B. Sc. 2nd Year, Paper 2, Unit 3
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LASER CONFOCAL MICROSCOPY

Mrs. Neetu Das
Assistant Professor (Microbiology)
Govt. V.Y.T. PG. Autonomous College , Durg , C.G.

Confocal microscopy (**confocal laser scanning microscopy** (**CLSM**) or **laser confocal scanning microscopy** (**LCSM**), is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation.

Confocal microscopes have superior resolution -- much higher than conventional optical microscopes -- and are primarily used in measurement applications. Confocal microscopes can be categorized as either reflection-type or transmission-type based on the type of illumination used for imaging.

Microscopes using a laser as light source are known as laser microscopes. They are also confocal microscopes. Since they capture a two dimensional image by scanning spot or line-shaped beams of light in XY directions, they are also a type of scanning microscope like scanning electron microscope (SEM) and scanning probe microscope (SPM).

In general, the performance of an optical microscope depends largely on the wavelength of light it uses and the numerical aperture (NA) of its objective lens.

No matter how small the wavelength becomes, unless we increase NA, we cannot get higher resolution. To observe fine patterns, we need a high-NA high-magnification objective lens.

However, if we use a high-NA high-magnification lens for observing a sloped or rough surface, it is not possible to get focus at every point throughout the field of view. This is because, as NA becomes higher, the depth of focus becomes shallower. There is a tradeoff between resolution and depth of focus. We cannot get both at the same time. This dilemma can be resolved, however, if we use a confocal microscope.

The answer lies in confocal optics used on the microscope.

1. High resolution image
Confocal optics offers a unique characteristic not available in conventional microscopes. On an image captured with confocal optics, areas in focus are highlighted. This is called optical sectioning. There is no interference of undesirable scattered light from out-of-focus areas at the highlighted sections.

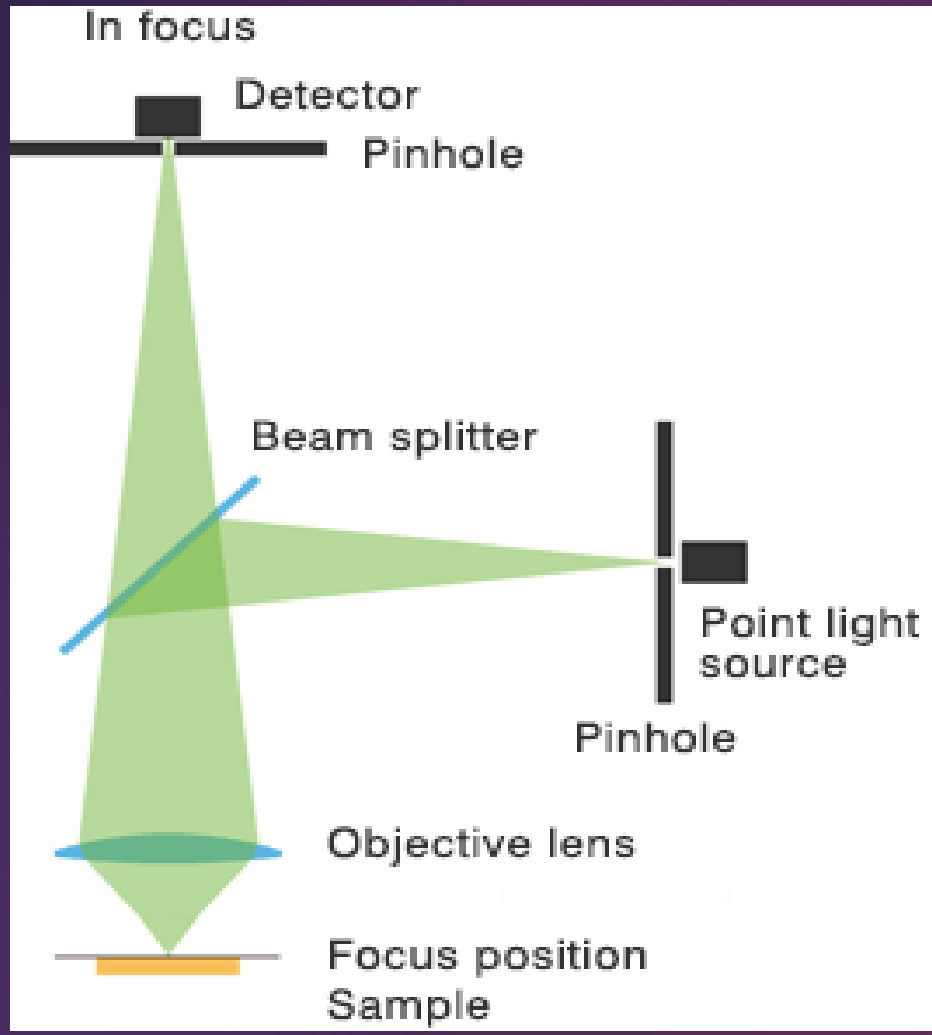


Figura1

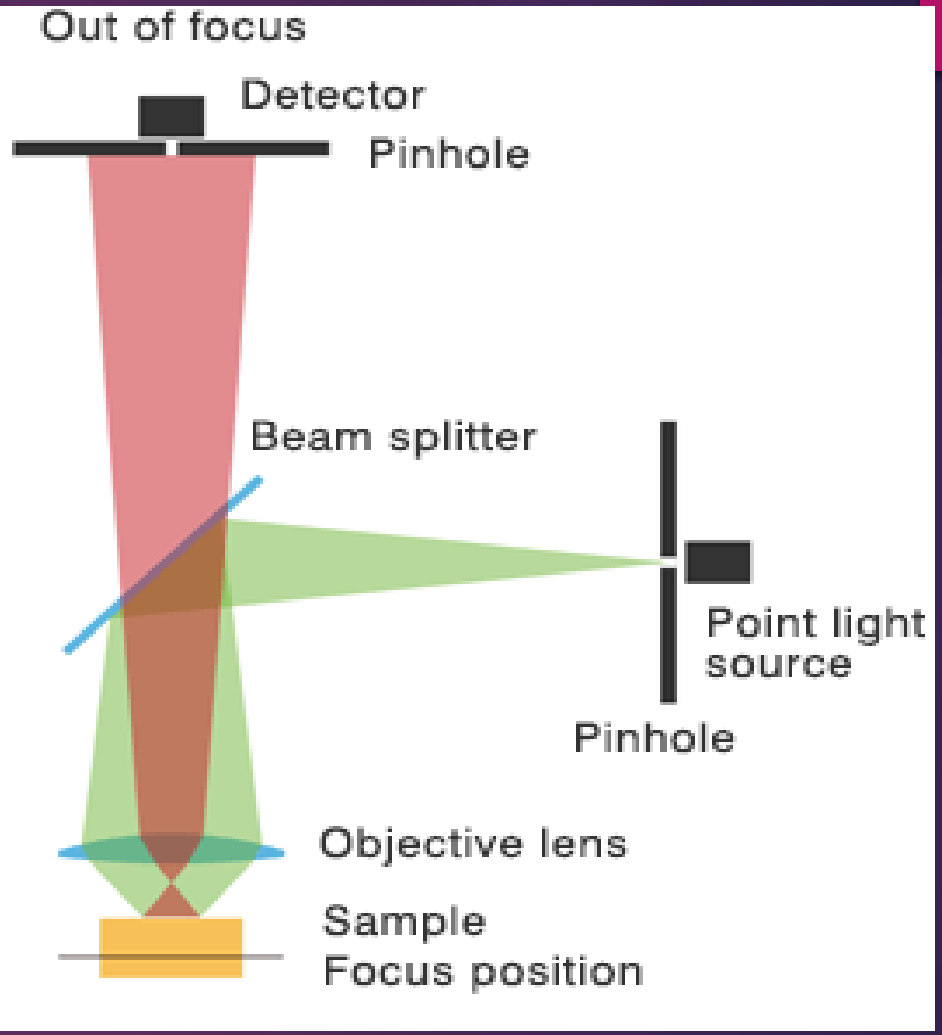
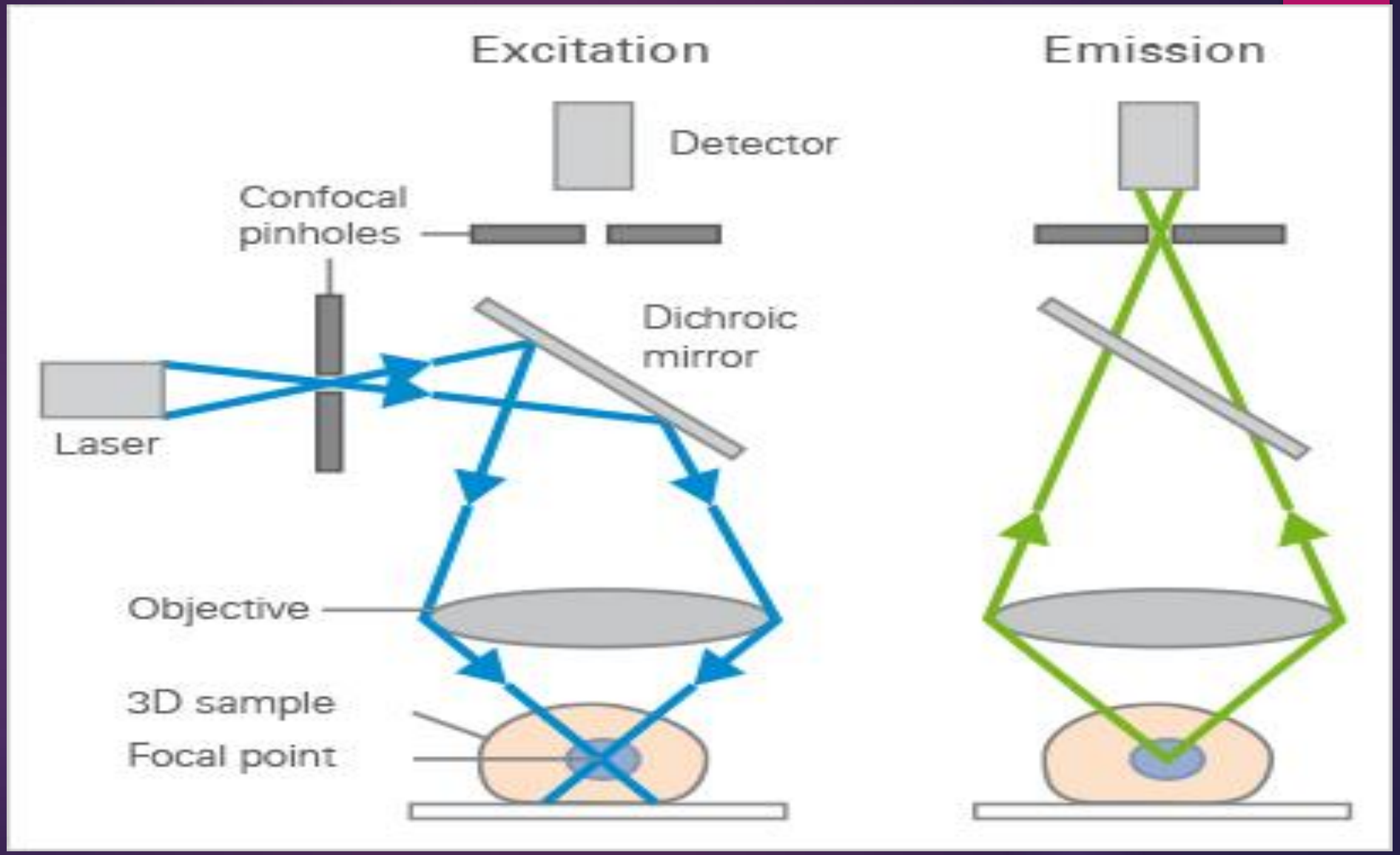


Figura2

Professor Marvin Minsky first employed the basic principle of confocal imaging in confocal microscope and he got this instrument patented in the year 1957.
The confocal scanning optical microscope , an extensive piece of instrumentation ,which illuminates the object with small beam of light in a point-by –point that is serial fashion, illuminated most of the photo –oxidation problems , permitting the observation of objects for extended periods at very high resolution with little loss of signal . The placement of a small aperture in the beam path generate a small depth of field and effectively eliminates the out –of –focus information in image formation.


Excitation
Emission
Detector
Confocal
pinholes
Dichroic
mirror
Laser
Objective
3D sample
Focal point

The confocal scanning optical microscope is designed to illuminate an object in a serial fashion , point – by – point , where a small beam of light (from a laser) is scanned across the object rapidly in an X-Y raster pattern. Thus , a bright spot of light scans across an object from top to bottom , line –by – line . The image is also generated point by point. The image formation is translated into intensities at each spot in the X-Y raster by a photomultiplier tube. The intensity information is digitized and store in a computer. A complex image processing software package permits visualization and manipulation of the images.

Resolution is limited by spot size for the Laser and approaches 0.12 - 0.15 μm for an ideal specimen and with the best available objective lenses . The microscope also includes a pinhole diaphragm at a very special place in the optical path, near to the site of the photomultiplier tube . This pinhole is situated in a plane where the light from the in-focus part of the image converges to a point. The light from object planes above or below the focused image does not converge at the spot in the optical path occupied by the pinhole . Because of this design , the out – of –focus image information is darkened to the extent that it is not detectable.

Consequently , all the out-of –focus information is removed from the image . The confocal image is basically an “optical section” of what could be a relatively thick object.

As the confocal images are stored in a computer , it is possible to stack them up and generate 3-dimentional reconstructions . The image-processing programmes also enable us to rotate this images and observe 3- Dimensional aspects of the cellular structure . The computer must be fast and powerful and is responsible for these image manipulations .

The biggest problem is the storage of image because even a single image can routinely occupy more than 1, 000,000 bytes of space. In rather short periods of use , it is easy to accumulate sufficient numbers of images to fill the largest of hard disk .

The confocal scanning technique is used to obtain 3 –Dimensional images of the entire cell and the cellular components . Beside this , it can be used to evaluate cellular physiology by monitoring the distributions and concentrations of substances such as ATP and calcimines .

In biomedical sciences a major application of confocal microscopy involves imaging either fixed or living cells and tissues that have usually been labelled with one or more fluorescent probes . A large number of fluorescent probes are available , specifically stains cellular organelles and structure for example dyes that label nucleus , golgi- apparatus , endoplasmic reticulum , mitochondria etc .

REFERENCES

Confocal Microscopy, from Wikipedia , the free encyclopedia

Ghosal Sabari , Srivastava A.K. , Fundamentals of Bio-analytical techniques and Instrumentation , PHI Learning Private limited , Delhi , 2013, Page-38 -40