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Review Article

Biotechnology Section

A Narrative Review on Confocal Laser Scanning Microscopy: Principle, Applications, Advancements and Challenges

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ABSTRACT

Confocal Laser Scanning Microscopy (CLSM) is an advanced imaging technique used in various fields. It offers several advantages over conventional microscopy due to its high resolution and ability to collect three-dimensional data using optical sectioning. While the resolution in confocal microscopy is comparatively lower than that of electron microscopy, it has effectively bridged the gap between these two imaging techniques. In the medical field, CLSM has a wide range of applications, including the diagnosis and treatment of corneal diseases, the study of biofilms and the diagnosis of various types of cancers, as well as the viral pathogenic cycle, including that of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). CLSM is also beneficial in food technology. The present review synthesises the current knowledge on CLSM, encompassing its applications, technological advancements and limitations. A comprehensive search of relevant databases identified studies and articles published by September 2023. The findings highlight CLSM's versatility, with applications ranging from cell biology to materials science, while also addressing technical challenges and future prospects.

Keywords: Electron microscopy, In-vivo confocal microscopy, Live cell imaging, Non invasive technique, Three-dimensional imaging

INTRODUCTION

Microscopes are instruments that magnify the images of very small objects, such as cells and microorganisms and are primarily used in science laboratories. A light microscope is one of the important tool for research conducted by cell biologists. Until recently, ultra-structural details could not be observed using light microscopes due to their low resolution. However, advancements in optical designs have opened new opportunities for microscopists to better understand and study cellular structures with greater visibility.

There are various types of microscopes, depending on the source of light used and the types of lenses involved. The confocal microscope is one such type, which uses lasers as a light source. In recent times, confocal microscopes have become available to researchers with advanced optical designs. Modern confocal microscopes offer increased optimised resolution, better differentiation and greater depth of field. Confocal microscopy allows for rapid, non invasive imaging of thick samples, producing high-resolution images and three-dimensional structures, especially in comparison to electron microscopy [1].

The present article provides a detailed overview of the principles of confocal microscopy, including its various applications, advantages and limitations associated with the laser scanning confocal microscope.

The CLSM is a type of fluorescence microscopy that scans a focused point of laser light in a diffraction manner around a sample to produce high-resolution images of objects stained with fluorescent probes [1]. The term "confocal" refers to having the same focus, meaning that the image plane and the confocal pinhole are conjugate. This configuration eliminates light from above and below the plane of focus, resulting in a crisp image with high resolution.

The original concept of confocal microscopy was developed by Marvin Minsky, who experimented with neural networks to view unstained preparations of brain tissue. However, the invention went unnoticed due to poor light sources [2]. Later, in the late 1970s and 1980s, significant improvements in computer and laser technology, along with the availability of digital image manipulation, led to a marked increase in interest in confocal microscopy [3,4].

Modern confocal microscopes are equipped with powerful lasers, wavelength selection options, one or more electronic detectors and a beam scanning assembly. The confocal microscope works by integrating all these components to produce a digital image. This advanced microscopy allows for the imaging and investigation of living tissues, cells and molecules, which were previously impossible [5].

Reflectance confocal microscopy is another type of optical imaging technique that provides a horizontal view of the specimen. It uses laser light at near-infrared wavelengths, which penetrates the tissue to illuminate a single spot [6-8].

Though there are many types of confocal microscopes, including laser scanning microscopy, spinning disk microscopes, programmable array microscopes and swept field microscopes, the present article mainly focuses on the laser scanning confocal microscope.

Search Methods

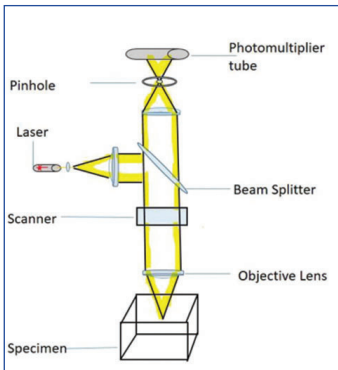
A search was conducted in electronic databases, including PubMed, Scopus, Web of Science and Google Scholar. The search terms included "confocal microscopy", "CLSM", and related variations. The inclusion criteria encompassed studies using CLSM that were published in peer-reviewed journals and available in English.

Study selection was performed after removing duplicates and assessing the eligibility of articles based on their titles, abstracts and full texts. Data extraction included study characteristics, CLSM applications, technical advancements and limitations. The extracted data were synthesised and analysed to provide a comprehensive overview of CLSM.

Principle of Confocal Microscopy

The basic principle of confocal microscopy is the generation of a high-resolution image using a focused spot of laser light directed through an objective lens onto a specific object plane of interest. Confocal microscopy also employs fluorescence optics, like wide-field fluorescence microscopy. In wide-field microscopy, the entire sample is illuminated at once, while in confocal microscopy, laser light is focused on a diffraction-limited spot at a specific depth within the sample [2,4].

Point illumination directs light precisely to a specific point, while out-of-focus light is eliminated by a pinhole located within the optical pathway. As a result, only fluorescence signals from the focused spot reach the light detector, forming a sharp image [Table/Fig-1].



CLSM components	Description
The laser	Laser lines can be chosen via a selection device and are matched with the fluorophores used in the experiment.
Beam splitter	This is a filter that separates the excitation from the emitted light in the fluorescence beam path of the microscope
Scanner	This is a unit based on two or more mirrors, which guide the focused laser beam across the specimen, pixel-by-pixel and line-by-line.
Objective lens	These are the heart of the microscope and mainly determine the optical image formation and the resolution of the system.
Z-control	This allows you to focus on any focal plane within your sample and the motorized Z-stepper allows movement in the axial direction in small step sizes (>10 nm) with high precision
Pinhole	This is an adjustable iris in the intermediate image plane. It allows exclusion of most of the out-of-focus light from the acquired image and thus provides optical sectioning capacity.
Photomultiplier tube	(PMT): These are highly sensitive detectors that collect the photons emitted by your sample. They basically transform the (light signal into an electrical one that is recorded by a computer.

[Table/Fig-1]: Components of CLSM.

The generation of CLSM images involves three process steps. Fluorescent molecules in the specimen are excited by laser light, resulting in fluorescence. The emitted fluorescent light is refocused in the objective image plane and out-of-focus light is removed from the image by passing the light through a pinhole. The fluorescence emitted by the scanned specimen is detected pixel by pixel with a Photomultiplier Tube (PMT), where a pixel represents a picture element. To capture a sharp image, the size of the confocal pinhole should be matched with the size of the Airy disk. The Airy disk is the central diffraction spot of light, having a finite diameter enclosed by the first minimum of the airy pattern, named after British astronomer GB airy [4]. At one airy unit, the system is diffraction-limited [4,5]. The pinhole may be opened for dim samples, but this comes at the cost of resolution. The brightness of the image depends on the number of photons acquired. Image resolution is improved with a higher number of pixels and a larger scanning area can be covered at a given pixel size [9,10]. The resolution is defined as the smallest distance between two points on a specimen that can be distinguished as two separate entities by an objective, while the numerical aperture refers to the light-gathering capacity of the objective.

Scanning speed is equally important in confocal imaging. A slower scanning speed can lead to photobleaching of the specimen, while increased speed decreases the pixel dwell time- the amount of time the laser spends on each pixel- thereby reducing the signal-to-noise ratio [5,11].

In a confocal microscope, different modes of light microscopy imaging can be collected using the transmitted light detector, which includes bright field, differential interference contrast and others. Images captured can be further deconvolved using software such as “Image J”, where deconvolution is an image processing method for reducing blurring in fluorescent imaging. This technique improves the contrast and resolution of the image through mathematical operations. However, deconvolution should be applied carefully and accurately [12].

Components of Confocal Laser Scanning Microscope

The components of a CLSM are depicted in [Table/Fig-1] and are expanded upon here. The laser serves as the source of light for the confocal microscope. The available laser lines depend on the model of the confocal microscope. The fluorophores used in the experiment are chosen according to the available laser lines. An ultraviolet laser range can be installed if it is not already provided with the confocal microscope. A beam splitter or dichroic mirror separates the excitation light from the emitted light in the fluorescence beam path of the microscope.

The scanning of the laser beam is guided by two or more scanning mirrors. The confocal microscope scans the sample point by point in a raster pattern. The objective lens of the microscope determines the optical image formation and resolution of the image. Among the various types of objectives, plan apochromatic objective lenses are considered the best type, as they correct for both spherical and chromatic aberrations. The pinhole present in the system excludes out-of-focus light from the focal plane, resulting in a sharper image. It is measured in airy units, with one airy unit being considered optimal for imaging. This measurement is calculated as the resolution of the lens multiplied by the magnification of the lens, multiplied by two.

The PMTs are the detectors that convert photons emitted from the sample into electrical signals. These detectors are very sensitive, fast and possess a large dynamic range. However, the quantum efficiency of PMTs is not as high when compared to Charge-Coupled Devices (CCDs), which can respond to incoming photon signals and convert them to measurable electron signals [4,5]. Hybrid detectors are also used as an alternative means to overcome the photobleaching of the fluorescent probe [13,14].

Sample Preparation

Sample preparation is a crucial step in confocal imaging. It includes the proper fixation of the sample, permeabilisation of tissue or cells for targeted intracellular antigen detection and labelling methods that preserve cellular morphology. Fixatives and permeabilising agents should be chosen according to the type of cells to be visualised, as each fixative or permeabilising agent has different advantages and drawbacks. When selecting a fluorophore, factors such as the quantum efficiency of the detector and the excitation and emission spectra of the probe should be considered [11].

The mounting medium should be chosen to maintain the desired information while providing significant signal strength, including antifading properties. Care should be taken to prevent damage to the sample. Coverslips are then sealed using colourless nail polish to avoid drying out the specimen. The specimen may be preserved in the dark in the refrigerator until acquisition [11,12].

Advantages and disadvantages of confocal microscopy [Table/Fig-2].

S. No.	Advantages of confocal microscopy	Limitations of confocal microscopy
1	High-resolution image, 3D images of the specimen by collecting serial optical sections.	Limited number of excitation wavelengths
2	Living and fixed cells can be used. Magnification is adjusted electronically by zoom factor.	Expensive to produce the ultraviolet rays used by the Confocal Microscopes and expensive to purchase
3	Very high sensitivity and better resolution with uniform scanning.	Phototoxicity of used probe

[Table/Fig-2]: Advantages and limitations of confocal microscope.

In confocal microscopy, out-of-focus light is suppressed during image formation, increasing axial resolution. The signal-to-noise ratio is improved in confocal imaging, making it suitable for both living and fixed cell imaging. Optical sections of thick specimens are collected in a confocal microscope using motorised Z-axis movement. The confocal laser scanning microscope generates various images of the Z focal planes through optical sectioning, which are referred to as a z-series. These images are collected by moving the specimen only along the Z-axis while keeping the position fixed (with no movement in the X-Y axis). The resulting data can be viewed as three-dimensional multicolour video sequences in real-time.

Time-lapse and live cell imaging are additional advantages of confocal microscopy. The single optical sections captured at preset points in time constitute time-lapse images. Time-lapse images are used to study the dynamics of living cells with increased resolution. The magnification of the confocal microscope can be adjusted electronically without changing the objectives by varying the scanned area. This feature is called the zoom factor and is used to adjust image resolution [12].

The disadvantages of confocal microscopy include the limited number of available excitation wavelengths, which do not encompass the ultraviolet range. However, this limitation can be addressed by installing a white light supercontinuum laser as a versatile substitute for the multiple monochromatic lasers typically used for fluorescence excitation, particularly in live cell imaging [10]. Confocal microscopes are comparatively expensive to purchase but can serve as centralised instruments for various departments. Phototoxicity of the probe is another disadvantage of CLSM, but hybrid detectors are available to mitigate photobleaching and monitor protein interactions in living cells [13-16].

Confocal microscopes have various applications in cell biology as well as in the food industry. These applications include techniques such as Fluorescence Recovery After Photobleaching (FRAP), Fluorescence Resonance Energy Transfer (FRET) and Fluorescence In-situ Hybridisation (FISH). FRAP is a technique used to study protein mobility in living cells; a specific area of a cell or tissue is photobleached using intense laser light and the recovery of fluorescence in that area is monitored.

The FRET allows for the determination of the proximity between two molecules within several nanometers, a distance sufficiently close for molecular interactions to occur. FRET can be employed to map protein-protein interactions. LysoTracker Red is a probe used to study apoptosis, which can be visualised using confocal microscopy [17,18]. In-vivo corneal confocal microscopy is gaining importance in the diagnosis of ocular diseases as it describes changes in corneal nerves and sublayers because of z-sectioning ability [19,20]. FISH detects specific Deoxyribonucleic Acid (DNA) sequences on chromosomes. FISH, when combined with CLSM, is also used for analysing biofilm systems in wastewater treatment research [21]. Green Fluorescent Protein (GFP) is utilised to study gene expression and intracellular signalling.

In food technology, CLSM is employed to monitor dynamic processes as they relate to various function of variables in the food industry. Changes in temperature, pH, concentration and pressure are monitored using fluorophores, which are crucial factors in the transportation of food products [22]. Commonly consumed wheat products, such as bread, wheat dough and pasta, are imaged using CLSM with fluorescent dyes, allowing for the acquisition of three-dimensional information on the cellular structure of yam parenchyma and on the properties of protein and starch networks in wheat products [23]. Reflection mode analysis enables the examination of surface properties of pasta.

Live cell imaging using CLSM is an efficient method for studying the physiology and pathophysiology of cells, including calcium dynamics. Calcium signals observed using CLSM can be detected in a large number of endocrine and exocrine cells with single-cell or even subcellular resolution [24-26].

The applications of CLSM have a wide range, including but not limited to:

1. Cell Biology: Visualisation of cellular structures, organelles and dynamic processes in living cells [23].
2. Neuroscience: Imaging of neuronal morphology and neural circuitry [24].
3. Materials science: Analysis of surface topography, microstructure and defects in materials [25].
4. Medicine: Diagnosis and monitoring of diseases, including cancer and ophthalmic disorders [27].
5. Environmental Science: Study of microorganisms and particulate matter in environmental samples [28].

Key limitations of CLSM include:

- Photobleaching and phototoxicity
- Limited penetration depth in thick specimens
- Equipment cost and complexity
- Data processing and storage requirements

Potential Uses of Confocal Microscopy

The confocal microscope is beneficial in a wide range of fields, including fundamental and medical research, nanoparticle imaging and quantum optics.

In the medical field, corneal confocal microscopy is widely used to diagnose ocular diseases. This modern, non invasive In-vivo ophthalmic imaging technique can accurately and rapidly measure the damage and repair of small fibers in Parkinson's disease [27]. Confocal microscopy is also utilised to develop and explore automated cell identification and segmentation methods for the morphometry of corneal epithelial cells [28,29]. Another common application of confocal microscopy is the examination of surface and near-surface features in biomaterials such as bone, dentin and enamel.

Microbial resistance, particularly in hospitalised patients, is a growing concern. CLSM plays a vital role in detecting biofilms produced by microbes, aiding in the identification of antibiotic-resistant strains and redirecting treatment. In recurrent urinary tract infections, biofilms formed by uropathogenic gram-negative bacteria in urothelial cells are significant [30,31]. Microarray techniques combined with CLSM facilitate biofilm characterisation [32,33].

In tuberculosis, treatment challenges arise due to dormant mycobacteria. CLSM, paired with immunofluorescence, enhances the detection of M. tuberculosis in lung histological samples, especially when Ziehl-Neelsen staining is inadequate [34]. Dormant bacilli, often undetectable by routine methods, are crucial to study as they contribute to latent infections and prolonged therapy. CLSM improves TB diagnosis by visualising dormant bacilli and studying their metabolism, offering insights into drug and vaccine efficacy [34-36]. The optical sectioning feature of CLSM allows for precise identification of infected host cells and the location of bacilli, making it a valuable tool in medical research and clinical diagnostics [37].

Recently, due to the Coronavirus 2019 (COVID-19) pandemic, the study of coronaviruses has become an important aspect of treatment and vaccine development. Studies on the SARS-CoV-2 virus can be performed by fluorescently tagging or immunolabelling viral proteins or Ribooxynucleic Acid (RNA) strands. Fluorescence microscopy has been used in studies of different viruses, so it can also be applied to identify coronaviral infections [38]. To investigate the endocytic pathway used by the virus, confocal microscopy is employed in some studies. Recent research has revealed the role of a secretory form of the Angiotensin-converting Enzyme 2 (ACE2) receptor (sACE2) in SARS-CoV-2 infection [38,39]. The replicative cycle and immunopathogenic mechanisms of this novel coronavirus are being studied using several high-resolution microscopy techniques to develop new treatments against this disease [11]. In experimental studies, immunofluorescence staining reveals abundant ACE2, which is the SARS-CoV-2 receptor and Transmembrane Protease Serine 2 (TMPRSS2) protein in the cells of differentiated human enteroids [39].

In the case of cancer, therapy depends on the stages of the disease and can become difficult and painful in advanced stages. Early diagnosis is crucial for all types of cancer. Confocal microscopy is a useful technique that can be utilised for diagnosing various types of cancers. Many types of skin cancers, from melanoma to basal and squamous cell carcinomas, can be detected using confocal microscopy. This technique visualises epidermal cells and superficial dermal cells in real time manner [40,41]. It minimises the number of skin biopsies and can be applied both in-vivo and in-vitro. In basic dermatology, this microscopy is beneficial for clinical practice as well as basic research [42,43]. Confocal microscopy can monitor post-skin cancer lesions without the need for further biopsies,

which provides patients with comfort and a more positive outlook about their care [44].

Cervical cancer is a preventable disease if diagnosed early. The diagnosis of cervical precancer can be improved using confocal microscopy, according to some studies. It has been observed that in the precancer state, epithelial fluorescence increases while stromal fluorescence decreases. By using the vital dye mitotracker orange, these changes can be observed. Confocal microscopy can provide information regarding epithelial changes through multiple images at various focal planes [45].

Nowadays, portable, automated models are available for in-vivo CLSM. In dermatology, this technology provides morphological characterisation of skin structures in the same area at different time points. It offers high-resolution images with a non invasive approach for real-time monitoring of diseases. Reflectance confocal microscopy is predominantly used for clinical diagnosis and research purposes in dermatology, while fluorescence confocal microscopy is also employed [44]. Portable models of confocal endomicroscopes are available for diagnosis as well. Cost-effective, convenient and robust models have been developed with automated alignment features. The clinical data obtained from such tools can be further evaluated. Nuclear morphology alterations in cervical lesions can be studied in real time, which aids in treatment decisions. Large-scale studies are necessary for further evaluation [44].

Currently, corneal confocal microscopy is gaining importance in the diagnosis of ocular diseases. Microbial keratitis is a common and blinding eye disease among contact lens wearers worldwide. In-vivo Confocal Microscopy (IVCM) has proven advantageous in the early diagnosis of fungal and Acanthamoeba keratitis. Disease monitoring is conducted according to the depth of Acanthamoeba cyst invasion, which informs antimicrobial therapy decisions. IVCM also assists clinicians in examining flap-related complications after refractive surgery and it describes changes in corneal nerves and sublayers [37]. Keratoconus, a corneal disease characterised by the development of a conical shape of the cornea, is diagnosed using confocal microscopy, where the loss of keratocytes is assessed by counting them with this technique.

Technological Advancements

Recent advancements in CLSM include:

1. Multi-photon microscopy: This technique offers enhanced penetration depth and reduced phototoxicity [45].
2. Super-resolution techniques: These methods improve spatial resolution beyond the diffraction limit [46,47].
3. Spectral imaging: This allows for the simultaneous acquisition of multiple fluorescent markers [48,49].
4. Light-sheet microscopy: This technique enables rapid imaging of large specimens with minimal photobleaching [49].

When combined with electron microscopy, confocal microscopy provides significant advancements in optical microscopy. Today, it is possible to achieve 4D imaging with new designs of confocal microscopy, capturing short-timescale dynamics. Such models include the LSM 980 with Airyscan, which allows for fast and gentle multiplex imaging.

CONCLUSION(S)

The CLSM is a very useful technique for imaging thick tissue samples. Considering some limitations of laser scanning microscopes, a technique that combines reflectance and fluorescence modes could be beneficial for obtaining additional information in various situations, such as the diagnosis of human skin morphology. More recently, live-cell confocal imaging has gained importance and portable models of confocal microscopes are now available for disease diagnosis. This technique is particularly useful for the early diagnosis of precancer stages in various types of cancers, helping to avoid repeated biopsies for the convenience of patients. The physiology and pathophysiology of various cell types can be observed using live-cell imaging in confocal microscopy. The CLSM remains a vital imaging technique with diverse applications across multiple scientific fields. Advancements in technology have expanded its capabilities, while ongoing research aims to overcome its limitations. As CLSM continues to evolve, it is likely to play an even more significant role in advancing our understanding of the micro and nano-scale world.

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