimen in focal planes away from the region of interest interferes with resolution of structures in focus, especially for those specimens that are thicker than 2 μm or so (**Fig. 1**). The confocal approach provides a slight increase in both lateral and axial resolution. It is the ability of the instrument to eliminate the "out-of-focus" flare from thick fluorescently labeled specimens that has caused the explosion in its popularity recently *(1)*. Most modern confocal microscopes are now relatively easy to operate and have become integral parts of many multiuser imaging facilities. Since the resolution achieved by the LSCM is a little better than that achieved in a conventional wide-field light microscope (theoretical maximum resolution of 0.2 μm), but not as great as that in the transmission electron microscope (0.1 nm), it has bridged the gap between these two commonly used techniques.

The method of image formation in a confocal microscope is fundamentally different from that in a conventional wide-field microscope where the entire specimen is bathed in light from a mercury or xenon source, and the image can be viewed directly by eye. In contrast, the illumination in a confocal microscope is achieved by scanning one or more focused beams of light, usually from a laser, across the specimen. An image produced by scanning the specimen in this way is called an optical section. This term refers to the noninvasive method of image collection by the instrument, which uses light rather than physical means to section the specimen. The confocal approach has thus facilitated the imaging of living specimens, enabled the automated collection

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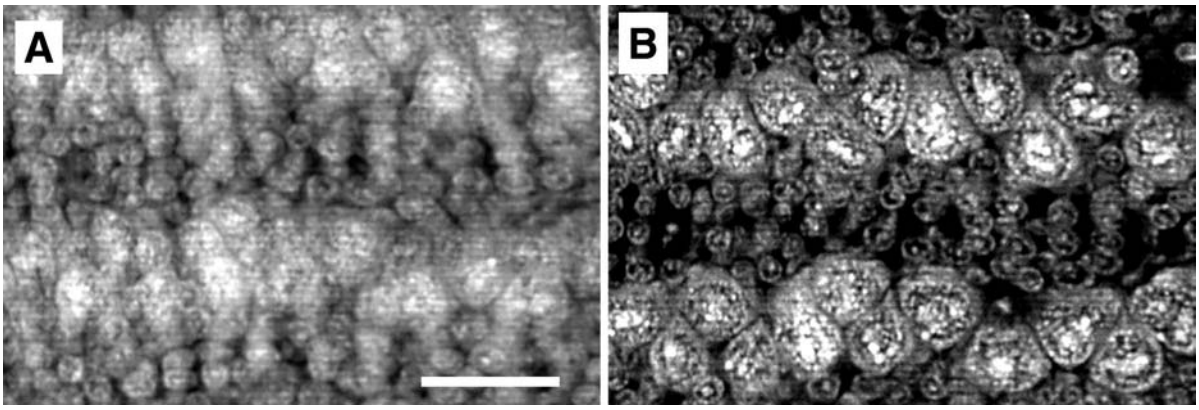

Fig. 1. Conventional epifluorescence image **(A)** compared with a confocal image **(B)** of a similar region of a whole mount of a butterfly (*Precis coenia*) pupal wing stained with propidium iodide. Note the improved resolution of the nuclear detail **(B)**.

of three-dimensional data in the form of Z-series, and improved the images of multiple labeled specimens ***(1)***.

Emphasis has been placed on the laser scanning confocal microscope (LSCM) throughout this article because it is currently the instrument of choice for most biomedical research applications, and it is therefore most likely to be the instrument first encountered by the novice user. Several alternative designs of confocal instruments occupy specific niches within the biological imaging field ***(2)***. Optical sections can be produced using other, nonconfocal, methods. For example, using deconvolution, which calculates the out-of-focus information in an image and removes it digitally ***(3)***, and multiple photon imaging ***(4)***, which uses the same method of scanning as the LSCM but uses a laser that only excites the fluorochromes that are imaged in the optical section itself.

2. Evolution of the Confocal Approach

The development of confocal microscopes was driven largely by a desire to image biological events as they occur in vivo. The invention of the confocal microscope is usually attributed to Marvin Minsky, who built a working microscope in 1955 with the goal of imaging neural networks in unstained preparations of living brains. Details of the microscope, and of its development can be found in an informative memoir by Minsky, and all of the modern confocal imaging systems employ the principle of confocal imaging that he patented in 1957 ***(5)***.

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 into the specimen, and light that passes through it is focused by a second objective lens at a second pinhole, which has the same focus as the first pinhole, i.e., it is confocal with it. Any light that passes through the second pinhole strikes a low-noise photomultiplier, which produces a signal that is directly proportional to the brightness of the light. The second pinhole prevents 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 light or "flare" in the specimen by spatial filtering. Minsky also described a reflected light version of the microscope that uses a single objective lens and a dichromatic mirror arrangement. This is the basic configuration of most modern confocal systems used for fluorescence imaging **(Fig. 2)**.

In order to build an image, the focused spot of light must be scanned across the specimen in some way. In Minsky's original microscope the beam was stationary and the specimen itself was moved on a vibrating stage. This optical arrangement has the advantage of always scanning on the

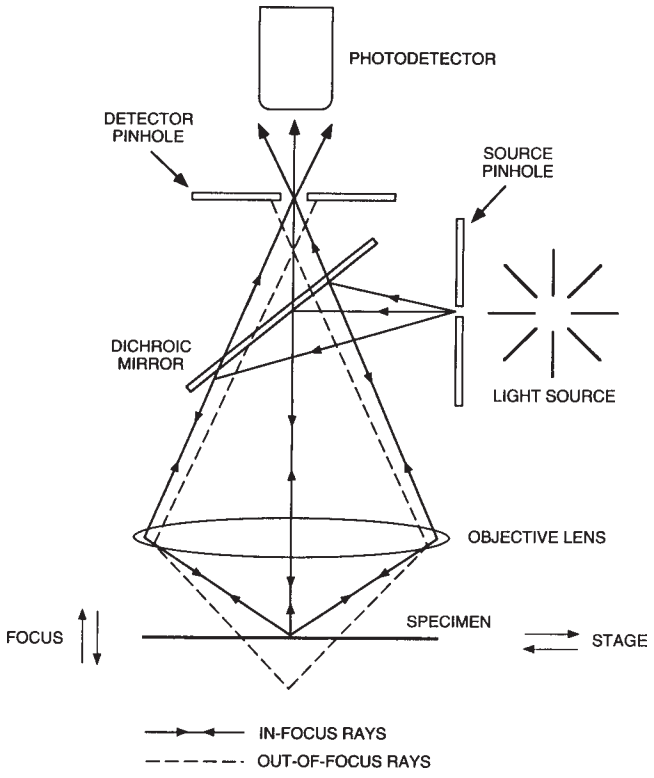

Fig. 2. The light path in a typical LSCM is based upon that of a conventional reflected-light wide-field epifluorescence microscope, but with pinholes placed in front of the light source (the point source is now a laser) and in front of the photodetector. The pinhole at the light source, the focused point in the specimen, and the pinhole in front of the detector are all confocal with one another.

optical axis, which can eliminate any lens defects. However, for many biological specimens, movement of the specimen can cause them to wobble and distort, which results in a loss of resolution in the image. Moreover, it is impossible to perform various manipulations such as microinjection of fluorescently labeled probes when the specimen is moving.

Finally an image of the specimen has to be produced. A real image is not formed in Minsky's original microscope but rather the output from the photodetector is 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 oscilloscope with no facility for hard copy. Minsky admitted at a later date that the image quality in his microscope was not very impressive because of the quality of the oscilloscope display and not because of lack of resolution achieved with the microscope itself.

It is clear that the technology was not available to Minsky in 1955 to fully demonstrate the potential of the confocal approach especially for imaging biological structures. According to Minsky, this is perhaps a reason why confocal microscopy did not immediately catch on with the biological community, who were, (as they are now) a highly demanding and fickle group when it came to the quality of their images. After all, at the time they could quite easily view and photograph their brightly stained and colorful histological tissue sections using light microscopes with excellent optics.

In modern confocal microscopes the image is either built up from the output of a photomultiplier tube or captured using a digital charge coupled device (CCD) camera, directly processed in a computer imaging system, and then displayed on a high-resolution video monitor and recorded on modern hard copy devices, with spectacular results.

The optics of the light microscope have not changed drastically in decades since the final resolution achieved by the instrument is governed by the wavelength of light, the objective lens and properties of the specimen itself. However, the associated technology and the dyes used to add contrast to the specimens have been improved significantly over the past 20 yr. The confocal approach is a direct result of a renaissance in light microscopy that has been fueled largely by advancements in modern technology. Several major technological advances that would have benefited Minsky's confocal design are now available (and affordable) to biologists. These advances include:

1. Stable multiwavelength lasers for brighter point sources of light.
2. More efficiently reflecting mirrors.
3. Sensitive low-noise photodetectors.
4. Fast microcomputers with image processing capabilities.
5. Elegant software solutions for analyzing the images.

6. High-resolution video displays and digital printers.
7. Brighter and more stable fluorescent probes.

These technologies have been developed independently, and since 1955, they have slowly been incorporated into modern confocal imaging systems. For example, Shinya Inoue and Robert Allen first effectively applied digital image processing to biological imaging in the early 1980s at Woods Hole. Their "video-enhanced microscopes" enabled an apparent increase in resolution of structures using digital enhancement of images captured using a low-light-level silicon-intensified-target (SIT) video camera mounted on a light microscope and connected to a digital image processor. Cellular structures such as the microtubules, which are just beyond the theoretical resolution of the light microscope, were imaged using differential interference contrast (DIC) optics and enhanced using such digital methods as background subtraction and contrast enhancement in real time. These techniques are reviewed in a landmark book titled *Video Microscopy* by Shinya Inoue, which has been recently updated with Ken Spring, and provides an excellent primer for the principles and practices of modern light microscopy ***(6)***.

Confocal microscopes are usually classified by the method in which the specimens are scanned. Minsky's original design was a stage scanning system driven by a primitive tuning fork arrangement that was rather slow to build an image. Stage scanning confocal microscopes have evolved into instruments that are used traditionally for materials science applications such as the microchip industry. Systems based upon this principle have recently become popular in biomedical applications for screening arrays of fluorescently labeled DNA oligonucleotides on glass wafer supports or "gene chips" ***(7)***.

An alternative to moving the specimen is to scan the beam across a stationary one, which is more practical for imaging biological samples, and is the basis of many systems that have evolved into the confocal imaging systems that are so prevalent today. The more technical aspects of confocal instrumentation have been covered elsewhere ***(2)***, but briefly there are two fundamentally different methods of beam scanning: multiple-beam scanning or single-beam scanning. The most popular way is currently single-beam scanning, which is typified by the LSCM. Here the scanning is most commonly achieved by computer-controlled galvanometer driven mirrors (1 frame/s), or in some systems, by an acoustooptical device for faster scanning rates (near video rates). The alternative is to scan the specimen with multiple beams (almost real time) usually using some form of spinning Nipkow disc. The forerunner of these systems was the tandem scanning microscope (TSM) and subsequent improvements to the design have become more efficient for collecting images from fluorescently labeled specimens.

There are currently two viable alternatives to confocal microscopy that produce optical sections in technically different ways. These are deconvolution ***(3)*** and multiple photon imaging ***(4)***, and as with confocal imaging they are based on a conventional light microscope. Deconvolution is a computer-based method that calculates and removes the out-of-focus information from a fluorescence image. The deconvolution algorithms and the computers themselves are now fast enough to make this technique a practical option for imaging. Multiple photon microscopy uses a scanning system that is identical to that of the LSCM but there is no need for the pinhole since the laser only excites at the point of focus in the specimen. This makes the technique more practical for imaging living tissue ***(4)***.

3. The Laser Scanning Confocal Microscope

The LSCM is built around a conventional light microscope, and uses a laser rather than a lamp for a light source, sensitive photomultiplier tube detectors (PMTs), and a computer to control the scanning mirrors and to facilitate the collection and display of the images. The images are subsequently stored using computer media and analyzed using a plethora of computer software either using the computer of the confocal system or a second computer (**Fig. 3**).

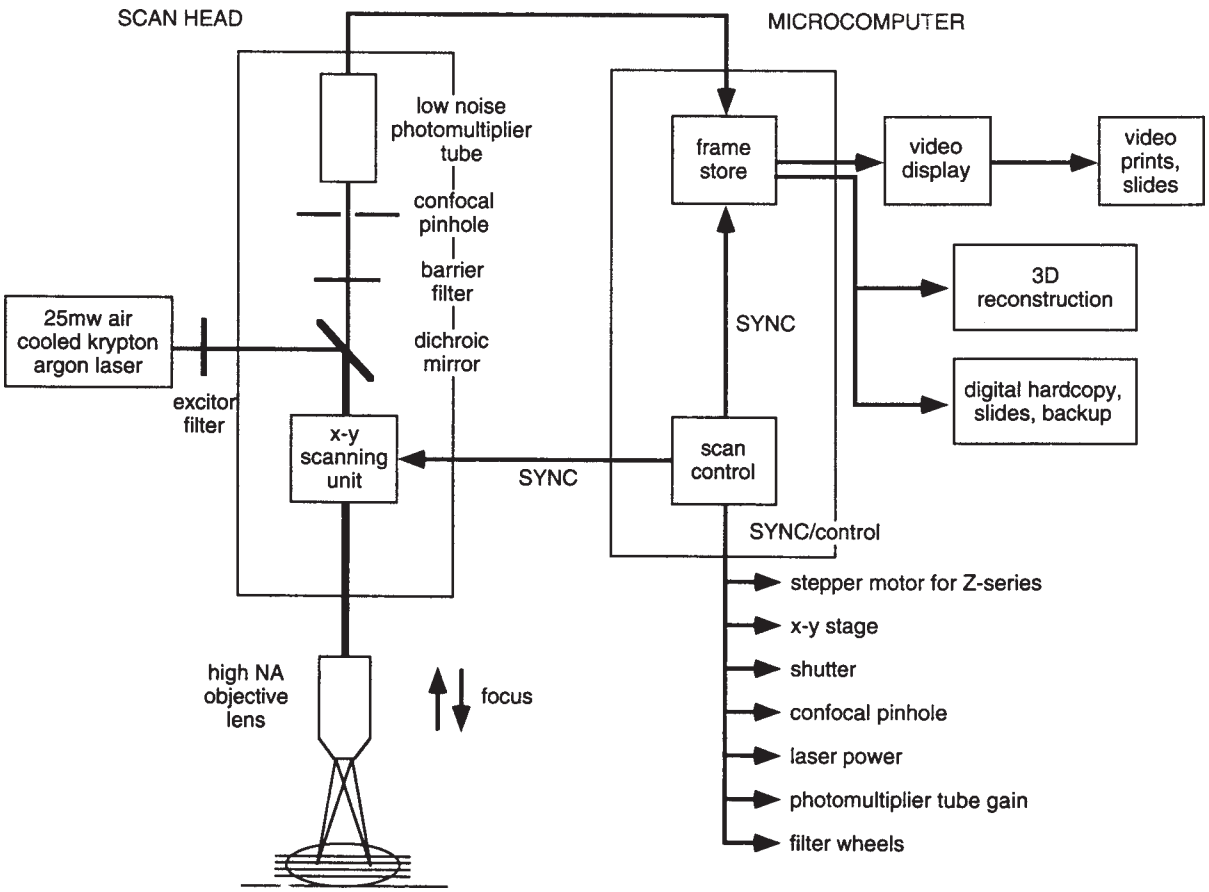

Fig. 3. Information flow in a laser scanning confocal imaging system.

In the LSCM, illumination and detection are confined to a single, diffraction-limited, point in the specimen. This point is focused in the specimen by an objective lens, and scanned across it using some form of scanning device. Points of light from the specimen are detected by a PMT, which is positioned behind a pinhole, and the output from the PMT is built into an image by the computer **(Fig. 2)**. Specimens are usually labeled with one or more fluorescent probes, or unstained specimens can be viewed when the light reflected back from the specimen is detected at the PMT.

One of the more commercially successful LSCMs was designed by White, Amos, Durbin, and Fordham ***(8)*** in order to tackle a fundamental problem in developmental biology, namely, imaging specific macromolecules in fluorescently labeled embryos. Many of the structures inside these embryos are impossible to image after the two-cell stage using conventional epifluorescence microscopy because as cell numbers rise, the overall volume of the embryo remains approximately the same, which means that increased fluorescence from the more and more closely packed cells out of the focal plane of interest interferes with image resolution.

When he investigated the confocal microscopes available to him at the time, White discovered that no system existed that would satisfy his imaging needs. The technology consisted of the stage scanning instruments, which tended to be slow to produce images (approx 10 s for one full frame image), and the multiple-beam microscopes, which were difficult to align at that time and the fluorescence images were extremely dim, if not invisible without extremely long exposure times! Using available lasers, electronics, and

digital image processors, White and his colleagues designed and built a LSCM that was suitable for conventional epifluorescence microscopy of biological specimens, for example, fluorescently labeled embryos, that has since evolved with improvements in technology into an instrument that is now used in a plethora of biomedical applications.

In a landmark paper that captured the attention of the cell biology community by the quality of the images published in it ***(9)***, White et al. compared images collected from the same specimens using conventional wide-field epifluorescence microscopy and their LSCM. Rather than physically cutting sections of multicellular embryos, their LSCM produced "optical sections" that were thin enough to resolve structures of interest and were free of much of the out-of-focus fluorescence that had previously contaminated their images. This technological advance allowed them to take full advantage of the specificity of immunofluorescence labeling to follow changes in the cytoskeleton in cells of early embryos at a higher resolution than was possible using conventional epifluorescence microscopy.

Simply adjusting the diameter of a pinhole in front of the photodetector could vary the thickness of the optical sections **(Fig. 2)**. This optical path has proven to be extremely flexible for imaging biological structures as compared with some other designs that employ fixed diameter pinholes. The image can be zoomed with no loss of resolution simply by decreasing the region of the specimen that is scanned by the mirrors, and placing the scanned information into the same size of digital memory or framestore. This imparts a range of magnifications to a single objective lens, and is extremely useful when imaging rare events when changing a lens may risk losing the region of interest.

This microscope and several other LSCMs developed during the same time period, were the forerunners of the sophisticated instruments that are now available to biomedical researchers from several commercial vendors ***(10)***. There has been a tremendous explosion in the popularity of confocal microscopy over the past 10 yr. Indeed many laboratories are purchasing the systems as shared instruments in preference to electron microscopes despite the reduced resolution, which is between the conventional light microscope and the electron microscope. The advantage of confocal microscopy lies within its great number of applications and its relative ease for producing extremely high-quality digital images from specimens prepared for the light microscope.

The first generation LSCMs were tremendously wasteful of photons in comparison to the new microscopes. The early systems worked well for fixed specimens but tended to kill living specimens unless extreme care was taken to preserve the viability of specimens on the stage of the microscope by minimizing the exposure to laser light. Nevertheless, the microscopes produced such good images of fixed specimens that confocal microscopy was fully embraced by the biological imagers.

Improvements have been made at all stages of the imaging process in the subsequent generations of instruments including the addition of more stable lasers, more efficiently reflecting mirrors, more sensitive low noise photodetectors, and improved digital imaging systems **(Fig. 3)**. The new instruments are much improved ergonomically so that alignment is easier to achieve (if at all necessary), the choice of different filter combinations, which is now controlled by software, motorized filter wheels and electronic filters, is much easier to achieve, and multiple fluorochromes can be imaged either simultaneously by frame or sequentially on a line-by-line basis ***(1)***. Furthermore, it is easier to manipulate the images using improved, more reliable software that has been developed over the ten or so years of experience with the LSCM, and using faster computers with more hard disk space and random access memory (RAM).

4. Confocal Imaging Modes

4.1. Single Optical Sections

The optical section is the basic image unit of the confocal microscope. Data are collected from fixed and stained samples in single-, double-, triple-, or multiple-wavelength modes **(Fig. 4)**.

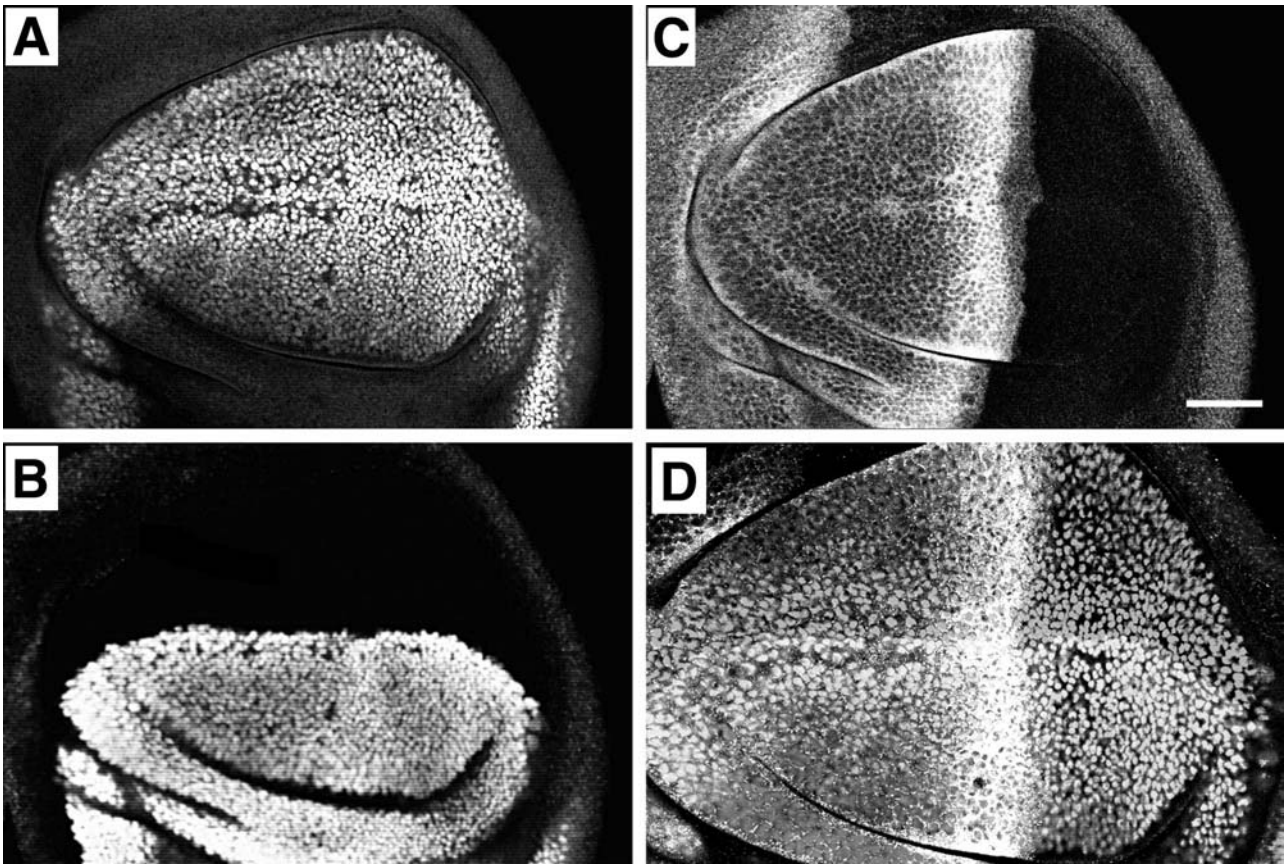

Fig. 4. Single optical sections collected simultaneously using a single krypton argon laser at three different excitation wavelengths—488 nm, 568 nm, and 647 nm—of a fruit fly third instar wing imaginal disc immunofluorescently labeled with antibody probes for three genes involved with patterning the developing wing; **(A)** vestigial (fluorescein 496 nm); **(B)** apterous (lissamine rhodamine 572 nm) and **(C)** CiD (cyanine 5 649 nm); with a grey-scale image of the three images merged **(D)**.

The resulting images are in register with each other and portray an accurate representation of the specimen, as long as an objective lens that is corrected for chromatic aberration is used. Registration can usually be relatively easily restored using digital methods. The time of image collection will also depend on the size of the image and the speed of the computer; for example, a typical 8-bit image of 768 by 512 pixels in size will occupy approx 0.3 MB. Using most LSCMs it takes approx 1 s to collect a single optical section, although several such sections are usually digitally averaged in order to improve the signal-to-noise ratio ***(6)***.

4.2. Time-Lapse and Live Cell Imaging

Time-lapse imaging refers to the collection of single optical sections at preset points in time, and it uses the improved resolution of the LSCM for studies of the dynamics of living cells **(Fig. 5)**. Imaging living tissues is perhaps an order of magnitude more difficult than imaging fixed ones using the LSCM **(Table 1)**. For successful live-cell imaging extreme care must be taken to maintain the cells in a healthy state on the stage of the microscope throughout the imaging process ***(11)***, and to use the minimum laser exposure possible because photodamage from the laser beam can accumulate over multiple scans. Antioxidants such as ascorbic

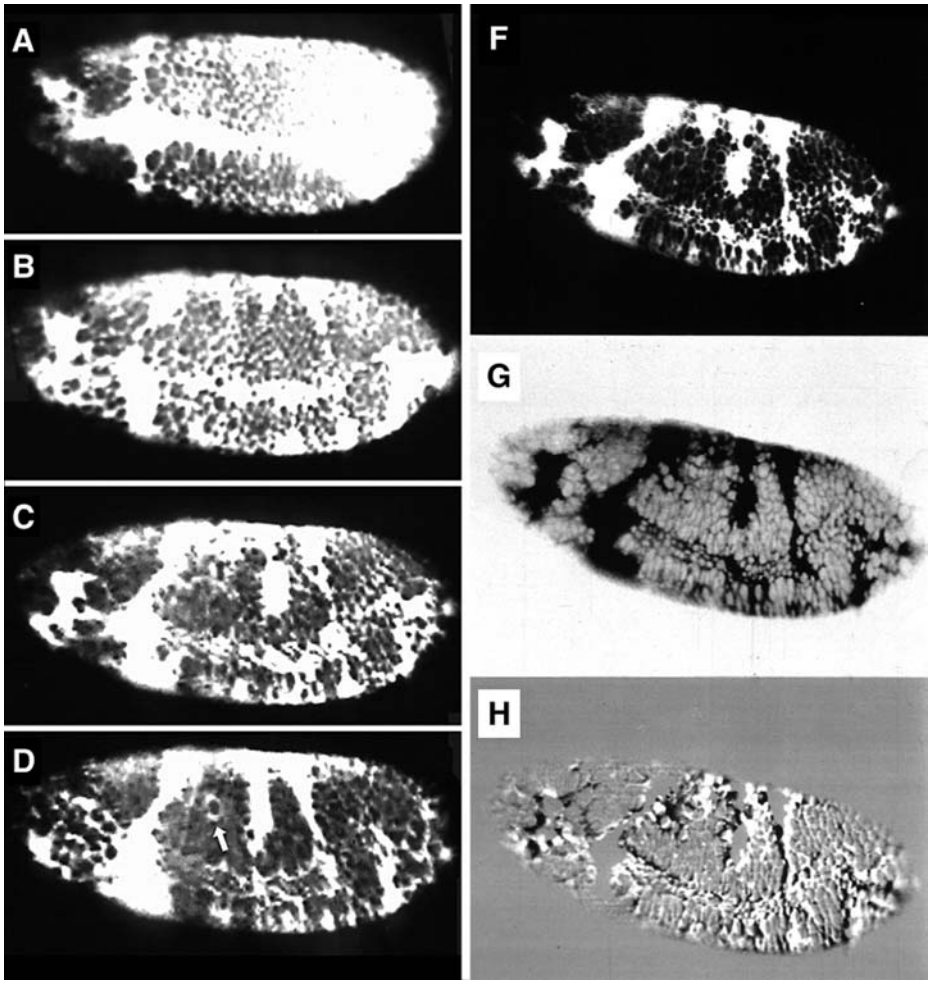

Fig. 5. Time lapse imaging of a living fruit fly embryo injected with Calcium green **(A–D)**. The larger outline of a dividing cell is shown—arrow in (D). One method of showing change in distribution of the fluorescent probe over time on a journal page is to merge a regular image of one time point **(F)** with a reversed contrast image of a second time point **(G)** to give a composite image **(H)**. The same technique can be used by merging different colored images from different time points.

acid can be added to the medium to reduce oxygen from excited fluorescent molecules, which can cause free radicals to form and kill the cells.

A series of preliminary control experiments is usually necessary in order to assess the effects of light exposure on the fluorescently labeled cells. A postimaging test of viability should ideally be performed; for example, an embryo should continue its normal development after imaging. Each cell type has its own specific requirements for life; for example, mammalian cells will require some stage heating device, and perhaps a perfusion chamber to maintain the carbon dioxide balance in the medium, whereas other cells such as insect cells are usually perfectly happy at room temperature in a relatively large volume of medium. Many experimental problems can be avoided by choosing a cell type that is more amenable to imaging with the LSCM. The photon efficiency of most modern confocal systems has been improved significantly over the early models, which were tremendously wasteful of photons, and when coupled with the availability of brighter objective lenses (including high NA water immersion lenses),

Table 1
Different Considerations for Imaging Fixed and Living Cells with the LSCM

	Fixed Cells	Living Cells
Limits of illumination	Fading of fluorophore	Phototoxicity and fading of dye
Antifade reagent	Phenylenediamine, etc.	NO!
Mountant	Glycerol (n = 1.51)	Water (n = 1.33)
Highest NA lens	1.4	1.2
Time per image	Unlimited	Limited by speed of phenomenon; light sensitivity of specimen
Signal averaging	Yes	No
Resolution	Wave optics	Photon statistics

and brighter and less phototoxic dyes, live-cell confocal analysis has become much more of a practical option.

The bottom line is to use the least amount of laser power possible for imaging and to collect the images quickly in order to reduce the time of exposure of the specimen to laser light. The pinhole may be opened wider than for fixed samples to speed up the imaging process and to collect the signal from all of the available photons for later image processing, for example, deconvolution may be used to improve the images.

Many physiological events take place faster than the image-acquisition speed of most LSCMs, which is typically on the order of a single frame per second. Faster scanning LSCMs that use an acoustooptical device and a slit to scan the specimen rather than the slower galvanometer-driven point scanning systems are marketed with physiological imaging in mind. These designs have the advantage of good spatial resolution coupled with good temporal resolution, i.e., full screen resolution 30 frames per s (near video rate). Using the point scanning LSCMs good temporal resolution is achieved by scanning a much-reduced area. Here frames at full spatial resolution are collected more infrequently ***(12)***. The disk scanning systems can also be used for imaging fast physiological events. The multiple-photon approach is now the method of choice for imaging living cells, however ***(4)***.

4.3. Z-Series and Three-Dimensional Imaging

A *Z*-series is a sequence of optical sections collected at different levels from a specimen **(Fig. 6)**. *Z*-series are collected by correlating the movement of the fine focus of the microscope with image collection usually using a computer-controlled stepping motor to move the stage of the microscope by preset distances **(Fig. 3)**. This is relatively easily accomplished using a macro program that collects an image, moves the microscope stage (focus) by a predetermined distance, collects a second image, moves the microscope stage (focus), and carries on in this way until several images through the region of interest have been collected. Often two or three images are extracted from such a *Z*-series and digitally projected to highlight some specific cells. It is also relatively easy to display a *Z*-series as a montage of images **(Fig. 6)**. These programs are standard features of most of the commercially available imaging systems.

Z-series are ideal for further processing into a three-dimensional (3D) representation of the specimen using volume visualization techniques ***(13)***. This approach is now used to elucidate the relationships between the 3D structure and function of tissues, because it can be conceptually difficult to visualize complex interconnected structures from a series of 200 or more optical sections taken through a structure with the LSCM. Care must be taken to collect the images at the correct *Z*-step of the motor in order to reflect the actual depth of the specimen in the image. Since the *Z*-series produced with the LSCM are in perfect register (assuming the specimen itself does not move during the period of image acquisition) and are in a digital form, they can relatively easily be processed into a 3D representation of the specimen **(Fig. 7)**.

There is sometimes confusion about what is meant by optical section thickness. This usually refers to the thickness of the section of the sample

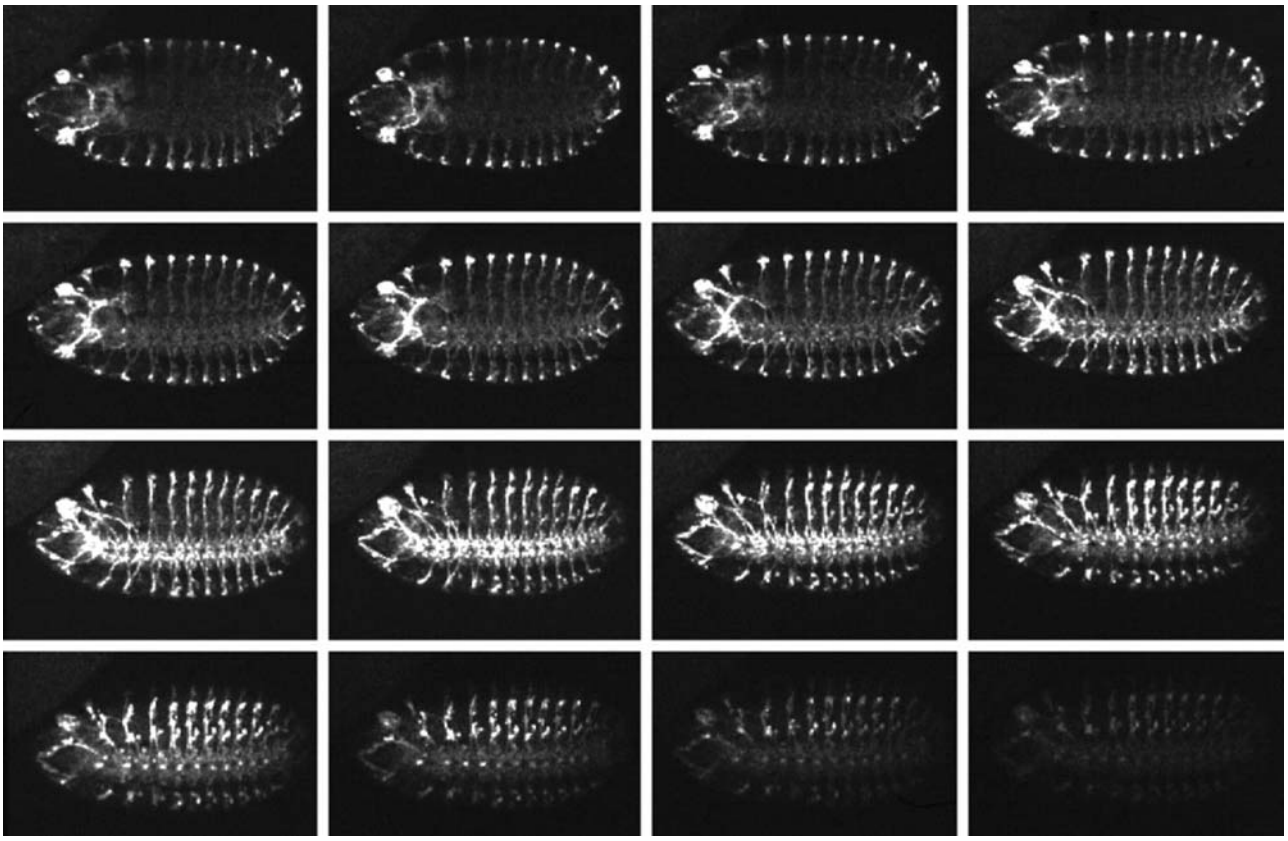

Fig. 6. A *Z*-series of optical sections collected from a fruit fly embryo labeled with the antibody designated 22C10, which stains the peripheral nervous system.

collected with the microscope and not to the step sizes taken by the stepper motor, which is set up by the operator. In some cases these can be the same however, and may be a source of the confusion.

The series of optical sections from a time-lapse run can also be processed into a 3D representation of the data set so that time is the *Z*-axis. This approach is useful as a method for visualizing physiological changes during development. For example, calcium dynamics have been characterized in sea urchin embryos ***(14)***. A simple method for displaying 3D information is by color coding optical sections at different depths. This can be achieved by assigning a color (usually red, green, or blue) to sequential optical sections collected at various depths within the specimen. The colored images from the *Z*-series are then merged and colorized using an image manipulation program such as Adobe Photoshop ***(15)***.

4.4. Four-Dimensional Imaging

Time-lapse sequences of *Z*-series can also be collected from living preparations using the LSCM to produce four-dimensional (4D) data sets, i.e., three spatial dimensions *X*, *Y*, and *Z* with time as the fourth dimension. Such series can be viewed using a 4D-viewer program, stereo pairs of each time point can be constructed and viewed as a movie or a 3D reconstruction at each time point is subsequently processed ***(16,17)***.

4.5. X-Z *Imaging*

An *X-Z* section is a profile of the specimen, and it can either be produced directly from the specimen using the LSCM by scanning a single line at different *Z* depths under the control of the stepper motor **(Fig. 8)** or indirectly in a 3D reconstruction program by extracting the *X-Z* profile from a *Z*-series of optical sections ***(13)***.

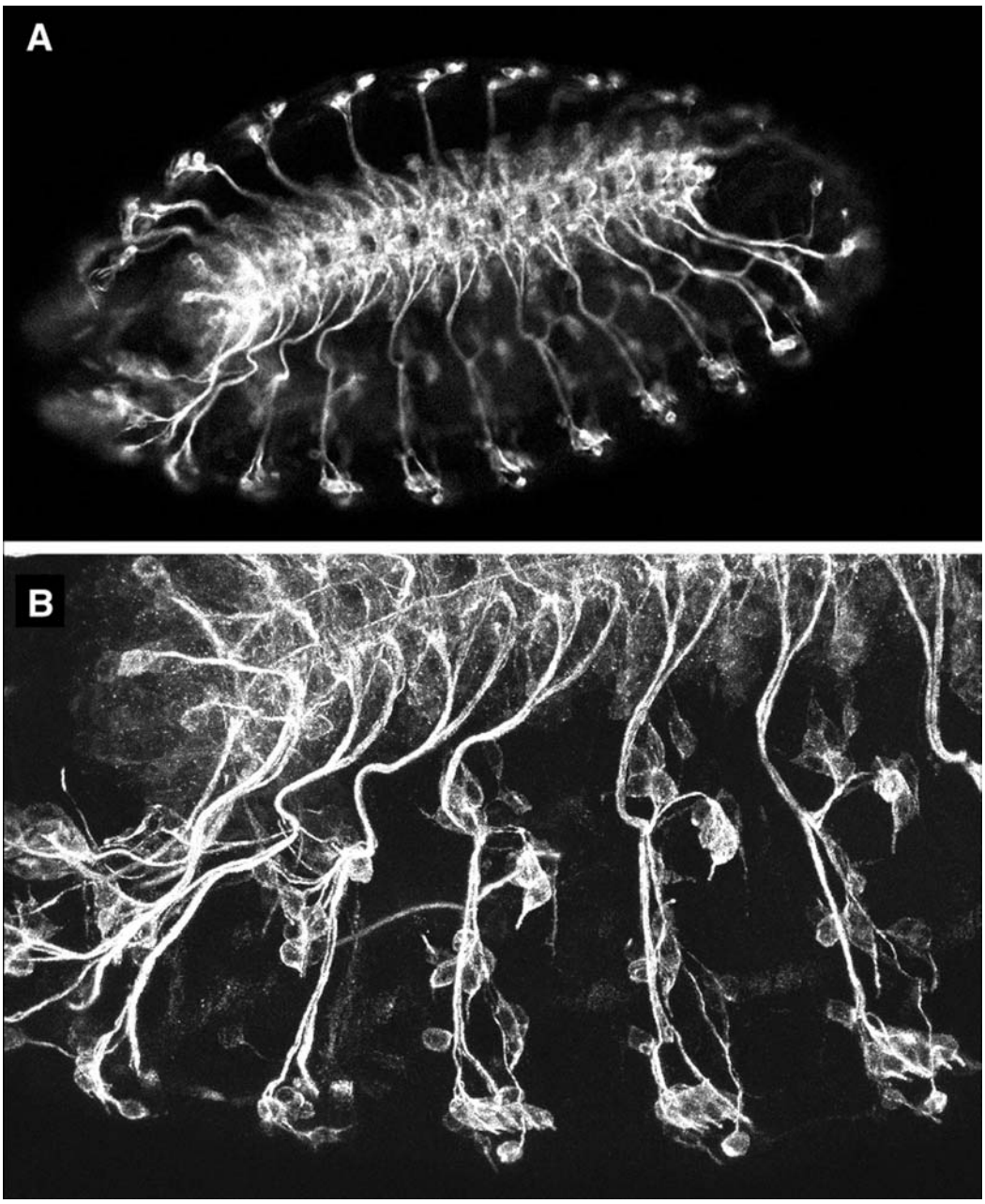

Fig. 7. A **(A)** single optical section compared with a **(B)** *Z*-series projection of a fruit fly peripheral nervous system stained with the 22C10 antibody.

4.6. Reflected-Light Imaging

Unstained preparations can also be viewed with the LSCM using reflected- (backscattered) light imaging. This is the mode used in all of the early confocal instruments **(Fig. 9)**. In addition, specimen can be labeled with probes that reflect light such as immunogold or silver grains ***(18)***. This method of imaging has the advantage that photobleaching is not a problem especially for living samples. Some of the probes tend to attenuate the laser beam, and in some LSCMs there can be a reflection from optical elements in the microscope. The problem can be solved using polarizers or by imaging away from the artifact, and

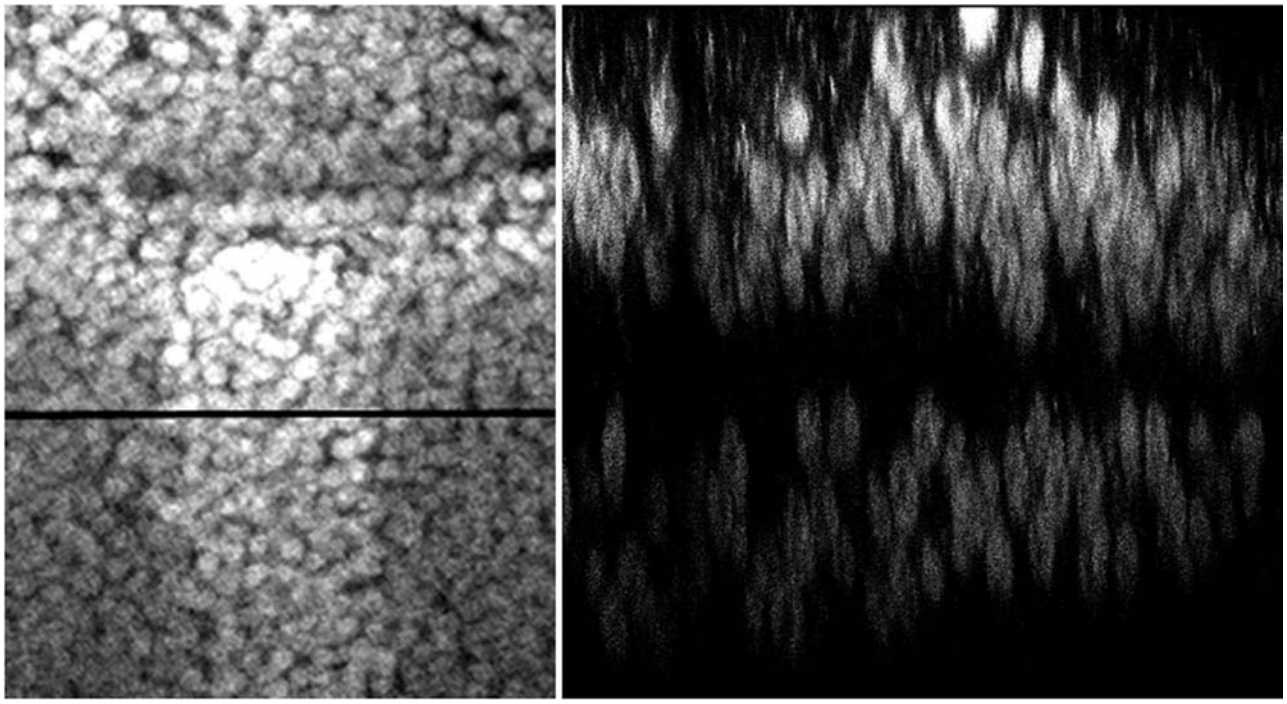

Fig. 8. *X-Z* imaging; the laser is scanned across a single line at different *Z* depths—black line in **(A)** and an *X-Z* image of all the scanned lines was constructed by the confocal imaging system **(B)**. Note that the butterfly wing epithelium is made up of two epithelial layers, and note that the fluorescence intensity drops off deeper into the specimen.

off the optical axis. The reflection artifact is not a problem using the slit- or multiple-beam scanning systems.

4.7. Transmitted Light Imaging

Any form of light-microscope image, including phase contrast, DIC, polarized light or dark field, can be collected using a transmitted light detector **(Fig. 8)**. This is a device that collects the light passing through the specimen and the signal from it is transferred to one of the PMTs in the scan head via a fiber optic. Since confocal epifluorescence images and transmitted-light images are collected simultaneously using the same excitation beam, image registration is preserved, so that the precise localization of labeled cells within the tissues is preserved when the images are merged using digital methods.

It is often informative to collect a transmitted, nonconfocal image of a specimen and to merge such a transmitted light image with one or more confocal fluorescence images of labeled cells. For example, the spatial and temporal components of the migration of labeled cells within an unlabeled population of cells can be mapped over hours or even years ***(19)***.

A real-color transmitted-light detector has been introduced into those systems that scan three channels simultaneously. The detector collects the transmitted signal in the red, the green, and the blue channels through red, green, and blue filters, respectively, and builds the real-color image in a similar way to some color digital cameras ***(6)***. This device is useful to pathologists who are used to viewing real colors in bright-field mode of their stained preparations, and also to combine them with confocal images taken of the same region of the specimen in fluorescence mode.

4.8. Correlative Microscopy

The aim of correlative or integrated microscopy is to collect information from the same region of a specimen using more than one microscopic technique. Confocal microscopy can be used in tandem

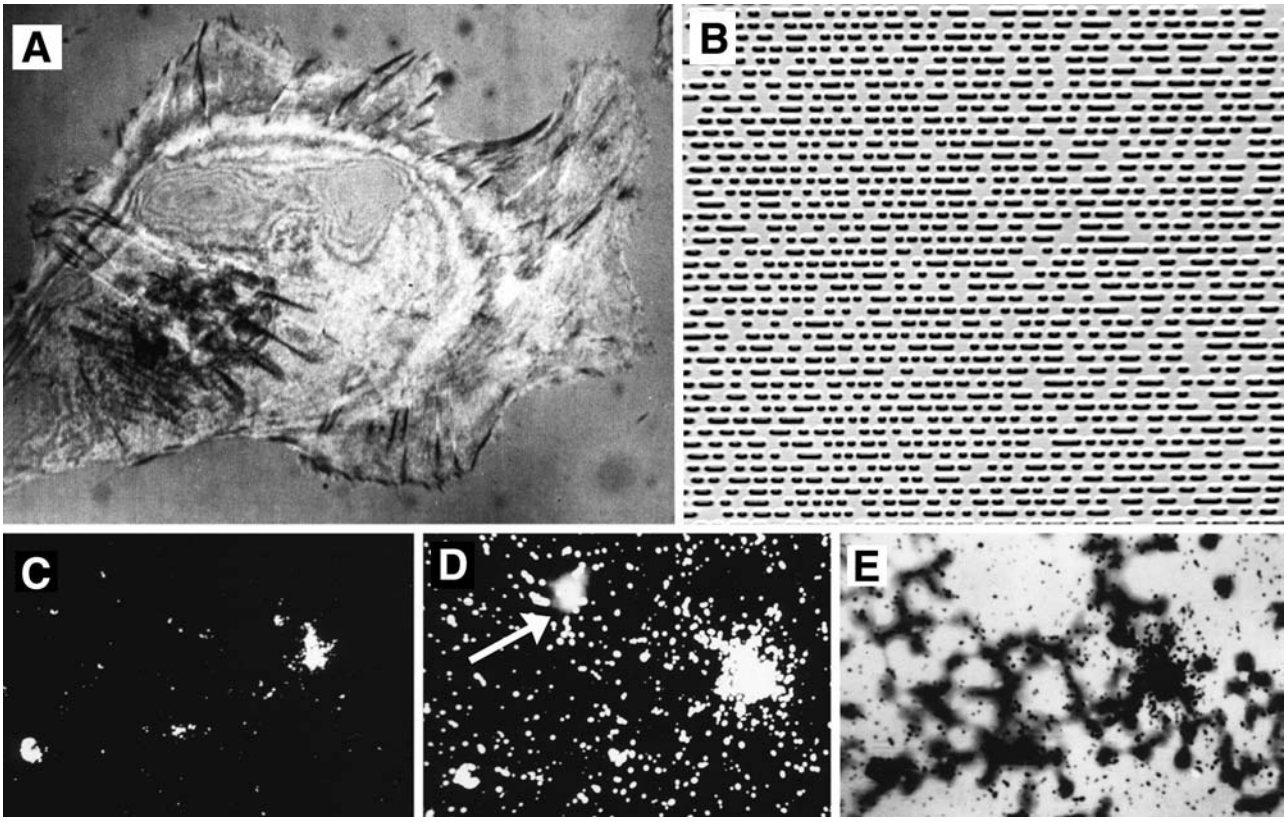

Fig. 9. Reflected-light and transmission imaging: Interference reflection microscopy in the LSCM demonstrates cell substratum contacts in black around the cell periphery **(A)**; confocal systems are used extensively in the materials sciences - here the surface of an audio CD is shown **(B)**; and **(C)** through **(E)** is an *in situ* hybridization of HIV infected blood cells. The silver grains can be clearly seen in the reflected light confocal image (C) and in the transmitted light dark-field image **(D)** and bright-field image (E). Note the false positive from the dust particle—arrow in (D).

with transmission electron microscopy (TEM). For example, the distribution of microtubules within fixed tissues has been imaged using the LSCM, and the same region was imaged in the TEM using eosin as a fluorescence marker in the LSCM and as an electron-dense marker in the electron microscope ***(20)***. Reflected light imaging and the TEM have also been used in correlative microscopy to image cell substratum contacts ***(21)***.

5. Specimen Preparation and Imaging

More details of specimen preparation and the confocal methods used to image them are available elsewhere ***(22–25)***. Most of the protocols for confocal imaging are based upon those developed over many years for preparing samples for the conventional wide field microscope. A good starting point for the development of a new protocol for the confocal microscope therefore is with a protocol for preparing the samples for conventional light microscopy, and to later modify it to the confocal instrument if necessary. Most of the methods for preparing specimens for the conventional light microscope were developed to reduce the amount of out-of-focus fluorescence. The confocal system undersamples the fluorescence in a thick sample as compared with a conventional epifluorescence light microscope, with the result that samples may require increased staining times or concentrations for confocal analysis, and may appear to be overstained in the light microscope. Moreover, whole mounts may be imaged success-

fully in the LSCM where it was only practical to view sections using a conventional wide field light microscope.

The illumination in a typical laser scanning confocal system is extremely bright, although millions of points are scanned per second. For example, a typical scan speed is one point per 1.6 μs so that the average illumination at any one point is relatively moderate, and generally less than a conventional epifluorescence light microscope. Many protocols include an antibleaching agent that protects the fluorophore from the bleaching effects of the laser beam. It is advisable to use the lowest laser power that is practical for imaging in order to protect the fluorochrome, and it may be possible to omit the antibleaching agents when using many of the more modern instruments.

The major application of the confocal microscope is for improved imaging of thicker specimens although the success of the approach depends on the specific properties of the specimen. Some simple ergonomic principles apply: for example, the specimen must physically fit on the stage of the microscope and the area of interest should be within the working distance of the lens. For example, a high-resolution lens such as a 60× NA 1.4 has a working distance of 170 μm, whereas a 20× NA 0.75 has a working distance of 660 μm. This means that resolution may have to be compromised in order to reach the region of interest and to prevent squashing the specimen and risking damage to the lens.

It is often necessary to take steps to preserve the 3D structure of the specimen for confocal analysis using some form of spacer between the slide and the coverslip, for example, a piece of coverslip or plastic fishing line. When living specimens are the subject of study, it is usually necessary to mount them in some form of chamber that will provide all of the essentials for life on the stage of the microscope, and will also allow access to the specimen by the objective lens for imaging without deforming the specimen.

Properties of the specimen such as opacity and turbidity can influence the depth that the laser beam may penetrate into it. For example, unfixed and unstained corneal epithelium of the eye is relatively transparent and therefore the laser beam will penetrate further into it (approx 200 μm) than into unfixed skin (approx 10 μm), which is relatively opaque and therefore scatters more light. The tissue can act as a neutral density filter and attenuate the laser beam. Many fixation protocols incorporate some form of clearing agent, which will increase the transparency of the specimen.

If problems do occur with depth penetration of the laser light into the specimen, then thick sections can be cut using a microtome; usually fixed specimens but also slices of living brain have been cut using a vibratome, and imaged successfully. The specimen can also be removed from the slide, inverted, and remounted, although this is often messy, and usually not too successful. Dyes that are excited at longer wavelengths, for example, cyanine 5, can be used to collect images from a little deeper within the specimen than those dyes excited at shorter wavelengths ***(26)***. Here the resolution is slightly reduced in comparison to that attained with images collected at shorter wavelengths. Multiple photon imaging allows images to be collected from more deeply within specimens ***(4)***.

5.1. The Objective Lens

The choice of objective lens for confocal investigation of a specimen is extremely important ***(27)***. This is because the numerical aperture (NA) of the lens, which is a measure of its light collecting ability, is related to optical section thickness and to the final resolution of the image. Basically, the higher the NA, the thinner the optical section.

The optical section thickness for the 60× (NA 1.4) objective with the pinhole set at 1 mm (closed) is on the order of 0.4 μm, and for a 16× (NA 0.5) objective, again with the pinhole at 1 mm, the optical section thickness is around 1.8 mm. Opening the pinhole (or selecting a pinhole of increased diameter) will further increase the optical section thickness **(Table 2)**. The vertical resolution is never as good as lateral resolution **(Fig. 8)**. For example, for a 60× NA 1.4 objective lens the horizontal resolution is around 0.2 μm and the vertical resolution is around 0.5 μm. Additional factors include chromatic aberration especially for imaging multilabel specimens at different wavelengths

Table 2
Optical Section Thickness (in μm)
for Different Objective Lenses Using
The BioRad MRC600 Laser Scanning
Confocal Microscope

Objective		Pinhole	
Magnification	NA	Closed (1 mm)	Open (7 mm)
60×	1.40	0.4	1.9
40×	1.30	0.6	3.3
25×	0.80	1.4	7.8
20×	0.75	1.8	10.0
4×	0.20	20.0	100.0

and flatness of field are of importance when choosing an objective lens for confocal imaging.

The lenses with the highest numerical apertures are generally the highest magnifications (and the most expensive), so that a compromise is often struck between the area of the specimen to be scanned and the maximum achievable resolution for the area **(Table 3)**. For example, when imaging *Drosophila* embryos and imaginal discs a 4× lens (NA 0.2) is used to locate the specimen on the slide, a 16× (NA 0.5) lens for imaging whole embryos, and a 40× (NA 1.2) or 60× (NA 1.4) lens for resolving individual cell nuclei within embryos and imaginal discs. In contrast for butterfly work where the structures of interest are much larger, the 4× lens is extremely useful for imaging whole wing imaginal discs in a single image, and for cellular resolution a 40× or a 60× objective lens is used **(Fig. 10)**. Some microscopes have the facility to view large fields at high resolution using an automated *X-Y* stage that can move around the specimen collecting images into a montage. Such montages can also be built manually and pasted together digitally.

A useful feature of most LSCMs is the ability to zoom an image with no loss of resolution using the same objective lens. This is achieved simply by decreasing the area of the specimen scanned by the laser by controlling the scanning mirrors and by placing the information from the scan into the same area of frame store or computer memory. In this way, several magnifications are imparted onto a single lens without moving the specimen

Table 3
Important Properties of Microscope Objectives
for Confocal Imaging[a]

Property	Objective 1	Objective 2
Design	plan-apochromat	CF-fluor DL
Magnification	60	20
Numerical aperture	1.4	0.75
Coverslip thickness	170 μm	170 μm
Working distance	170 μm	660 μm
Tube length	160 mm	160 mm
Medium	oil	dry
Contrast technique	None or DIC	Ph3
Color correction	Best	Good
Flatness of field	Best	Fair
UV transmission	None	Excellent

[a]An aid for choosing the correct lens for imaging. Objective 1 would be more suited for high-resolution imaging of fixed cells, whereas Objective 2 would be better for imaging a living preparation.

(Fig. 10). However, when possible, a higher NA lens should be used for the best resolution.

Many instruments have an adjustable pinhole. Opening the pinhole gives a thicker optical section and reduced resolution but it is often necessary to give more detail within the specimen or to allow more light to the photodetector. As the pinhole is closed down the section thickness and brightness decreases, and resolution increases up to a certain pinhole diameter, when resolution does not increase but brightness continues to decrease. This point is different for each objective lens ***(28)***.

5.2. Probes For Confocal Imaging

The synthesis of novel fluorescent probes for improved immunofluorescence localization continues to influence the development of confocal instrumentation ***(29)***. Fluorochromes have been introduced over the years that have their excitation and emission spectra more closely matched to the wavelengths delivered by the lasers supplied with most commercial LSCMs **(Table 4)**. Improved probes that can be conjugated to antibodies continue to appear. For example, the cyanine dyes are alternatives to more established dyes; cyanine 3 is a brighter alternative to rhodamine and cyanine 5 is useful in triple-label strat-

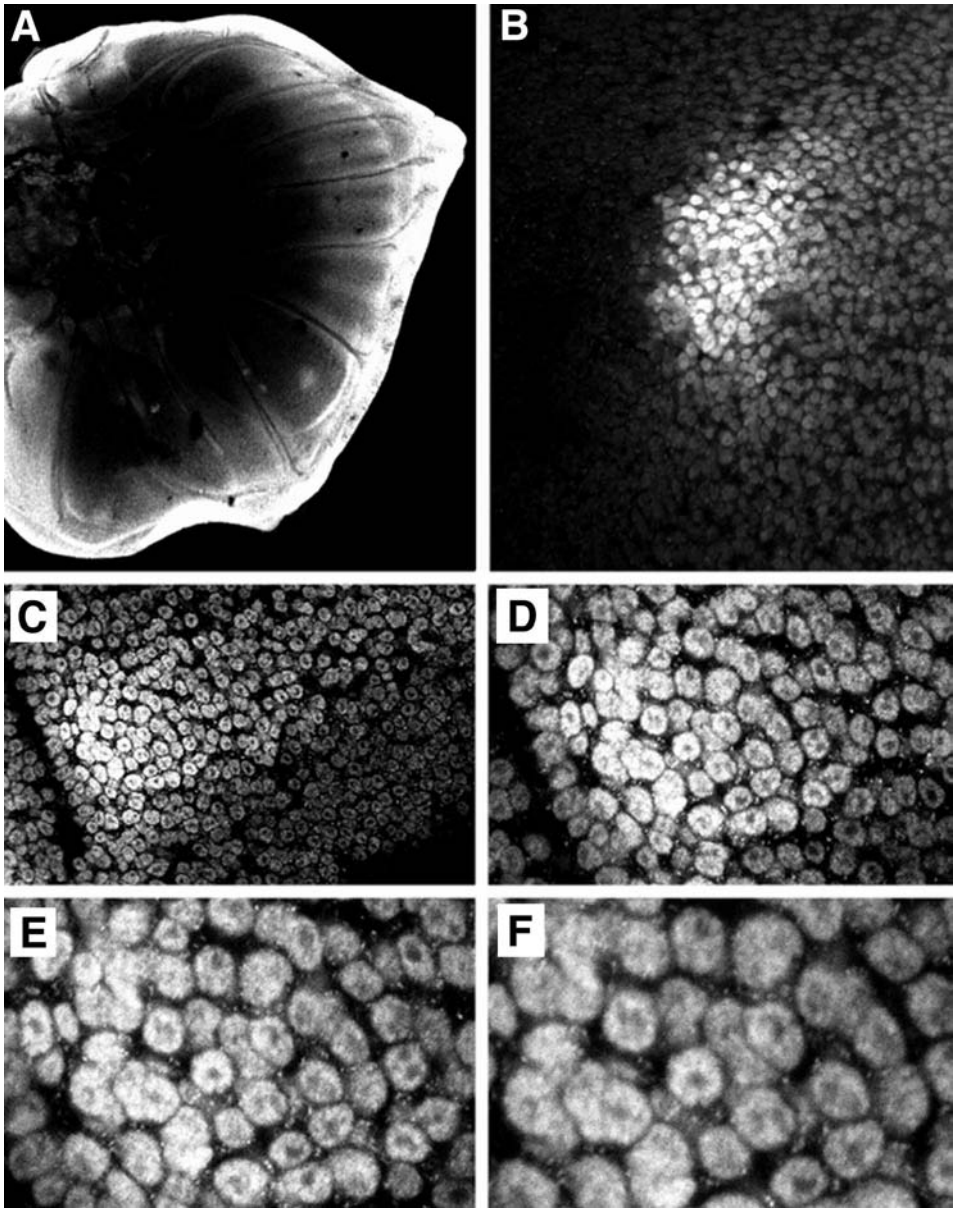

Fig. 10. Different objective lenses and zooming using the same lens. The 4× lens **(A)** is useful for viewing the entire butterfly fifth instar wing imaginal disc although the 16× lens **(B)** gives more nuclear detail of the Distal-less stain. The 40× lens gives even more exquisite nuclear detail **(C)**, and zoomed by progressive increments (**D**, **E**, and **F**). This is achieved by scanning smaller regions of the same area of the specimen, and in this way, several magnifications are imparted onto the same objective lens.

egies. More recently, the brighter Alexa dyes have been introduced by Molecular Probes ***(29)***.

Fluorescence *in situ* hybridization (FISH) is an important approach for imaging the distribution of fluorescently labeled DNA and RNA sequences in cells. Improved protocols have been developed for imaging specimens prepared by FISH, for example, the tyramide amplification system ***(30)***. In addition, brighter probes are now available as counterstains for imaging the total DNA in nuclei and isolated chromosomes using the LSCM. For example, the dimeric nucleic acid

Table 4
Peak Excitation and Emission Wavelengths of Some Commonly Used Fluorophores and Nuclear Counterstains

Dye	Excitation Max (nm)	Emission Max (nm)
Coumarin	350	440
GFP	470	508
FITC	496	518
Bodipy	503	511
CY3	554	568
Tetramethylrhodamine	554	576
Lissamine rhodamine	572	590
CY3.5	581	588
Texas red	592	610
CY5	652	672
CY5.5	682	703
CY7	755	778
Nuclear Dyes		
Hoechst 33342	346	460
DAPI	359	461
Acridine orange	502	526
Propidium iodide	536	617
ToPro	642	661

dyes TOTO-1 and YOYO-1, dyes such as Hoechst 33342 and DAPI, have excitation spectra (346 nm and 359 nm) that are too short for most of the lasers and mirrors that are supplied with the commercially available LSCMs, although these dyes can be imaged using a HeNe laser/UV system ***(31)*** or multiple photon microscopy. The latter technique does not require specialized UV mirrors and lenses, because red light from a pulsed Ti-Sapphire laser is used for illumination ***(4)***.

Many fluorescent probes are now available that stain specific cellular organelles and structures in relatively simple protocols ***(24)***. This includes a plethora of dyes that stain nuclei **(Fig. 11)**, mitochondria, the Golgi, and the endoplasmic reticulum, and also dyes such as the fluorescently labeled phalloidins that label polymerized actin in cells ***(29)***. Phalloidin is used to image cell outlines in developing tissues, because the peripheral actin meshwork appears as bright fluorescent rings in the optical section **(Fig. 11)**. These dyes are extremely useful in multiple-labeling strategies in order to locate antigens of interest to specific compartments in the cell ***(24)***. For example, using a combination of phalloidin and a nuclear dye with the antigen of interest in a triple-labeling scheme, the labeled protein can be mapped to a specific cellular compartment. In addition, antibodies to proteins of known distribution or function in cells, for example, antitubulin, is a useful tool for mapping in multilabel experiments.

When imaging living cells, it is most important to be aware of the effects of adding fluorochromes to the system. Such probes can be toxic to living cells especially when they are excited with the laser. Adding ascorbic acid to the medium can reduce such effects. The region of the cell labeled can also affect its viability; for example, nuclear stains tend to have a more deleterious effect than vital staining in the cytoplasm. One trick is to use a fluorescent dye, which does not enter the cells at all, but rather fluoresces in the medium surrounding the cells. Here an outline or negative image of the sample is produced. Probes that distinguish between living and dead cells are also available. Most of these assays rely on the fact that the membranes of dead cells are permeable to many dyes that cannot cross them in the living state. Such probes include acridine orange, and various "cell-death" kits are available commercially ***(29)***.

Dyes are available that change their fluorescence characteristics in the presence of ions such as calcium. These dyes can be imaged using visible wavelengths used in the LSCM, for example, fluo-3 or rhod-2 ***(14,23,24)***. New reporter probes for imaging gene expression have been introduced. For example, the jellyfish green fluorescent protein (GFP) allows gene expression and protein localization to be observed in vivo. For example, GFP has been used to monitor gene expression in many different cell types including living *Drosophila* oocytes, in mammalian cells, and in plants using the 488 nm line of the LSCM for excitation ***(32,33)***. Moreover, spectral mutants of GFP are now available for multilabel experiments and are also useful for avoiding problems with autofluorescence of living tissues.

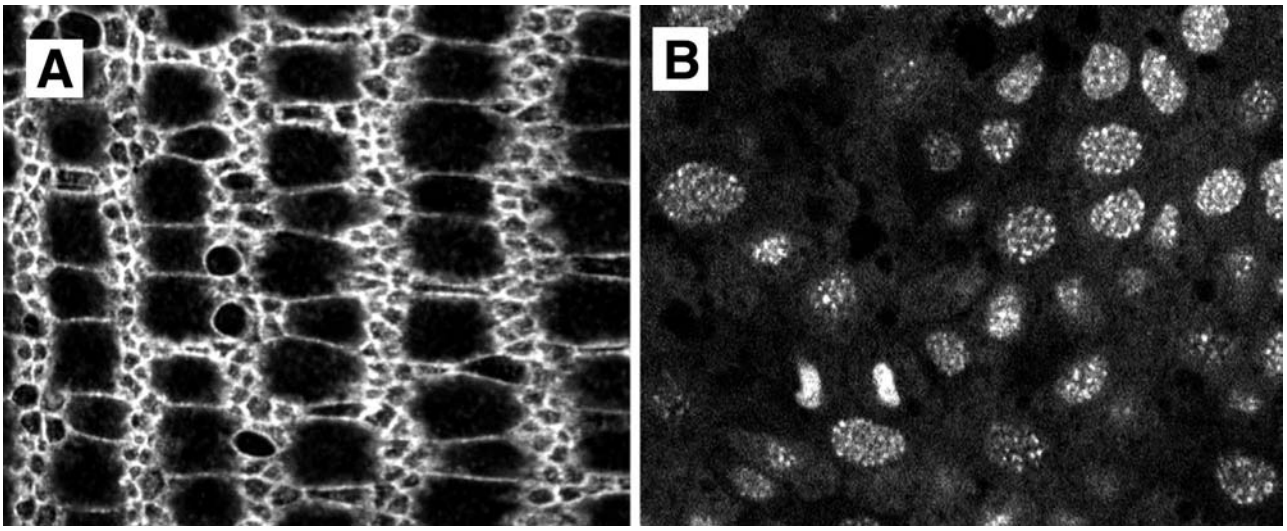

Fig. 11. Examples of dyes used for labeling cellular features. Cell outlines can be labeled with fluorescently labeled phalloidin **(A)** or nuclei using ToPro **(B)**. Both samples are whole mounts of butterfly (Precis coenia) pupal wing imaginal discs.

5.3. Autofluorescence

Autofluorescence can be a major source of increased background when imaging some tissues. Tissue autofluorescence occurs naturally in most cell types, and especially in yeast and in plant cells, for example, chlorophyll fluoresces in the red. Some reagents, especially glutaraldehyde fixative, are sources of autofluorescence, and it can be reduced using borohydride treatment. Autofluorescence can be avoided by using a wavelength for excitation that is out of the range of natural autofluorescence. For example, cyanine 5 excitation is often out of the range of the autofluorescence of many tissues.

An idea of the amount of autofluorescence can be gained by viewing an unstained specimen at different wavelengths, and taking note of the PMT settings of gain and black level together with the laser power **(Fig. 12)**. A relatively simple solution is to collect the experimental images at settings above those recorded for autofluorescence. Autofluorescence may also be bleached out using a quick flash at high laser power or flooding the specimen with the mercury lamp to bleach it out where the fluorescence of labeled structures is protected by antifade reagents. A more sophisticated method of dealing with autofluorescence is to use time-resolved spectroscopy. Autofluorescence can also be removed digitally by image subtraction. Although it is more often a problem, tissue autofluorescence can be utilized as a low level background signal for imaging overall cell morphology in multiple-labeling schemes **(Fig. 4)**.

5.4. Collecting the Images

The novice user can gain experience of confocal imaging from several sources. The manual provided with the confocal imaging system will usually include a series of simple exercises necessary for getting started. The person responsible for running the instrument will usually be only too happy to provide a short orientation session, and in most multiuser facilities, the manager will usually require a short training session and demonstration of a certain competence level before solo imaging is allowed. The novice should pay particular attention to the house rules of the facility. Other useful sources of information are the training courses run by the confocal companies, workshops on light microscopy, and various publications ***(24)***.

It is essential to be familiar with the basic operation of the imaging system before working with experimental slides. It is usually recommended, for the novice at least, to start imaging with a relatively easy

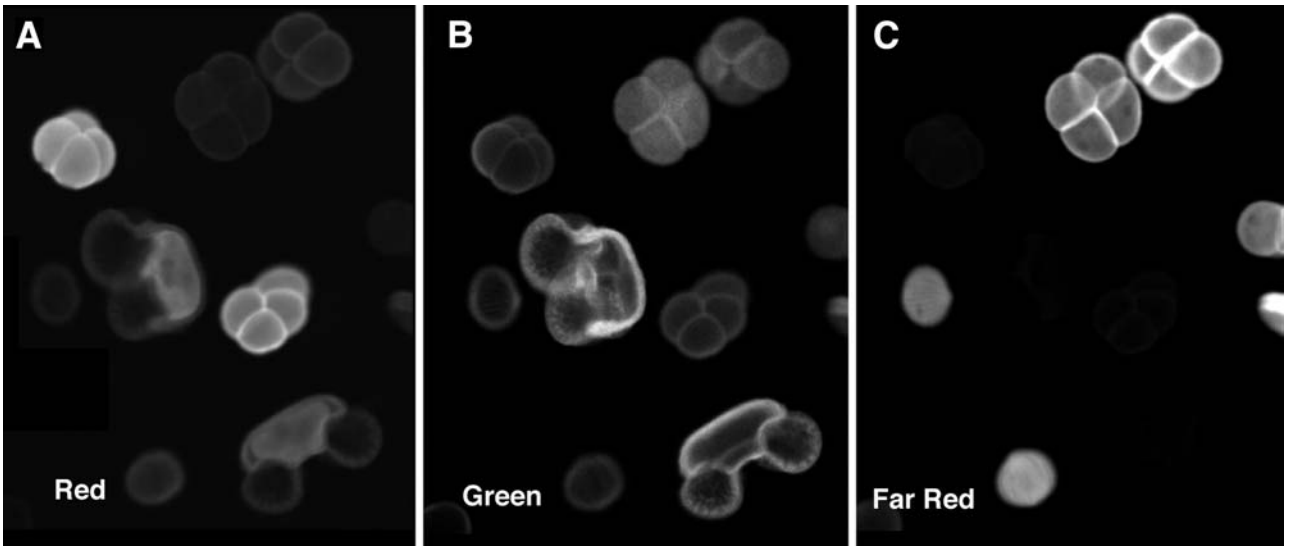

Fig. 12. Example of tissue autofluorescence in pollen grains. Note that different types of pollen fluoresce at different excitation wavelengths and some at different intensities in different channels—these images were collected simultaneously at the same settings of gain, black level and pinhole diameter.

specimen rather than with a more difficult experimental one. Some good test samples include paper soaked in one or more fluorescent dyes or a preparation of fluorescent beads, which are both bright, and relatively easy specimens to image with the confocal microscope. A particular favorite of mine is a slide of mixed pollen grains that autofluoresce at many different wavelengths (**Fig. 12**). Such slides are available from biological suppliers such as Carolina Biological (www.Carolina.com) or can be easily prepared from pollen collected from garden plants. These specimens tend to have some interesting surface features and hold up well in the laser beam. A relatively reliable test specimen for living studies can be prepared from onion epithelium or the water plant *Elodea* sp., using autofluorescence or staining with DiOC6.

The aim should always be to gain the best possible performance from the instrument, and this starts with optimal alignment, especially when imaging with older model confocal instruments. The alignment routine depends on the specific instrument, and is usually best performed by the person responsible for the instrument. Alignment should definitely not be attempted before training and permission from the microscope owner has been granted. This is because the beam can be lost completely, and in some instruments it may require a service visit to rectify the situation.

The basic practices of light microscopic technique should be followed at all times ***(6)***. For example, all glass surfaces should be clean, because dirt and grease on coverslips and objective lenses is a major cause of poor images. Care should be taken to mount the specimen so that it is within the working distance of the objective lens. The refractive index between the objective lens and the specimen should be matched. For example, a coverslip of correct thickness for the objective lens should be used, especially for higher-power lenses, which will require a #1 or #1.5 coverslip, and not a #2 coverslip. The coverslip should be sealed to the slide in some way, and mounted flat—use nail polish for fixed specimens making sure that it's dry before imaging, and some form of nontoxic sealing agent for live specimens, for example, a Vaseline, beeswax, and lanolin mixture works well although for viewing living cells some form of specimen chamber is much preferred to a coverslip/slide arrangement. Taking great care with the simple basics of cleanliness at this stage can save a lot of time and effort.

One of the secrets of successful confocal imaging is in mastering the interplay between lens NA, pinhole size, and image brightness using the lowest laser power possible to achieve the best image. The new user should play with these parameters using the test specimen and several different objective lenses of different magnifications and numerical apertures in order to get a feel for the capabilities of the instrument before progressing to the experimental specimens. Try zooming using the zoom function and compare these images with those obtained using a different objective lens.

The specific imaging parameters of the microscope should be set up away from the region of interest to avoid photobleaching of valuable regions of the specimen. This usually involves setting the gain and the black levels of the photomultiplier detectors together with the pinhole size in order to achieve a balance between acceptable resolution and adequate contrast using the lowest laser power possible to avoid excessive photobleaching. Many instruments have color tables that aid in setting the correct dynamic range within the image. Such look-up tables are designed so that the blackest pixels, around zero, are pseudocolored green and the brightest pixels, around 255 in an 8-bit system, are colored red. The gain and black levels (and the pinhole) are adjusted so that there are a few red and green pixels in the image thus ensuring the full dynamic range from 0 to 255 is utilized. These adjustments can also be made by eye. It is not always practical to collect an image at full dynamic range because full laser power cannot be used or the specimen has uneven fluorescence so that a bright region may obscure a dimmer region of interest in the frame.

As the specimen is scanned, an image-averaging routine is usually employed that filters out random noise from the photomultiplier and enhances the constant features from labeled structures in the image. An image equalization routine can be applied directly after collection of the images so that the image is scaled to the full dynamic range. This routine should not be applied if measurements of fluorescence intensity are to be made unless a control image is included in the same frame as the rest of the experimental images before applying the equalization routine. If space on the hard disk allows, it is often a good strategy to save raw unprocessed images alongside any processed ones.

The image is usually saved to the hard disk of the computer and later backed up onto a mass-storage device, for example, onto CDs. In general it is advisable to collect as many images as possible during an imaging session, and, if necessary, to cull out the bad ones in a later review session. It is quite surprising how a seemingly unnecessary image at first sight suddenly becomes valued at a later date after further review—especially with one's peers! It is much harder to prepare another specimen, and often harder still to reproduce the exact parameters of previously prepared specimens, and hard disk space should be more plentiful.

A strategy for labeling image files should be mapped out before imaging, and during imaging many notes should be taken or placed on the image file along with the image if this facility is available. Users should conduct a test to ensure that the information is accessible after imaging and remember that it can be lost when the images are subsequently transferred to image manipulation programs such as NIH Image or Adobe Photoshop on other computers. It is hard to replace a well-ordered note book preferably using a table of image file names with facility for comments and details of the objective lens and the zoom factor for calculating scale bars at a later date. Many modern systems incorporate an image database that will keep track of file names, location of the files and include a thumbnail of the images ***(34)***.

5.5. Troubleshooting

A protocol will sometimes inexplicably cease to work, and there is often an initial reflex to blame the instrument rather than the sample. A good test is to view the sample with a conventional epifluorescence microscope, and if some fluorescence is visible by eye, then the signal should be very bright on the confocal system. If the sample is visible by eye but not detected with the LSCM, it might be prudent to run through

some checks of the confocal system. It is advisable to use a known test specimen rather than the experimental one for testing the system. A digital file of an image of the test specimen should be accessible to all users together with all of the parameters of its collection including laser power, gain, black level, pinhole diameter, zoom, and objective lens used.

It is advisable to seek help from an expert who may have prior experience of the problem. If all else fails, do not panic, each of the confocal companies should have a good help line whose number will usually be posted close to the microscope. The golden rule is, if you are not sure of something, ask or at least step back from the problem before attempting to fix something.

Problems with the protocols themselves are usually caused by degradation of reagents, and a series of diagnostic tests should be performed. It is usually best to make up many of the reagents fresh yourself or to your recipe, or at least, "borrow" them from a trusted lab mate! Antibodies should be aliquoted in small batches from the frozen stock, and stored in the refrigerator. They should only be re-used if absolutely necessary, although this is sometimes unavoidable with expensive or rare reagents, and often does not present a problem.

Bleed through can occur from one channel into another in multiply labeled specimens. This can result from properties of the specimen itself or from problems with the instrument. The causes and remedies of bleed through have been reviewed in much detail elsewhere *(35)*. A good test of the instrument is to view a test sample with known bleed through properties using both the multiple-labeling settings and the single-label settings. It is advisable to collect an image of the test specimen and record the settings of laser power, gain, black level, and pin hole diameter so that when problems do occur, one can return to these settings with a test sample and compare the images collected with those of the stored test images collected when the instrument was operating in an optimal way.

Additional tests include a visual inspection of the laser color and the anode voltage of the laser, for example, if the beam from a krypton/argon laser appears blue and not white when scanning on a multiple-label setting, then this suggests that the red line is weak. If this is the case, then the anode voltage will usually be high, and can usually be reduced to an acceptable level by adjusting the mirrors on the laser (often called "walking the beam"—this should usually be left to the person responsible for the instrument). If it is not possible to reduce the voltage, a new or refurbished laser may be required.

Sometimes the antibody probes may have degraded or need to be cleaned *(36)*. Older specimens may have increased background fluorescence and bleed through caused by the fluorochrome coming off the secondary antibody and diffusing into the tissue. Always view freshly prepared specimens if at all possible. Changing the concentration and/or the distribution of the fluorochromes often helps. For example, if fluorescein bleeds into the rhodamine channel, then switch the fluorochromes so that rhodamine is on the stronger channel because the fluorescein excitation spectrum has a tail that is excited in the rhodamine wavelengths. The concentration of the secondaries can be reduced in subsequent experiments. The use of an acoustooptical tunable filter for the excitation beams can help to fine tune the strength of each channel independently *(37)*.

5.6. Image Processing and Publication

Confocal images are collected as digital computer files, and they can usually be manipulated using the proprietary software provided with the confocal imaging system or using secondary software, for example, Adobe Photoshop *(38)*. One of the most dramatically improved features of the LSCM has been in the display and analysis of confocal images. This part of the process is extremely important because it is fine to achieve improved resolution using the LSCM but this improvement is of little value if it cannot be displayed and reproduced in hard copy format.

Even 5 yr ago most laboratories used darkrooms and chemicals for their final hard copy. Color images were even harder to reproduce because an independent printer who had little idea

of the correct color balance usually printed them. For hard copies, images are now exported to a slide maker, a color laser printer, or a dye sublimation printer for publication quality prints. Photographs were taken directly from the screen of the video monitor. Moreover, movie sequences can be published on the World Wide Web.

The quality of published images has also improved drastically as most of the journals are able to accept digital images for publication. This means that the resolution displayed by the computer of the confocal imaging system is more faithfully reproduced in the final published article. Many journals publish their articles in CD ROM format or they are available on the World Wide Web. This means that the images viewed by the reader should be exactly the same as those collected using the confocal microscope. These technological advances are especially useful for color images where the intended resolution and color balance can be accurately reproduced by the journals, and, theoretically, at a much lower cost to the author.

6. Conclusions

The LSCM has found a niche in many biomedical imaging laboratories, because it produces improved images of all specimens prepared for fluorescence microscopy including those labeled by immunofluorescence and FISH ***(24)***. The confocal systems currently available, while they are still based on Minsky's long-expired patent and a conventional wide-field epifluorescence microscope, have been developed into sophisticated digital imaging systems that are relatively easy to operate especially if the user has some experience using an epifluorescence microscope and a microcomputer.

Today's instruments are largely computer-controlled, and they have greatly facilitated the collection of multidimensional images from fluorescently labeled specimens. These dimensions include the *X* and the *Y* dimension of the optical section, the *Z* dimension of a through focus series of optical sections (*Z*-series), the dimension of wavelength so that multiple fluorochromes are imaged either simultaneously or sequentially in the same region of the specimen ***(38)***, the dimension of time for imaging living tissues by collecting optical sections of the same region or *Z*-series (4D imaging) in a time-lapse mode ***(39)***, and the dimension of position in the specimen where several locations are imaged sequentially using a motorized stage that can be controlled by the computer.

The technology continues to improve at a rapid rate as evidenced by the introduction of practical multiple photon imaging, which gives improved viability of living cells, deeper penetration into intact specimens, and the possibility of multiple wavelength imaging ***(40)***. Instruments are now available with several laser sources so that confocal and multiple photon imaging modes can be used, and when combined with improved probes, gives a powerful means of imaging the spatial distribution and behavior of macromolecules in cells ***(41)***.

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References

1. Paddock, S. W. (1999) Confocal laser scanning microscopy. BioTechniques **27,** 992–1004.
2. Pawley, J. B (1995) *Handbook of Biological Confocal Microscopy*, 2nd ed., Plenum, New York.
3. Chen, H., Hughes, D. D., Chan, T. A., Sedat, J. W., and Agard, D. A. (1996) IVE (Image Visualization Environment): a software platform for all three-dimensional microscopy applications. *J. Struct. Biol.* **116,** 56–60.
4. Potter, S. M. (1996) Vital imaging: two photons are better than one. *Curr. Biol.* **6,** 1595–1598.
5. Minsky, M. (1988) Memoir on inventing the confocal scanning microscope. *Scanning* **10,** 128–138.
6. Inoue, S. and Spring, K. S. (1997) *Video Microscopy: The Fundamentals,* 2nd ed. Plenum, New York.
7. DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M. et al. (1996) Use of a cDNA microarray to analyze gene expression patterns in human cancer. *Nature Genetics* **14,** 457– 460.
8. White, J. G., Amos, W. B., Durbin, R., and Fordham, M. (1990) Development of a confocal imaging system for biological epifluorescence application, in *Optical Microscopy For Biology,* Wiley-Liss, New York, pp. 1–18.
9. White, J. G., Amos, W. B., and Fordham, M. (1987) An evaluation of confocal versus conventional imag-

ing of biological structures by fluorescence light microscopy. *J. Cell Biol.* **105,** 41–48.
10. Pawley, J. B. (1995) Light paths of current commercial confocal microscopes for biology, in *Handbook of Biological Confocal Microscopy,* 2nd ed. (Pawley J. B., ed.) Plenum, New York, pp. 581–598.
11. Terasaki, M. and Dailey, M. E. (1995) Confocal microscopy of living cells, in *Handbook of Biological Confocal Microscopy,* 2nd ed. (Pawley J. B., ed.), Plenum, New York, pp. 327–346.
12. Cheng, H., Lederer, W. J., and Cannell, M. B. (1993) Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* **262,** 740–744.
13. White, N. S. (1995) Visualization systems for multidimensional CLSM, in *Handbook of Biological Confocal Microscopy,* 2nd ed. (Pawley J.B., ed.) Plenum, New York, pp. 211–254.
14. Stricker, S. A., Centonze, V. E., Paddock, S. W., and Schatten, G. (1992) Confocal microscopy of fertilization-induced calcium dynamics in sea urchin eggs. *Dev. Biol.* **149,** 370–380.
15. Paddock, S. W., Hazen, E. J., and DeVries, P. J. (1997) Methods and applications of three color confocal imaging. *BioTechniques* **22,** 120–126.
16. Thomas, C. F., DeVries, P., Hardin, J., and White, J. G. (1996) Four dimensional imaging: computer visualization of 3D movements in living specimens. *Science* **273,** 603–607.
17. Mohler, W. A. and White, J. G. (1998) Stereo-4-D reconstruction and animation from living fluorescent specimens. *Biotechniques* **24,** 1006–1012.
18. Paddock, S. W., Mahoney, S., Minshall, M., Smith, L. C., Duvic, M., and Lewis, D. (1991) Improved detection of *in situ* hybridization by laser scanning confocal microscopy. *BioTechniques* **11,** 486–494.
19. Serbedzija, G. N., Bronner-Fraser, M., and Fraser, S. (1992) Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* **116,** 297–307.
20. Deerinck, T. J., Martone, M. E., Lev-Ram, V., Green, D. P. L., Tsien R. Y., Spector, D. L., et al. (1994) Fluorescence photooxidation with eosin: a method for high resolution immunolocalisation and *in situ* hybridization detection for light and electron microscopy. *J. Cell Biol.* **126,** 901–910.
21. Paddock, S. W. and Cooke, P. (1988) Correlated confocal laser scanning microscopy with high-voltage electron microscopy of focal contacts in 3T3 cells stained with Napthol Blue Black. *EMSA Abs* **46,** 100–101.
22. Sheppard, C. J. R. and Shotten, D. M. (1997) Confocal laser scanning microscopy. Royal Microscopical Society Handbook Series #38, Bios scientific publishers, Oxford, UK.
23. Matsumoto, B. (2000) Cell Biological Applications of Confocal Microscopy, 2nd ed. *Methods In Cell Biology,* Academic Press, San Diego, CA, in press.
24. Paddock, S. W. (1998) Protocols In Confocal Microscopy. *Methods In Mol. Biol.* 122. (J. Walker, ed.) Humana, Totowa, NJ.
25. Spector, D. L., Goldman, R., and Leinwand, L. (1998) *Cells: A Laboratory Manual, vol. II Light Microscopy and Cell Structure*. Cold Spring Harbor Press, Cold Spring Harbor, NY.
26. Cullander, C. (1994) Imaging in the far-red with electronic light microscopy: requirements and limitations. *J. Microsc.* **176,** 281–286.
27. Keller, H. A. (1995) Objective lenses for confocal microscopy, in *Handbook of Biological Confocal Microscopy,* 2nd ed. (Pawley, J. B., ed.), Plenum, New York, pp. 111–126.
28 Wilson, T. (1995) The role of the pinhole in confocal imaging system, in *Handbook of Biological Confocal Microscopy,* 2nd ed. (Pawley, J. B., ed.), Plenum, New York, pp. 167–182.
29. Haugland, R. P. (1999) *Handbook of Fluorescent Probes and Research Chemicals.* 7th ed. Molecular Probes Inc., Eugene, Oregon. (www.probes.com).
30. Wilkie, G. S. and Davis, I. (1998) High resolution and sensitive mRNA *in situ* hybridization using fluorescent tyramide amplification. Tech. Tips Online t01458 (www.biomednet.com).
31. Bliton, C., Lechleiter, J., and Clapham, D. E. (1993) Optical modifications enabling simultaneous confocal imaging with dyes excited by ultra-violet and visible-wavelength light. *J. Microsc.* **169,** 15–26.
32. Chalfie, M. and Kain, S. (1998) *Green Fluorescent Protein: Properties, Applications and Protocols.* Wiley-Liss, New York.
33. Sullivan, K. F. and Kay, S. A. (1998) Green fluorescent proteins. Meth. Cell Biol. Vol. 58. Academic, San Diego, CA.
34. Karten, H. J. (1998) Information management of confocal microscopy images. *Meth. Mol. Biol.* **122,** 403–420.
35. Brelje, T. C., Wessendorf, M. W., and Sorenson, R. L. 1993. Multicolor laser scanning confocal immunofluorescence microscopy: practical applications and limitations. *Meth. Cell Biol.* **38,** 98–177.
36. Brown, N. L. (1998) Imaging gene expression using antibody probes. *Meth. Mol. Biol.* **122,** 75–91.
37. Sharma, D. (1999) The use of an AOTF to achieve high quality simultaneous multiple label imaging. BioRad Tech. Note 04. www.microscopy.bio-rad.com38.
38. Halder, G. and Paddock, S. W. (1998) Presentation of confocal images. *Meth. Mol. Biol.* **122,** 373–384.
39. Bornfleth, H., Edelmann, P., Zink, D., Cremer, T., and Cremer. C. (1999) Quantitative motion analysis of subchromosomal foci in living cells using four-dimensional microscopy. *Biophys. J.* **77,** 2871–2886.
40. Centonze, V. E. and White, J. G. (1998) Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. *Biophys. J.* **75,** 2015–2024.
41. Fan, G. Y., Fujisaki, H., Miyawaki, A, Tsay, R.-K., Tsien R. Y., and Ellisman, M. H. (1999) Video-rate scanning two-photon excitation fluorescence microscopy and ratio imaging with cameleons. *Biophys. J.* **76,** 2412–2420.