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Confocal Microscopy and its Implementation in Different Biological Aspects

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Abstract

Biophysical tools and its techniques are in high demand for the study of molecular mechanisms underlying cell biology, immunology and biomedical research including several diagnostics techniques. Biophysical tools basically include microscopy and imaging hydrodynamics, electrophysiology, modeling and simulation, single molecule techniques and spectroscopy. Among these, microscopy is one of the streams of optics that not only presents a magnified view of cellular structures of bacteria, germs, organs etc. but also ensures micro to nano elaboration of organisms or their parts. In this regard, confocal microscopy has become an essential technique in all the fields of biomedical research including life sciences that produce optical sections of cells and tissues that are totally free from fluorescence. In this review, we described the importance and applications of confocal microscopy along with its implementation in different biological aspects.

Keywords biomedical research, confocal microscopy, fluorescence microscopy, microbiology

Introduction

One of the popular microscopic techniques, confocal microscopy is used for imaging fluorescently labelled specimens with significant three-dimensional structure and has shown various applications in clinical sciences and life sciences comprising imaging of the structural distribution of macromolecules in living cells, three dimensional programmed assortment of data, imaging of numerous labelled specimens and the assessment of physiological movements in living cells [1, 2]. The invention of the confocal microscope [2, 3, 4] is related to the target imaging of neural networks in living brains (unstained preparation). The confocal approach is the direct result of modifications in light microscopy that has been largely encouraged by the advancements in modern technology. The comparative difference of fluorescent microscopy and confocal microscopy is shown in Table 1.

In the past, a major aim of the research has been to observe the general spatial distribution of cell populations in living or fixed samples containing one or two fluorescently labelled structures [5]. However, now a days, researchers can compare two closely spaced structures within a specific cell organelle using different microscopic technique e.g. telomeric and centromeric sequences have been localized to the chromosomes of human sperm to reveal a well-defined structure of chromatin in the sperm nucleus [5, 6] (Figure 1).

Fluorescence microscopy is a crucial tool (biophysical) in both basic and applied biomedical research because of its molecular specificity and optical sensitivity [7, 8]. Similarly, confocal microscopic technique has an enormous potential in many fields of medical research and life sciences [9, 10]. However, its molecular specificity and optical sensitivity is much better than fluorescence microscopy [9, 10]. It is basically divided into four types as follows:

- ***Confocal Laser Scanning Microscopy (CLSMs)***: that uses a sharply focused laser that scans over the sample.

- ***Spinning disk (Nipkow Disk) confocal microscopy***: that uses a disk with pinholes and split into it and finally displayed in the form of spiral.

- ***Reflectance confocal microscopy (RCM)*:** that works on the basis of reflected light rather than fluorescence.

- ***Programmable array microscopes (PAM)***: that

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Table 1. Comparative difference of fluorescent microscopy and confocal microscopy

	Fluorescence microscopy	Confocal microscopy
Source	Mercury lamps	Laser beams
Mechanism	Each section of the biological specimen is excited by light rays and the subsequent fluorescence is perceived through microscope (photo detector or CCD camera).	This microscope is based on the point illumination and thus, due to the detection of limited fluorescence, optical resolution of the images better in comparison to the wide-field microscope.
Resolution	Traditional fluorescence microscopes have resolution limitations.	Theoretical limit up to 0.1 to 0.2 μm and delivers a substantial imaging perfection over conventional microscopes.
Optics	Mercury lamp is used for illumination and delivering ultra violet light. A dichroic mirror reflects short wavelength (λ) and transmits longer wavelength. The fluorescence emitted from the sample passes back through dichroic mirror, however the ultra-violet light is stopped. An excitation filter in front of the mirror will control the excitation λ. An emission filter in front of the eyepiece will control the λ of the emitted light.	In confocal microscopy, a laser beam is focused on a fluorescent specimen through the objective lens. The reflected and emitted light is captured by the same objective. Reflected light is deviated by the mirror while the emitted fluorescent light passes through a pinhole to decrease the "out of focus" light. The focused light passes via emission filter and proceeds to the photomultiplier. Then the single point scanned in an X-Y manner as the laser focus is moved over the specimen for entire image generation.

works like spinning disk microscopes, except that pinholes in the PAM can be opened and closed by the user. All the above mentioned confocal microscopes result in an image, but the spinning disk and PAM systems induce a much higher frame per secondary image.

Technically, confocal approach gives a slight increase or enhancement in resolution (lateral and axial) and it has the capacity to eliminate flare (out-of-focus) from thick specimens (fluorescently labelled). These features have largely added to its popularity in recent years as the resolution achieved by the laser scanning mode of confocal microscope is much better than that of the conventional, wide-field light microscope (theoretical maximum resolution of 0.2 μm). Most of the modern confocal microscopes are now relatively easy to operate and become an essential part of several multiuser imaging facilities [9, 10]. Typically, fluorescent molecules reduce the image contrast and resolution because of the presence of the sample object outside the plane of focus. Thus, specimens with substantial tissue thickness or depth are difficult to study with wide-field of microscopy. With the help of laser scanning confocal microscopy, detection pinhole rejects light (out-of-focus) and final image shows a thin slice through the specimen with greatly improved axial resolution. As shown in Figure 2, the light entering through the tube lens from out of focus plane is blocked by the pinholes in front of the detector and allow light coming from the in-focus plane. Improvements in confocal microscopy have paralleled the rapid advances in conventional (wide-field fluorescence) microscopy [8, 9, 10]. With the current technological advances, confocal microscopy has opened a new world to biologists interested in the dynamic complexities of the cell.

Applications in Microbiology

Microscope is one of the most crucial tools for microbiologists. Antony van Leeuwenhoek (in 1600s) built microscope using a simple model comprising of a tube, magnifying lens and stage to observe first bacteria (i.e. smallest, unicellular, prokaryotic and microscopic organisms) and circulating blood cells. Further, using advanced technologies, microbiologists concluded the differences in the genetic, biochemical, physiological or behavioral properties of microbial cells [11, 12]. In this regard, recent advances in analytical methods and technologies have enabled

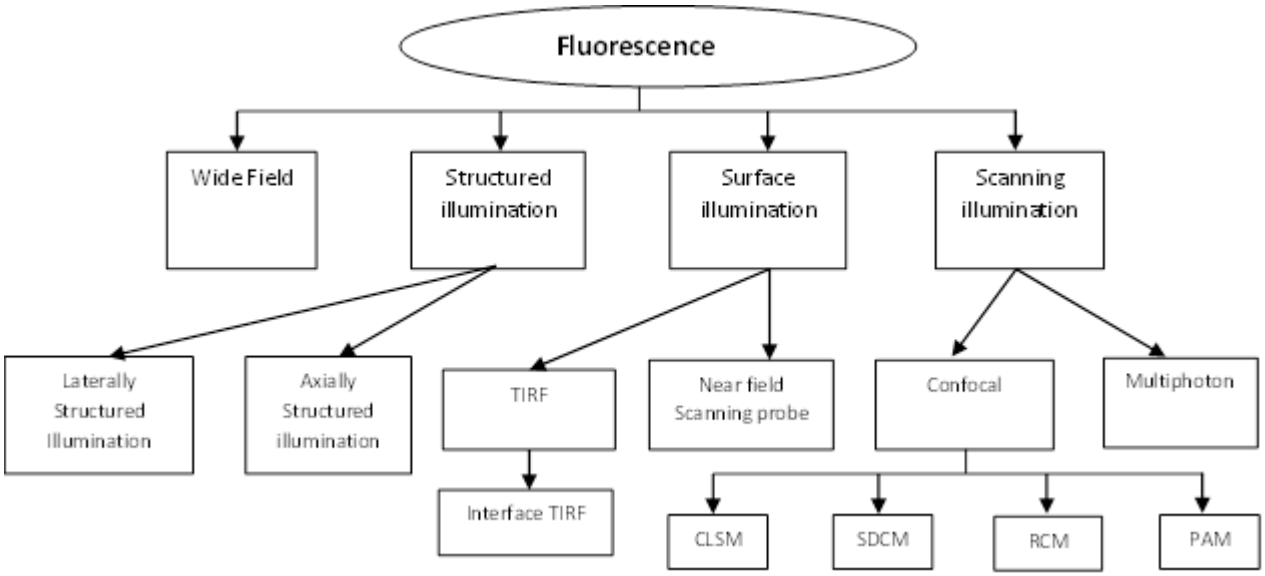

Figure 1. Differential types of microscopy

microbiologists including medical researchers to resolve the individual cellular differences and morphology. This technique has provided a lot of basic information and fundamental insights into the inner mechanism and working of the microbes and their relatedness with the environment or with other organisms.

In advance studies of microorganisms, confocal microscopy depends on its ability to be used on living specimens and to generate a 3-D image. Laser scanning of the confocal microscopy enables to recognize the living microbes within their habitat e.g. microorganisms (99% or more) are found on the surface of food and are generally accepted in natural and industrial habitats in food microbiology [12, 13]. Once they are attached to the surface, these microorganisms generate or propagate and build a spatially (space) organized biological assemblage called biofilm [14]. Laser scanning of the confocal microscopy is the technique of choice to analyze the dynamics of antimicrobials within the biofilms using dead or live staining procedure [12, 13]. As this technique enables the visualization of microorganism on the surface and also opaque material which is of particular interest in variable types of food matrices that allowed microbial multiplication such as cheese, it is really helpful to avoid food born infections.

Biomedical research

The major use of confocal microscopy in the biomedical research is for imaging either fixed or living tissues that have been usually tagged with one or more fluorescent probes and it is being increasingly used as a basic tool in biomedical research [15]. It allows the assemblage of thin optical sections, without the requirement for

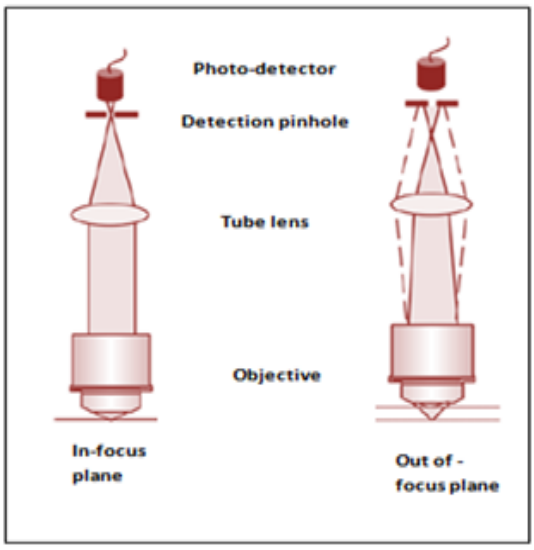

Figure 2. The principle of confocal fluorescence microscopy Light coming from out-of-focus planes is largely blocked by a pinhole in front of the detector

physical sectioning of the tissue. Moreover, confocal microscopes usually produce high quality images with greater sensitivity, contrast and resolution than those produced with normal light microscopes. In combination with immune fluorescence histochemistry based methods, confocal microscopy can now be used to examine complex 3-D distributions of well-shaped structures within the tissues including nerves (airways) [15]. Additionally, confocal microscopy (ultraviolet) allows the assessment of both dynamic and static phenomena in living tissues and cells. In general, imaging of fluorescence is normally correlated with the structural elements and these confocal microscopes can be quantitatively applied in order to evaluate the distribution including flux of intracellular ions like calcium [16].

Most of the successful achievements in biomedical research especially related to developmental biology (imaging microtubules in C. elegans embryos) can be attributed to the usage of laser scanning confocal microscope. Using conventional microscopy, it was most difficult to capture image of the two celled stage of the developing embryos due to the enhancement of cell number while volume of the embryo remains the same [17]. Laser scanning confocal microscopy has emerged as a new high-tech method for exploring developmental biology including stem cells and cardiovascular sciences and solving similar issues.

Entomology

Confocal microscopy has been first reported in arthropods and is now routinely used in zoological research especially entomology, limnology and human genetics [18]. Mostly, confocal microscope is used for imaging morphological structures including tissues and internal structures of selected taxa. It has also been demonstrated to be useful for the three-dimensional reconstructions of the insect exoskeleton in cockroaches and flies. Laser scanning confocal microscopy provides a detailed morphological overview of insects; however it is expensive to prepare material for such studies [18].

Immunology and Cell biology

Fluorescent (e.g. Annexin V, Sytox Green etc.) markers are widely used to evaluate cell death (apoptosis) in various immune cell types using confocal microscopy. In case of apoptotic cells, phospholipids (e.g. phosphatidylserine) are associated with the surface of plasma membrane that can be measured or evaluated through confocal microscopy employing phosphatidylserine binding protein (e.g. Annexin V) conjugated to fluorochromes [19].

Coincidentally, fluorescent marker, Sytox Green dye (stains dead cells with intense green fluorescence by binding to cellular nucleic acids) is similar to propidium iodide in its function, but can effectively distinguish between the dead cells and necrotic cells that are intact with the plasma membrane [19]. So, confocal microscope can provide proper measurement of apoptotic markers (Annexin, Sytox Green etc.).

Conclusion

Confocal microscopy can provide high quality images of various biological samples or specimens. As per the literature, fluorescence is related to high energy source, light excites various molecules and emit a ray longer wavelength. With the advancement in the field of confocal microscopy, excitation beams from one or more lasers are focused on the specimen at different focal planes. This result a fluorescence that passes through a dichromatic mirror and get detected by a photomultiplier tube through a second pinhole. Cells, tissues and other biological samples labelled with various fluorescent dyes, or fluorophores are commonly observed, viewed and examined through confocal microscopy.

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