

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 structures¹⁻⁷. 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 deconvolution³. 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