Module -4:

Confocal Microscopy

4.1. Confocal Microscopy

Confocal microscopes produce sharp images of an object which would appear blurred in the conventional microscopes. Confocal microscopes only include the light from the object which is from microscope's focal plane, thereby excluding most of the light coming from the object. The obtained images have less haze along with better contrast than achievable by the normal microscopes and represent a fine cross-section of the object. Therefore, in addition to better observation of fine details, this technique also allows to build three-dimensional (3D) reconstructions of a volume of the object by accumulating a series of thin slices taken along the vertical axis.

Marvin Minsky discovered the basic idea of confocal microscopy in mid 1950s while working at Harvard University. Approximately 10 years later, Egger and Petran (Egger et al., 1967; Petrfifi et al., 1968) developed a spinning disk, multiple-beam confocal microscope. They employed this technique to examine unstained brain sections and ganglion cells. Working in this field, Egger developed earliest mechanically scanned confocal laser microscope and took first recognizable images of cells in 1973. Scanning confocal microscope was devised by G. Fred Brakenhoff in 1979. By providing explanation for image formation, Colin Sheppard greatly contributed to the development of the technique. Confocal Microscopy equipment was first commercialized in 1987.

4.1.1. Principle and working:

Confocal microscopes work by exciting the fluorescent labels present in a specimen. The excitation is provided by lasers, as against the use of mercury and xenon lamps in standard epifluorescence technique. The laser beam is focused onto a point and then scanned across the specimen in a point-by-point or line-by-line manner, and the emitted fluorescence light at each scanned point is collected by an objective. The collected fluorescent light is then passed through an aperture and a photomultiplier tube or PMT detects it. Subsequently, a pixel containing appropriate brightness is stored in the memory and displayed on adisplay screen, e.g., monitor. Typically, an array comprising 512 x 512 pixels forms an image, leading to 256 kB imagesize. Iris, or pinhole, is crucial to confocal effect. It is a controllable aperture which avoids the detection of out of focus light by PMT. Thus, only a thin piece of the sample is imaged, such a piece/slice of sample is often termed as an 'optical section'.The iris controls the resolution and brightness of the image such that a closure of iris results in a thin optical slice and better resolution, whereas when iris is opened results in a thick slice and increases image brightness.

By eliminating the signals from out of focus plane, confocal fluorescence microscopes achieve true three-dimensional resolution. To achieve this, iris (or pinhole) is used in front of detector (**Figure 1**). The function of iris is to pass the light coming from in-focus plane and to block the light from out of focus planes.

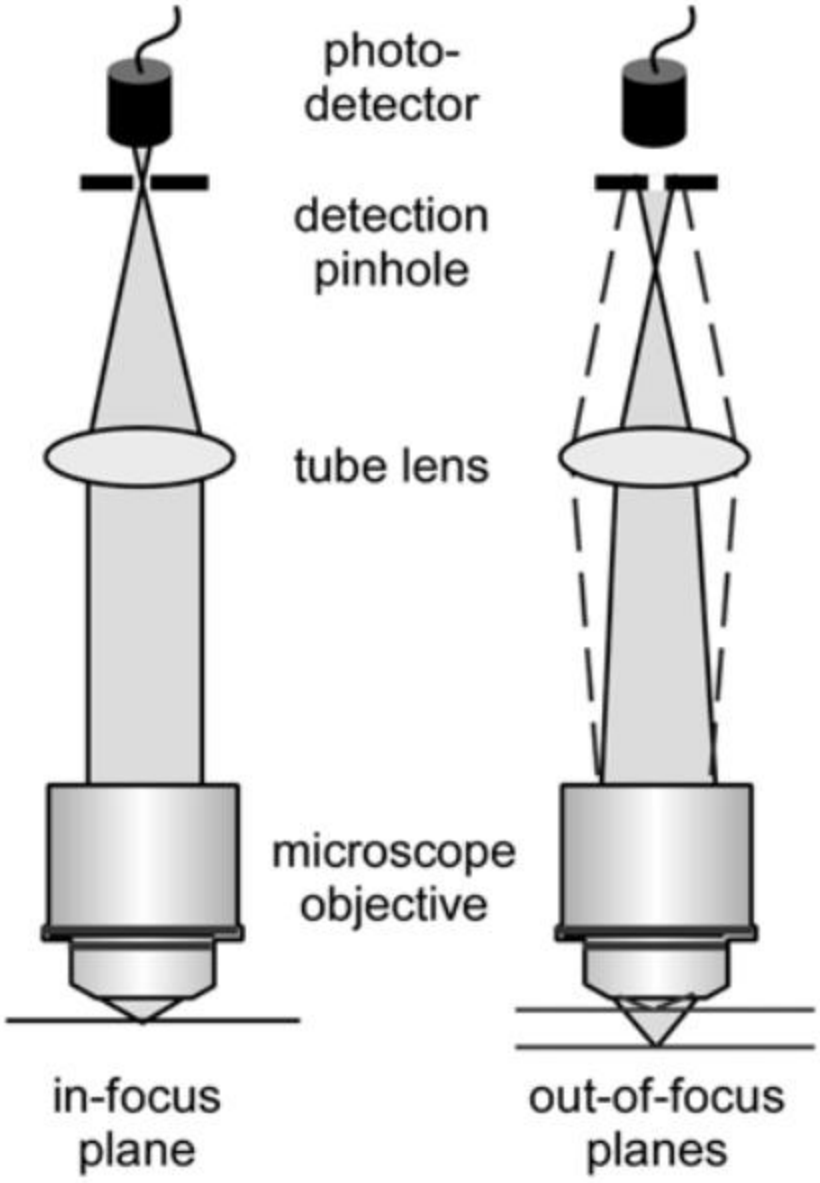

Figure 1Confocal fluorescence microscope. Light originating from out-of-focus planes getseliminatedby an iris (or pinhole)placed in front of detector.

- Confocal means having same focus. A point like light source, generally a laser is used. This point source is derived by passing the light through a pin hole, which can be conveniently achieved by using a fiber -optic connector. This is directed to the specimen through a beam splitter and an objective, which illuminates a spot. The point of illumination can be moved across the sample by a scanner and there are several ways by which this can be done. The fluorescence light emitted from the sample generally passes from the detecting pinhole and forms a point like image on the detector.

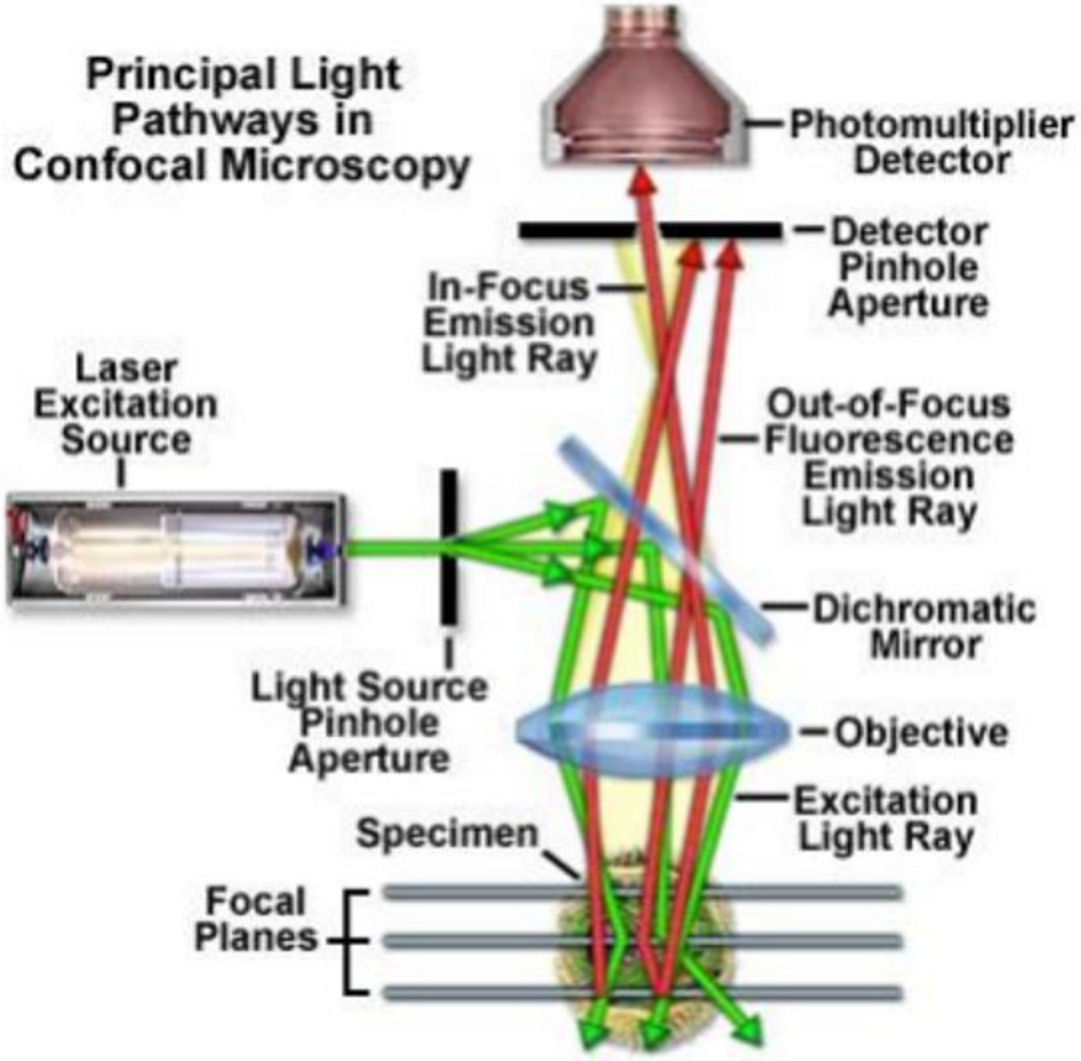

Figure 2 Schematics of optical path and principal components of a laser scanning confocal microscope.

- The light is scanned across the sample to obtain a two-dimensional image or the depth from which the light is collected is varied by moving either the objective or the sample thus giving a three dimensional image of the sample. All these three points, namely the illumination pin hole, sample spot and detector pin hole are optically conjugated together, thus giving the confocal microscope.
- The confocal microscope is therefore a confocal scanning optical microscope. The optical sectioning aspect is the most important advantage of confocal microscope. The sections can be as thin as the wavelength of light and its spatial resolution of the microscope is the best that can be achieved by using optical microscopy. Collecting the imaging involves scanning the light.
- The simplest approach would be to move the sample as the optical system is optimized. The other approach is to have a set of two mirrors to scan the laser in the x-y plane, by first doing an x-scan and then making a y shift, then an x- scan again and so on. The laser beam itself can be split into several smaller beams and all the beams may be simultaneously used for imaging. In this way, each beam needs to be moved only for a short distance for imaging the entire sample. All the beams are focused by the objective simultaneously and the light coming out from the sample is

collected through the pinholes and micro lenses detected parallel. The holes can be arranged in a spiral fashion so that the entire space can be scanned by rotating the disk.

- Increasing the speed of rotation will increase the speed of imaging. The signal collected from the sample is confined to specific illumination volume by the use of an aperture. Thus, the aperture sits at the same focal point of the objective, rejecting all light that comes from other region. This facilitates localization of the illumination volume. Thus, direct localization of the illumination volume smaller than the resolution of light microscopy is possible in the confocal technique.

Figure 3shows various images comparing specific view-fields in a conventional wide-field and that obtained from a laser scanning confocal fluorescence microscopy.

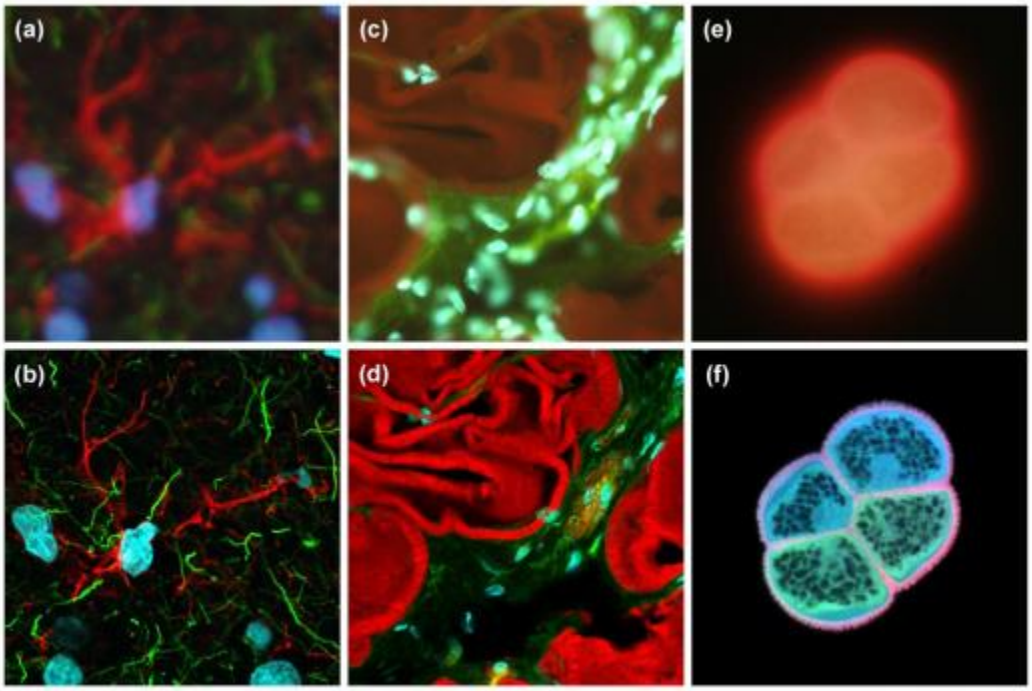

Figure 3Comparative images obtained from a conventional wide-field (a,c,e) and laser scanning confocal fluorescence microscope (b,d,f). (a) and (b) mouse brain hippocampus thick section treated with primary antibodies to glial fibrillary acidic protein (GFAP; red), neurofilaments H (green), and counterstained with Hoechst 33342 (blue) to highlight nuclei. (c) and (d) thick section of rat smooth muscle stained with phalloidin conjugated to Alexa Fluor 568 (targeting actin; red), wheat germ agglutinin conjugated to Oregon Green 488 (glycoproteins; green), and counterstained with DRAQ5 (nuclei; blue). (e) and (f) Sunflower pollen grain tetrad auto-fluorescence.

4.1.2. Types of confocal microscopes:

There are three types of confocal microscopes:

1. Confocal laser scanning microscopes: A pair of mirrors is used across the fixed iris(or pinhole) and detector for scanning the laser across the specimenas well as for de-scanning the image.A separate mirror is used for X-axis and Y-axis.
2. Spinning disk Nipkow disk confocal microscopes: A series of moving irises or pinholes on a disc is used to scan a spot of light.

3. Programmable array microscopes: Spatial light modulator is used to produce a set of moving irises or pinholes. The light modulator can be controlled electronically.

4.1.3. Specimen Preparation:

Poorly prepared samplesresult in distorted three-dimensional images of the sample (Blatter, 1999; Wright and Schatten, 1991; Wright et al., 1993). Fixation and extraction techniquesused intraditional fluorescence microscopesusually result in flattening of specimens especially by the weight of the coverslip (Wright et al., 1990; Wright and Schatten, 1991). Fixation and dehydration protocols also introduce distortional artifacts. Hence, samples for three-dimensional imaging should be prepared very cautiously. An important consideration in such imaging is how far the sample can be focused along its z-axis, since the focusing of sample along z-axis causes great loss in contrast and often results in shadowing effects. This leads to a differential brightness in vertical direction of the image obtained such that focal planes lying at the top appear brighter than those lying at the bottom. This can be avoided by increasing theintensity of illumination; however, an increase in illumination intensity also increases photobleaching of the specimen. This necessitates the use of antioxidants while acquiring large data sets, so as to minimize photobleaching. Heterogeneity inherent in specimens can also create problems in acquiring a Z-series (Cheng and Kriete, 1995). The intensity of the excitation light as well as emitted fluorescence can be attenuated by featureslocated between the focal plane of interest and the objective. This causes self-shadowing of structures and considerably reduces contrast of the image in some areas of the specimen.

4.1.4. Advantages-

- The fielddepth can be controlled.
- The principal advantage of confocal microscopy is the image contrast, which is achieved by rejecting light that comes from other focal planes of the sample. These signals if not eliminated, degrade the obtained image. For this purpose, a slit if used such thatthe smaller the slit, larger is the rejection, but the overall signal quality decreases in this way.
- By using optical sectioning, this technique avoids artifacts occurring while physical sectioning and fluorescent staining of samples (such as tissues) as employed in conventional microscopes.
- Serial optical sections can be collected from thick samples.
- By reducing background fluorescence and improved signal-to-noise ratio, the contrast and definition are greatly enhanced in comparison to widefield technique.
- Owing to the non-invasive nature of optical sectioning, this technique can be used to examine living as well as fixed samples in several different conditions with improved clarity.
- The magnification can be adjusted electronically only by tuning the area scanned by the laser, and does not require any change in objective.

4.1.5. Disadvantages-

- The intensity of the laser beam can damage the living cells. Thus, laser light must be aptly attenuated before applying on the sample.
- Depending on the setup, a can be over 10 times the price of a typical conventional fluorescence light microscope.
- Another disadvantage of confocal microscope systems is that they often take larger space than traditional fluorescence microscopes as additional space is required for laser, scan head and computer hardware.

4.1.6. Applications-

- The most significant application of confocal microscopes in nanoscience is in the investigation of the interactions of Nano systems with biological components. There are numerous examples of this kind wherein nanoparticles, Nano shells, nanotubes and such other objects are made to interact with cells, bacteria, viruses etc.

- Penetration behavior of various restorative materials can be studied under confocal microscopes. Resin infiltration of natural caries lesions has been examined by confocal microscope as well as transverse microradiographs. The results obtained indicated towards lower penetration depth of adhesive than the infiltrant.
- The Candida albicans' susceptibility towards photodynamic therapy has also been studied by confocal microscopes. The observations reveal that photodynamic therapy is a potential alternative to present-day antiCandida therapy, particularly, for superficial infections which canrespond toward illumination.
- Functional behavior of dental tissues towardsslow-moving cutting instruments(emulating chisels)as well as high-speed instruments (e.g., rotary burs, air-propelled abrasive particles, soft powder slurries, etc.), has been examined by confocal microscopy.
- Non-destructive imaging of plaques is possible by confocal laser scanning microscopy (CLSM). This is done by using a vitality staining approach to characterize the immediate bactericidal effect of chlorhexidine (CHX) on biofilm, therebyvisualizing and quantifying the biofilm left on the substrate on which it was grown, after treatment with antimicrobial agent.
- Determining the localization ofions,macromolecules (proteins, RNA, etc.), cytoskeletal elements, organelles, etc. within the cells.
- Tracing particular cells or structures through a tissue.
- Producing optical sections for stereo image production, three-dimensional reconstruction, and 4-D imaging.

Self-assessment

Question

1. Confocal laser scanning microscopy is a fluorescence-based microscopy technique.
    a) True
    b) False
2. Briefly explain the principle and working of confocal microscopy.
3. Explain in brief about different Types of confocal microscopes.
4. What are the limitations of confocal microscope?
5. List the significance of confocal microscope.
6. What are the advantages of using confocal microscopes?
7. What are the application of confocal microscopes?