Confocal microscopy: A powerful technique for biological research

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Confocal microscope permits the generation of three-dimensional images of biological and nonbiological specimens. The efficacy of this technique lies in the elimination of out-of-focus glare by spatial filtering, utilizing a point source of light for excitation, and a pinhole confocal with the excitation pinhole in front of the detector. A combination of transverse resolution with noninvasive optical sectioning results in very high quality images of biological specimens. Several combination of lasers can be coupled to the fibre optics of the scanning unit in order to increase the number of excitation wavelengths. Powerful softwares that display and analyse 3-D data are currently available. Laser scanning confocal microscopy has proved to be most suitable for the analysis of structural details of thick specimens and promises to be of great potential in providing 3-D volume renderings of living cells and tissues over time.

CONVENTIONAL light microscopy allows the observation of living as well as fixed cells and tissues to generate two-dimensional images. The out-of-focus information often obscures the ultrastructural details, especially in thick specimens with overlapping structures[1–7]. The earliest available light microscopy visualized the objects in hydrated state in two-dimensions during their temporal development. The emergence of electron microscopy (EM) provided superb resolution of ultrastructural details, but it was applicable only for objects in the dehydrated state and thereby potentially introducing handling artefacts[1–2,8]. The usefulness of optical methods, however, has been limited by the poor depth discrimination. Often, the fluorescence and reflectance images are severely degraded by the scattered- or emitted-light from tissue structures outside the plane of focus. These limitations have been partially overcome by video image processing[1–2,4,5] and deconvolution[3]. Laser scanning confocal microscopy (LSCM) overcomes the above difficulties and produces improved light microscopic images of fixed as well as living cells and tissues[2,3,4,9,10]. In terms of resolution of the image, the confocal microscope occupies a position in between the light and electron microscopes[7,11,12].

Confocal microscope generates information from a well-defined optical section rather than from the entire specimen, thereby eliminating the out-of-focus glare and increasing the contrast, clarity and detection sensitivity[2,12,13]. Optical sectioning is noninvasive and less time consuming compared to reconstruction algorithms to give 3-D images[4,11,14–16]. Optical sectioning is achieved not only in the *xy* plane (perpendicular to the optical axis of the microscope) but also vertically in the *xz* or *yz* plane (parallel to the optical axis)[2,3,10]. With vertical sectioning, cells are scanned laterally (*x* or *y* axis) as well as in depth (*z* axis). Stacks of optical sections taken at successive focal plane (known as *z* series) are then reconstructed to generate a 3-D version of the specimen. The 3-D image can be directly visualized where each data point represents the quantity of specific contrast parameter used at a certain point in space. The image processing can be additionally used to enhance the confocal images. It is widely used in the fluorescence mode for different specimens and in bright field reflection mode for objects of different forms.

Principle of confocal microscope

The principle of confocal microscope was introduced by Minsky[17,18]. The method of image formation in confocal microscope differs fundamentally from conventional wide field microscope (Figure 1). In conventional microscopy, the entire specimen is illuminated uniformly and simultaneously along the plane in which the objective lens is focused (Figure 1 *a*). This results in an out-of-focus blur from areas above and below the focal plane of interest, thereby reducing the contrast and the resolution of image. Illumination in confocal microscopy, on the other hand, is not simultaneous but sequential[2,15,18]. The illumination from a laser source is focused as a spot on one volume element of the specimen at a time (Figure 1 *b*). A point light source is imaged in the plane of the object and the fluorescence emitted from the object is directed to a photomultiplier through a detector pinhole. A computer displays the point as a pixel on screen. In order to produce a complete image, the

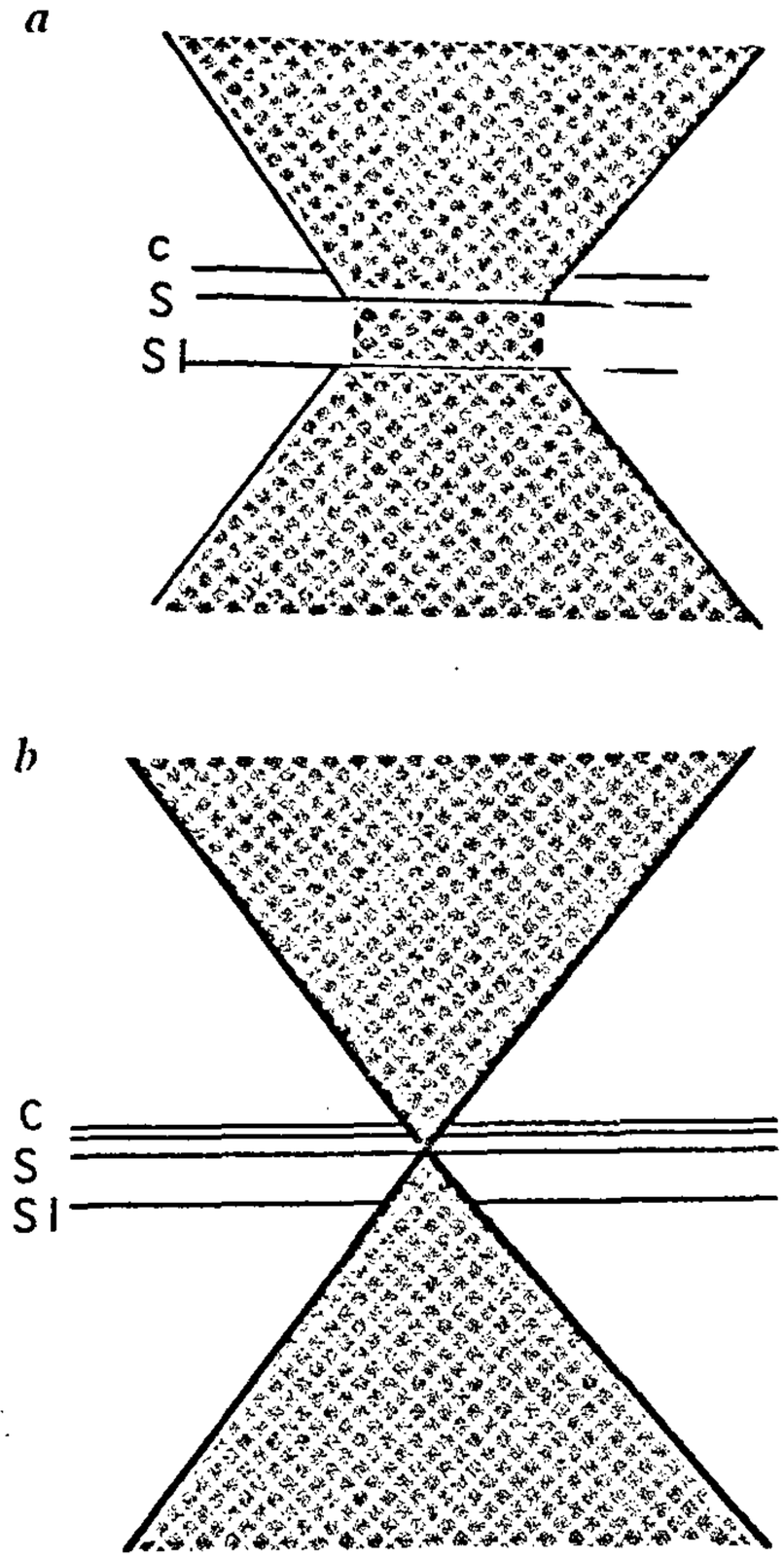

Figure 1. Illumination of specimen in conventional and laser scanning confocal microscopy. In conventional microscopy (*a*), the entire depth of specimen is illuminated continuously which results in the detection of out-of-focus and in-focus signals together, causing loss of resolution. In LSCM, the specimen is illuminated sequentially covering specific points at a time (*b*). These images of points which are devoid of out-of focus signals are then added to form a complete in-focus image of the specimen. (C, coverslip; S, specimen; Sl, slide). (Adapted from Ref. 15)

light point is moved over the entire object line by line, by means of a mirror. The image is generated simultaneously on a monitor. The arrangement of the detector pinhole, confocal with the illumination pinhole, ensures that the information from only the focal plane reaches the detector. Light from out-of-focus elements is virtually eliminated (Figure 2). This accounts for the unique ability of the confocal microscope to create images of sections through a sample. The focusing stage and the computer allows the generation of a whole series of sectional images which can be stored. These data records are used to create 3-D images in a number of ways. Topographic measurements can also be made easily.

There are at least three different technical setups of the confocal microscopes: (i) True-point scanning system using point illumination and detection,

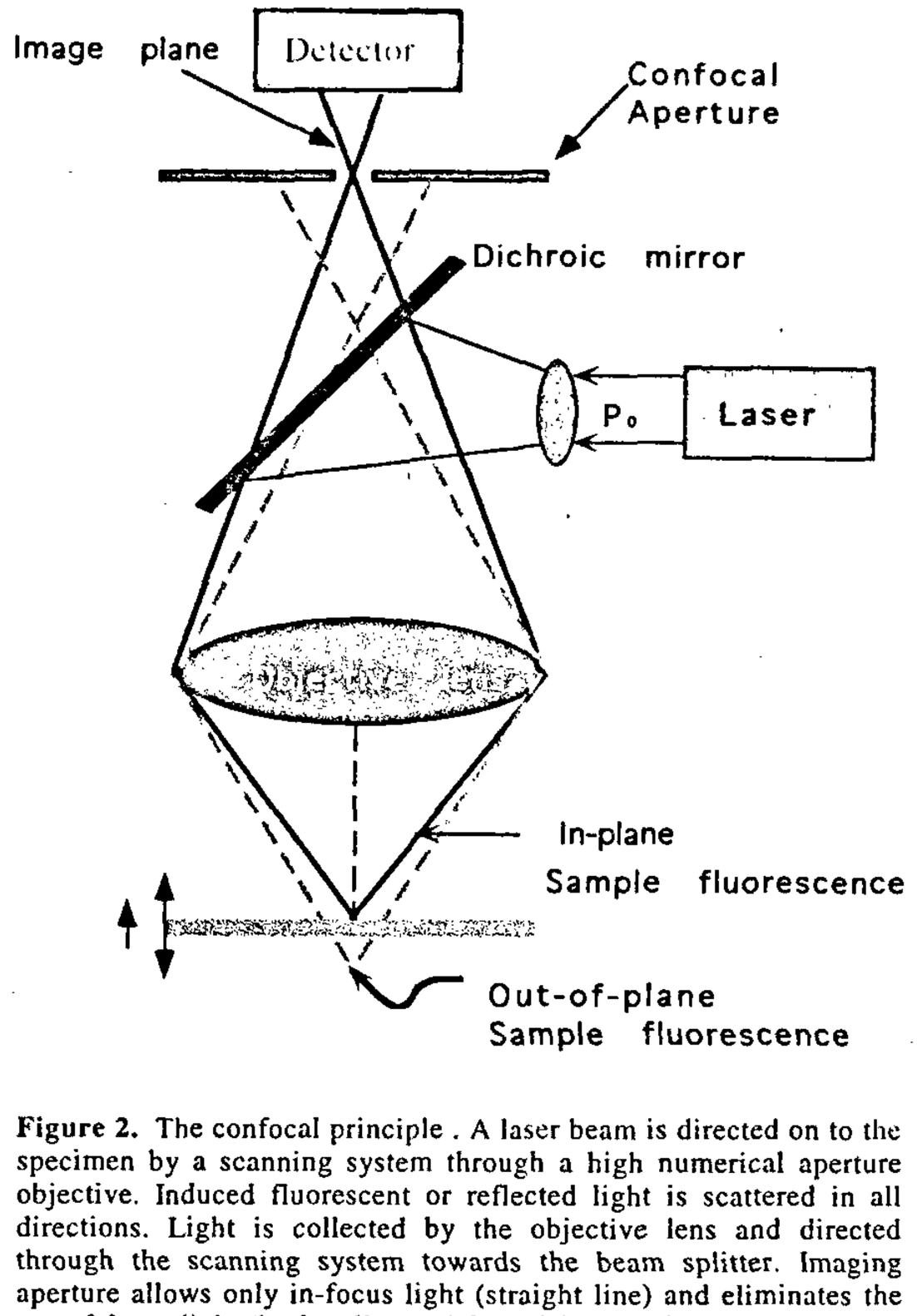

Figure 2. The confocal principle . A laser beam is directed on to the specimen by a scanning system through a high numerical aperture objective. Induced fluorescent or reflected light is scattered in all directions. Light is collected by the objective lens and directed through the scanning system towards the beam splitter. Imaging aperture allows only in-focus light (straight line) and eliminates the out-of-focus light (broken line). (Adapted from Ref. 65)

(ii) Slit-scanning systems using a slit instead of the pinhole in front of the detector, and (iii) Disk scanners using multiple pinholes on a rotating disk for scanning and detection. True-point system has high sensitivity and the theoretical *x*-, *y*- and *z*-resolution. Slit- and disk-scanning systems provide the benefit of real time observation of the specimen but there are drawbacks and compromises with respect to *z*- and *x*-resolution, higher backgrounds and fluorescence yields. Confocal microscope achieves a dramatic 1.4-fold increase of resolution by physical means compared to the normal optical microscopes[5,15].

Historical perspective

The confocal microscope was invented specifically for studying neural networks in the living brain[17,18]. Zirconium arc was used as a light source with a pinhole in front of it. The ray of light was focused by an objective lens into the specimen. The light transmitted through the specimen was focused by a second objective lens into a

Box 1. Confocal microscope

Confocal microscope is a versatile tool which permits the generation of 3-D images of biological and nonbiological specimens. A schematic presentation of the confocal microscope is shown in Figure 2. The instrument uses a high numerical aperture (NA) objective to focus the specific wavelength of a laser to a diffraction-limited spot on or in the specimen. An ordinary epifluorescence microscope is used for the purpose, with a semi-silvered mirror (for reflectance) or a dichroic mirror (for fluorescence) serving to introduce the illuminating beam into the microscope optical axis[4]. A pair of microcomputer-controlled galvanometer mirrors beyond the dichroic or semi-silvered mirror steer the spot over the object being viewed. Reflected or fluorescent light from the illuminated spot is returned and descanned by the same galvanometer mirrors, passed by the dichroic mirror, and focused on a pinhole in front of the detector – a low-noise photomultiplier tube (Figure 3). A beam splitter and a second photomultiplier permit dual wavelength fluorescence imaging and ratio measurements. Images are built up from the digitized photomultipliers. The images can be collected more rapidly by reducing the number of pixels and can be summed, subtracted from or added on to the stored images. A wide range of digital-image-enhancement methods including contrast stretching, false colour intensity coding and various image convolutions leading to edge enhancement or other forms of spatial frequency filtering can be applied. By integrating successive images, weakly fluorescent structures can be recorded with a sensitivity comparable to that of a silicon intensified camera.

The beam steering and photodetector apparatus are attached to the microscope in much the same way as an ordinary video camera. The microscope is also equipped with a routine mercury arc epifluorescence system to facilitate rapid location of the areas of interest by direct visual observation of a fluorescence image. A light collector can be mounted below the substage condenser, directing the transmitted laser light to a photomultiplier tube (PMT) through a fibre optic conduit (Figure 3). This way, ordinary bright field, phase contrast or differential interference contrast images of thin specimens can be collected for direct comparisons with confocal fluorescence or reflectance images.

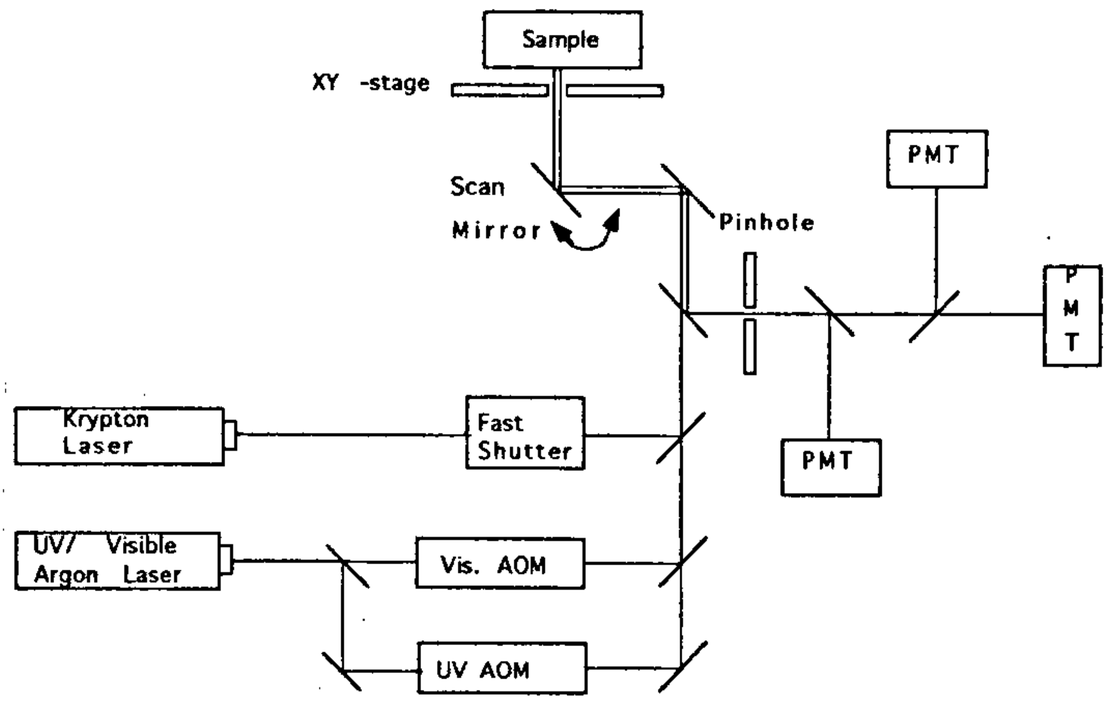

Figure 3. The organization of the confocal microscope and the path of the laser beam.

second pinhole which was confocal (i.e. at the same focus) with the first hole. The light passing through the second hole was detected by a low-noise photomultiplier.

The basic configuration of the modern confocal microscope is a lighter version of Minsky's microscope[17,18], where the beam was stationary and the specimen was moved on a vibrating stage. This optical arrangement had the advantage of scanning always on the optical axis, eliminating lens defects. However, this was used mainly in materials science and only to a limited extent in biological research because the vibratory movement caused wobble and distortion of the image[19]. An alternative to moving the specimen is to scan the beam across the specimen which can be performed either by multiple beam scanning or by single beam scanning. The flying-spot microscope[20] and the scanning-mirror microscope[21] are nonconfocal forerunners of beam-scanning confocal microscopes.

The pioneers of multiple beam technology were interested in imaging the growing nerve cells in intact brain[22]. The tandem scanning reflected light microscope uses a bright white light source and a modified Nipkow disk to scan the specimen with hundreds of points of light. The apertures of this disk were designed to match the excitation and emission of the light path. The disk spins rapidly and acts as a point source, and a spatial filter prevents the out-of-focus light from reaching the photodetector, which can be the human eye or a sensitive video camera. The image viewed is the same as in conventional microscope and shows true colours of the specimen[23].

Fluorescence microscopy, using tandem scanning reflected light microscope, can be a practical option because of the presence of Nipkow disk and the improved efficiency of light[24]. It is also easy to select different wavelengths of light, including UV and IR excitations, by optical filtering for multiparameter fluorescent imaging[25]. The microscope scans in almost real time so that events such as exocytosis during fertilization could be observed[26].

Tandem scanning confocal microscope produces extremely high resolution images, scanning fast enough to make the full image visible. Highly reflective or fluorescent specimens and extremely bright light sources could only be used because the aperture excludes a high proportion of light. This difficulty is overcome by the use of laser illumination with a mechanically scanned specimen[10] which, however, imposes limits on the mass and stability of the preparation and on the speed of the

scan. These limitations have been largely overcome through the development of beam-steering methods to scan the illuminating spot within fixed microscope optics[14].

Advantages of laser scanning confocal microscopy

Confocal microscopy provides two major advantages over the conventional light microscope: (i) the amount of glare from the specimen is reduced by the spatial filtering of the pinhole. In general, by reducing the diameter of pinhole the amount of specimen sampled is reduced to provide a thinner optical section, but there is an optimal pinhole size for every objective lens[27], and (ii) the image resolution is improved by a factor of two if the input and output apertures are both imaged as a diffraction limited spot. These factors vary with the objective lens and with the biological specimen. It is better to use a planapochromat objective lens with the highest possible numerical aperture and absence of phase rings[28]. The system should have flexibility in image generation to overcome the limitations imposed by the biological specimens. For instance, the adjustable pinhole should not only allow confocal conditions ideally required for imaging but also the 'not-quite-confocal' ones to capture images from relatively dim specimens.

LSCM provides dramatic improvement in image quality in thick and brightly-labelled fluorescent specimens such as eggs, embryos and whole tissues. For instance, in embryonic gene expression studies on mulberry silkworm, *Bombyx mori*, epifluorescence microscopy failed to resolve the expression patterns in embryos as they are thickly encased under the vitellophages[29]. Light from fluorescent microscope was unable to pass through the thick embryos, resulting in blurred images, but this problem was overcome by LSCM. The optical sectioning of the embryos permitted visualization of the domains of gene expression in germband (Figure 4). Further, LSCM has been extensively applied in developmental genetic studies in *Drosophila*. A representative example of this application presented here (Figure 5), shows the pattern of gene expression, *patched* (*ptc*) and *wg* (Figures 5 *a*, *b*) respectively, during embryonic development. Optical sectioning improves the resolution bringing out the cellular details even in such specimen as the imaginal discs (Figure 5 *c*). LSCM not only permits imaging of single label (Figures 5 *a*, *b*, *c*) and multiple labels (Figure 5 *d*), but also the superimposition of these images helps in better interpretation and analysis.

White *et al.*[14] have compared images of the same region from a range of specimens using conventional epifluorescence microscope and LSCM. In most cases confocal microscopy provided 3-D structures with significant reduction in the out-of-focus glare. Samples in general, are overstained for LSCM compared to staining for conventional epifluorescence microscopy because the total fluorescence within the entire specimen is not sampled in the former. Further, various antifade agents are used to combat photobleaching of fluorophore by the laser beam[30–32].

Applications of LSCM

The confocal microscope provides novel and better understanding of cellular structures and processes. These include: (i) cellular localization of organelles, cytoskeletal elements and macromolecules such as proteins, RNA and DNA, (ii) tracing specific cells through a tissue, (iii) producing optical sections for stereo image production and three-dimension reconstruction, (iv) imaging in four dimensions, and (v) ion imaging and fluorescence recovery after photobleaching[33]. The confocal microscope can also be used in the reflectance mode, which allows the reduction in out-of-focus blur from nonfluorescent labels such as the diaminobenzidine reaction products formed in cytochemical detection or the silver grains present in autoradiograms during *in situ* hybridization[34]. Various applications of LSCM are summarized in Table 1.

Confocal fluorescence microscopy

The confocal principle is especially valuable in fluorescence microscopy since it almost completely eliminates the stray light coming from the nonfocal plane. Thus, fluorescence images with optimum clarity and resolution of specific details are produced. The use of fluorescent dyes and labels has revolutionized LSCM and a vast collection of such dyes is currently available[5,29,35,36] (Table 2). Optical sectioning power of LSCM has been exploited in many studies to produce images of fixed specimens labelled with single fluorochrome (e.g., studies on neurons[37], assembly and movement of plant viruses[38] and cell lineage and differentiation of corneal cells[39]).

Confocal microscopy allows the observation of two or more structures simultaneously in the same cell or tissue using multiple labelling (Figure 5 *d*). One of the ways is to use the 514 nm excitation line of Argon laser to excite both fluorescein and rhodamine. The light emitted from the two flurochromes on excitation is split into two by filters which is then directed to separate photomultipliers. Alternatively, a Krypton–Argon laser, which has a better separation of fluorescein and rhodamine signals, or two individual lasers can be utilized to produce the same effect. A great advantage of simultaneously collecting images in the same framestore is that they can be merged to map the distribution of the two labels in a tissue. For instance, one of the images can be coloured green and the second image can be coloured red and the regions of overlap will then appear yellow (Figure 5 *d*). The double-labelled specimens collected

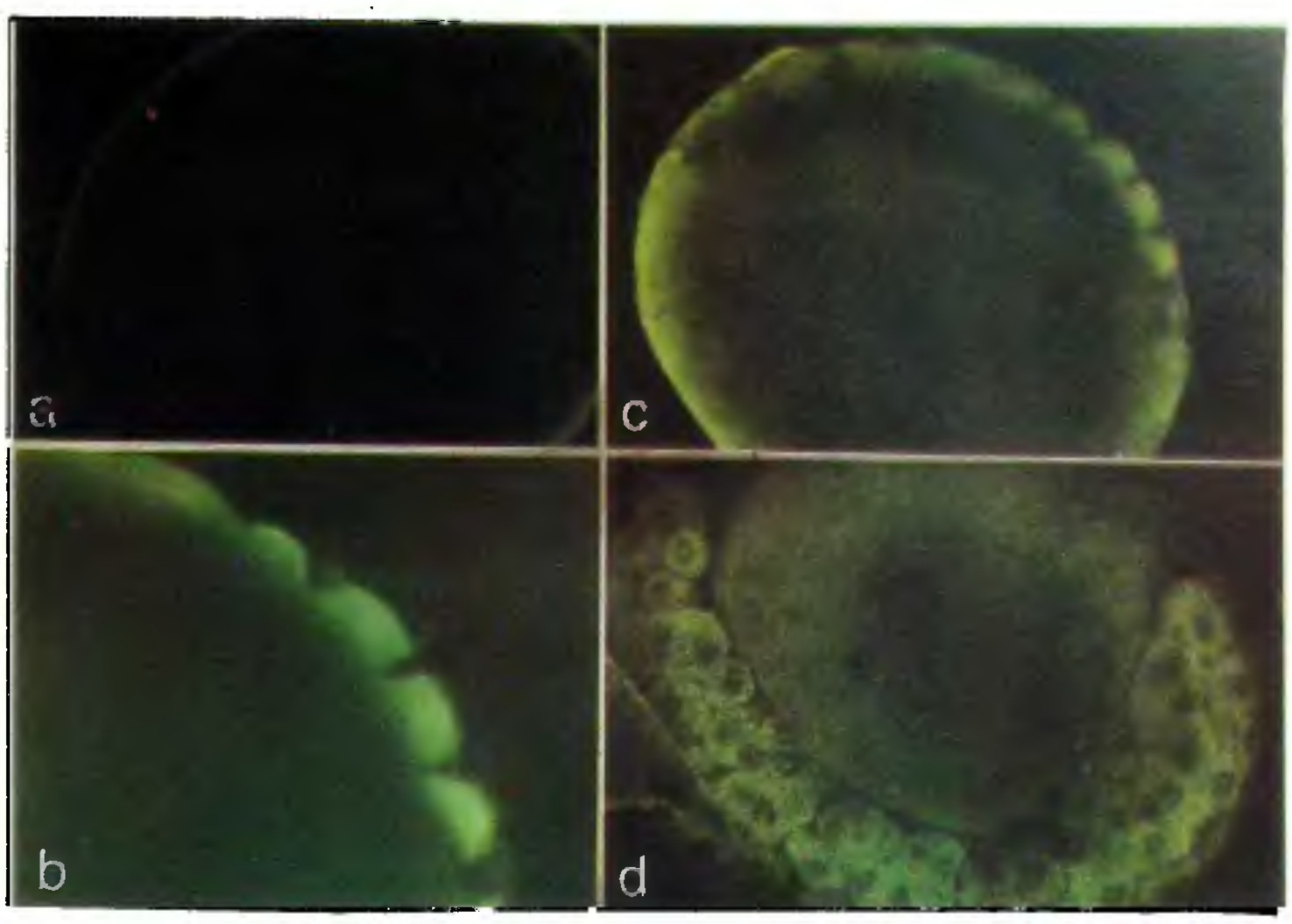

Figure 4. Visualization of gene expression in silkworm embryos. The expression of proliferating cell nuclear antigen (*pcna*) gene in silkworm embryos has been visualized by confocal microscopy, following antibody staining. *Bombyx mori* embryos were dechorionated by treatment with hypochlorite bleach-heptane[29] followed by treatment with primary antibodies and stained using FITC conjugated secondary antibodies. The samples were examined under an argon laser of confocal microscope (Biorad MRC 1000). (*a*) Expression at the syncitial blastoderm stage, at 10 h after egg laying (AEL) (10 ×). (*b*) Developing germband with formation of the segments, at 20 h AEL. (*c*) Magnified view of the developing germband (20 ×) and (*d*) Embryo with well-developed germband, at 35 h AEL. Note the expression is uniformly distributed in the entire germband with several pockets of rapidly dividing cells.

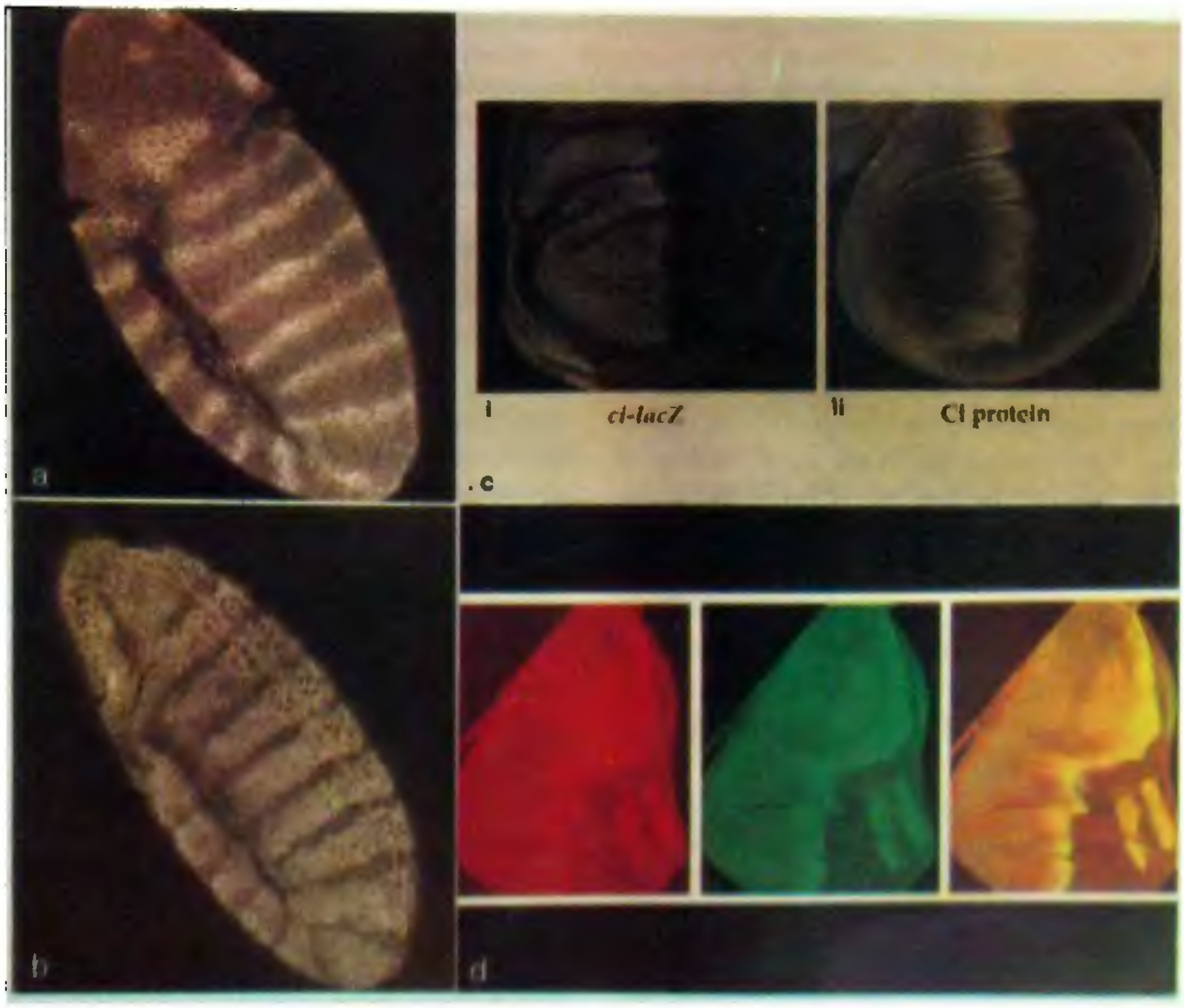

Figure 5. Confocal micrographs of *Drosophila* embryos and imaginal disc. The specimen were treated with primary antibody [*patched (ptc), wingless (wg)* and *cubitus interruptus (ci)]* followed by FITC-tagged secondary antibody and observed under confocal microscope. (*a, b*) *ptc* and *wg* expression in stage 10 embryos. (c) Localization of *ci* using (i) anti βgal antibody in ci^{Plac} enhancer trap line (ii) anti ci antibody in wing imaginal discs. (*d*) Multiple labelling in somatic clones generated by flip recombinase technique in wing imaginal discs [*ci*- (red), *ptc* (green) and *ci* and *ptc* superimposed (yellow)]. Photodocumentation can be done on both black and white and coloured films. (Courtesy: Dr Robert Holmgren)

Table 1. Applications of the laser scanning confocal microscope

Technique	Application	Reference
Confocal reflected light microscopy	Imaging unstained specimen	36, 54–56
Confocal fluorescence microscopy	FISH in thick samples and sections	35, 45–49
	Single label	29, 37–39
	Multiple label	40–42
	Cytogenetics and chromosomal analysis	50, 51
	3-D reconstruction of live tissue	16,40,47,57–60
	Time lapse	62, 63
	Photoactivation of caged compounds	68
	FRAP	69
	Cell–cell communication	70
Laser scissors and tweezers	Chromosome surgery/micro-dissection	78
	Mitosis and motility of cell	
	Cell membrane mechanics and cell fusion	
	Reproductive biology:	
	Sperm and oocyte manipulation	
	Preembryo manipulation,	
	In vivo manipulation of internal cell organelles	79
Atomic force microscopy	Macromolecular imaging	70
Interactive laser cytometer	Membrane potential in single cells	71
	Anisotropy in cell membranes	74
	Diffusion of membrane components and cytoplasmic structures	75
	Ion concentration and imaging in cells	64, 65, 67
	Ratiometric ion measurements in cells	32
	Statistical analysis of immunofluorescence at the level of single cells	78
	Cell sorting	32
	Multi well screening for fluorescence quantitation in cell cultures	
	Analysis of anchored cells for enzymatic induction *in situ*	79

this way are prone to bleed through from one channel to the other and therefore require careful specimen preparation. However, this can be rectified in a variety of ways by digitally subtracting the bleed through of one image in the other, or by using newer dyes whose excitation and emission spectra are better suited to the laser[32]. This method has been successfully applied for the localization of human growth hormone to a subset of cytoplasmic vesicles in PC12 cells[40] and the determination of gene expression during wing development of *Drosophila*[41].

Another improvement for double-labelling is to use two different excitation wavelengths which are sufficiently separated. This is possible either by using a single laser and changing filter blocks, and digitally realigning the images[42] or by using two Krypton–Argon lasers which give good separation of fluorescein and rhodamine[43]. Moreover, the Krypton–Argon laser has a third major line at 647 nm (red) and, therefore, different fluorochromes can be imaged simultaneously with a single laser. In fact, new dyes that are excited around 647 nm line of the Krypton–Argon laser are currently available[32,44]. Imaging at these longer wavelengths, provides improved viability of live samples and deeper penetration into the sample[5].

In situ hybridization technique has been exploited in various model systems for the detection of nucleic acids within the cells and tissues. LSCM has been successfully employed in conjunction with *in situ* hybridization to analyse the spatio-temporal profile of gene expression[35,45–49], genetic diversity[47], cytogenetic and chromosome analysis[50,51].

A nonconfocal transmitted light imaging can also be generated with LSCM[52]. This is extremely useful for image display purposes where a fluorescence image can be merged into a transmitted light image. In many cases, the fluorescence image alone is difficult to interpret without reference to the transmitted light image. The transmitted light image produced by the LSCM can be bright field, phase contrast, DIC or darkfield. The transmitted light detector collects light that passes through the specimen and then pipes it up on to the

Table 2. List of the laser sources and the emission by compatible dyes

Laser	Blue 450nm	Green 530nm	Yellow 560nm	Orange 590nm	Red 610nm	Far Red 640nm
UV-Argon	Cascade blue Hoechst DAPI AMCA Monochlorobimane Indo-1					
Argon		FITC Rhodamine 123 Bodipy FL NBD-PC Fluo-3 Acridine Orange DiOC6 BCECF	Cy3 POPO-3 Phycoerythrin	Acridine orange Bodipy581 Nile Red Hexylrhodamine	Propidium-iodide Red613	Fura red Quantum red
Argon 514 nm				Hexylrhodamine DiL TRITC		
Krypton 568 nm					Lissamine Rhodamine Texas red DiA Mitotracker	
Krypton 647 nm						Cy5 TOTO-3 TO-PRO-3

The dyes listed here include conjugates, DNA-, lipid-, membrane- and cell organelle binding agents, pH , Ca^{+2} and glutathione measuring dyes. (For details see ref. 32).

second photomultiplier tube by means of fibre optics. The double label fluorescence transmitted light technique has been used to study phagocytosis of fluorescent microspheres ingested by murine macrophages[53]. By optical sectioning of the fluorescence image and superimposing the transmitted light DIC image, it is possible to determine the number of fluorescent beads phagocytosed by individual macrophages. This technique is especially useful for cells that tend to form 3-D clumps.

Confocal reflected light microscopy

Reflected light confocal microscopy can be used as an alternative to interference reflection microscopy[54]. LSCM produces improved images of the unstained specimen that reflects enough light to produce contrast in the image. Under conventional light microscope, these specimens scatter so much light that the structural details are obscured. LSCM eliminates much of this scattered light and produces extremely clear images (e.g. for observing cell adhesions to the substratum in tissue culture). In this mode of reflected light, LSCM specular reflection can appear in the image as a series of diffraction rings due to the reflection from eyepiece in the microscope but this can be eliminated by inserting two polarizing filters and a quarter wave plate in the optical path[11]. Reflected light LSCM improves visualization of the silver grains in autoradiographs of specimens prepared by *in situ* hybridization[36]. This method has been utilized in observing samples of human immunodeficiency virus infected peripheral blood cells and tissue sections of placenta[55] and to detect viruses[56].

Confocal microscopy of living tissues and 3-D reconstruction

Minsky's dream of *in situ* observation of live neurons is rapidly being approached by the currently available confocal microscopes in conjunction with the newly developed vital fluorescent dyes. The early LSCM was extremely light inefficient, whereas the recent developments have improved the light budget so that live cell imaging is now a practical option[4,5]. A major prerequisite for successful live cell imaging is to reduce the number of photons that interact with specimen and then

to use all the photons leaving the specimen to produce an image. This is because living cells, especially when fluorescently labelled, are prone to photodamage[57]. The improvements include scanning mirrors with better light efficiency (99%), and enhanced digitized images with a fast photon-counting mode.

Different approaches for observing living cells and tissues using the LSCM have been developed. One of them is to include static imaging of unfixed material either with reflected light or using epifluorescence. This technique has been applied for tracing the pathway of labelled peptides through the cornea[40], visualization of the endoplasmic reticulum in the plants using vital dye DiOC6[58], and the movement of antiproliferative heparin derivatives into rat smooth muscle cells[59]. Another approach is the use of reporter genes. The introduction of the green fluorescent protein (GFP) as a reporter coupled with LSCM has revolutionized the scenario of live-cell-imaging in the modern biological research. GFP has generated enormous interest among researchers studying gene expression in transgenic plant and animal model systems.

There is a tremendous advantage in 3-D image construction from optical sections of LSCM compared to the physically sectioned specimens. The 3-D data sets can be transferred directly into a 3-D reconstruction programme that runs on a graphic workstation. After a relatively trivial file reorganization, they are processed into a 3-D image which can be rotated or dissected on the screen of the graphics computer in a short time[16,47,60]. There are two basic methods currently applicable to the display of confocal data sets – volume reading[13] and geometric surface rendering[61]. The 3-D reconstruction permits the imaging of vitally stained tissues without damaging the specimen by physically cutting sections.

Time lapse has been used for imaging changes in structure of single optical sections or z series with subsequent graphic reconstruction of each time point, sometimes referred to as 4-D imaging. Programmes are now available which automatically collect optical sections over time and store them on a large hard disk or optical disk recorder. These programmes also allow the entire series or individually selected optical sections to be played back over time. This is useful in following a particular structure that moves in the z plane over time. An example of time lapse using the LSCM is the recording of Golgi dynamics in cultured rat hippocampal astrocytes which are labelled with a vital dye which showed both tubulovesicular processes, and small submicron particles emerging from the trans Golgi and migrating along the microtubules[62]. Further, laser photobleaching experiments showed that tubulovesicular processes can provide direct pathways for the diffusion of membrane lipids between joined trans Golgi elements[63].

Confocal ion imaging

Confocal microscopy is advantageous for imaging ions such as calcium because the narrow depth of field allows better visualization of intracellular details by eliminating out-of-focus signals. Besides, the measurement errors due to path length variation are minimized since only a small and constant volume of the cell is sampled through the focal plane. Fast events are imaged using a smaller region, or a point in the specimen. Various fluorescent dyes, which change their fluorescence intensity relative to the concentration of free ions especially calcium, are now available[64]. The dyes available earlier (e.g. FURA-2), were excited at relatively short wavelengths but most of the earlier versions of LSCMs were not equipped with UV source. However, excitation of the shorter wavelength dyes was achieved using two-photon laser scanning fluorescence microscopy[6,7] although the laser tends to be prohibitively expensive for routine work[64,65].

Two-photon excited fluorescence is another major advancement in microscope-based laser manipulation[6,7]. In the conventional fluorescence microscopy, the absorption of a photon excites each fluorescing molecule to a higher energy state, from which the emission of one photon occurs when it returns to ground state. In the two-photon mode there is simultaneous absorption of two laser photons at the desired wavelength from the intended fluorophore. The only point in the cell/organelle at which the photon intensity is high enough to result in two-photon absorption is the intense focal point of the laser beam. Since the molecule absorbs two photons virtually simultaneously, it behaves as if a single photon at half the wavelength of the impinging photons is absorbed, leading to fluorescence emission at a wavelength shorter than excitation wavelength. The two-photon fluorescence was initially developed as an analytical fluorescence technique but it can be used to induce photochemical events at the cellular and subcellular levels. Multiphoton-induced focal plane specific photochemistry could become a powerful tool in future to induce or to suppress site-specific photochemical processes in cells.

Various ion-sensitive dyes which are excited at longer wavelengths and better matched to the argon laser are now available. They include FLUO-3, RHOD-2, calcium green, calcium orange, calcium crimson, FURA-Red for calcium, SNAFL, SNARF, and BCECF for pH measurements[66,67].

Other applications

LSCM also permits photoactivation of caged compounds[68], which provides a rapid method for releasing a specific compound as a discrete spot within a single cell. A short burst of UV breaks the chemical bond of the

caged probe, releasing thereby the biologically active substance. Because UV is required for the photolysis, cells are often colabelled with a fluorescent probe, which can be monitored subsequent to the uncaging. Many of the current caged probes such as Nitr5, Diazo-2, IP3 and EGTA induce calcium responses which can be monitored by colabelling with Fluo-3, calcium green and fura red.

Fluorescence redistribution after photobleaching (FRAP) is another important application of LSCM for monitoring the recovery of fluorescence following the photochemical destruction of fluorophores within a small localized area[69]. FRAP enables the monitoring of lateral diffusion of fluorescently-labelled molecule in or on a single cell, and is frequently used to quantitate the rate and extent of diffusion of fluorescently-labelled lipids or proteins across the cell- or nuclear-membranes.

LSCM is also used in measuring the efficiency of cell–cell intercommunication[70]. This is based on the principle that intercellular gap junctions allow low-molecular weight molecules to diffuse between two live cells which are in direct contact with each other. There will be recovery of fluorescence within a cell following photochemical destruction of the fluorochrome. Photobleached cells, which are connected to nonphotobleached cells by functional gap junctions, will exhibit fluorescence recovery.

LSCM has also been exploited in various cell biological approaches such as the study of membrane potential[71], immunofluorescence[72] at single cell level, anisotropy of cell membranes[73], diffusion of membrane components and cytoplasmic structures[74] and *in situ* analysis of enzymatic induction in anchored cells[75].

Limitations

A limitation of the present-day confocal microscope is the speed of data acquisition (time for scanning the laser across the preparation and for writing the data to the disk) and therefore, unsuitable for the study of phenomena changing in a rapid time scale. Cell development in cultures can be followed with the 3-D image acquisition in 0.5 to 1 min and in two dimension at considerably faster rates. Although a laser scanner can form an image in 1 to 4 sec, nearly all biological samples require multiple scans before the signal to noise ratio becomes good enough to produce high quality images. Thus the real time for image acquisition in LSCM may be 1 min or longer. The speed of laser scanning will probably increase to acquire as many as 30 images per sec. The choice will be the white light-based systems because they offer a wide range of excitation wavelengths and necessary speed of data acquisition.

Another basic problem of LSCM is that the UV epifluorescence objectives are designed to maximize UV transmission and are made achromatic for visible wavelengths. When a laser beam is scanned through an objective, this inherent limitation causes lateral chromatic aberration, a situation in which objective focuses the UV laser illumination off to the side with respect to the visible fluorescence deletion spot (Figure 6 *a*). As the laser beam is scanned off-axis, both signal intensity and resolution decrease[76] effectively, limiting the useful field of view.

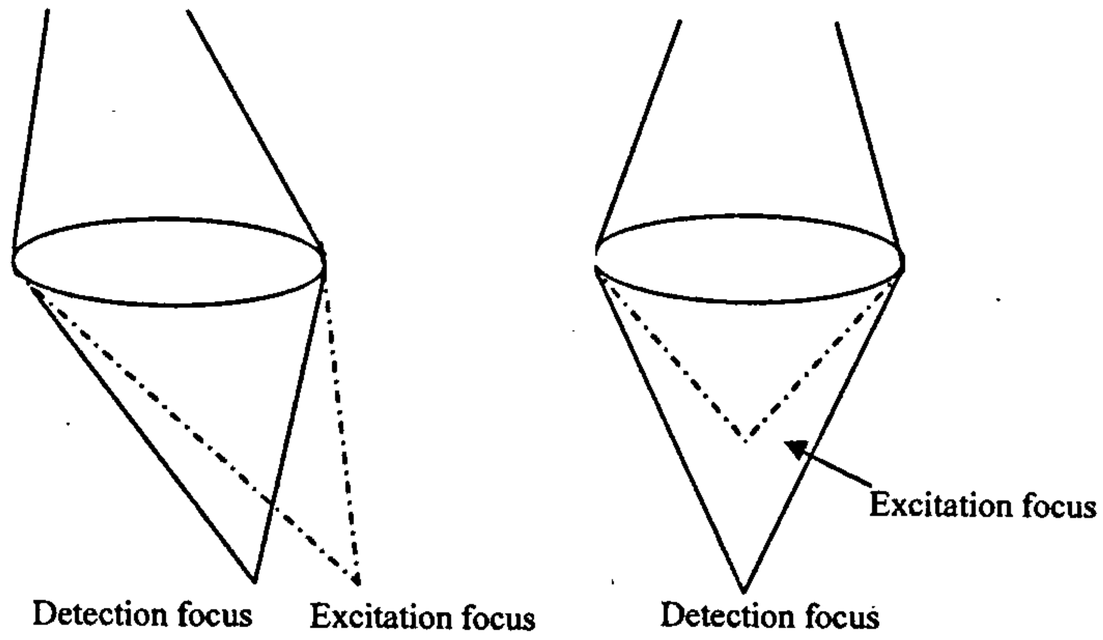

Figure 6. The limitations faced by LSCM. Line drawing demonstrating the phenomenon of (*a*) Lateral- and (*b*) Axial-chromatic aberration.

LSCM can also suffer from axial chromatic aberration, a vertical displacement of the UV laser illumination spot in relation to the point of visible fluorescence detection[76]. Since all the objectives used in LSCM focus the UV excitation light at a different depth than the visible detection light, the signal originating from the point of excitation will not be in proper focus for detection. The situation results in a marked loss of sensitivity, becoming worse as thinner and thinner optical sections are selected for imaging (Figure 6 *b*).

Application in biomedical field and future developments

A versatile microscope workstation known as confocal ablation trapping system (CATSZ), composed of two trapping laser beams and one ablation beam, has been developed around a confocal microscope. The current generation of instruments are used in novel ways with potential for development into clinical instruments including confocal opthalmoscope and confocal screening system for *in situ* hybridizations involved in karyotyping. The confocal images could be helpful in more accurate diagnosis. Optical sectioning property of confocal microscope is coming up as a replacement to the cryo- or ultrathin-sections in pathology laboratories. The optimal use of the 3-D confocal data is believed to form the basis for structural analysis of the biological objects in the future. The property of equal imaging conditions

for all data points in on-axis confocal systems may prove to be very important for that. diagnostic research, which at present extensively depends on sectioning of the material coupled with histochemical examination, will rely on LSCM. Another possible application of LSCM in future can be in optical biopsy (the use of laser-induced fluorescence and other spectroscopic methods to identify biochemical constituents in tissues) and in monitoring photosensitizers immediately before or during photodynamic cancer therapy procedures.

Advances may include more sophisticated computational and imaging software that will aid in manipulating the enormous data to construct volumes rendered three dimensionally in real time for living specimens. The areas of application of LSCM are expanding with the introduction of new probes for biomedical research. Further developments in the design of sensitive dyes and specific probes for use in confocal microscope will permit cell biologists to explore subtle relationships between cellular structures and function quantitatively in a dynamic volume. Less expensive and more compact laser equipment will possibly become available in the near future.

Nondamaging, high resolution techniques for imaging molecular structures in aqueous solutions continue to be developed and improved. Scanning probe microscopy (e.g. atomic force microscopy) is emerging as an important technique for macromolecular imaging and manipulations to enable imaging structures and to examine their mechanical properties[77].

Mention should be made about laser tweezers and laser scissors, a laser trap device that enables the application of known forces to micrometer to submicrometer particles even within the cells. These two noninvasive techniques can be combined to provide cell biologists with the capabilities to hold (tweezers) and to cut (scissors) individual cell and organelles[78]. Their earliest combined use was to induce cell fusion by first holding and positioning two cells with laser tweezers, and then cutting the adjoining cell membranes with laser scissors. The other critical applications are in the area of biophysical and molecular mechanics including motor functions and polymer unfolding. Laser scissors and laser tweezers are currently available to do microsurgical ablations and chromosome surgery or dissections[78], oocyte and sperm motility studies and preembryo manipulations[79]. They can also be used to spatially organize components *in vitro* to reconstitute cellular functions which require correct juxtaposition of the components. Greatest changes and optimization in the techniques are expected in the next ten years, which might allow real time analysis even in complex samples.

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