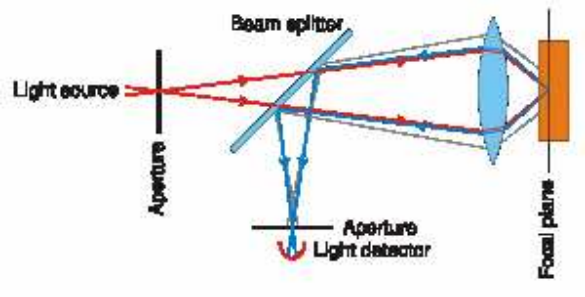
Confocal microscopy

Confocal microscopy is an optical imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane. This technique has been gaining popularity in the scientific and industrial communities. Typical applications include life sciences and semiconductor inspection.



Principle of Confocal microscopy

Basic concept

The principle of Confocal imaging was patented by Marvin Minsky in 1957. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded in light from a light source. Due to the conservation of light intensity transportation, all parts of the specimen throughout the optical path will be excited and the fluorescence detected by a photodetector or a camera. In contrast, a Confocal microscope *uses point illumination and a pinhole in an optically conjugate plane in front of the detector* to *eliminate out-of-focus information*. Only the light within the focal plane can be detected, so the image quality is much better than that of wide-field images. As only one point is illuminated at a time in Confocal microscopy, 2D or 3D imaging requires scanning over a regular raster (i.e. a rectangular pattern of parallel scanning lines) in the specimen. The thickness of the focal plane is defined mostly by the square of the numerical aperture of the objective lens, and also by the optical properties of the specimen and the ambient index of refraction.

Types

Three types of Confocal microscopes are commercially available: Confocal laser scanning microscopes, spinning-disk (Nipkow disk) Confocal microscopes and Programmable Array Microscopes (PAM). Confocal laser scanning microscopy yields better image quality than Nipkow and PAM, but the imaging frame rate was very slow (less than 3 frames/second) until recently; spinning-disk Confocal microscopes can achieve video rate imaging—a desirable feature for dynamic observations such as live cell imaging. Confocal laser scanning microscopy has now been improved to provide better than video rate (60 frames/second) imaging by using MEMS based scanning mirrors.

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM or LSCM) is a technique for obtaining high-resolution optical images. The key feature of Confocal microscopy is *its ability to produce in-focus images of thick specimens*, a process known *as optical sectioning*. *Images are acquired point-by-point and reconstructed with a computer*, allowing three-dimensional reconstructions of topologically-complex objects. The principle of Confocal microscopy was

originally patented by Marvin Minsky in 1957, but it took another thirty years and the development of lasers for CLSM to become a standard technique toward the end of the 1980s.

Image formation

In a Confocal laser scanning microscope, a laser beam passes through a light source aperture and then is focused by an objective lens into a small (ideally diffraction limited) focal volume within a fluorescent specimen. A *mixture of emitted fluorescent light as well as reflected laser light* from the illuminated spot is then recollected by the objective lens. A **beam splitter** separates the light mixture by allowing only the laser light to pass through and reflecting the fluorescent light into the detection apparatus. After passing a pinhole, the fluorescent light is detected by a **photodetection device (**a photomultiplier tube (PMT) or avalanche photodiode), transforming the light signal into an electrical one that is recorded by a computer.

The detector aperture obstructs the light that is not coming from the focal point. The *out-of-focus light is suppressed: most of their returning light is blocked by the pinhole, resulting in sharper images than those from conventional fluorescence microscopy techniques*, and permits one to obtain images of various z axis planes (also known as z stacks) of the sample.

The detected light originating from an illuminated volume element within the specimen represents *one pixel* in *the resulting image*. As the laser scans over the plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected fluorescent light. The beam is scanned across the sample in the horizontal plane by using one or more (servo controlled) oscillating mirrors. This scanning method usually has low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better contrast and higher resolution. Information can be collected from different focal planes by raising or lowering the microscope stage. The computer can generate a three-dimensional picture of a specimen by assembling a stack of these two-dimensional images from successive focal planes.

Confocal microscopy provides the capacity *for direct, noninvasive, serial optical sectioning* of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution. Because CLSM depends on fluorescence, a sample usually needs to be treated with fluorescent dyes to make objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create organisms that produce their own fluorescent chimeric molecules (such as a fusion of GFP, green fluorescent protein with the protein of interest).

Resolution enhancement

CLSM is a scanning imaging technique in which the resolution obtained is best explained by comparing it with another scanning technique like that of the scanning electron microscope (SEM). Do not confuse CLSM with phonograph-like imaging—AFM or STM, for example, where the image is obtained by scanning with an atomic tip over a surface.

In CLSM a fluorescent specimen is illuminated by a point laser source, and each volume element is associated with discrete fluorescence intensity. Here, the size of the scanning volume is determined by the spot size (close to diffraction limit) of the optical system because the image of the scanning laser is not an infinitely small point but a three-dimensional diffraction pattern. The size of this diffraction pattern and the focal volume it defines is controlled by the numerical aperture of the system's objective lens and the wavelength of the laser used. This can be seen as the classical resolution limit of conventional optical microscopes using wide-field illumination. *However, with Confocal microscopy it is even possible to improve on the resolution limit of wide-field illumination*

techniques because the confocal aperture can be closed down to eliminate higher orders of the diffraction pattern. For example, if the pinhole diameter is set to 1 Airy unit then only the first order of the diffraction pattern makes it through the aperture to the detector while the higher orders are blocked, thus improving resolution at the cost of a slight decrease in brightness. In practice, the resolution limit of Confocal microscopy is often limited by the signal to noise ratio caused by the small number of photons typically available in fluorescence microscopy. One can compensate for this effect by using more sensitive photodetector or by increasing the intensity of the illuminating laser point source. Increasing the intensity of illumination later risks excessive bleaching or other damage to the specimen of interest, especially for experiments in which comparison of fluorescence brightness is required.

Uses

CLSM is widely-used in numerous biological science disciplines, from cell biology and genetics to microbiology and developmental biology.

Clinically, CLSM is used in the evaluation of various eye diseases, and is particularly useful for imaging, qualitative analysis, and quantification of endothelial cells of the cornea. It is used for localizing and identifying the presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy. Research into CLSM techniques for endoscopic procedures is also showing promise.

CLSM is also used as the data retrieval mechanism in some 3D optical data storage systems and has helped determine the age of the Magdalen papyrus.