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An Introduction to Confocal Imaging

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1. Introduction

The major application of confocal microscopy in the biomedical sciences is for imaging either fixed or living tissues that have usually been labeled with one or more fluorescent probes. When these samples are imaged using a conventional light microscope, the fluorescence in the specimen away from the region of interest interferes with resolution of structures in focus, especially for those specimens that are thicker than approx. 2 μm (**Fig. 1**). The confocal approach provides a slight increase in both lateral and axial resolution, although 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 in recent years. Most modern confocal microscopes are now relatively easy to operate and have become integral parts of many multiuser imaging facilities. Because the resolution achieved by the laser scanning confocal microscope (LSCM) is somewhat 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 in which 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. The images produced by scanning the specimen in this way are called optical sections. This 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 facilitated

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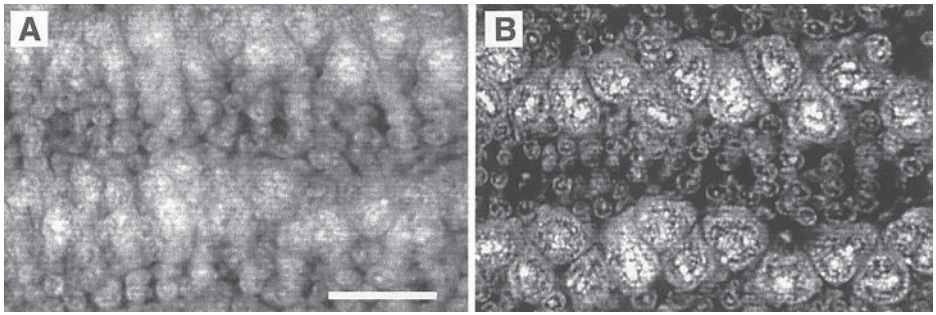

Fig. 1. Conventional epifluorescence image **(A)** compared with a confocal image **(B)** of a similar region of a whole mount of a butterfly pupal wing epithelium stained with propidium iodide. Note the improved resolution of the nuclei in **(B)**, due to the rejection of out-of-focus flares by the LSCM.

the imaging of living specimens, enabled the automated collection of three-dimensional (3D) data in the form of Z-series, and improved the images of multi-labeled specimens.

Emphasis has been placed on the LSCM throughout the book because it is currently the instrument of choice for most biomedical research applications, and 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 ***(1)***. Most of the protocols included in this book can be used, albeit with minor modifications, to prepare samples for all of these confocal microscopes, and to related, but not strictly confocal, methodologies that produce perfectly good optical sections including deconvolution techniques ***(2)*** and multiple-photon imaging ***(3)***.

The protocols in this book were chosen with the novice user in mind, and the authors were encouraged to include details in their chapters that they would not usually be able to include in a traditional article. This first chapter serves as a primer on confocal imaging, as an introduction to the subsequent chapters, and provides a list of more detailed information source. The second chapter covers some practical considerations for collecting images with a confocal microscope. Because fluorescence is the most prevalent method of adding contrast to specimens for confocal microscopy, the third chapter contains essential information on fluorescent probes. The next eight chapters cover protocols for preparing tissues from a range of the "model" organisms currently imaged using confocal microscopy. The following six chapters emphasize live cell analysis with the confocal microscope including methods of imaging various ions and green fluorescent protein as well as a novel method of imaging the changes in the 3D structure of living cells. The last section of the book focuses on the analysis and

presentation of confocal images. The field of confocal microscopy is now extremely large, and it would be impossible to include every protocol here. This current edition has been designed to give the novice an introduction to confocal imaging, and the authors have included sources of more detailed information for the interested reader.

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 its development can be found in an informative memoir by Minsky ***(4)***. All modern confocal microscopes employ the principle of confocal imaging patented in 1957 ***(5)***.

In Minsky's original confocal microscope the point source of light was produced by a pinhole placed in front of a zirconium arc source. The point of light was focused by an objective lens into the specimen, and light that passed through it was focused by a second objective lens at a second pinhole, which had the same focus as the first pinhole, i.e., it was confocal with it. Any light that passed through the second pinhole struck a low-noise photomultiplier, which produced a signal that was related to the brightness of the light. The second pinhole prevented light from above or below the plane of focus from striking the photomultiplier. This is the key to the confocal approach, namely eliminating out-of-focus light or "flare" in the specimen by spatial filtering. Minsky also described a reflected light version of the microscope that used 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**).

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 optical axis, which can eliminate any lens defects. However, for biological specimens, movement of the specimen can cause wobble and distortion, which results in a loss of resolution in the image. Moreover, it is impossible to perform 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 was not formed in Minsky's original microscope but rather the output from the photodetector was translated into an image of the region of interest. In Minsky's original design the image was built up on the screen of a military surplus long persistence oscilloscope with no facility for hard copy. Minsky wrote at a later date that the image quality in his microscope was not very impressive because

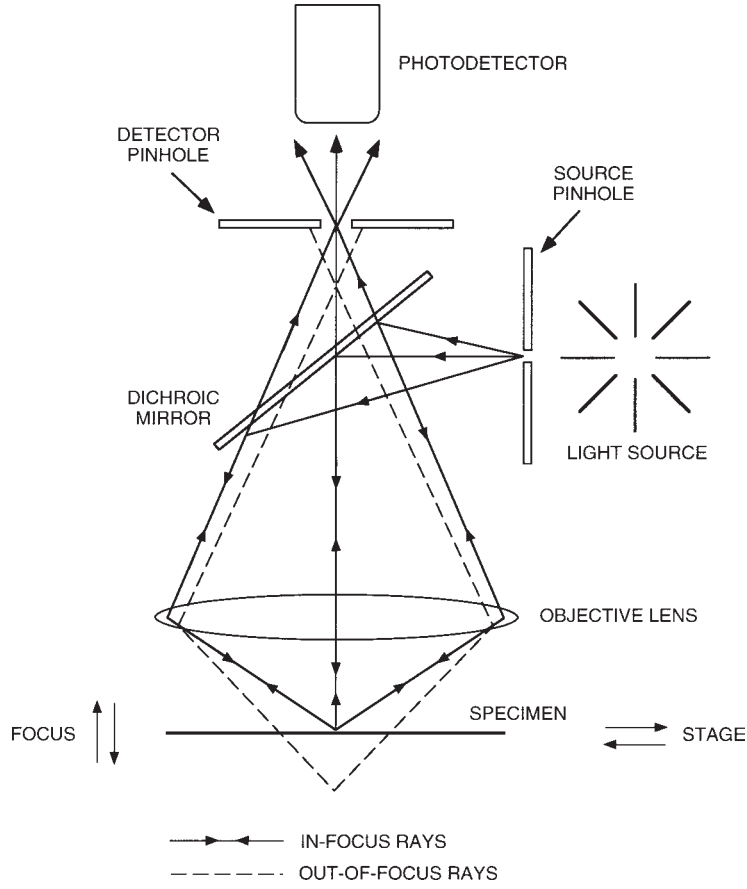

Fig. 2. Light path in a stage scanning LSCM.

of the quality of the oscilloscope display and not because of lack of resolution achieved with the microscope itself ***(4)***.

It is clear that the technology was not available to Minsky in 1955 to demonstrate fully the potential of the confocal approach especially for imaging biological structures. According to Minsky, this is perhaps a reason why confocal microscopy was not immediately adopted by the biological community, who were, as they are now, a highly demanding and fickle group concerning 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 and high resolution film.

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 outstanding results.

The optics of the light microscope have not changed drastically in decades because 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 years. 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 have gradually become available to biologists. These 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

These technologies were developed independently, and since 1955, they have been incorporated into modern confocal imaging systems. For example, digital image processing was first effectively applied to biological imaging in the early 1980s by Shinya Inoue and Robert Allen at Woods Hole. Their "video-enhanced microscopes" enabled an apparent increase in resolution of structures using digital enhancement of the images which were 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 contract (DIC) optics and the images were further enhanced using digital methods. 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 on the principles and practices of modern light microscopy ***(6)***.

Confocal microscopes are usually classified using the method by 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 in materials science applications such as the microchip industry. Systems based upon this principle have recently gained in popularity for biomedical applications for screening DNA on microchips ***(7)***.

An alternative to moving the specimen is to scan the beam across a stationary specimen, which is more practical for imaging biological specimens. This is the basis of many systems that have evolved into the research microscopes in vogue today. The more technical aspects of confocal microscopy have been covered elsewhere ***(1)***, but in brief, there are two fundamentally different methods of beam scanning; multiple-beam scanning or single-beam scanning. The more popular method at present is single-beam scanning, which is typified by the LSCM. Here the scanning is most commonly achieved by computer-controlled galvanometer-driven mirrors (one frame per second), or in some systems, by an acoustooptical device or by oscillating mirrors 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: deconvolution ***(2)*** and multiple-photon imaging ***(3)***, 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 much faster, with the result that this technique is a practical option for imaging. Multiple-photon microscopy uses a scanning system that is identical to that of the LSCM but without the pinhole. This is because the laser excites the fluorochrome only at the point of focus, and a pinhole is therefore not necessary. Using this method, photobleaching is reduced, which makes it more amenable to imaging living tissues.

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 by means of a plethora of computer software either using the computer of the confocal system or a second computer (**Fig. 3**).

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 photomultiplier behind a pinhole, or in some designs, a slit, and the output from this is built into an image by the computer (**Fig. 2**). Specimens are usually labeled with one or more fluo-

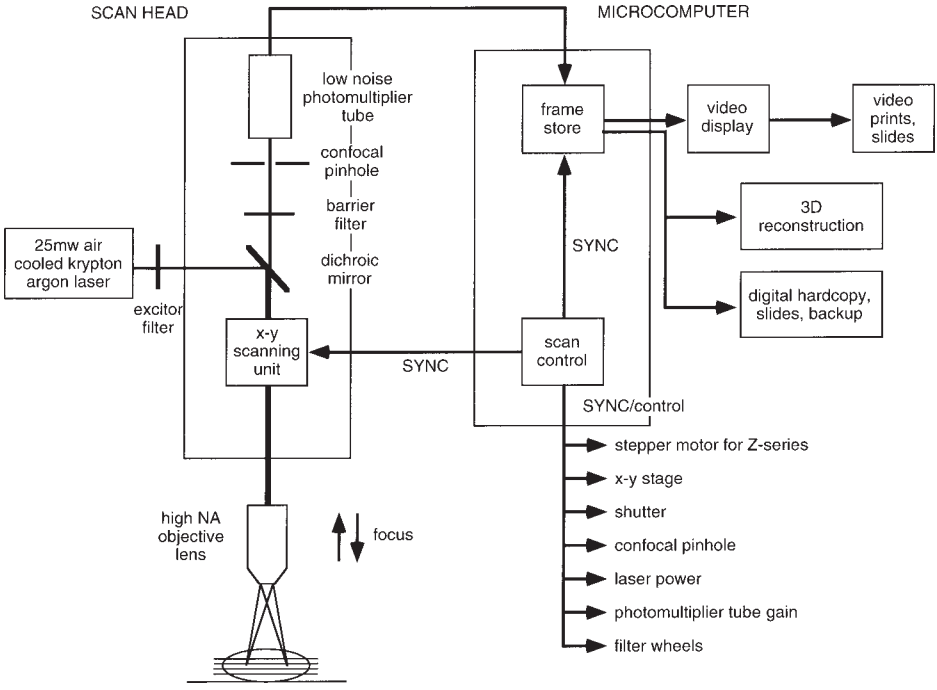

Fig. 3. Information flow in a generic LSCM.

rescent probes, or unstained specimens can be viewed using the light reflected back from the specimen.

One of the more commercially successful LSCMs was designed by White, Amos, Durbin, and Fordham *(8)* to tackle a fundamental problem in developmental biology: imaging specific macromolecules in immunofluorescently 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 increase, 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 not practical for fluorescence imaging at the time. White and his colleagues designed a LSCM that was suitable for conventional epifluorescence microscopy that has since evolved into an instrument that is used in many different biomedical applications.

In a landmark paper that captured the attention of the cell biology community ***(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 from much of the out-of-focus fluorescence that previously contaminated their images. This technological advance allowed them to follow changes in the cytoskeleton in cells of early embryos at a higher resolution than was previously possible using conventional epifluorescence microscopy.

The thickness of the optical sections could be varied simply by adjusting the diameter of a pinhole in front of the photodetector. 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 by 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 to another lens may risk losing the region of interest.

This microscope together with 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 years. Indeed many laboratories are purchasing the systems as shared instruments in preference to electron microscopes. The advantage of confocal microscopy lies within its great number of applications and its relative ease for producing extremely high-quality 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. 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 more stable lasers, more efficient mirrors and photodetectors, and improved digital imaging systems (**Fig. 3**). The new instruments are much improved ergonomically so that alignment, choosing filter combinations, and changing laser power, all of which are often controlled by software, is much easier to achieve. Up to three fluorochromes can be imaged simultaneously, and more of them sequentially, and it is easier to

manipulate the images using improved, more reliable software and faster computers with more hard disk space and cheaper 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** and Color Plates I and II, following page 372). The images collected from multiple-labeled specimens will be in register with each other as long as an objective lens that is corrected for chromatic aberration is used. Registration can usually be restored using digital methods. Using most LSCMs it takes approximately 1 s to collect a single optical section although several such sections are usually averaged to improve the signal-to-noise ratio. The time of image collection will also depend on the size of the image and the speed of the computer, e.g., a typical 8-bit image of 768 by 512 pixels in size will occupy approx. 0.3 Mb.

4.2. Time-Lapse and Live Cell Imaging

Time-lapse confocal imaging uses the improved resolution of the LSCM for studies of living cells (**Fig. 5**). Time-lapse imaging was the method of choice for early studies of cell locomotion using 16 mm movie film with a clockwork intervalometer coupled to the camera, and more recently using a time-lapse VCR, OMDR, digital imaging system, and now using the LSCM to collect single optical sections at preset time intervals.

Imaging living tissues is perhaps an order of magnitude more difficult than imaging fixed ones using the LSCM (**Table 1**), and this approach is not always a practical option because the specimen may not tolerate the rigors of live imaging. It may not be possible to keep the specimen alive on the microscope stage, or the phenomenon of interest may not be accessible to the objective lens or the specimen may not physically fit on the stage of the microscope. For example, the wing imaginal disks of the fruit fly develop too deeply in the larva, and when dissected out they cannot be grown in culture, which means that the only method of imaging gene expression in such tissues is currently to dissect, fix, and stain imaginal disks from different animals at different stages of development.

For successful live cell imaging extreme care must be taken to maintain the cells on the stage of the microscope throughout the imaging process ***(11)***, and to use the minimum laser exposure necessary for imaging because photodamage from the laser beam can accumulate over multiple scans. Antioxidants such as ascorbic acid can be added to the medium to reduce oxygen from excited fluorescent molecules, which can cause free radicals to form and kill

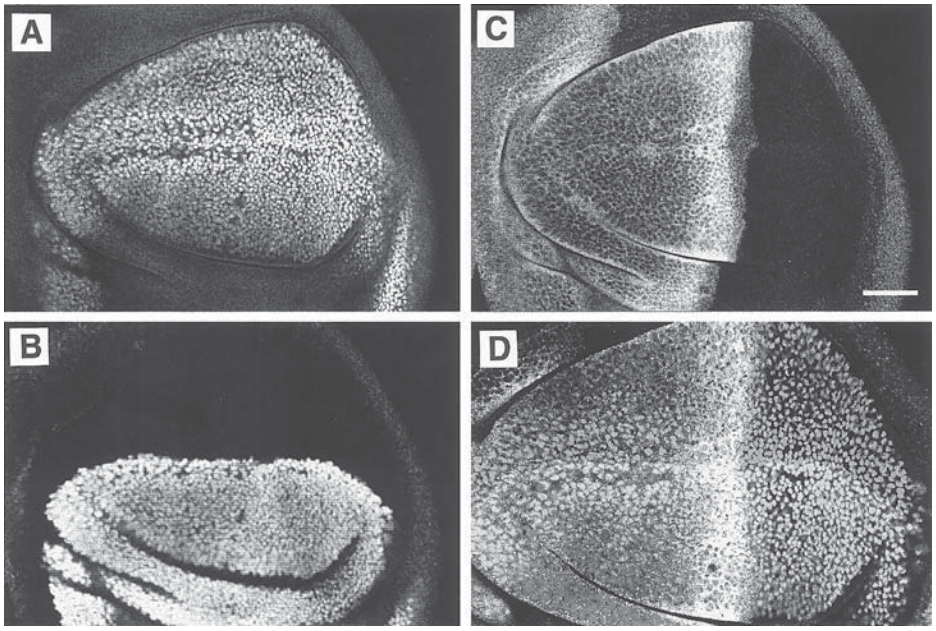

Fig. 4. Single optical sections collected simultaneously using a single krypton/argon laser at three different excitation wavelengths—488 nm, 568 nm and 647 nm—of a fruit fly third instar wing imaginal disk labeled for three genes involved with patterning the wing: **(A)** vestigial (fluorescein 496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

the cells. An extensive series of preliminary control experiments is usually necessary to assess the effects of light exposure on the fluorescently labeled cells. It is a good idea to note down all of the details of the imaging parameters—even those that appear to be irrelevant. A postimaging test of viability should be performed. Embryos should continue their normal development after imaging; for example, sea urchin embryos should hatch after being imaged. Any abnormalities that are caused by the imaging process or properties of the dyes used should be determined.

Each cell type has its own specific requirements for life, e.g., most cells will require a stage heating device, and perhaps a perfusion chamber to maintain the carbon dioxide balance in the medium (*see* Chapter 13), whereas other cells such as insect cells usually can be maintained at room temperature in a relatively large volume of medium (*see* Chapter 14). 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

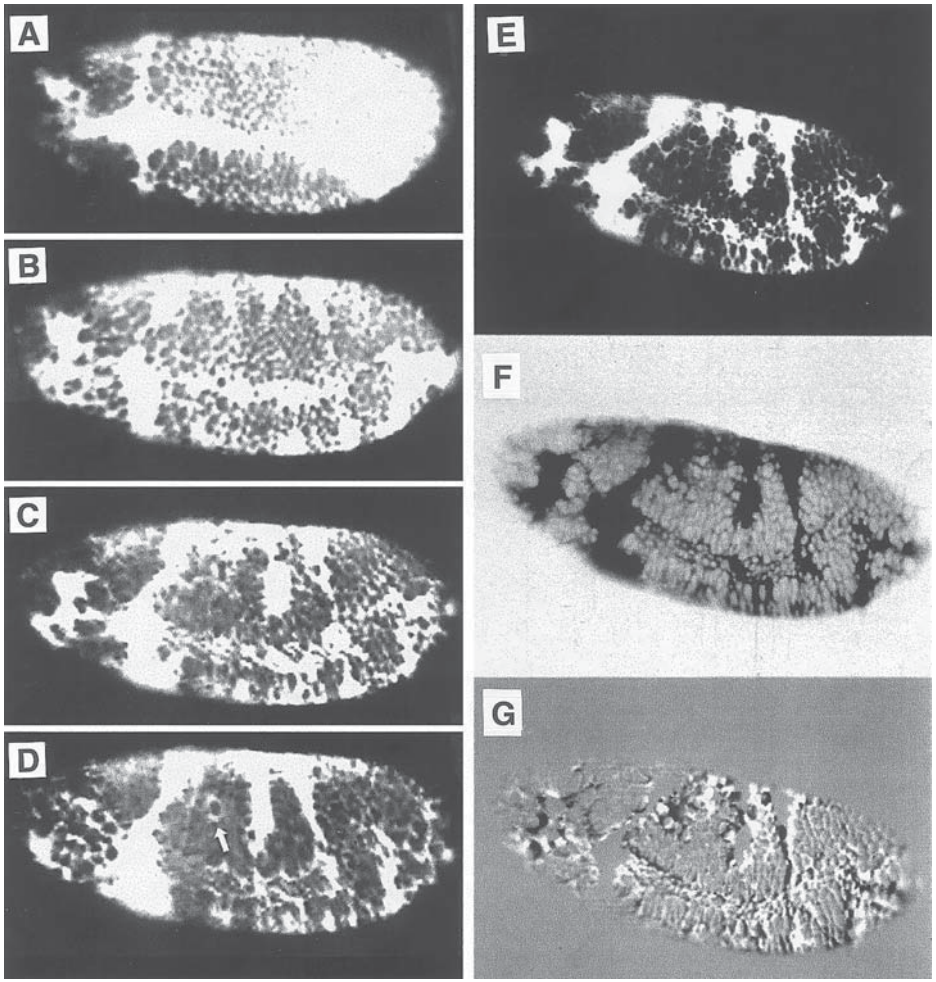

Fig. 5. Time-lapse imaging of a living fruit fly embryo injected with Calcium green **(A–D)**. One method of showing change in distribution of the fluorescent probe over time on a journal page is to merge a regular image of one time point **(E)** with a reversed contrast image of a second time point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

systems has been improved significantly over the early models, and when coupled with brighter objective lenses and less phototoxic dyes, has made live cell confocal analysis a practical option. The bottom line is to use the least amount of laser power possible for imaging and to collect the images quickly. The pinhole may be opened wider than for fixed samples to speed

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

up the imaging process, and deconvolution may be used later 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 more practical for physiological imaging. This design has the advantage of good spatial resolution coupled with good temporal resolution, i.e., full screen resolution of 30 frames per second (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 and oscillating mirror systems can also be used for imaging fast physiological events.

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 coordinating the movement of the fine focus of the microscope with image collection, usually using a computer-controlled stepping motor to move the stage by preset distances. This is relatively easily accomplished using a macro program that collects an image, moves the focus by a predetermined distance, collects a second image, stores it, moves the focus again, and continues 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 merged to highlight cells of interest. 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.

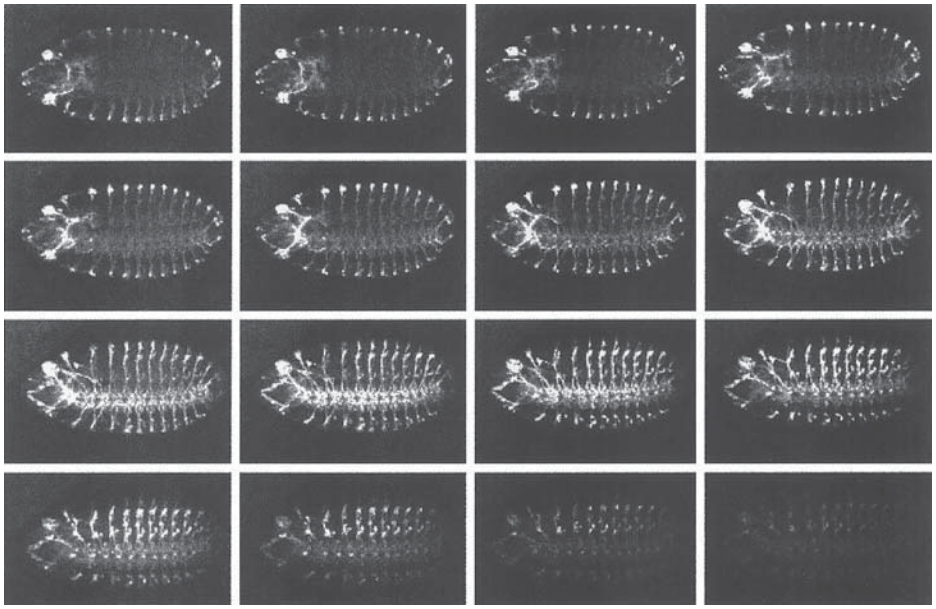

Fig. 6. A Z-series of optical sections displayed as a montage collected from a fruit fly embryo labeled with the antibody designated 22C10, which stains the peripheral nervous system.

Z-series are ideal for further processing into a 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 (*see* **Chapter 18**), as 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. Because 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 be processed relatively easily 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 collected with the microscope and depends on the lens and the pinhole diameter, and not to the step size taken by the stepper motor, which is set up by the operator. In some cases these have the same value, however, and may be a source of the confusion.

The Z-series file is usually exported into a computer 3D reconstruction program. These packages are now available for processing confocal images and run either on workstations at extremely high speeds or using more affordable,

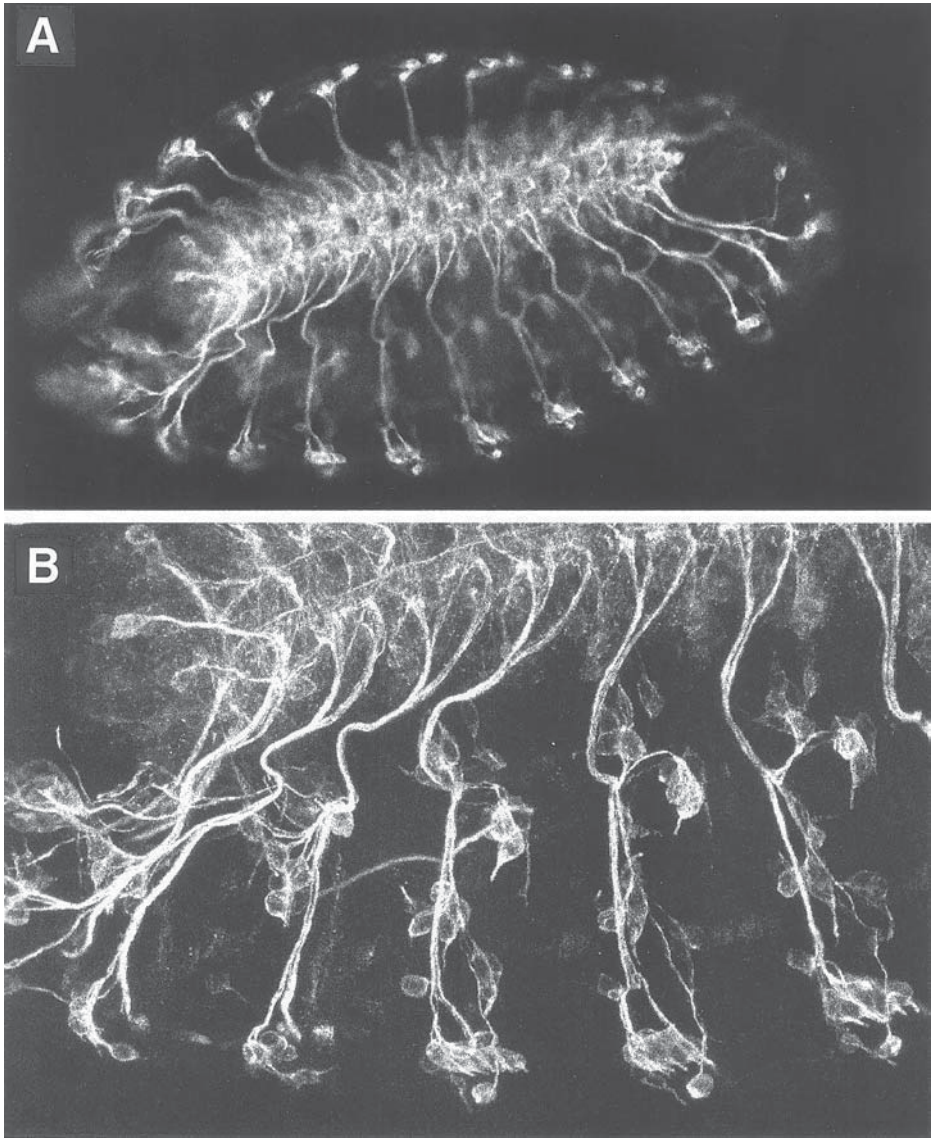

Fig. 7. A single optical section **(A)** compared with a Z-series projection **(B)** of a fruit fly peripheral nervous system, stained with the antibody 22C10.

personal computers. With the introduction of faster computer chips and the availability of cheaper RAM, 3D reconstructions can be produced quite effectively on the workstation of the confocal microscope. The 3D software packages produce a single 3D representation or a movie sequence compiled

from different views of the specimen. Specific parameters of the 3D image such as opacity can be interactively changed to reveal structures of interest at different levels within the specimen, and various length, depth, and volume measurements can be made.

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 when this method of displaying the data was used ***(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 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 and viewed as a movie or montage ***(16,17)***.

4.5. X–Z Imaging

An *X–Z* section produces a profile of the specimen, e.g., a vertical slice of an epithelial layer (**Fig. 8**). Such X–Z profiles can be produced either by scanning a single line at different *Z* depths under the control of the stepper motor or by extracting the profile from a Z-series of optical sections using a cut plane option in a 3D reconstruction program.

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, the 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 reflection artifact and off the optical axis. The reflection artifact is not present in the slit or multiple-beam scanning systems.

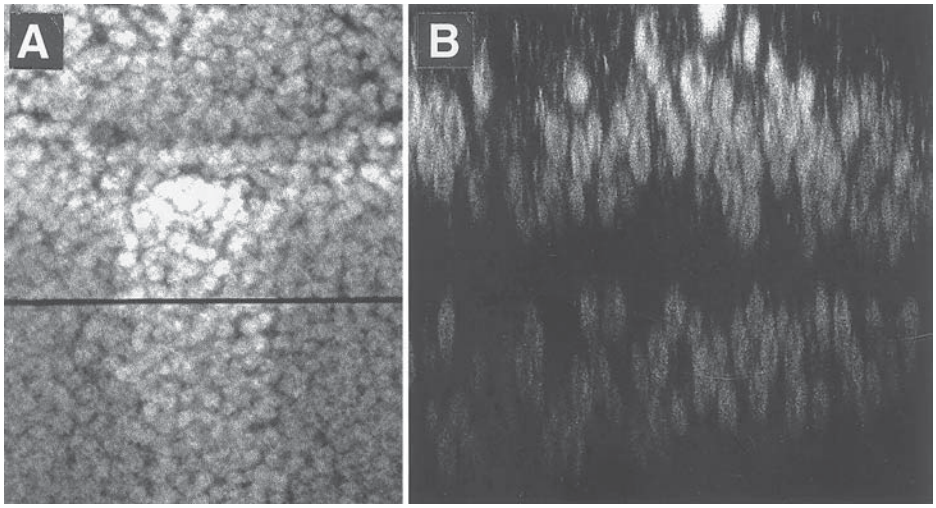

Fig. 8. *X–Z* imaging; the laser was scanned across a single position in the sample (marked by the horizontal black line in **(A)**) at different *Z* depths. An *X–Z* image was built up and displayed in the confocal imaging system **(B)**. Note that the butterfly wing epithelium is made up of two epithelial layers, and since the fluorescence intensity drops off deeper into the specimen, only the upper layer is visualized.

4.7. Transmitted Light Imaging

Any form of transmitted-light microscope image, including phase-contrast, DIC, polarized light, or dark field can be collected using a transmitted light detector (**Fig. 9**), which is a device that collects the light passing through the specimen in the LSCM. The signal is transferred to one of the PMTs in the scan head via a fiber optic cable. Because confocal epifluorescence images and transmitted light images are collected simultaneously using the same beam, image registration is preserved, so that the precise localization of labeled cells within the tissues can be mapped when the images are combined using digital methods.

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

A real color transmitted light detector has recently been introduced that collects the transmitted signal in the red, the green, and the blue channels to build the real color image in a similar way to some color digital cameras. This device is useful to pathologists who are familiar with viewing real colors in transmitted light and overlaying the images with fluorescence.

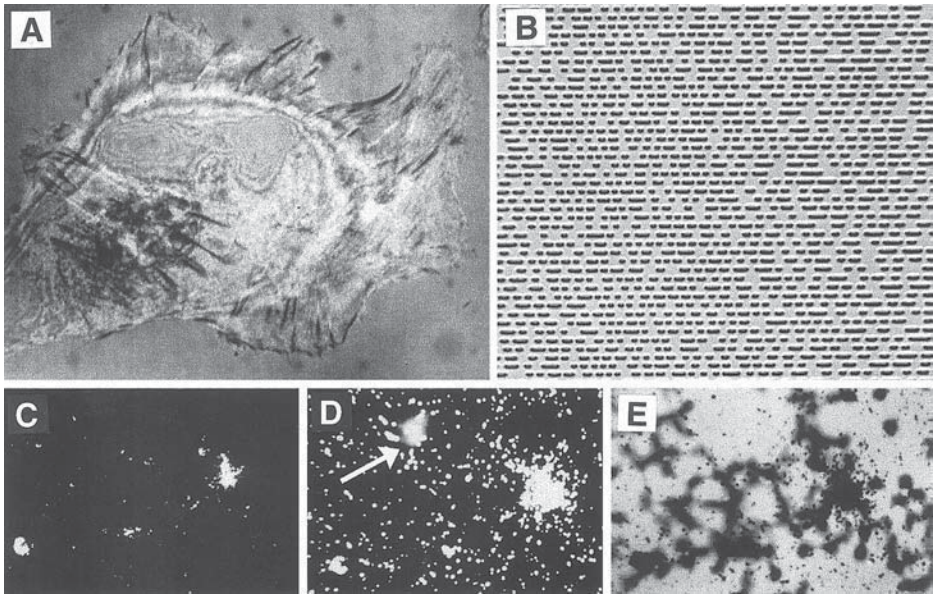

Fig. 9. Examples of reflected light **(A,B,C)** and transmission imaging **(D,E)**: Interference reflection microscopy in the LSCM demonstrates cell substratum contacts in black around the cell periphery **(A)**; confocal systems are used extensively in the materials sciences—here the surface of an audio CD is shown **(B)**; **(C)** through **(E)** show 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)**], which is not present in the optical section **(C)**.

4.8. Correlative Microscopy

The premise of correlative or integrated microscopy is to collect information from the same region of a specimen using more than one microscopic technique. For example, the LSCM can be used in tandem with the transmission electron microscope (TEM). 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 TEM *(20)*. Reflected light imaging and the TEM have also been used in correlative microscopy to image the cell substratum contacts in the LSCM (**Fig. 9A**) and in the TEM *(21)*.

5. Specimen Preparation and Imaging

Most of the protocols for confocal imaging are based upon those developed over many years for preparing samples for the conventional wide field micro-

scope ***(22–25)***. 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 for 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.

The illumination in a typical laser scanning confocal system appears to be extremely bright although many 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 in 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 antibleaching agents may not be required when using many of the more modern instruments (*see* Chapter 3).

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; e.g., 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× numerical aperture (NA) 1.4 has a working distance of 170 µm whereas a 20× NA 0.75 has a working distance of 660 µm Occasionally, resolution may have to be compromised in order to reach the region of interest, and to prevent squashing the specimen with the lens and risking damage to it.

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

Properties of the specimen such as opacity and turbidity can influence the depth into the specimen that the laser beam may penetrate. 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, for example, into unfixed skin (approx. 10 µm), which is relatively opaque and therefore scatters more light. The tissue acts like a neutral density filter and attenuates the laser beam. Many fixation protocols incorporate some form of clearing agent that 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 of 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 very successful. Dyes that are excited at longer wavelengths, e.g., cyanine 5, can be used to collect images from a somewhat deeper part of the specimen than with 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 deeper areas within specimens because red light is used for excitation.

5.1. The Objective Lens

The choice of objective lens for confocal investigation of a specimen is extremely important ***(27)***, as the NA of the lens, which is a measure of its light-collecting ability, is related to optical section thickness and to the final resolution. Basically, the higher the NA is, the thinner the optical section will be. The optical section thickness for the 60× (NA 1.4) objective lens 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 approx. 1.8 mm. Opening the pinhole (or selecting a pinhole of increased diameter) will increase the optical section thickness further (**Table 2**). These values were measured from the BioRad MRC600 LSCM. The vertical resolution is never as good as lateral resolution. For example, for a 60× NA 1.4 objective lens the horizontal resolution is approx. 0.2 μm and the vertical resolution is approx. 0.5 μm. Chromatic aberration, especially when imaging multilabeled specimens at different wavelengths, and flatness of field are additional factors to consider when choosing an objective lens ***(6)***.

The lenses with the highest NAs are generally those with the highest magnifications, and 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 disks a 4× lens 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 disks. For large tissues, for example, butterfly imaginal disks, the 4× lens is extremely useful for whole wing disks, and for cellular resolution 40× or 60× 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, and collecting images into a montage. Such montages can also be built manually and pasted together digitally.

Table 2
Optical Section Thickness (in microns) for Different Objective Lenses Using the Bio-Rad 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
40×	0.55	1.4	4.3
25×	0.80	1.4	7.8
4×	0.20	20.0	100.0

Table 3
Important Properties of Microscope Objective Lenses for Confocal Imaging. An Aid for Choosing the Correct Lens for Imaging.

Property	Objective 1	Objective 2
Design	Plan-apochromat	CF-fluor DL
Magnification	60	20
Numerical aperture	1.4	0.75
Coverslip thickness	170 um	170 um
Working distance	170 um	660 um
Tube length	160 mm	160 mm
Medium	Oil	Dry
Color correction	Best	Good
Flatness of field	Best	Fair
UV transmission	None	Excellent

*a*Objective 1 would be more suited for high-resolution imaging of fixed cells whereas Objective 2 would be better for imaging a living preparation stained with a UV dye.

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 framestore or computer memory. Several magnifications can be imparted onto a single lens without moving the specimen (**Fig. 10C,D,E,F**). However, when possible a lens with a higher NA should be used for the best resolution, rather than zooming a lens of lower NA.

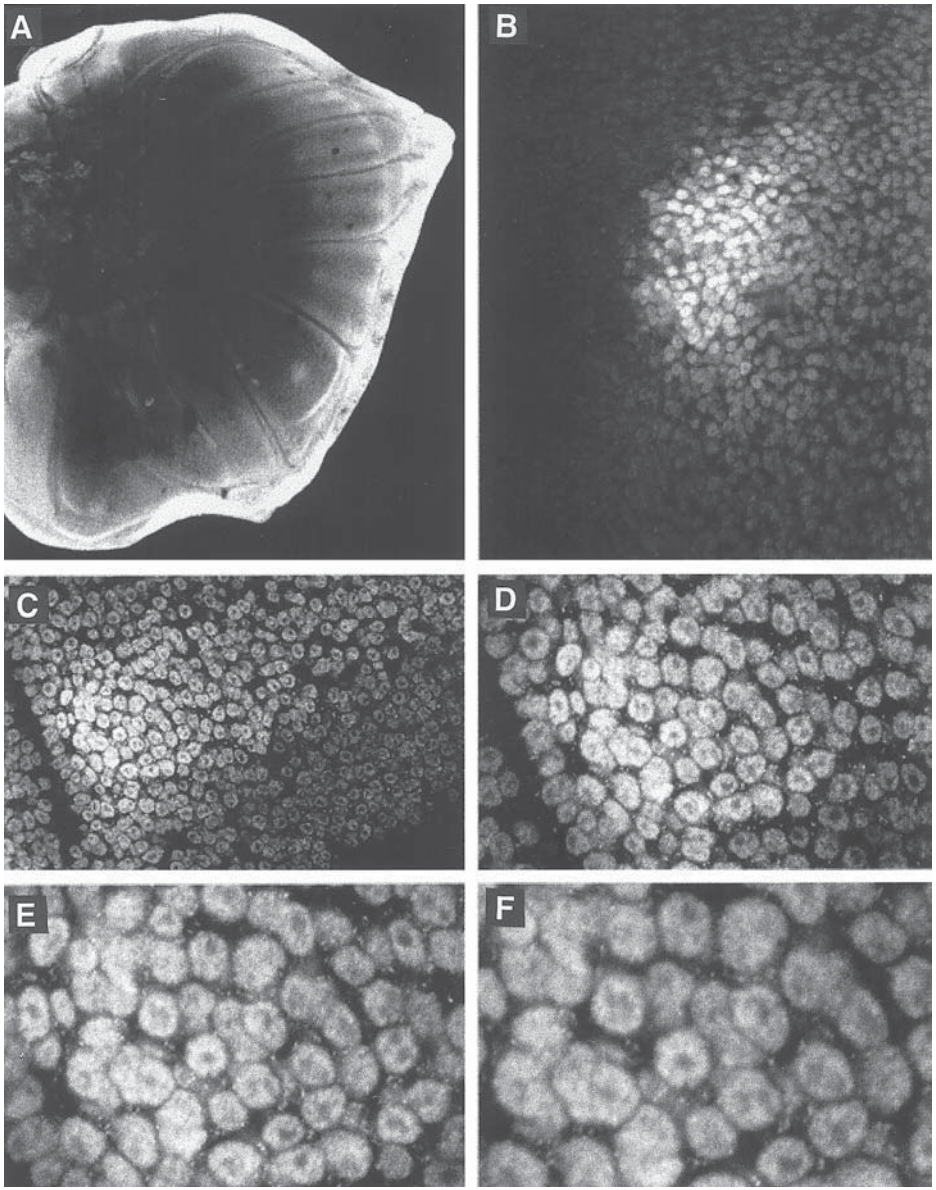

Fig. 10. Different objective lenses and zooming using the same lens. The 4× lens **(A)** is useful for viewing the entire butterfly fifth instar wing imaginal disk although the 16× lens **(B)** gives more nuclear detail of the distal-less stain. The 40× lens gives even more exquisite nuclear detail **(C)**, and zoomed by progressive increments **(D,E,F)**.

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

Dye	Exc. Max. (nm)	Em. Max. (nm)
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
Nuclear dyes		
Hoechst 33342	346	460
DAPI	359	461
Acridine orange	502	526
Propidium iodide	536	617
TOTO3	642	661

Many instruments have an adjustable pinhole. Opening the pinhole gives a thicker optical section and reduced resolution but it is often necessary to provide more detail within the specimen or to allow more light to strike the photodetector. As the pinhole is closed the section thickness and brightness decrease, and resolution increases up to a certain pinhole diameter, at which 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)*** and *see* Chapter 3. Fluorochromes have been introduced over the years with 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 strategies.

Fluorescence in situ hybridization (FISH) is an important approach for imaging the distribution of fluorescently labeled DNA and RNA sequences in cells ***(30)*** and *see* Chapter 5. In addition, brighter probes are now available for

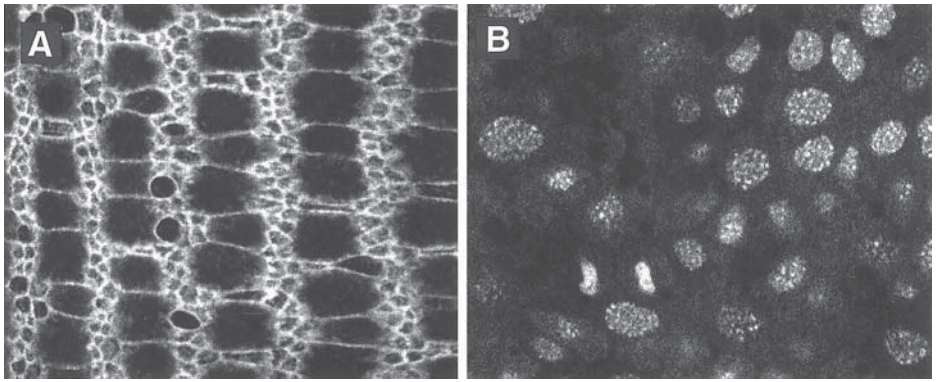

Fig. 11. Examples of dyes used for labeling cellular features. Cell outlines can be labeled with fluorescently labeled phalloidin **(A)** or nuclei using ToPro **(B)**. Both samples are whole mounts of butterfly pupal wing imaginal disks.

imaging total DNA in nuclei and isolated chromosomes using the LSCM. For example, the dimeric nucleic acid dyes TOTO-1 and YOYO-1 and dyes such as Hoechst 33342 and 4,'6-diamidino-2-phenylindale (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 it uses red light for excitation with a pulsed Ti-Sapphire laser for illumination ***(3)***.

Many fluorescent probes are available that stain, using relatively simple protocols, specific cellular organelles and structures. These probes include a plethora of dyes that label nuclei, mitochondria, the Golgi apparatus, 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, as the peripheral actin meshwork is labeled as bright fluorescent rings (**Fig. 11**). These dyes are extremely useful in multiple labeling strategies to locate antigens of interest with specific compartments in the cell, for example, a combination of phalloidin and a nuclear dye with the antigen of interest in a triple labeling scheme (**Fig. 11**). In addition, antibodies to proteins of known distribution or function in cells, e.g., antitubulin, are useful inclusions 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. These effects can be reduced by adding ascorbic acid to the medium. The cellular component labeled can also affect its viability during imaging, e.g., nuclear stains tend to have a more

deleterious effect than cytoplasmic stains. One way to overcome this problem is to include a fluorescent dye in the medium around the cells. Probes that distinguish between living and dead cells are also available and can be used to assay cell viability during imaging. Most of these assays are based upon the premise that the membranes of dead cells are permeable to many dyes that cannot cross them in the living state. Such probes include acridine orange; various kits are available from companies such as Molecular Probes ***(29)***.

Many dyes, for example, Fluo-3 and rhod-2, have been synthesized that change their fluorescence characteristics in the presence of ions such as calcium. New 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. GFP has been used to monitor gene expression in many different cell types including living *Drosophila* oocytes, mammalian cells, and plants using the 488 nm line of the LSCM for excitation ***(32)***. Spectral mutants of GFP are now available for multi-label experiments and are also useful for avoiding problems with autofluorescence of living tissues ***(33*** and *see* Chapter 15).

5.3. Autofluorescence

Autofluorescence can be a major source of increased background when imaging some tissues. Tissue autofluorescence occurs naturally in many cell types. In yeast and in plant cells, for example, chlorophyll fluoresces in the red spectrum. In addition, some reagents, especially glutaraldehyde fixative, are sources of autofluorescence, which can be decreased by borohydride treatment. Autofluorescence can be avoided by using a wavelength for excitation that is out of the range of natural autofluorescence. The longer wavelength excitation of cyanine 5 is often chosen to avoid autofluorescence at shorter wavelengths.

The amount of autofluorescence can be assessed 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**). Autofluorescence may be bleached out using a quick flash at high laser power or flooding the specimen with light from the mercury lamp. A more sophisticated method of dealing with autofluorescence is using time resolved fluorescence imaging. Autofluorescence can also be removed digitally by image subtraction. Although it is more often a problem, tissue autofluorescence can be utilized for imaging overall cell morphology in multiple-labeling schemes.

5.4. Collecting the Images

The novice user can gain experience in confocal imaging from several sources. The manual provided with the confocal imaging system usually

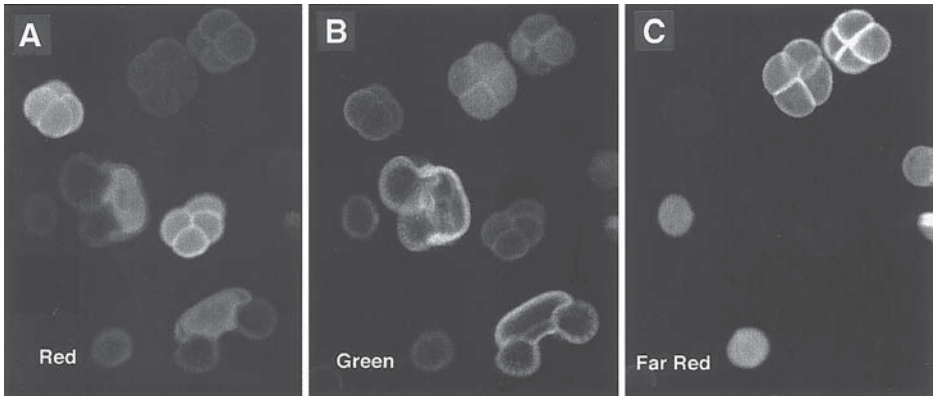

Fig. 12. Example of tissue autofluorescence. Note that different types of pollen fluoresce at different excitation wavelengths; these images were collected simultaneously at the same settings of gain, black level, and pinhole diameter.

includes a series of simple exercises necessary for getting started. The person responsible for operating the instrument may 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 conducted by the confocal companies, workshops on light microscopy, and various publications.

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 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 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 ***(11)***. Many examples of test specimens are covered further in Chapter 2.

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 the case of 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 are major causes 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 lens and the specimen should be matched correctly. For example, use the correct immersion oil for a particular NA and use a coverslip of correct thickness for the objective lens, especially for higher power lenses, which will require a No. 1 or No. 1.5 coverslip, and not a No. 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; e.g., a Vaseline, beeswax, and lanolin mixture works well. Much time and effort can be saved by taking great care with the simple basics of cleanliness at this stage.

A region of interest is located using either bright field or conventional epifluorescence microscopy, preferably using the microscope of the confocal system. It can be extremely difficult for the novice to find the correct focal plane using the confocal imaging mode alone (*see* Chapter 2). If conventional imaging is not available then structures of interest can be located using a separate fluorescence microscope and their positions marked using a diamond marker mounted on the microscope, a sharpie, or by recording the coordinates from the microscope stage. The ability to preview samples with the actual microscope of the confocal imaging system using the epifluorescence mode is especially useful when attempting to image a rare event such as a gene expressed at a specific stage of development in a sample containing hundreds of embryos of different ages. This can save much time in scanning many specimens using the confocal mode. Many instruments have a low-resolution fast-scanning mode that alleviates some of these problems. It is far easier, however, to scan slides using a conventional microscope when searching for rarely occurring events, and then immediately switching to the confocal mode to collect the images.

The secret to 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 vary these parameters using the test specimen and several different objective lenses of

different magnifications and NAs to gain a sense of 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 an objective lens of higher NA.

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 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 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 to filter out random noise from the photomultiplier and to enhance the constant features 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 (*see* Chapter 20). If space on the hard disk allows it is often a good strategy to save raw unprocessed images in addition to any processed ones.

The image is usually saved to the hard disk of the computer and later backed up onto a mass storage device. In general it is advisable to collect as many images as possible during an imaging session, and, if necessary, to cull out the unsatisfactory 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.

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 determine if this information is accessible after imaging and remember that it can be lost when the images are subsequently transferred to image manipula-

tion programs such as NIH Image or Photoshop on other computers. It is hard to replace a well-ordered notebook, or perhaps a laptop computer file, preferably with 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. Most confocal imaging systems do not automatically keep track of the lens used; this is important for calculating the scale bars for publication. In addition, some computer systems will accept up to nine characters for their file labels; and beware of using periods in the file names that can sometimes be confused by the software. For example, STEVE.NEW.PIC may be read by the imaging system as STEVE.NEW rather than as .PIC image file. Many modern systems incorporate an image database that will keep track of file names and location of the files, and may also include a thumbnail of the images (*see* Chapter 23).

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. The authors have been encouraged to include tips on such eventualities in the *Notes* sections of their chapters, and this is covered in more detail in Chapter 2. A good test is to view the sample on a conventional epifluorescence microscope, and if some fluorescence is visible by eye then the signal should be very bright on the confocal system. If this is the case, it might be time to run through some checks of the confocal system using a known test specimen and not the experimental one. 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 is usually posted close to the microscope, and can be accessed through websites listed at the end of this chapter. As a rule, if you are not sure of something ask, or at least step back from the problem before attempting to remedy it.

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, at least, “borrow” them from a trusted co-worker. Antibodies should be aliquoted in small batches from the frozen stock, and stored in the refrigerator. They should be reused only if absolutely necessary although this is sometimes unavoidable when using expensive or rare reagents, and often does not present a problem.

Bleedthrough can occur from one channel into another in multilabeled specimens. It can be caused by properties of the specimen itself or can result from

problems with the instrument. The causes and remedies of bleedthrough have been reviewed in much detail elsewhere ***(34)***. A good test of the instrument is to view a test sample with known bleedthrough properties using both the multiple-label 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 pinhole 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, e.g., 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; 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 up. Older specimens may have increased background fluorescence and bleedthrough caused by the fluorochrome separating from 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.

5.6. Image Processing and Publication

Confocal images are usually collected as digital computer files, and they can usually be manipulated using the proprietary software provided with the confocal imaging system. One of the most dramatically improved features of the LSCM has been in the display of confocal images. This part of the process is extremely important because although it is good to achieve improved resolution using the LSCM, this improvement is of little value if it cannot be displayed and reproduced in hard copy format.

Even 5 years ago most laboratories used darkrooms and chemicals for their final hard copy. Color images were even harder to reproduce because they were usually printed by an independent printer who had little idea of the correct color balance. For hard copies, images are now exported to a slide maker, a color laser printer, or to a dye sublimation printer for publication quality

prints. Photographs are taken directly from the screen of the video monitor. Moreover, movie sequences can be published on the worldwide web.

The quality of published images has also improved dramatically as most journals are able to accept digital images for publication. This means that the resolution achieved within the computer of the confocal imaging system is more faithfully reproduced in the final published article. Some journals also publish their articles on CD ROM, which means that the images 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. Information Sources

6.1. Websites

6.1.1. Good General Sites

www.videomicroscopy.com Superb magazine on video and digital imaging; excellent links to many websites that pertain to confocal technology and imaging. Good basic tutorials and sources of instrumentation including hard copy devices

www.ou.edu/research/electron/mirror/web-org.html A directory of microscopy websites listed by organization

www.patents.ibm.com The IBM patents webserver is a useful database of patents and contains those patents that pertain to confocal imaging. The entire patent including diagrams can be accessed through this server.

www.bocklabs.wisc.edu/imr/home2.htm Useful site for basic principles of confocal, two-photon, and four-dimensional imaging. Lists meetings and workshops, and a booking form for reserving time on the instruments in Madison.

6.1.2. Confocal Microsxope Companies

www.microscopy.bio-rad.com Bio-Rad Microscopes: Information on their laser scanning, real-time, and two-photon systems. Many useful application notes can be downloaded and a database of papers can be accessed here.

www.leica.com Details on light microscopes including a laser scanning confocal system. Tutorials on confocal imaging

www.nikon.com Microscopic products and technical information.

www.noran.com Noran Instruments: Details of a real-time scanning system and image analysis software

www.olympus.co.jp Microscopes and confocal imaging systems.

www.lasertec.co.jp Lasertec

www.optiscan.com.au Optiscan

www.technical.com Technical Instruments

www.lsr.co.uk Life Science Resources

www.mdyn.com. Molecular Dynamics. Good application notes.

www.zeiss.com Website for Carl Zeiss in the USA with details of light microscopes including real-time and laser scanning systems

6.1.3. Filters

www.chroma.com Useful handbook of optical filters.
www.omegafilters.com
www.image1.com

6.1.4. Dyes

www.jacksonimmuno.com Fluorescent probes and antibodies.

www.probes.com The "Molecular Probes" website is great for details of most of the fluorescent probes used for imaging

6.1.5. Confocal Methodology

www.bioimage.org Details of 3D microscopy.

www2.uchc.edu/htterasaki Live cell imaging

6.1.6. Courses and Societies

www.mbl.edu Marine Biological Laboratory at Woods Hole, which runs two excellent courses on basic light microscopy including sessions on confocal imaging. A good place to see many confocal microscopes at the same site.

www.cshl.org Cold Spring Harbor laboratory web page; details of various courses and CSH Press publications

msa.microscopy.com Web site of the Microscopy Society of America contains useful links to other sites and microscopy societies. Details of their annual conference and of other meetings pertaining to all forms of microscopy including confocal microscopy.

www.rms.org.uk Website of the Royal Microscopy Society of the UK, and links to the *Journal of Microscopy*

6.2. Listservers

One of the most useful confocal resources is the confocal e-mail group based at SUNY, Buffalo and started by Robert Summers. The confocal listserver was set up some years ago as a discussion group for Bio-Rad users, and it has developed into a discussion group on all forms of confocal microscopy and related technologies. An extremely useful aspect of the group is that previous messages are archived for reference purposes.

To join the group send the e-mail message "Subscribe Confocal (your name)" to the confocal microscopy list Confocal@listserv.acsu.buffalo, or for help contact the current listowner, Paddock@facstaff.wisc.edu.

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