Dr. Bibha Verma
PG Department of Zoology
Maharaj College, Ara .

Confocal Microscopy

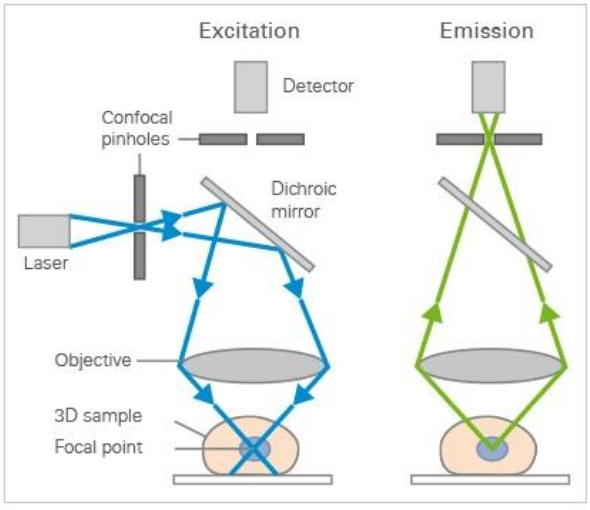
Introduction

Over the past several years, fluorescence microscopy has become a primary quantitative tool in the armamentarium of the biomedical research scientist. In the past, the technology was limited to easily quenched fluorophores such as fluorescein and rhodamine, non-optimized objectives, filters consisting of little more than colored glass, and film as a recording media. At the present time, designer fluorophores span the entire visible spectrum to the near infrared, objectives are specifically designed to maximize throughput from fluorescent specimens, filters are made from specifically designed dielectric-coated glass, and modern cameras allow detection of vanishingly low numbers of photons such that a high-quality image may be recorded in a fraction of a second.

Improvements in confocal microscopy have paralleled the rapid advances in wide-field fluorescence microscopy. The goal of this review is a brief history and comparison of confocal technologies, as well as a few examples of the primary applications of confocal approaches.

Whereas confocal microscopes were initially only able to image one or two colors at relatively low speed, the modern confocal microscope can collect high-quality multispectral images across the visible range. The image shown in Figure 1 illustrates the high-power, high-resolution, and multicolor capabilities of the modern confocal microscope. Furthermore, specialized instruments work at very high speed and are thereby able to image living specimens with little photodamage. In this review we discuss the basic technology inherent to all confocal microscopes, the applications in which the confocal is an essential tool, with attention to the specialized use of confocal systems with in vivo preparations and provide a limited comparison of the applications of specific subtypes of confocal microscopes.

Ray Diagram



Excitation and emission light pathways in a basic confocal microscope configuration.

Working

Similar to the widefield microscope, the confocal microscope uses fluorescence optics. Instead of illuminating the whole sample at once, laser light is focused onto a defined spot at a specific depth within the sample. This leads to the emission of fluorescent light at exactly this point. A pinhole inside the optical pathway cuts off signals that are out of focus, thus allowing only the fluorescence signals from the illuminated spot to enter the light detector.

By scanning the specimen in a raster pattern, images of one single optical plane are created. 3D objects can be visualized by scanning several optical planes and stacking them using a suitable microscopy deconvolution software (z-stack). It is also possible to analyze multicolor immunofluorescence staining using state-of-the-art confocal microscopes that include several lasers and emission/excitation filters.

Uses

As the imaging parameters can be maintained as a constant, the confocal microscope is capable of sampling and reporting intensity in single voxels. Importantly, this quantitation is highly reproducible. Variance in the imaged substrate is more likely to be due to variance in the sample than in the imaging system. The confocal microscope is therefore able to collect reproducible signals from specimens and reproducible z-axis slices with the only limitation being that, because of scattering and absorbance of light, the data should be sampled ideally at the same depth and in the same specimen. The confocal microscope can only penetrate and generate quantitative data for 20–50 µm in single photon mode, but when used appropriately, it is extremely quantitative. In this regard, the confocal microscope can be conceived as a flow cytometer, but with the added advantage of maintaining morphology so that cellular location can be correlated with signal intensity. This is an invaluable feature in examining the abundance of proteins or specific cell types within a specimen. One example would be in comparing cells within a tumor relative to those in the surrounding nonmalignant tissue.

Benefits

Confocal microscopy provides many advantages over conventional widefield microscopy for life sciences applications. It allows control of depth-of-field and the ability to collect serial optical sections from thick specimens. Confocal microscopy can be used to create 3D images of the structures within cells. Examining these structures can help researchers observe the internal workings of cellular processes.

In conventional widefield optical microscopes, secondary fluorescence emitted from a specimen often occurs through the excited volume and obscures resolution of features that lie in the objective focal plane. Thicker specimens exhibit such a high degree of fluorescence that much of the detail is lost. Spatial filtering techniques eliminate out-of-focus light in specimens whose thickness exceeds the immediate plane of focus.

Limitations

Because the confocal microscope is now an extraordinarily easy tool to use, there is a tendency to misuse the instrument. For example, the axial thickness of optical sections depends on the inverse square of the NA. As such, when low NA, low magnification objectives are used, the optical section may be 7 µm or more. As cryostat sections and many adherent cells are of this thickness or less, there is no rational reason for using the confocal microscope in these situations. Furthermore, confocal imaging requires a compromise between resolution, scan time, and photo destruction of the specimen. The higher the resolution, the more time required for the scan, and the longer the fluorophore is exposed to the laser. In many situations, enhancing resolution does not result in an increase in useful biological information about the specimen. This is illustrated in Figure 4. The left panel in Figure 4 shows a data set taken with a confocal microscope (shown as a 3-D reconstruction). In the right panel in Figure 4, the wide-field image of the same cell is shown. There is little difference in the two images, although the confocal image took 10 s to collect, whereas the wide-field image took 300 ms to collect.

Nuclei were labeled using cytox green (green). Actin filaments were visualized using Cy™3-labeled phallodin (red), and tubulin staining is shown in blue. The left panel shows a data set taken with a confocal microscope (shown as a three-dimensional reconstruction). In the right panel, the wide-field image of the same cell is shown.

References

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