

Laser Scanning Confocal Microscopy: History, Applications, and Related Optical Sectioning Techniques

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Abstract

Confocal microscopy is an established light microscopical technique for imaging fluorescently labeled specimens with significant three-dimensional structure. Applications of confocal microscopy in the biomedical sciences include the imaging of the spatial distribution of macromolecules in either fixed or living cells, the automated collection of 3D data, the imaging of multiple labeled specimens and the measurement of physiological events in living cells. The laser scanning confocal microscope continues to be chosen for most routine work although a number of instruments have been developed for more specific applications. Significant improvements have been made to all areas of the confocal approach, not only to the instruments themselves, but also to the protocols of specimen preparation, to the analysis, the display, the reproduction, sharing and management of confocal images using bioinformatics techniques.

Key words Confocal, Optical section, Resolution, Laser, Fluorescence, Illumination, Microscopy, Living, Fixed, Digital image, Informatics

1 Introduction

The major application of confocal microscopy in the biomedical sciences is for imaging 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 the resolution of structures in focus, especially for those specimens that are thicker than 2 μm .

When compared with the conventional wide field light microscope, the confocal microscope provides an increase in both maximum lateral resolution (0.5 μm vs. 0.25 μm) and maximum axial resolution (1.6 μm vs. 0.7 μm). However, it is the ability of the instrument to eliminate the “out-of-focus” brightness from images collected from thick fluorescently labeled specimens at a range of magnifications that has made it an invaluable instrument for most applications in biomedical imaging (Fig. 1).

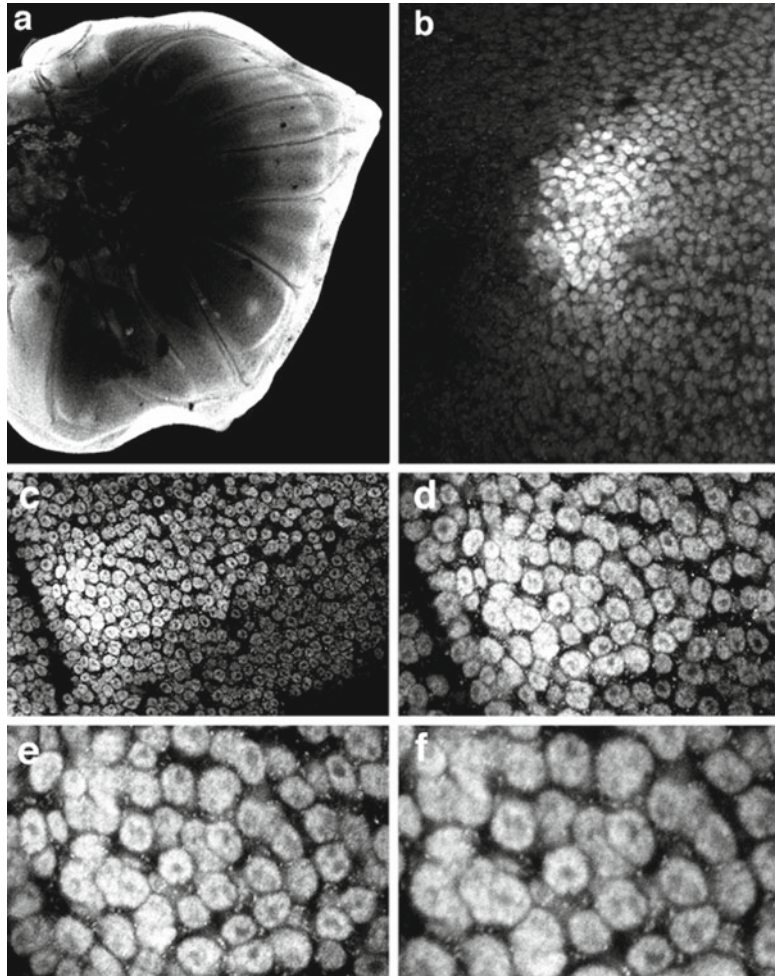


Fig. 1 Examples of Single Optical Sections from the same Specimen. Optical sections can be collected using different objective lenses or using the same lens in combination with the optical zoom function of the LSCM. In this example a fifth instar butterfly wing imaginal disk has been fixed and labeled with *distalless* antibodies and secondary fluorescein-labeled antibodies, and imaged in the LSCM. A single optical section of the entire imaginal disk is imaged using a 4 \times lens (**a**), whereas a 16 \times lens is used for improved resolution of an eyespot field (**b**). A 40 \times lens is required for cellular resolution (in this case resolution of nuclei since *distalless* is a transcription factor) (**c**). Improved nuclear resolution is achieved by using the optical zoom (**d–f**) in conjunction with the 40 \times objective lens. This strategy is often useful when imaging at high magnifications when switching to a higher power lens will risk losing the field of interest or damaging the specimen

The method of image formation in a confocal microscope is fundamentally different from that in a conventional wide field epifluorescence microscope where the entire specimen is bathed in light from a mercury or xenon source. 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. Images produced by scanning the specimen in this way are called optical sections [1]. This refers to the noninvasive method of image construction by the instrument, which uses light, rather than a physical method such as a microtome, to section the specimen.

The popularity of the confocal microscope has increased dramatically over the past ten years since the publication of the first edition of this book [2]. This is due in part to the increased number of confocal applications and increased accessibility of the technology and specifically to the introduction of fluorescence reporter techniques that have simplified the imaging of living cells [3].

Confocal technology has been developed to a level where most research institutions and many individual laboratories house one or more confocal instruments. In addition, instruments that produce optical sections for more specific applications continue to be developed as modifications of the confocal design [4, 5]. While the second edition, like the first, is focused on the laser scanning confocal microscope, many of the featured protocols are suitable for use with these new methods of optical sectioning [6, 7].

2 History of Confocal Instrumentation

2.1 *Marvin Minsky's Microscope*

The development of confocal microscopes was, and continues to be, driven by the desire to image biological events as they occur in vivo. The invention of the confocal microscope is attributed to Marvin Minsky who built a working scanning optical microscope in 1955 with the goal of imaging neural networks in unstained preparations of living brains. Details of Minsky's microscope, and of its development, can be found in his memoir, "On inventing the confocal scanning microscope" [8]. All modern confocal microscopes, by definition, employ the principle of confocal imaging that he patented in 1957 [9], although the term confocal was not introduced in this context until later [10].

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 passing through the pinhole. The second pinhole prevents light from above or below the plane of focus from striking the photomultiplier (Fig. 2).

The key to the confocal approach is the elimination of out-of-focus light (sometimes called flare) by scanning a point source of light across the specimen and using a pinhole to eliminate the

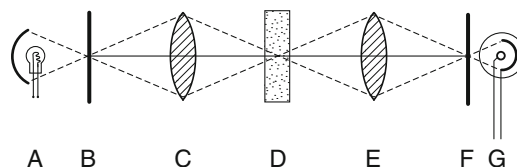


Fig. 2 Schematic of Marvin Minsky's confocal microscope—transmitted light version. A point of light is produced by a zirconium light source (a) and a pinhole placed in front of it (b). This is focused by an objective lens (c) into the specimen (d), and light that passes through it, is focused by a second objective lens (e) at a second pinhole (f), 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 detector (g), which produces a signal that is proportional to the brightness of the light passing through the pinhole. The second pinhole prevents light from above or below the plane of focus from striking the photomultiplier. The image is built up by moving the specimen (d) Image drawn by Leanne Olds

out-of-focus light from the detector. Minsky also described a reflected light version of the microscope that used a single objective lens and a dichromatic mirror arrangement (Fig. 3). This arrangement eliminated the considerable problem of aligning the two objective lenses in the transmitted light version since a single objective lens is used for both the excitation and the emission paths. This epi-illuminated design is the basic configuration of most modern confocal systems that are used for fluorescence imaging today.

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 optical axis, which can eliminate any lens defects. However, for biological specimens, movement of the specimen can cause them to wobble, which results in a loss of resolution in the final image.

Finally an image of the specimen has to be recorded. 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 that the quality of the final images collected from his microscope was not very impressive. This was most likely due to the inferior quality of the oscilloscope display and sensitivity of the photodetector and not by the lack of resolution achieved with the microscope itself.

The images produced by Minsky's instrument at this time were unremarkable. It is clear that the technology was not available to him in 1955 to fully demonstrate the potential of the confocal approach to the biomedical imaging community. This may have

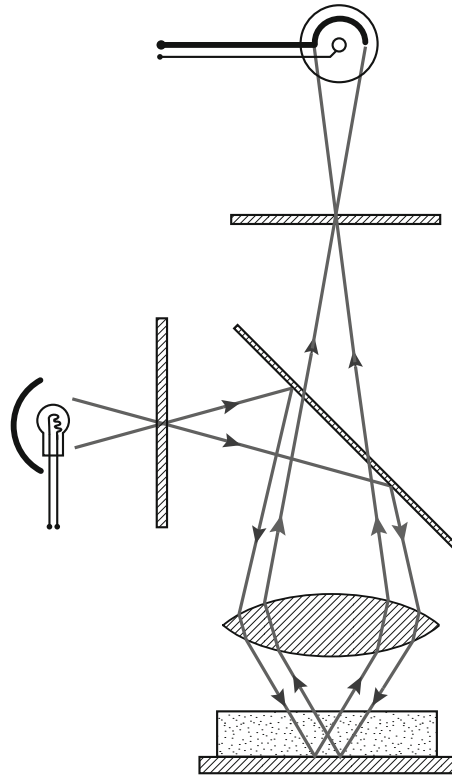


Fig. 3 Schematic of Marvin Minsky's confocal microscope—reflected light version. A zirconium light source (a) and a pinhole (b) produces a point source of light (a). This is reflected by a dichromatic mirror (c) and focused by an objective lens (d) onto the specimen (e). Longer wavelength light is reflected back from the specimen, is subsequently focused by the same objective lens (d), passes through the dichromatic mirror (c), and is focused onto a second pinhole (f) in front of the photodetector (g). Both source and detector pinholes are confocal with the focused point of light in the specimen. The image is built by moving the specimen (e). Image drawn by Leanne Olds

been why confocal microscopy did not immediately catch on. After all, at this time, biologists were used to viewing and photographing their brightly stained and colorful histological tissue sections using light microscopes with excellent optics, and in real color. Confocal imaging of living tissues would have to wait.

2.2 Subsequent Technological Innovation

Several major technological advances that would have benefited Minsky's confocal design have become available to biologists during the years since 1955. These include;

1. Bright and stable laser light sources.
2. Efficiently reflecting mirrors and more precise filters.
3. Improved methods of scanning and electronics for data capture.

4. High quantum efficiency low noise photodetectors.
5. Improved methods of specimen preparation.
6. Fast computers with image processing capabilities.
7. Elegant software solutions for analyzing the images.
8. High-resolution digital displays and color printers.
9. Bioinformatics methods for managing the images.

The introduction of practical confocal microscopes was largely dependent upon the development of efficient methods of scanning the excitation spot within the specimen. Confocal microscopes are typically classified using the method by which the specimens are scanned. Minsky's original design was a stage scanning system driven by a tuning fork arrangement that was rather slow to build an image. It was also extremely difficult to locate a region of interest in the specimen, and even harder to focus, using this system.

The 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 also been used for screening DNA sequences on microchip arrays.

An alternative to moving the specimen (stage scanning) is to scan the beam across a stationary specimen (beam scanning). This configuration is more practical for imaging biological specimens, and is the basis of those systems that have developed into the current generation of research microscopes.

More details of the technical aspects of confocal microscopes are covered elsewhere [11], but briefly there are two fundamentally different methods of beam scanning; single beam scanning or multiple beam scanning. Single beam scanning continues to be the most commonly used method at this time, and is epitomized by the laser scanning confocal microscope (LSCM). Here the scanning is achieved using computer-controlled galvanometer-driven mirrors to direct a single beam of excitation light across the sample.

The alternative to single beam scanning is to scan the specimen with multiple beams (almost real time). Point-scanning LSCM, when used with high numerical aperture lenses, has an inherent speed limitation in fluorescence. This arises because of a limitation in the amount of light that can be obtained from the small volume of fluorophore contained within the focus of the scanned beam. This can be overcome with parallel or multiple laser excitation approaches. This is most commonly achieved using some form of spinning Nipkow disk; a design adapted from the early days of television transmission. The forerunner of the spinning disk systems was the tandem-scanning microscope (TSM), and subsequent improvements to the design have resulted in instruments that collect acceptable images from fluorescently labeled living specimens. The modern Nipkow or other spinning disk based variants have a

much higher speed potential than conventional LSCMs because the spinning disk based parallelism avoids fluorophore saturation enabling higher levels of excitation to be used. As these systems typically optically reconstruct the image, this allows the use of high sensitivity CCD detectors giving extended red response of great advantage for many of the newly developed fluorophores. Spinning disk based confocal systems have been very popular for applications where close to real time capture is needed such as tracking calcium ion transients in cell environments.

In modern confocal microscopes the image is either built up from the output of a photomultiplier tube or captured using a 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. Moreover, vastly improved methods of specimen preparation, especially using fluorescent reporters of gene activity, have enabled the realization of Minsky's dream of imaging living neurons in vivo.

2.3 The Laser Scanning Confocal Microscope

The LSCM continues to be the instrument of choice for most routine biomedical research applications, and it is, therefore, most likely to be the instrument first encountered by the novice user. As in the first edition, emphasis has been placed on the LSCM in this edition.

The LSCM is built around a conventional epifluorescence light microscope either in an upright configuration popular with neuroscientists and physiologists or an inverted configuration seen commonly for cell culture and developmental biology applications (Fig. 4).

The conventional light microscope is essential for efficiently finding the region of interest in the specimen by eye before scanning in the confocal mode. This is extremely useful since one of the great strengths of the confocal microscope, i.e., the elimination of out-of-focus information, can make it extremely difficult to locate a region of interest in the specimen in the confocal mode. This configuration is also very stable, especially when mounted on an anti-vibration air table. Any vibration results in a loss of resolution in the image, and can show up in the image as irregular horizontal lines.

The modern LSCM typically uses a laser rather than a lamp for a light source, acousto-optic tunable filters (AOTFs) for selecting specific excitation wavelengths, dichroics for multichannel emission discrimination, sensitive photomultiplier tube detectors (PMTs) and a computer to control the scanning mirrors and to facilitate the collection and display of the images. Modern LSCMs can excite and detect multiple fluorophores simultaneously typically through the use of multiple lasers and multiple detectors for each channel. Images are subsequently stored as digital image files and can be further analyzed using additional software.

In the LSCM, illumination and detection are confined to a single, diffraction-limited, point in the specimen. This point is

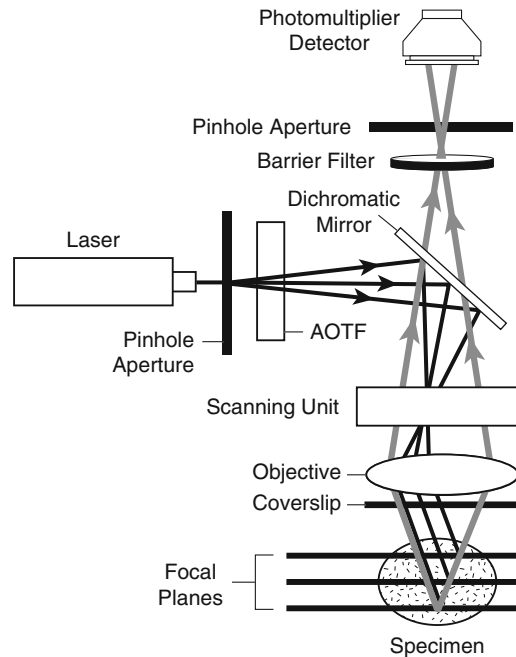


Fig. 4 The main components of a modern laser scanning confocal microscope-reflected light, upright version. Light from one or more lasers passes through a pinhole, attenuated through an AOTF, bounces off a dichromatic mirror, and passes into the scanning unit. A scanned beam enters the back focal plane of the objective lens, which focuses the light at a point in the specimen. Any light coming back from the excitation of a fluorochrome at this point inside the specimen passes back through the objective lens and the scanning unit. Since this light is of longer wavelength than the excitation light, it passes through the dichromatic mirror, is further cleaned up by a barrier filter and it is eventually focused at the second pinhole. Any light that passes through the pinhole strikes a low noise photomultiplier detector, the signal from which subsequently passes to the computer imaging system of the confocal microscope. This configuration is very similar to that of Minsky's reflected light schematic of Fig. 3. Image drawn by Leanne Olds

focused 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, and the output from this is built into an image by the computer. Specimens are usually labeled with one or more fluorescent probes (fluorescence mode). Unstained specimens can be viewed using the light reflected back from the specimen (reflected light mode).

One of the more commercially successful LSCMs was designed in the late 1980's at the Medical Research Council laboratories in Cambridge, England by the team of White, Amos, Durbin and Fordham. They set out to tackle a fundamental problem in developmental biology; namely imaging specific macromolecules in fluorescently labeled embryos [12, 13]. They were specifically

interested in imaging microtubules in *C. elegans* embryos. Many of the cells inside developing 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 the fluorescence signal increases from the more and more closely packed cells out of the focal plane of interest, and interferes with resolution of those structures in the focal plane of interest.

When he investigated the live cell imaging microscopes available to him in the mid-1980s including early confocal designs, John White discovered that no system existed that would solve his resolution issues caused by the increased signal brightness from the increased cell packing as the embryos developed over time. Technology at this time consisted of the stage scanning instruments, which tended to be impossible to focus and painfully slow to produce images (approximately 10 s for one full frame image that was often out-of-focus), and the multiple beam microscopes, which were difficult to align and the fluorescence images were extremely dim, if not invisible without extremely long exposure times!

The Cambridge team led by John White and Brad Amos designed a LSCM that was suitable for conventional epifluorescence microscopy applications and since evolved into an instrument that has been used in many different biomedical applications over the years [14]. The breakthrough came with the development of more efficient methods of scanning the beam using first a single galvanometer-driven mirror and a spinning polygon mirror design, and subsequently settling upon a dual galvanometer-driven mirror arrangement. It was also necessary to incorporate relatively new computer-based imaging technology and control electronics using a framestore card and analog to digital conversion to coordinate and keep track of the position of the scanning mirrors with the acquisition of the images into the computer. This required the development of software that was reliable and easy to use.

In a landmark paper that captured the attention of the cell biology community because of the vastly improved quality and resolution of the images of a diverse range of familiar specimens, White et al. compared images collected from the same specimens using conventional wide field epifluorescence microscopy and using their LSCM [15]. 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 previously contaminated their images. This technological advance allowed them to follow and record changes in the cytoskeleton in the increasing numbers of cells in early embryos at a higher resolution than using conventional epifluorescence microscopy.

The thickness of the optical section could be adjusted simply by changing the diameter of a pinhole in front of the

photodetector. The image could be zoomed with no loss of resolution simply by decreasing the region of the specimen that was scanned by the mirrors simply by placing the scanned information into the same number of pixels in the image. This imparted a range of magnifications to a single objective lens, and was extremely useful when imaging rare events when changing a lens may have risked losing the region of interest during the experiment (Fig. 1).

This design has proven to be extremely flexible for imaging biological structures as compared with some of the other designs that employed fixed diameter pinholes. This microscope together with several other instruments introduced by others during the same time period, were the forerunners of the sophisticated instruments that are now available to biomedical researchers from several commercial vendors.

The advantage of the LSCM lies within its versatility and large number of applications combined with its relative user-friendliness 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. This meant that photobleaching and photodamage to specimens were often problematic in the older instruments. The early systems tended to work well for brightly labeled and fixed specimens but tended to quickly kill many living specimens unless extreme care was taken to preserve the viability of specimens on the stage of the microscope by limiting the laser power for imaging. Nevertheless the microscopes produced such excellent images of fixed and fluorescently labeled specimens that confocal microscopy was fully embraced by the biological imagers.

Improvements have been, and continue to be, made to all parts of the imaging process. These include more stable lasers, more efficient mirrors, more sensitive photodetectors, electronic filters (AOTFs), improved methods for multichannel collection such as spectral based capture, and improved digital imaging systems. The new instruments have been improved ergonomically so that alignment is much easier to achieve and preserve. Filter combinations are now controlled by software and AOTFs and multiple fluorochromes can be imaged simultaneously with instrumentation for correcting for bleed through and autofluorescence (Fig. 5).

The development and commercial availability of fluorescent probes with improved levels of photostability and specificity for improved localization continues to influence the development of confocal instrumentation. The fluorophores include synthetic fluorochromes, for example, the Alexa dyes and quantum dots and naturally occurring fluorescent proteins, for example the green fluorescent protein (GFP) and its derivatives, for example CFP and YFP. Many of the new fluorescent probes have been designed to

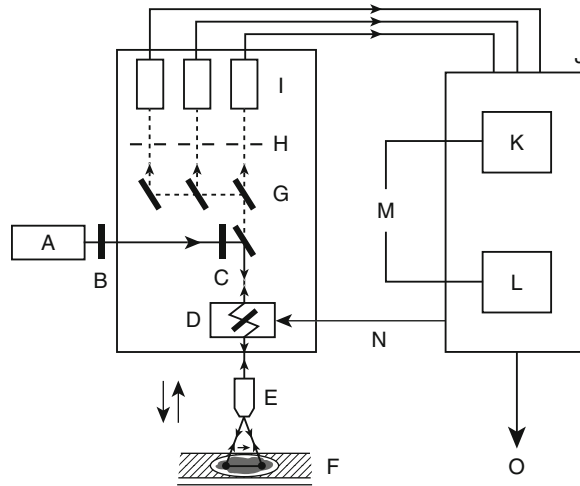


Fig. 5 The information flow in a generic laser scanning confocal microscope. Light from one or more lasers (a) passes through a neutral density filter or AOTF (b) and an exciter filter or AOTF (c) on its way to the scanning unit (d). The scanning unit produces a scanned beam at the back focal plane of the objective lens (e), which focuses the light at the specimen (f). The specimen is scanned in the X and the Y in a raster pattern and in the Z direction by fine focusing (arrows). Any fluorescence from the specimen passes back through the objective lens and the scanning unit and is directed via dichromatic mirrors (g) to three pinholes (h). The pinholes act as spatial filters to block any light from above or below the plane of focus in the specimen. This means that only distinct regions of the specimen are sampled. Light that passes through the pinholes strikes the PMT detectors (i) and the signal from the PMT is built into an image in the computer (j). The image is displayed on the computer screen often as three grayscale images together with a merged color image of the three-grayscale images. The computer synchronizes (n) the scanning mirrors (d) with the buildup of the image in the computer frame store or memory (k). The computer also controls a variety of peripheral devices. For example, the computer controls and correlates movement of a stepper motor connected to the fine focus of the microscope with image acquisition in order to produce a Z-series. Furthermore the computer controls the area of the specimen to be scanned by the scanning unit so that zooming is easily achieved by scanning a smaller region of the specimen. In this way, a range of magnifications is imparted to a single objective lens so that the specimen does not have to be moved when changing magnification. Images are written to the hard disk of the computer or exported to various devices for viewing, hard copy production or archiving (o). Final images are produced in the computer by synchronizing input from the scan head with the video card (m). Image drawn by Leanne Olds

have their excitation and emission spectra closely matched to the wavelengths delivered by the lasers supplied with most commercial LSCMs (Table 1). The instruments continue to be improved as new technologies from diverse sources are added to the existing LSCM designs.

Table 1
Peak excitation and emission wavelengths of some commonly used fluorophores

Alexa Fluors	350 thru 680	442 thru 702	He–cadmium
Cyanines	489 thru 710	506 thru 805	He–cadmium
DAPI	350	470	He–cadmium
Fluorescein	496	518	Argon ion
GFP	395/475	510	Blue diode
Qdot	350 thru 600	525 thru 655	Blue diode
Rhodamine B	540	625	Green He–Ne
DsRed	558	583	Green He–Ne
X-Rhodamine	580	605	Krypton–argon
TOTO3	642	661	Krypton–argon

3 Confocal Imaging Modes

The value of the LSCM for biomedical imaging is due to the ability of the instrument to both scan and detect a point of light under extremely fine control in the X, the Y and the Z direction within a fluorescently labeled specimen at various time and wavelength resolutions. The basic imaging modes of the instrument will be described.

3.1 Single Optical Sections

The basic output of all confocal microscopes is the optical section. This is a single image of a discrete region of a three dimensional cellular structure with any contribution from fluorescence from above and below the focal plane of interest removed. The resolution and the thickness of the optical section is related to the numerical aperture (NA) of the objective lens chosen for imaging and the diameter of the pinhole in front of the photodetector [16]. Higher NA lenses and narrower pinhole diameters achieve higher resolution images and produce thinner optical sections (Fig. 6).

There is an optimal pinhole setting for each objective lens chosen, which is calculated by the software of the confocal imaging system (after an initial calibration for each objective lens is entered into the software). However, there is a trade-off between the theoretically achievable resolution and the practical constraints imposed by the specimen itself in order to collect an acceptable image.

It is essential to choose the correct objective lens for the specific confocal imaging application (Table 2). Specific objective lenses are available for both high magnification/high resolution imaging and low magnification/high resolution imaging (Fig. 7). While most emphasis has been placed on high resolution and high

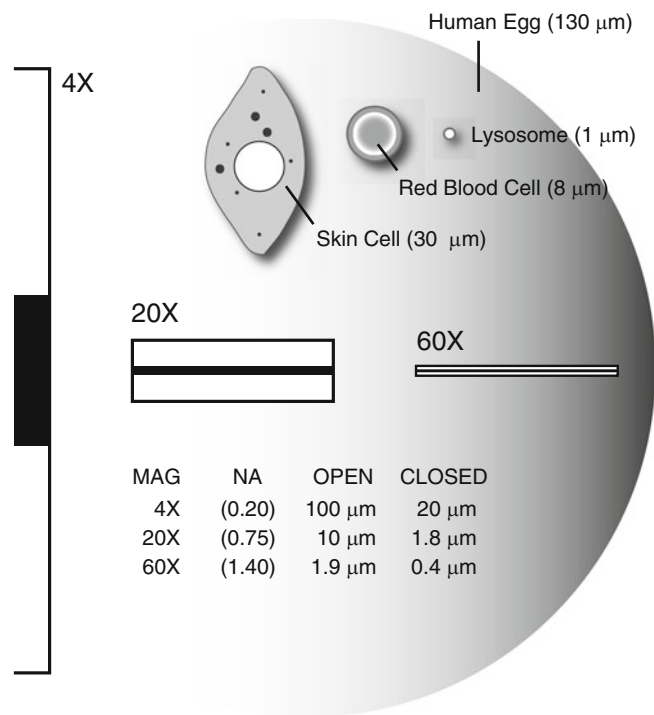


Fig. 6 The thickness of optical sections produced by the LSCM is a function of the numerical aperture of the objective lens chosen for imaging and the diameter of the confocal pinhole. The understanding of the relationship between these two factors is essential for efficient image capture. Some common biological specimens including a human egg, a skin cell, a red blood cell, and a lysosome have been represented in relation to the optical section thicknesses sampled from such biological specimens using a 4× lens, a 20× lens, and a 60× objective lens either with the pinhole open or with the pinhole set at an optimal diameter (filled areas). The maximum theoretical resolution for each lens and for each setting of the pinhole is included in the table. Image drawn by Leanne Olds

Table 2
Properties of microscope objectives for confocal 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

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
Medium	Oil	Dry
Color correction	Best	Good
Flatness of field	Best	Fair

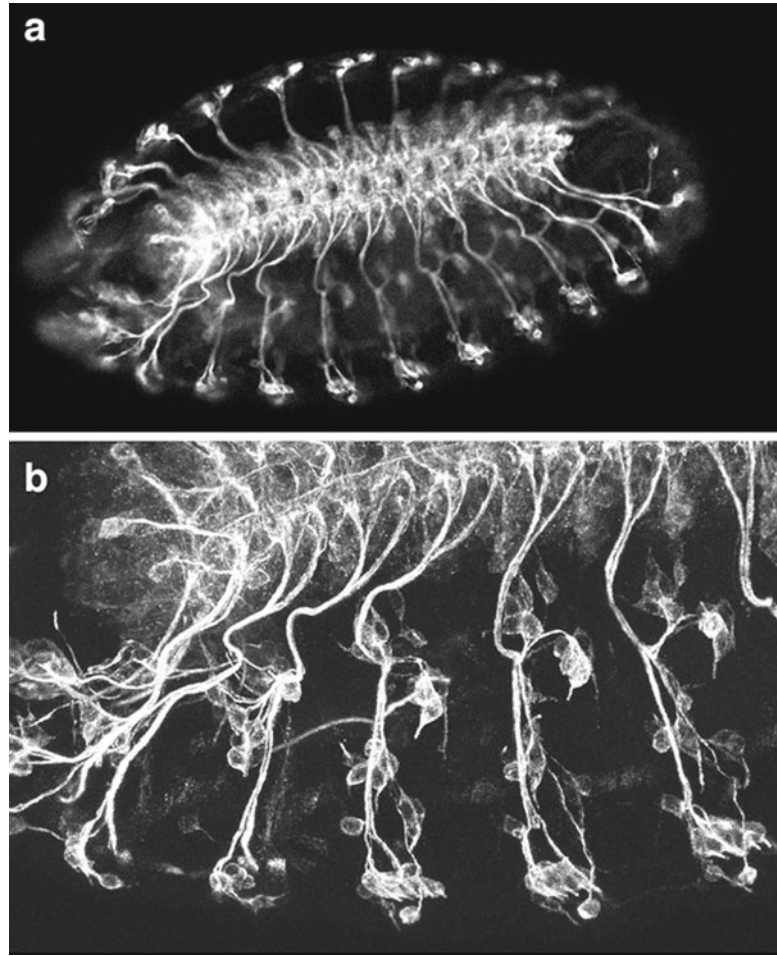


Fig. 7 Optical at the same zoom setting sections of the same specimen produced by two different objective lenses (**a**) 20 \times NA 0.75 and (**b**) 60 \times NA 1.4. The specimen is a late stage *Drosophila* embryonic peripheral nervous system labelled with the 22C10 antibody

magnification imaging, low power confocal imaging is also extremely useful in many biomedical applications. In order to attain maximal resolutions at low power it is usually necessary to collect images from several different regions of the specimen with high magnification high numerical aperture (NA) objectives and subsequently “stitch” the images together digitally. This is due to the lack of resolution in conventional low magnification lenses. This is changing however with several recent commercial macroconfocal systems that provide low magnification and relatively high NA. This includes a recent development by Brad Amos and colleagues at the MRC; the “mesolens” produces a full 3D image of large objects (up to 5 mm) such as mouse embryo with cellular detail in a single image.

Using most LSCMs it takes approximately 1 s to scan and collect a single optical section, a frame per second. Several scans are

usually averaged or integrated in order to improve the signal to noise ratio. The time to collect the image of a single optical section depends on the size of the image and the speed of the computer. For example, a typical image of 768 by 512 pixels in size will occupy approximately 0.3 MB. Larger images, e.g., $1,024 \times 1,024$, will occupy more space and take longer to collect.

An area for speed improvement in LSCMs is the galvo scanning approach. Galvo based systems are driven with a control signal at the rate of several microseconds per pixel, which is often the rate-limiting step in high-speed confocal acquisition. There have been two general strategies for improving the speed. The first has been to use line scanning based approaches where a row of pixels along a single axis of the specimen is collected very quickly and these rows can be then assembled into a image as needed. Line scanning has been proven to be quite useful for tracking dynamic fast phenomena such as calcium sparks but has been proven to be problematic for weak heterogeneous signals that are distributed spatially.

The second has been to explore alternative technologies for directing the beam. Several groups have developed confocals that use acoustical optical deflection (AOD) for beam steering. AOD based confocal designs with their precision and no moving parts allow for highly accurate saw tooth raster scans but typically suffer from poor axial resolution and reduced sensitivity as compared to conventional LSCMs. More recently vendors have developed systems that retain the galvanometer based scanning but rather than using the conventional servo-controlled galvos that are inherently limited to about a frame per second in most configurations are instead using a new class of resonant based galvanometers. These resonant based scanners use vibrational energy to move the mirror and can produce scanning acquisition speeds of up to 30 frames per second.

The value of optimal specimen preparation protocols cannot be overemphasized. There is usually a period of “fine-tuning” the specimen protocol to the constraints imposed by the physical characteristics of the confocal instrument available in order to collect the most information from the specimen in the most efficient way.

3.2 Multiple Wavelength Images

Modern confocal instruments are capable of detecting fluorescence emissions ranging from 400 to 700 nm. This covers a wide range of commonly available fluorescent probes. Spectral imaging systems either via multiple filters in a filter wheel or array of detectors with a spectral grating further aid the detection of probes with overlapping emission spectra and the imaging of more technically challenging specimens that may be compromised by autofluorescence that overlaps the emission wavelength of the probes of interest (Fig. 8). The AOTF is an invaluable aid to imaging multiple wavelength specimens since it affords fine control of both the intensity and the illumination wavelength at a high rate.

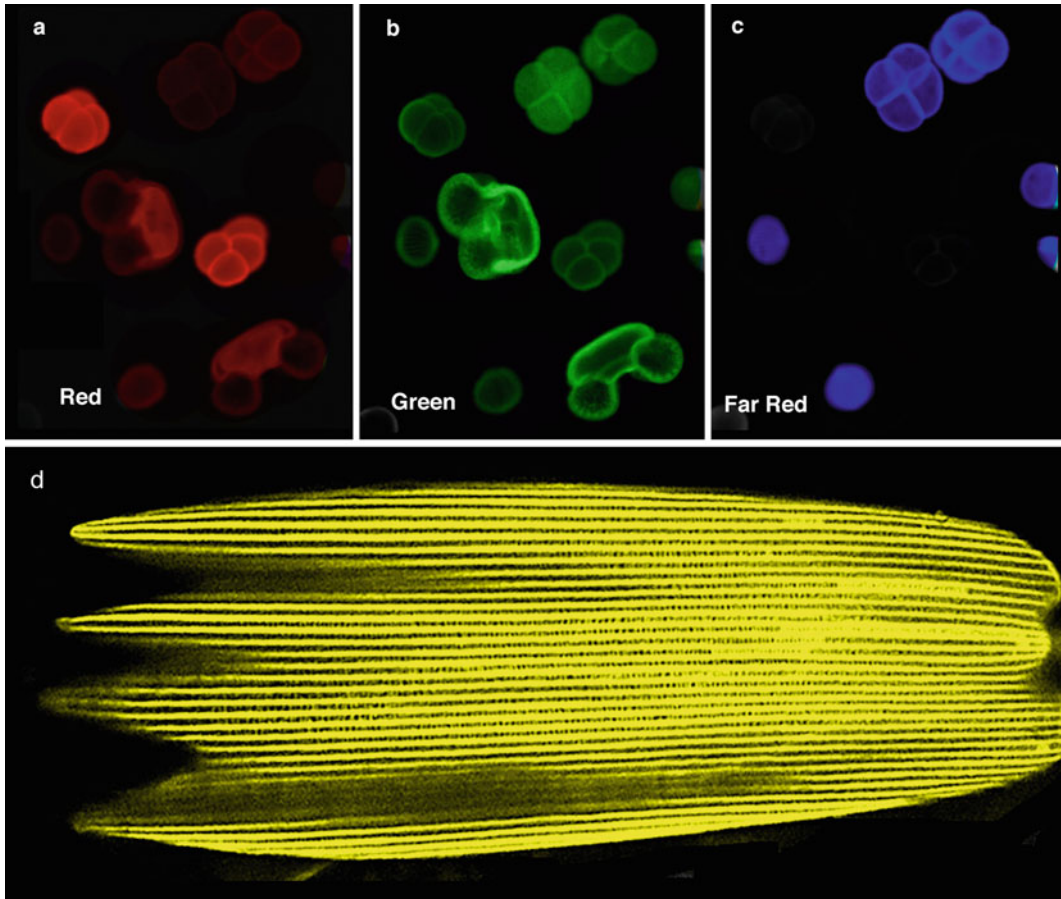


Fig. 8 Example of tissue autofluorescence. Many tissues have endogenous autofluorescence, and it is essential to map the amount of autofluorescence at different excitation wavelengths by imaging an unstained control sample in order to avoid false positive results. It is advisable to make a note of the excitation wavelengths of autofluorescence and the levels of gain and black level required to produce the images. When such autofluorescence is a problem it is best to choose a fluorochrome with an excitation maximum away from the autofluorescence. Autofluorescence can be filtered using a spectral imaging system. Autofluorescence can be an advantage for imaging cell outlines. In this case a sample of pollen grains is imaged in the *red* (a), the *green* (b) and the *far red* (c). Pollen grains from different plants have different autofluorescent characteristics. A single butterfly wing scale exhibits autofluorescence in all three channels (d). Such specimens are very convenient test specimens for imaging with the LSCM

Data are collected from either fixed-and-stained samples or living samples in single, double, triple, or multiple wavelength modes [17]. The resulting images will be in register with each other as long as an objective lens that is corrected for chromatic aberration is used and the specimen does not move while all of the emission wavelengths are collected (Fig. 9). Should it be necessary, registration of the images may be restored using digital methods.

Multiple wavelength confocal imaging protocols include those for direct labeling of cellular structures, for example mitochondria, nuclei and stress fibers (cell outlines), immunofluorescence techniques

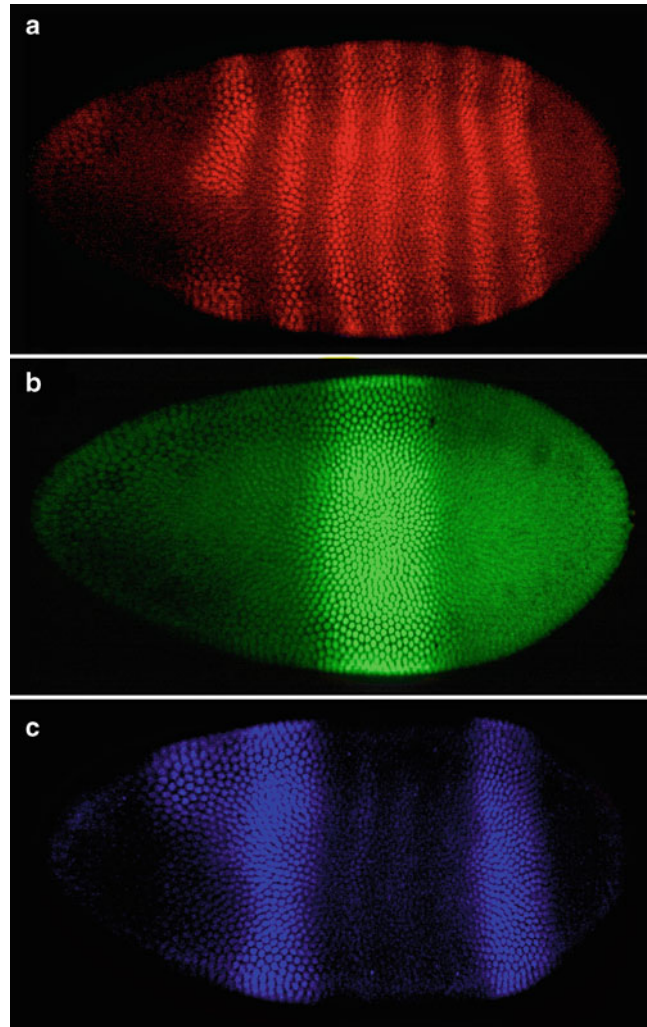


Fig. 9 Single optical sections of a triple labeled *Drosophila* embryo at the cellular blastoderm stage. The three optical sections were collected simultaneously using a single krypton argon laser at three different excitation wavelengths; 488, 568, and 647 nm. The embryo has been labeled for three genes involved with patterning the wing; (a) hairy (lissamine rhodamine 572 nm, Emission 590 nm); (b) Kruppel (fluorescein 496 nm, Emission 518 nm); and (c) giant (cyanine 5 649 nm, Emission 672nm)

(usually fixed specimens), fluorescence *in situ* hybridization (FISH), fluorescent reporter technology, and combinations of these techniques. FISH is used for imaging the distribution of fluorescently labeled DNA and RNA sequences in cells [18].

Specimens prepared by single, double and triple labeling protocols are now relatively routine for most modern confocal imaging systems [19]. The number of different fluorescent probes that can be imaged in a single preparation continues to increase (Fig. 10). Any additional channels will generally require more specialized

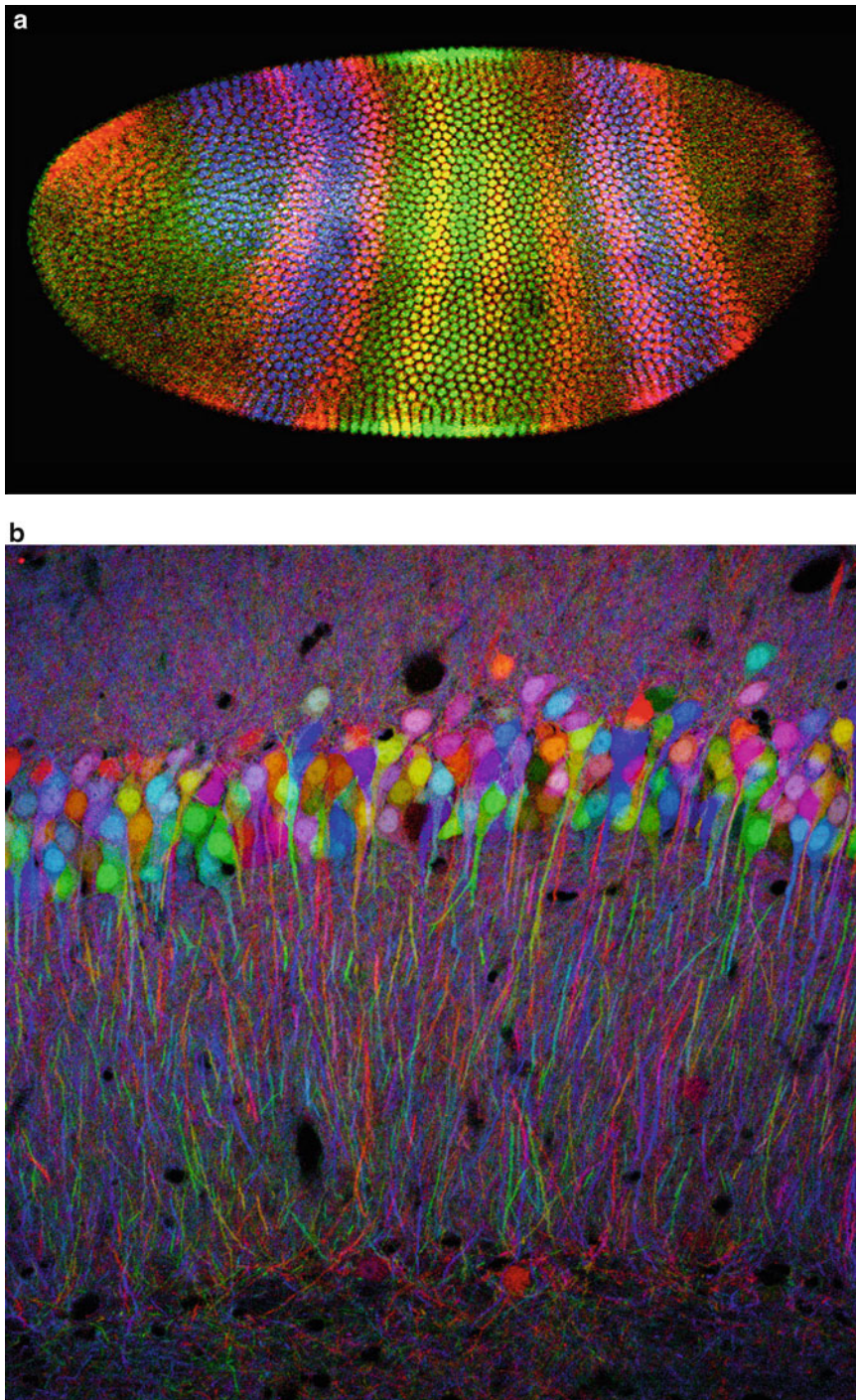


Fig. 10 Multiple Wavelength Imaging. **(a)** Three Color Image of a *Drosophila* embryo. This image was constructed by merging the three grayscale images from Fig. 8 by pasting each image into the *red* (*r*), the *green* (*g*), and the *blue* (*b*) channels of an RGB image using Adobe PhotoShop. Additive color combinations are useful for viewing biological information. For example the two *yellow* hairy stripes in the *blue* Kruppel domain represent nuclei that are expressing the two genes at the same time. Different color combinations for aesthetic and informational purposes can be made simply by rearranging and copying the images to different channels. **(b)** Ninety-nine color image of the hippocampus of a Brainbow transgenic mouse brain. Multiple copies of *red*, *green* and *blue* transgenes are randomly inserted into different cells to give one out of a possible 99 different colors (panel **(b)** was kindly reproduced with the permission of Dr. Katie Matho and Dr. Jean Livet)

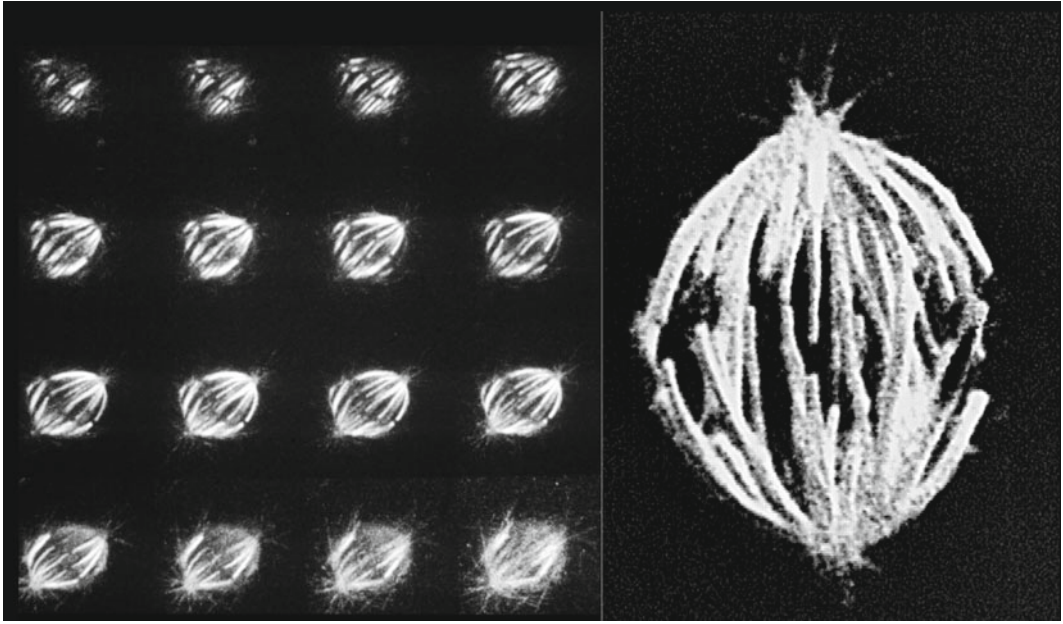


Fig. 11 A Z-series of optical sections collected from a fixed and immunofluorescently labeled mitotic spindle from a HeLa cell. Sixteen optical sections were collected at $0.2\ \mu\text{m}$ intervals from the top of the spindle to the coverslip surface using a $60\times$ NA 1.6 oil immersion objective lens. The optical sections were subsequently processed into a 3D reconstruction

specimen preparation techniques, more specialized imaging protocols and more specialized methods of image presentation and analysis. The current maximum number of colors detected in a single specimen using a standard LSCM is ninety-nine using the “Brainbow” technique where cells are randomly labeled with various combinations and concentrations of different colored fluorescent reporter probes [20].

3.3 Three-Dimensional Imaging

The capacity of confocal instruments to collect optical sections at precisely defined levels in the specimen has facilitated the production of three-dimensional images [21]. This is often necessary in order to glean any information from the images since a single optical section may appear rather abstract and not contain enough information for any meaningful interpretation. For example, single optical sections of fluorescently labeled neurons appear as abstract lines and spots whereas a 3D reconstruction appears as a network.

A Z-series is a sequence of optical sections collected at successive depths from within a specimen (Fig. 11). It is collected by coordinating the movement of the fine focus of the microscope electronically using a stepper motor with image acquisition. This is relatively easily accomplished using a macro program that instructs the LSCM to collect an image, move the focus by a predetermined distance, collect a second image, move the focus and continue until several images at consecutive levels through the region of interest have been collected.

Care must be taken to collect the images at the correct Z-step of the motor in order to calibrate for the actual depth of the specimen in the image. The XY pixel size of the image must match the Z pixel size of the image. This means that there is an optimal Z-step for each objective lens used. This is usually calculated by the confocal acquisition software, which must be calibrated for each objective lens available on the microscope.

The optical sections collected as a Z-series with the LSCM are usually in register with one another (this assumes that the specimen itself does not move during the period of image acquisition) and are output in a digital form. Z-series are ideal for further processing into a 3D representation of the specimen using 3D reconstruction software or volume visualization techniques. The Z-series file is usually processed into a single 3D representation or a movie sequence compiled from different views of the specimen. This appears as a 3D representation rotating or rocking.

Specific parameters of the 3D image such as opacity can be interactively changed in order to reveal structures of interest deep within the specimen. Measurements of length (distance between points in a 3D volume), depth and volume can be made. This approach is used to elucidate the 3D relationships of structures within cells and tissues since it can be conceptually difficult to visualize complex interconnected structures from a 2D montage of 200 or more optical sections.

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 rather than depth. This approach is useful as a method for visualizing physiological changes during development.

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 or NIH ImageJ or FIJI. These color based assignments can also be used to colorize different channels based on intensity color maps or look up tables (LUTs) that can be assigned to allow for improved discrimination of different fluorophores or changes in intensity.

3.4 X-Z Imaging

An X-Z section is usually produced by scanning a single line at successive Z depths under the control of the stepper motor (Fig. 12). It is essential to collect the line scans at stepper motor increment that is calibrated to the objective lens chosen so that the resulting pixel size is proportional to the Z-dimension of the specimen. Resolution in the Z dimension (0.7 μm maximum) is not as good as in the X-Y (0.25 μm maximum) and images tend to be a little blurry especially if the Z calibration is not calculated correctly.

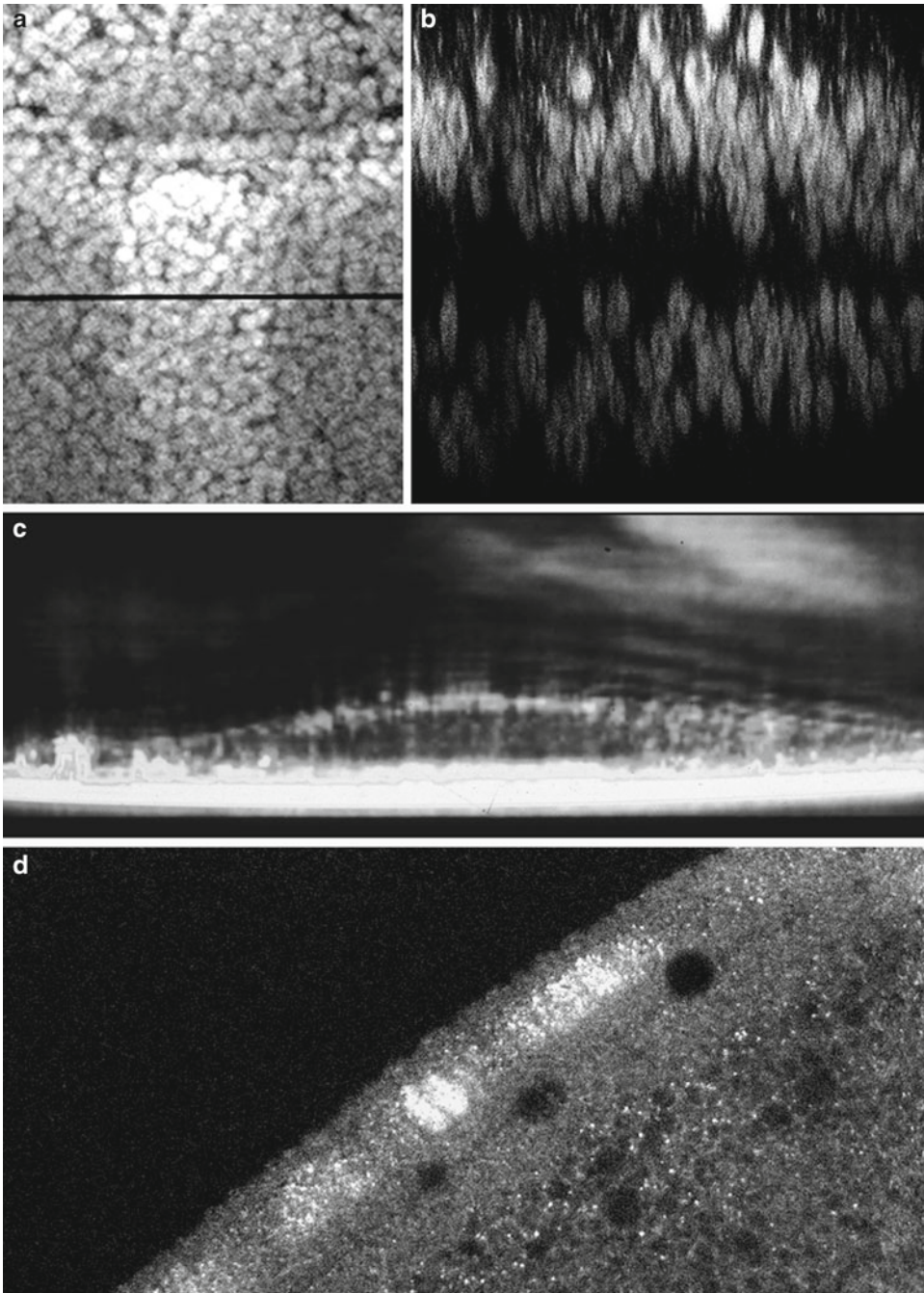


Fig. 12 X-Z imaging; the laser was scanned across a single line at different Z depths-black line in (a) and an X-Z image was built up from the line scans in the confocal imaging system (b). Note that the butterfly wing epithelium is made up of two epithelial layers, and note that the fluorescence intensity drops off deeper into the specimen. This is an artifact caused by attenuation of the signal by the optical properties of the specimen. (c) X-Z sectioning in reflected light of an unstained living cell growing on a glass coverslip in tissue culture. The coverslip is visible as a saturated region (*white*) beneath the cell profile. (d) Profiles of cells can also be produced by orientation of the specimen in the scanning beam. Here the edge of a *Drosophila* embryo at the cellular blastoderm stage is imaged

An alternative method of producing an X–Z image is to extract the profile from a Z-series of optical sections using a cut plain option in a 3D reconstruction program.

3.5 *Live Cell Imaging*

Major advancements have been made in the ability to image living cells using confocal microscopy [22]. The photon efficiency of most modern confocal systems has been improved significantly over the early models, and when coupled with high throughput objective lenses and brighter less phototoxic dyes, these improvements have made live cell confocal analysis a practical option. Images are usually collected using a time-lapse mode [23]. Image collection is at pre-selected time intervals, and the images are placed into a single image file, which is usually viewed as a movie (Fig. 13).

Imaging living tissues is perhaps an order of magnitude more difficult than imaging fixed ones using the LSCM [24]. For successful live cell imaging extreme care must be taken to preserve the viability of cells on the stage of the microscope throughout the imaging process (Table 3). Minimal laser powers should be used since harmful levels of light exposure can accumulate over multiple scans, and will eventually cause photo damage to the cells. Cells generally stay healthier for longer time periods when exposed to brief pulses of light. Longer wavelengths (infra-red) of excitatory light are generally less phototoxic than the shorter wavelengths (UV).

Cells from different sources have widely different requirements for imaging in the living state. For example, mammalian cells have more stringent temperature and pH requirements than those from most invertebrate sources. There are a array of microscope incubators available now that can provide precise control of the environment on the microscope stage including control for humidity, CO₂ levels and temperature. The choice of the best exposure time for any given tissue is a matter for experimentation with any given experimental set-up. It is necessary to check on the health of the cells after each imaging run. Simple tests might be a comparison with adjacent cells in the same preparation or following the subsequent development of observed cells as compared with a control group of cells after an imaging session. Markers of cell viability are also commercially available that can be used in addition to live dead stains.

New and improved probes for imaging gene expression in living cells continue to be introduced [25, 26]. These reporter probes avoid complicated and potentially harmful methods of loading cells with fluorescent probes by microinjection, chemical or electroporation, since the fluorescent reporter probes are genetically engineered into the cells at the site of protein action.

A commonly used reporter is the green fluorescent protein (GFP). This is used to determine the location, concentration, interactions or the dynamics of target proteins in living cells and tissues [27]. The excitation and emission spectra of enhanced GFP (a genetic

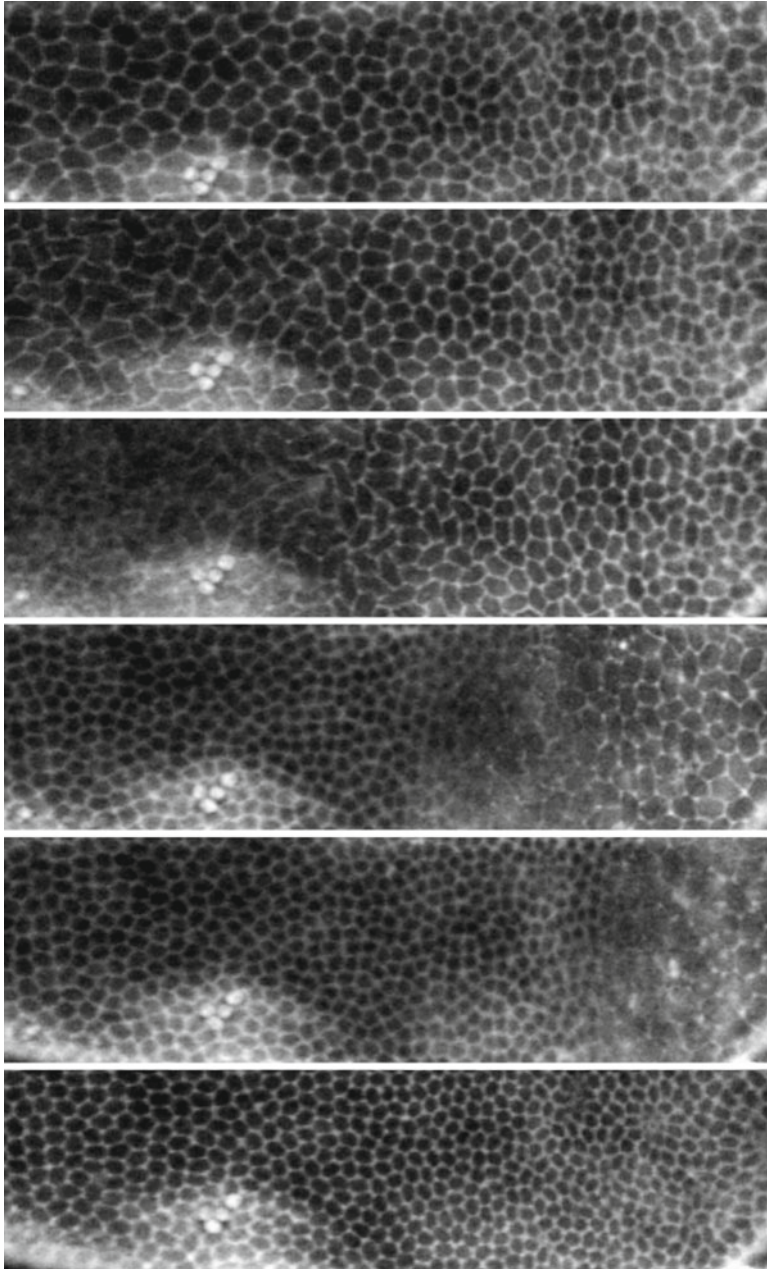


Fig. 13 Time lapse imaging of a living *Drosophila* embryo injected with Calcium green (a–d). Here Calcium green is used as a marker of cell outlines rather than a calcium indicator dye. A wave of cell divisions (mitotic wave) passing across the embryo is viewed as reduction in cell size and an increase in cell numbers. Image drawn by Leanne Olds

Table 3
Different considerations for imaging fixed and living cell with the LSCM

	Fixed cells	Living cells
Limits of illumination	Fading of fluorophore	Phototoxicity fading of dye
Anti-fade 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

derivative) have maxima at 489 and 508 nm, respectively. This is conveniently close to the excitation maxima and minima of fluorescein so that no modifications are required for the confocal instrumentation when GFP is the chosen reporter. Spectral variants of GFP including blue, yellow and cyan fluorescent proteins and other proteins such as DsRed (from *Discosoma* sp. Red) are now available for multiple wavelength imaging [28]. A new technique, called optogenetics allows the use of light to control behavior [29].

3.6 Multidimensional Imaging

As confocal instrumentation has been improved, the collection of multidimensional images has become more practical (Fig. 14). 4D data sets are Z-series of optical sections collected over time from living preparations [30]. It is important that the phenomenon of interest is not faster than the time it takes to collect each image stack for each time point in the series of images. Multidimensional data sets can be huge and becomes computationally challenging to manage. Extra “dimensions” continue to be added. For example, the collection of multiwavelength images as Z-series over time has been called “5D imaging”. Methods are available for the analysis and visualization of multidimensional data [31].

3.7 Reflected Light Imaging

Unstained preparations can be viewed with the LSCM using reflected (backscattered) light imaging [32]. This mode is often overlooked, and can often provide additional information from a specimen with relatively little extra effort (Fig. 15). Reflected light imaging usually requires a different filter combination to be inserted into the scan head. Specimens can be labeled with probes that reflect light such as immunogold or silver grains [33]. Intrinsic proteins such as collagen can also be imaged using this method.

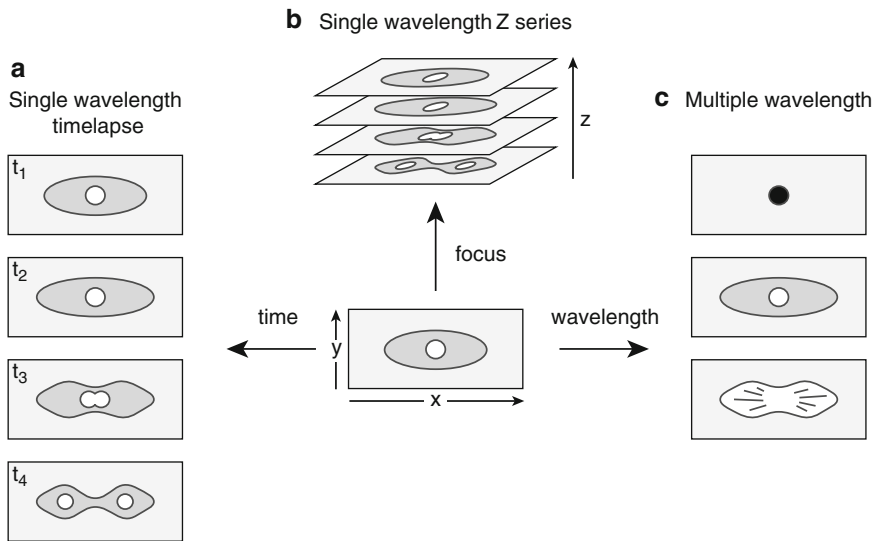


Fig. 14 Multidimensional imaging. (a) Single wavelength excitation over time 2D imaging; (b) Z-series or single wavelength over depth (3D imaging). The combination of (a and b) 3D over time is 4D imaging. (c) 3D multiple wavelength imaging. Over time is 5D imaging

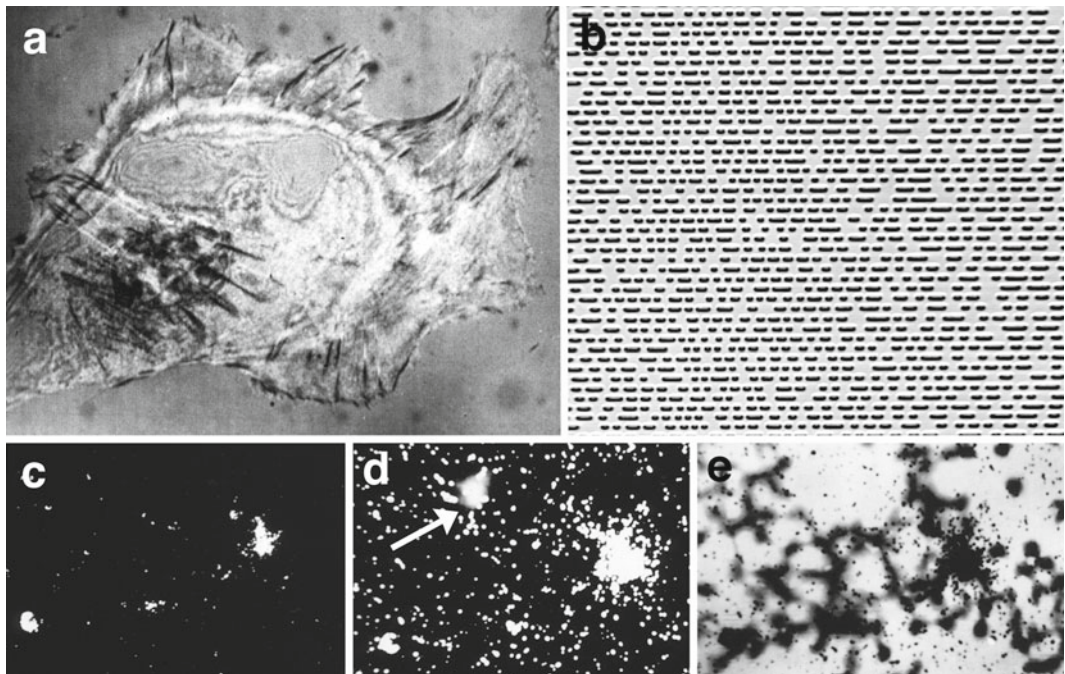


Fig. 15 Reflected light confocal and transmitted light non confocal imaging: (a) Reflected light image of an unstained living 3T3 cell focused at the interface of the cell with the coverslip. Such images are similar to those of cell substratum contacts produced by interference reflection microscopy. Here the contacts appear as *black* streaks around the cell periphery. (b) Confocal microscopes are used extensively in the materials sciences—here the surface of an audio CD is shown and represents a convenient test specimen. (c–e) In situ hybridization of HIV infected blood cells. The silver grains are 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 caused by reflection from a dust particle out of the focal plane of interest. The reflection is visible in the transmitted light dark field image (arrow in d) but not in the confocal reflected light image (c)

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 by inserting polarizers into the light path of the LSCM or by electronically zooming away from the artifact, and off the optical axis. The reflection artifact is not present in the slit or multiple beam scanning systems.

3.8 Transmitted Light Imaging

Any form of wide field light microscope image, including bright field, phase contrast, DIC, polarized light or dark field can be collected using the LSCM equipped with a transmitted light detector (Fig. 15). This device collects light that passes through the specimen and through the condenser of the light microscope. It essentially reverses the imaging process by using the objective lens as the condenser and the condenser as the lens. This results in a non-confocal image.

The signal is usually transferred to one of the PMTs in the scan head via a fiber optic. Since the confocal fluorescence images and transmitted light images are collected simultaneously using the same excitation beam image registration is preserved. It is often informative to collect a transmitted, non-confocal 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 (confocal image) within an unlabelled population of cells (non confocal transmitted light image) have been imaged.

An alternative to using a transmitted light detector is to collect an image with a conventional digital video camera attached to a side port of the light microscope.

3.9 Correlative Microscopy

Correlative, or “integrated microscopy”, is an approach where images are collected from the same region of a specimen using more than one microscopic technique [34]. The most usual combination of instruments is to use the light microscope to image living cells, and then to take advantage of the improved resolution of transmission electron microscopy to image the same region after fixation.

Confocal microscopy has been used in combination with transmission electron microscopy (TEM) to image the same region of the cell. For example, the distribution of microtubules within fixed tissues has been imaged using the LSCM, and the same region was imaged in the TEM [35]. Here, eosin was used both as a fluorescence marker in the LSCM and as an electron dense marker in the electron microscope. Reflected light confocal imaging and TEM have also been used in correlative microscopy to image focal adhesions in living cells growing on a glass coverslip in culture using the LSCM and the same region at higher resolution using the TEM. Rapid specimen preparation techniques such as high pressure freezing (HPF) that can preserve cellular structure for subsequent observation by high resolution TEM have proven to be very powerful for correlative experiments.

4 Measurements

Improvements in confocal instrumentation and the development of new fluorescent reporter probes of biological activity have enabled a new level of precision when the confocal microscope is used as a tool for quantitative imaging of biological events within living cells. Most measurements are based on the confocal instrument's ability to accurately record the brightness of and the wavelength emitted from a fluorescent probe within a sample over time at high spatial resolution.

4.1 Intensity

Intensity measurements are made either by using the software provided with the confocal imaging system or a secondary software, for example the public domain ImageJ or FIJI software package. The brightness of the fluorescence from the probe is calibrated to the amount of probe present at any given location in the cell. For example, the concentration of calcium is measured in different regions of living embryos using calcium indicator dyes whose fluorescence intensity is in proportion to the amount of free calcium in the cell. Many probes have been developed for making such measurements in living tissues. Controls are a necessary part of such measurements since photobleaching and dye artifacts during the experiment can obscure the true measurements of physiological levels of calcium inside cells.

4.2 Fluorescence Resonance Energy Transfer (FRET)

The multichannel feature of the LSCM is used for fluorescence resonance energy transfer (FRET) measurements of protein-protein interactions inside cells [36]. FRET occurs between two fluorophores when the emission of the first one (the donor) serves as the excitation source for the second one (the acceptor). FRET only occurs when the donor and the acceptor molecules are extremely close to one another, at a distance of 60 angstroms or less. In this way, sub-resolution molecular measurements are made [37]. For example, the excitation of a cyan fluorescent protein CFP-tagged protein has been used to monitor the emission of a yellow fluorescent protein YFP-tagged protein. YFP fluorescence will only be observed under the excitation conditions of CFP if the proteins are close enough together for excitation. Since this can be monitored over time, FRET has been used to measure direct binding of proteins or protein complexes.

4.3 Fluorescence Lifetime Imaging (FLIM)

Measurement of fluorescence excited-state lifetimes can provide another dimension of information from a fluorophore that is essentially independent of the energy (wavelength) of the emitted photons, and can therefore be used to distinguish photons from different fluorophores that have similar wavelengths [38, 39]. FLIM is a measure of how long an excited fluorophore stays in the excited state before decay. Furthermore changes in the microenvironment

including pH, proximity to other proteins and hydrophobic regions can affect lifetime. Thus lifetime can be used as a noninvasive read-out of cellular interactions and microenvironment changes [40].

There are two ways to measure fluorescence lifetime, in frequency or the time domains. Many live cell imaging biologists favor time domain measurements because they can use a specific time domain method, Time Correlated Single Photon Counting (TCSPC) system that minimizes the effects of noise sources such as multiplier gain noise in photodetectors. These systems are readily available on many modern confocal microscopes. However, limited photon-counting rates of currently available FLIM systems reduce the dynamic range of measurements and necessitate the use of long exposure times. Further development of FLIM in both the frequency and time domain is underway to allow for faster acquisition.

4.4 Fluorescence Recovery After Photobleaching (FRAP)

This technique uses the high light flux from a laser to locally destroy fluorophores labeling specific macromolecules to create a photobleached zone [41]. The observation and recording of the subsequent movement of undamaged fluorophores into the bleached zone using confocal microscopy gives a measure of molecular mobility.

4.5 Photoactivation

A second technique related to FRAP, photoactivation, uses a probe whose fluorescence can be induced by a flash of short wavelength (UV) light. The method employs “caged” fluorescent probes that are locally activated (uncaged) by a pulse of UV light [42]. More recently, variants of GFP have been expressed in cells and selectively photoactivated. The activated probe is imaged using a longer wavelength of light. Photoactivation has the advantage of a superior signal to noise ratio to FRAP.

5 Alternatives to Confocal Microscopy for Producing Optical Sections

The simplest method of producing optical sections is using a conventional light microscope equipped with differential interference contrast (DIC) optics. This technique is useful for imaging unstained and relatively transparent living specimens, for example sea urchin eggs and embryos. DIC lacks the signal to noise ratio and specificity of the fluorescence technique, however. DIC has been used mainly in the transmitted light function of the LSCM to map specific fluorescence to landmarks in the DIC image.

5.1 Structured Illumination

This technique uses conventional epifluorescence microscopy with a grid structure inserted into the illumination path. Several images are collected with the grid in different positions. Optical sections are subsequently calculated from the images using a computer

program. This is the basis of the ApoTome microscope, which is a relatively inexpensive option for producing optical sections [43].

5.2 Deconvolution

Deconvolution is a computer-based method that calculates and removes the out-of-focus information from an image after a stack of fluorescence images has been collected [44]. The method uses images collected from a conventional epifluorescence microscope equipped with a stepper motor attached to the fine focus control so that images are collected at precisely defined intervals between focal planes in the specimen. This method is used for routine analysis and is especially suited for imaging smaller specimens such as yeast and bacteria where there is insufficient signal for imaging with the LSCM.

5.3 Multiphoton

Multiphoton microscopy uses a scanning system that is identical to that of the LSCM [45, 46]. There is no need for a pinhole, however, because the long wavelength infrared laser only excites at the point of focus, and therefore a pinhole is not necessary (Fig. 16). Fluorophores in the specimen are simultaneously excited by two or three photons to produce excited state transitions that are equivalent to single-photon fluorescence. This is called nonlinear excitation. For example, two and three photon excitation at 900 nm is equivalent to excitation by higher energy photons of 450 and 300 nm, respectively.

Cell viability is generally improved using multiphoton microscopy as compared with confocal microscopy since the excitation wavelengths are in the longer infrared range and the wavelengths utilized are past many of the known UV check-points for biological damage.

Multiphoton microscopy enables penetration 2–3 times deeper into thick specimens than confocal microscopy although this figure is very specimen dependent based on changes in refractive index and scattering properties. This allows investigations on thick living tissue specimens that would not otherwise be possible with conventional imaging techniques [47]. Multiphoton imaging is usually chosen for imaging living cells and tissues both in vitro and in vivo. Multiphoton is also compatible with other nonlinear optical methods such as Second Harmonic Generation (SHG) that can be used to look at intrinsic proteins with a non-centrosymmetric ordered structure such as collagen [48].

5.4 Scanned Light Sheet Microscopy

This method uses a thin sheet of laser light for optical sectioning with an objective lens and CCD camera detector system oriented perpendicular to it (Fig. 16). This technique was developed to improve the penetration of living specimens and enables the imaging of live samples from many different angles at a cellular resolution. The technique has been realized by selective plane illumination microscopy (SPIM) where the specimen itself is rotated in the

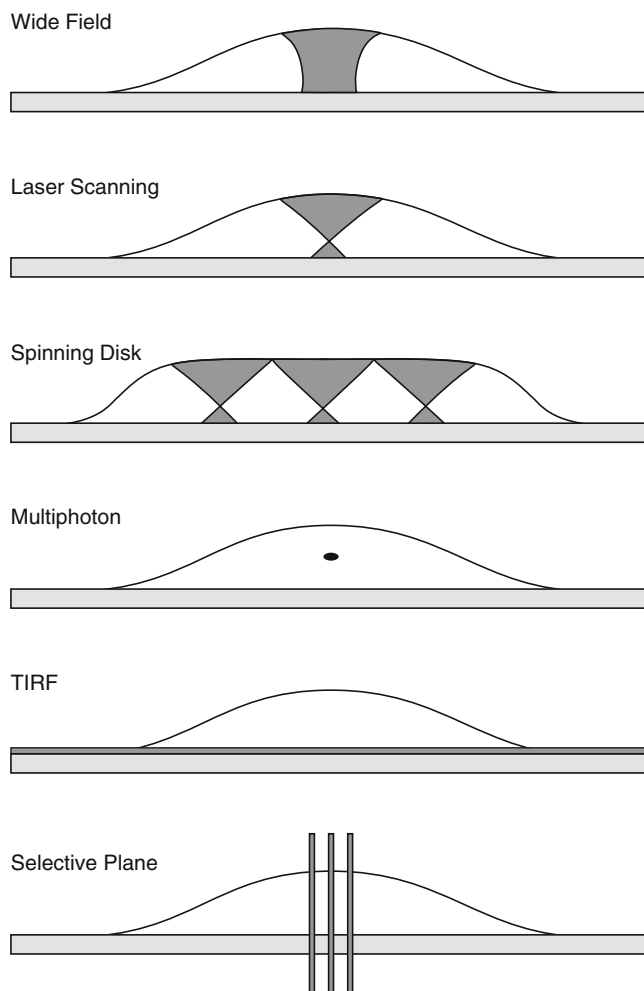


Fig. 16 Illumination profiles in different modes of optical sectioning microscopy. (a) Wide field epifluorescence microscopy; (b) Laser scanning confocal microscopy; (c) Spinning disk confocal microscopy; (d) Multiphoton microscopy; (e) Total internal reflection (TIRF) microscopy, and (f) Selective Plane illumination microscopy. The diagram shows a schematic of a side view of a fluorescently labeled cell on a coverslip. The shaded grey areas in each cell depict the profiles of fluorescent excitation produced by each of the different microscopes. Conventional epifluorescence microscopy (a) illuminates throughout the cell. In the laser scanning (b) and spinning disk (c) confocal microscopes, the fluorescence illumination is throughout the cell but is focused at one (b) or multiple (c) points in the specimen. In the multiphoton microscope (d), excitation only occurs at the point of focus where the light flux is high enough. In TIRF (e), a 100 nm thick region of excitation is produced at the glass water interface, and for selective plane illumination (f), a plane of laser light is produced that is perpendicular to the axis of viewing, and the specimen itself is moved in this beam. Image drawn by Leanne Olds

beam. Advantages of the technique include low phototoxicity and high acquisition speed. The technique has been used to image every nucleus in zebra fish embryos over 24 hours of development at stunning resolution. Multiphoton SPIM is under development for greater depth penetration [49, 50].

5.5 Total Internal Reflection Fluorescence Microscopy

This technique, usually referred to under the acronym, TIRF, is designed to probe the surface of fluorescently labeled living cells [51]. An evanescent wave is generated by a light beam traveling between two media of differing refractive indices. In practice, an incident laser beam is reflected at a critical angle (total internal reflection) when it encounters the interface between a microscope glass slide and the aqueous medium containing the cells (Fig. 16). Fluorophores within a few nanometers of the surface are excited by the evanescent wave, while those farther away are unaffected. TIRF gives much improved resolution in the Z-axis—TIRF 0.3 μm vs. confocal 0.7 μm vs. wide field fluorescence 1.6 μm .

The technique is commonly employed to investigate the interaction of molecules with surfaces, an area that is of fundamental importance to a wide spectrum of disciplines in cell and molecular biology.

5.6 Super Resolution Methods

Several methods are now challenging the resolution limit of the light microscope [52]. Up until relatively recently, the dogma was that the limit of resolution of the light microscope was dependent on the wavelength of light used, and was fixed at around 150–200 nm. Higher resolutions could only be achieved using electron microscopy, and therefore only fixed specimens were imaged.

New “super resolution” light microscopes are able to achieve resolutions down to 20–30 nm in the lateral dimension and 60–70 nm in the axial direction, and in living cells. Such techniques include fluorescence photoactivation localization microscopy (FPALM) with a resolution of 20–30 nm, stimulation emission depletion microscopy (STED) with a resolution of 30–80 nm, stochastic optical reconstruction microscopy (STORM) with a resolution of 20–30 nm and 3D structured illumination (SIM) with a resolution of 100 nm. These are all exciting improvements for live imaging of sub-cellular structures and are becoming commercially available.

5.7 Optical Projection Tomography

Optical projection tomography (OPT) is useful for imaging specimens that are too big to be imaged using other microscope-based imaging methods, e.g., vertebrate embryos [53]. Here the resolution is better than that achieved using magnetic resonance imaging (MRI) but not as good as confocal microscopy. OPT can take advantage of some of the similar dyes used in confocal microscopy.

5.8 Whole Animal Methods

Various instruments have been designed over the years for imaging cells in living animals [54]. There are two main approaches; mini microscopes that can be mounted on an animal for long term observations or hand-held probes that can be pressed against an animal for immediate diagnostic imaging. This continues to be an area of active research with the development of new lenses for efficient light capture in vivo and fiber based endoscopes that can capture the signal in vivo.

6 The Final Image

Confocal microscopy is routinely used to produce high-resolution images of single, double and triple labeled fluorescent samples. The images are collected as single optical sections (2D imaging), as Z-series (3D imaging), as time-lapse series (2D over time), as Z-series over time (3D over time or 4D imaging), or as multiwavelength, 3D over time (5D imaging). Since the images collected by a confocal instrument are confocal instruments in a digital format, they can be further manipulated using a range of software.

6.1 Recording the Image

Minsky's original microscope suffered from a problem with the final images. The instrument produced a ghostly image on a low-resolution oscilloscope screen. Moreover, it was not possible to record the images in a publication. In contrast, the images produced and published by the Cambridge group from their first LSCM drew the attention of the biological research community to the true potential of confocal microscopy because the final images were so impressive on the journal page.

Most of the confocal images produced at this time were single label grayscale images that were recorded as hard copies by photographing the screen of the computer monitor using a 35 mm camera or using a video printer. Single colors were added digitally using a color look up table (LUT). Color images of double label specimens were produced as red green images, and were again recorded by photographing the screen of the computer using color film in a 35 mm camera.

The current generation of confocal instruments takes advantage of modern methods of digital image display and reproduction so that images produced by the microscope are exactly the same as those delivered to the publisher for reproduction on the printed page and for access on journal Web pages.

Selected images are usually prepared for publication using an intermediary program, for example Adobe PhotoShop or ImageJ or FIJI. Such programs are useful for cropping and arranging images into a plate for publication. Images can be matched for brightness and contrast levels. Most of these manipulations were previously achieved using long hours of skillful chemical manipulation using photographic methods in a darkroom.

Such digital programs are capable of much more, and open up the possibility of unethical image manipulations. Many journals now publish guidelines and best practices for digital image preparations. Some basic practices should be followed, for example always keep all of the original raw data from each experimental run and keep notes of what operations were performed to produce the final published image.

6.2 Presenting the Image

Most of the information contained in a confocal image of a biological specimen is related to the spatial distribution of various macromolecules. Images of different macromolecules are collected at different wavelengths. At the present time images collected at three or four different excitations are routine using the LSCM.

A convenient method for the display of two or three colored images is to use the red, green and blue channels of an RGB color image within PhotoShop where any overlap (colocalization of fluorescent probes) is viewed as a different additive color when the images are colorized and merged into a single three-color image [55].

Several simple applications of this three color merging protocol include the mapping color to depth in Z-series, mapping color to time in a time-lapse series, the production of red/green or red/blue stereo anaglyphs from Z-series and merging confocal and transmitted light images (Fig. 17).

The combination of colors within a three color merged image is important for clearly conveying the biological information collected by the microscope. The true emission colors of two of the most commonly used fluorophores, rhodamine and fluorescein, are, conveniently, red and green, respectively, and overlapping domains of expression are yellow. Also some of the commonly used nuclear dyes that are excited in the near UV, such as Hoechst 33342, emit in the blue. These are the colors observed by eye in a conventional epifluorescence microscope equipped with the appropriate filter sets for simultaneous double label imaging. However, the third channel in a triple-label sample prepared for confocal analysis usually emits in the far red, e.g., Cyanine 5, which is conveniently shown as blue in digital images whereas the real Cyanine 5 emission is often extremely difficult to visualize by eye and not so easily depicted in a digital image. By rearranging the grayscale images, the best combination of colors that conveys the maximum amount of information, and best color balance can be achieved.

Additional images at different wavelengths are theoretically possible given enough lasers and filter combinations. However, many such multiparameter images rapidly become complex and difficult to interpret when more than three of them are colorized and merged unless the images contain many regions of nonoverlapping structures, for example, chromosomes painted with fluorescently labeled DNA probes or individual neurons labeled with specific combinations of dyes.

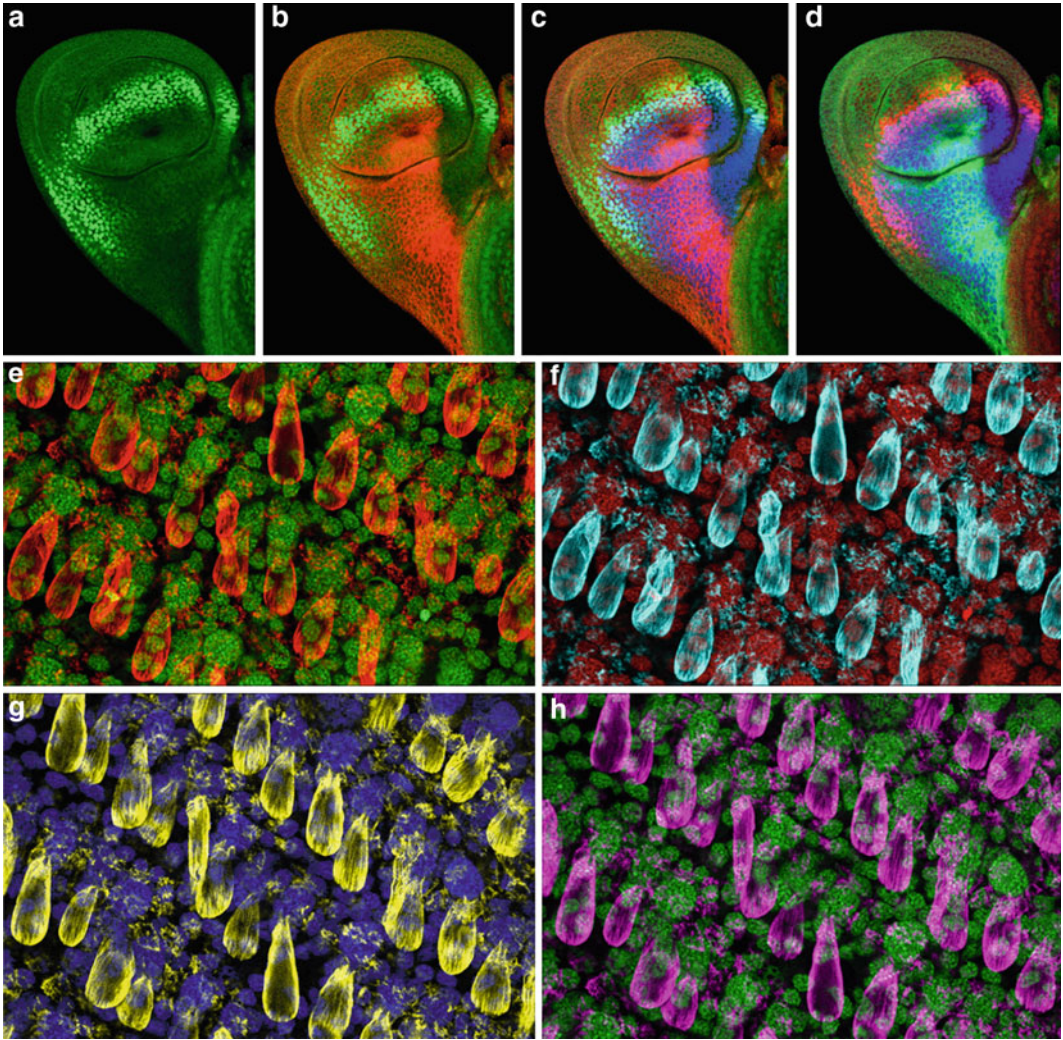


Fig. 17 Image presentation. Using PhotoShop it is a relatively simple task to experiment with various color combinations for optimizing informational quality by rearranging the grayscale images into different *red*, *green*, or *blue* color channels. A single label image is colored *green* (a), a double label image is colored *red* and *green* (b) and a triple labeled image is colored *red*, *green*, and *blue* in two different color combinations (c, d) simply by experimenting with cutting and pasting grayscale images. The specimen is a triple labeled third instar *Drosophila* haltere imaginal disk. In addition to displaying the relative distribution of up to three different macromolecules within cells, this method of combining the three images can be used as an alternative to 3D reconstruction for displaying depth information within a specimen (e–h). Here developing butterfly wing scales are viewed in *red*, *blue*, *yellow*, or *purple* growing out of the pupal wing epithelium colored *green*, *red*, *blue*, or *green*

6.3 Making Movies

The problems of presenting time-lapse series in a publication have been largely solved by the ability to publish QuickTime movie files on the Web pages of various journals or on a dedicated YouTube page. Photoshop also provides a bridge to additional image processing. For example sequences of confocal images of different

stages of development have been manipulated using Photoshop, and subsequently transferred to a commercially available morphing program such as Morpheus, and processed into short animated sequences of development. These sequences can be further edited and compiled using Final Cut Pro, and viewed as a digital movie using QuickTime software directly on the computer or exported to DVD for presentation purposes.

Since all of the images are in a digital form it is relatively easy to export them into presentation software such as PowerPoint and Keynote.

6.4 Image Storage

It is not usually advisable to store image files on the computer hard disk of the confocal microscope for a long period of time or even on a server since space can be limited on a multiuser confocal instrument and also hard disks are notorious for unpredicted crashes especially on computers with multiple users. Many laboratories are now using redundant array based servers (RAID) but even these don't necessarily offer a long-term storage solution and for many labs may be outside their accessibility. It is therefore a good practice to archive image files as quickly as possible after acquiring them. There are several options for archiving image files, including DVD writers and long-term offline redundant hard drive backup. Ideally copies of the most valued files should be stored in at least two different locations.

6.5 Image Informatics

While much emphasis has been placed on the development of specimen preparation techniques and confocal instrumentation for collecting optical sections, the ability to cope with large numbers of images and correspondingly large datasets has been somewhat overlooked in the past [56]. With the increasing use of digital image capture microscopy in the biomedical sciences, it has become a major challenge to locate, view, and interpret large numbers of images collected in a diversity of formats [57].

Many biological research laboratories have a pressing need to archive and annotate vast numbers of images collected by video, laser-scanning microscopy and other photonic-based imaging techniques [58]. Multidimensional images, such as four-dimensional images from multifocal plane time-lapse recordings, or images from spectral and lifetime microscopy, make the challenge even greater. Without careful organization, important research data can be difficult or impossible to find, much less visualize and analyze effectively. This need has spawned the field of "Image Informatics" to develop tools to aid in the management, sharing, visualizing, and the analysis of datasets collected using many different biological imaging platforms with a major emphasis on confocal microscopy [59, 60].

A prominent example of an image informatics platform is the Open Microscopy Environment (OME). OME is a consortium of companies and academics with the mission of developing open

source tools for biological image data management [61]. A unique and important emphasis of OME is the priority it places on having tools that not only can analyze and share the binary image data but the full metadata, which can include instrument, user and experimental information. Unfortunately there are currently over 150 proprietary microscopy formats in use and OME tools like Bioformats enable the full reading and open sharing of these formats in many programs. As confocal microscopy has become increasingly quantitative and the need and interest to analyze and annotate data from other sources increases, the importance of metadata is only going up. The need for tools like OME to analyze and share the original pixel and metadata information has become more vital.

6.6 Resources

This introductory chapter serves as a primer of confocal microscopy and related optical sectioning techniques and more-detailed information on specific topics can be found in subsequent chapters of this book. The field is huge and continues to grow. Here we provide references to several books and review articles [2, 6, 7, 11, 62–66] together with a list of some of our favorite Web sites (Table 4) as a starting point for gathering more-detailed information and specific protocols.

Table 4

A selection of popular Web sites (active at the time of printing) on all aspects of confocal microscopy from the technical to the artistic

“Microscopy U—The Source for Microscopy Education” http://www.microscopyu.com/
“Molecular Expressions—Exploring the World of Optics and Microscopy” http://www.microscopy.fsu.edu/
“Microscopy Society of America” http://www.msa.microscopy.org
“The Royal Microscopical Society” http://www.rms.org.uk
“The Open Microscopy Environment” http://www.openmicroscopy.org
“ImageJ” http://rsb.info.nih.gov/ij/
“Fiji” http://fiji.sc/Fiji
“Wellcome Trust Microscopy Resource” http://www.well.ox.ac.uk/external-website-links
“Nikon Small World” http://www.nikonsmallworld.com/
“Olympus BioScapes” http://www.olympusbioscapes.com/

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