INSTRUMENTAL TECHNIQUE

Confocal Microscopy

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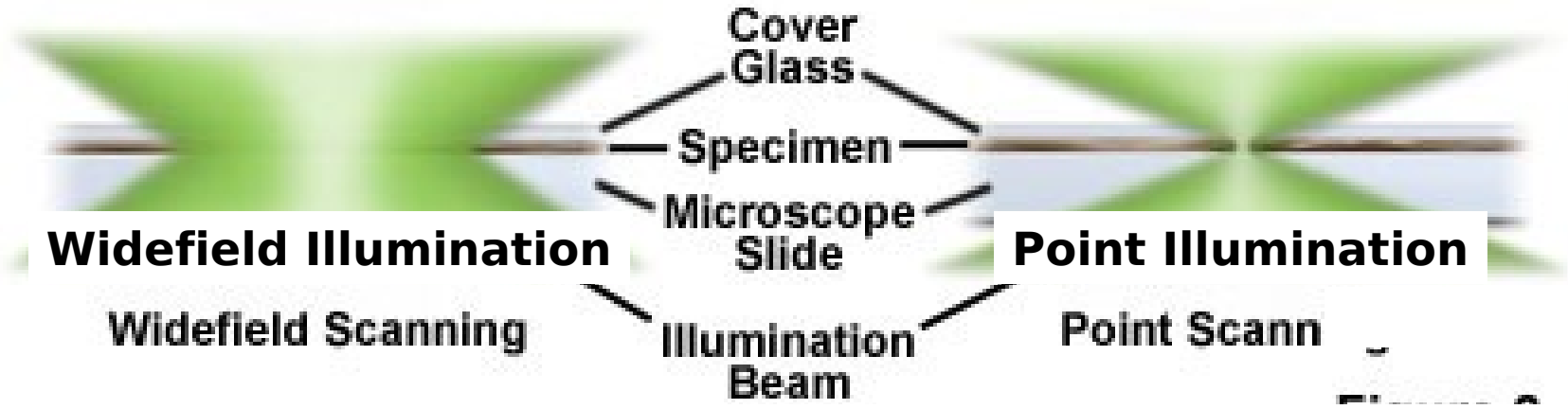
Widefield fluorescence imaging

❖ In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded evenly in light from a light source.

❖ All parts of the specimen in the optical path are excited at the same time and the resulting fluorescence is detected by the microscope's photodetector or camera including a large unfocused background part.

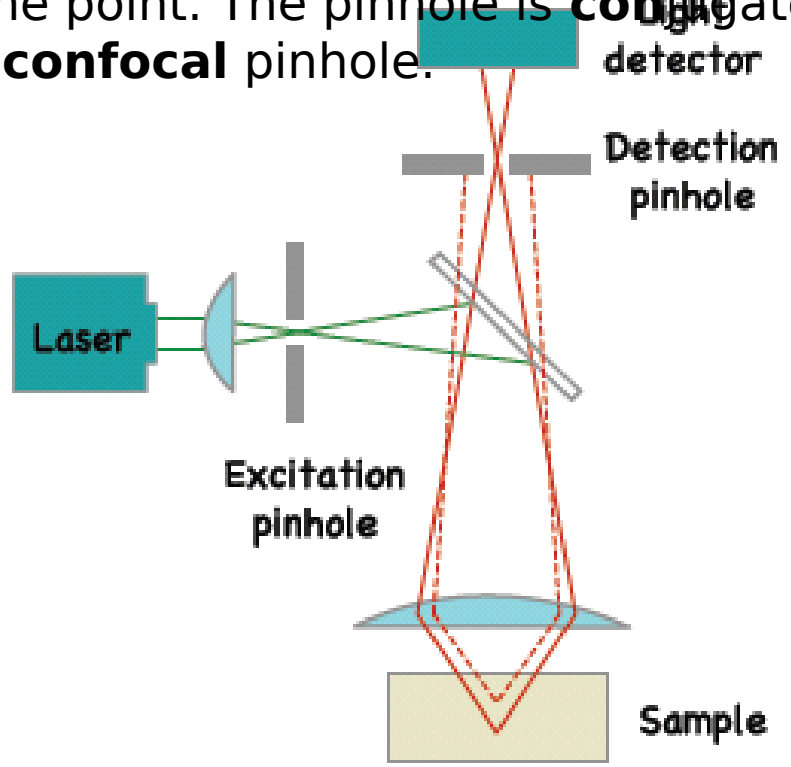


Confocal microscope

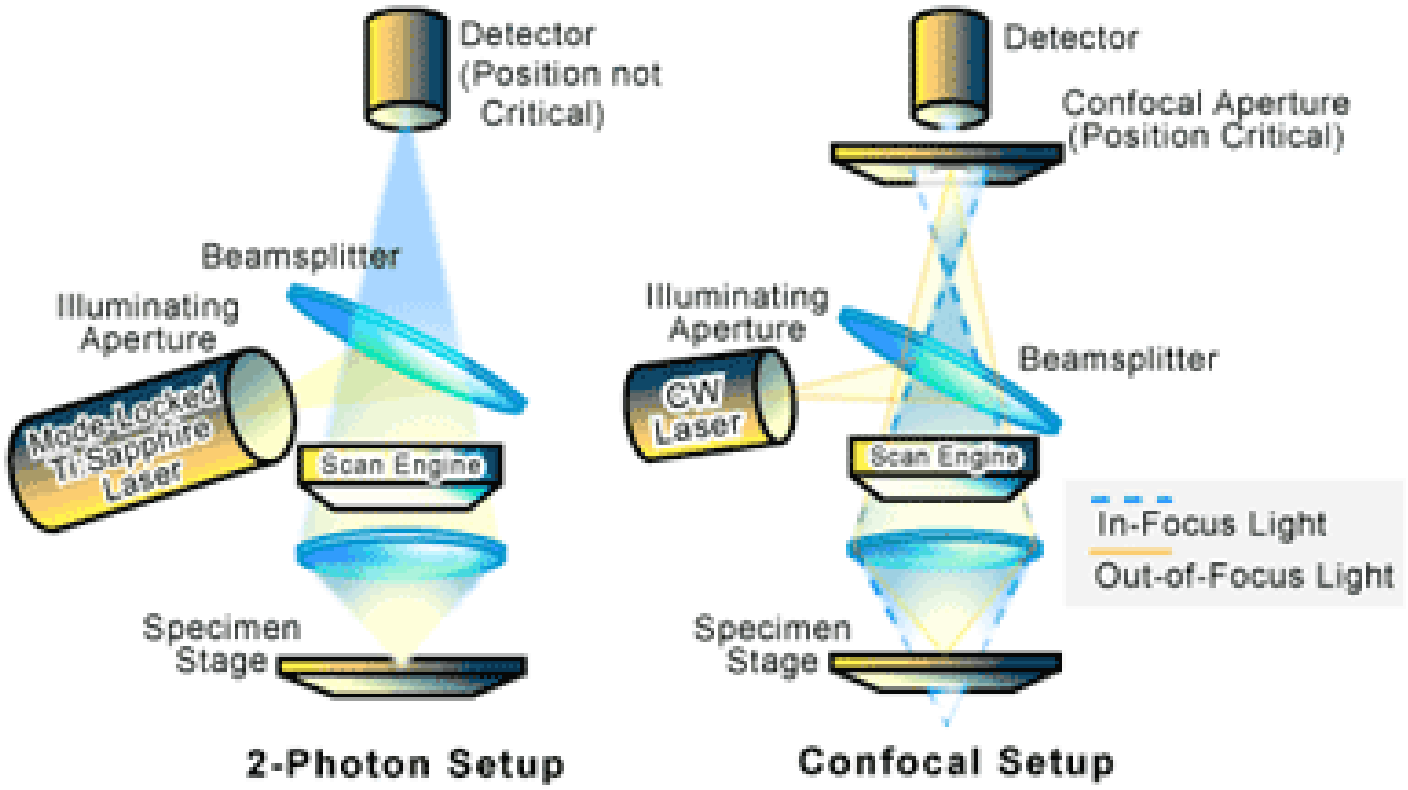
❖ The principle of confocal imaging was patented in 1957 by Marvin Minsky and aims to overcome some limitations of traditional wide-field flluorescence microscopes .

❖ The major optical difference between a conventional microscope and a confocal microscope is the presence of the confocal pinholes, which allow only light from the plane of focus to reach the detector.

❖ The term confocal refers to the condition where two lenses are arranged to focus on the same point. The pinhole is **con**jugate to the **focal** point of the lens, thus it is a **confocal** pinhole.



Different Types of Confocal Microscopes



- The laser beam excites a point on the specimen. It also inadvertently excites other points on the specimen.
- Only the In-Focus emission light is allowed to be detected by the PMT.
- The Light detected by the PMT is associated to a pixel (picture element) on the monitor.
- The laser beam then moves to the next point and another pixel is

Pros and Cons of Confocal Microscopy

Pros	Cons
Allows for higher resolution	Limited EX peaks on lasers
Allow collection of stacks of image planes and 3D reconstruction	Phototoxicity (up to a 40º C temp jump at focal point)
Laser penetrates somewhat thick sections	Loss of image intensity
Better control of bleed through/autofluorescence	Fairly expensive
Faster than deconvolution	Prone to Photobleaching
Precise Laser Positioning (FRAP)	Confocal does not fix scattering, refractive index mismatch, or everything else that can go wrong – it only removes out of focus light

Spinning Disk Confocal

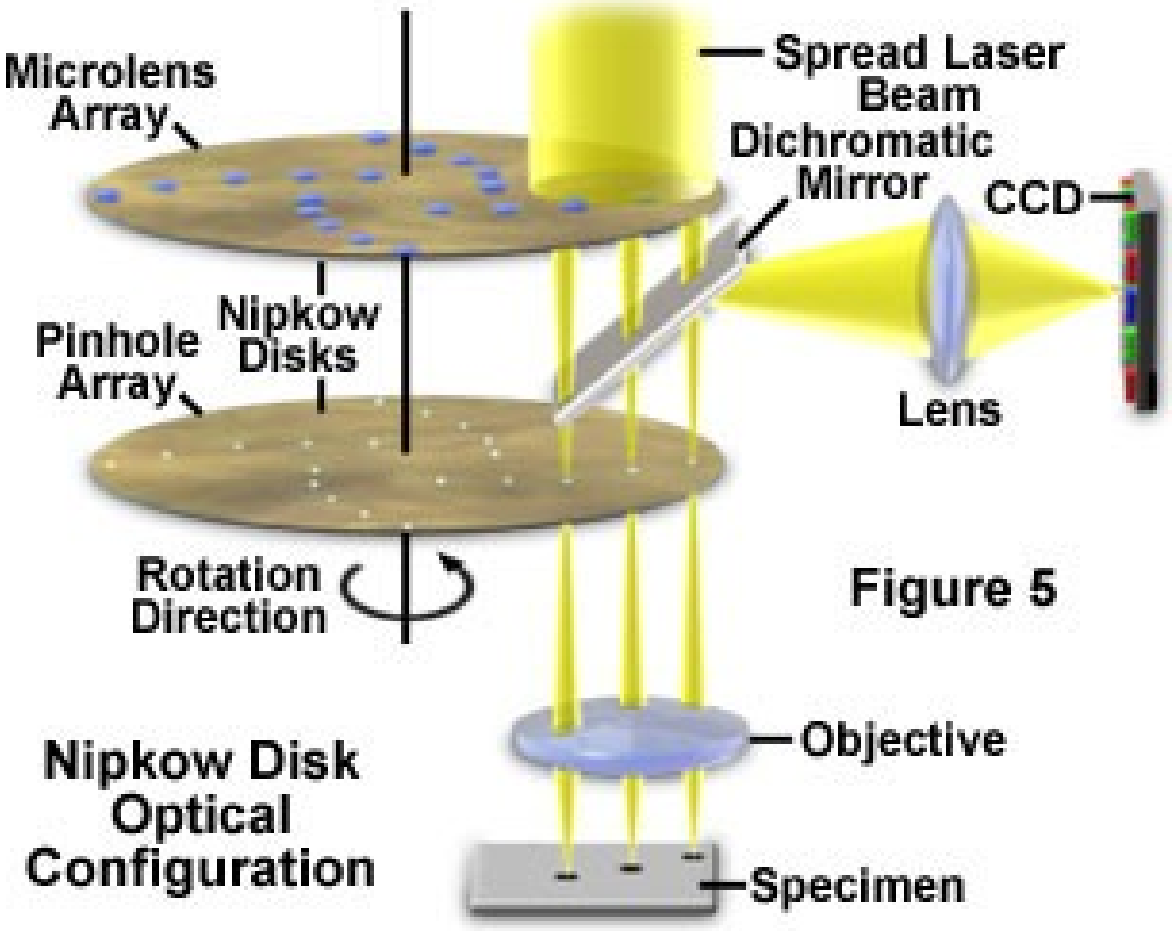


Image with many pinholes at once, so fast

Use CCD as detector, so much higher QE

Pros/Cons of spinning disk

- Fast – multiple points are illuminated at once
- Photon efficient – high QE of CCD
- Gentler on live samples – usually lower laser power

- Fixed pinhole – except in swept-field
- Small field of view (usually)
- Crosstalk through adjacent pinholes limits sample thickness

Multi-Photon Confocal Microscopy

Characteristics

1. Attosecond pulses only properly strike at the In-Focus plane of the specimen.
2. Multiple short pulses of longer wavelength light have the same amount of energy as one long pulse of shorter wavelength.

Pros and Cons of Multi-Photon Microscopy

Pros	Cons
More penetrating – up to hundreds of micron	Best excitation wavelength is not always obvious
No out of focus light	Cost (Very Expensive)
Laser is tunable. Can get range of wavelengths including UV dyes.	Laser is POWERFUL. Can damage cells, slides and even walls.
Better for Living cells if you're careful.	Difficult to align and use (Less so now)
Can also kill off specific cells/organelles.	

How big should the pinhole be?

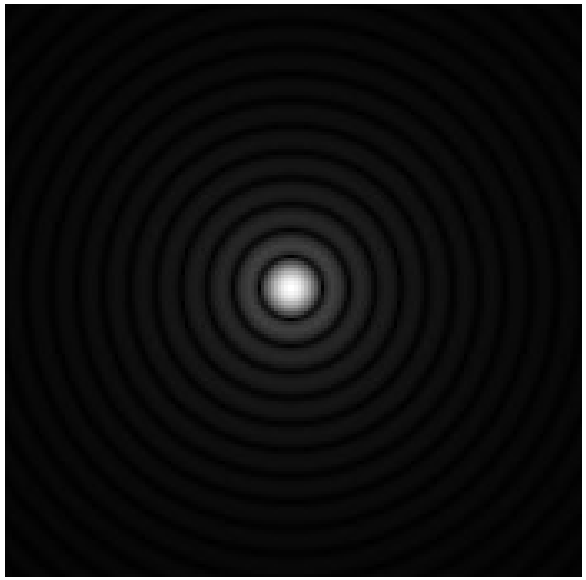
Resolution is limited by **the point-spread function**

❖ The ideal point spread function (PSF) is the three-dimensional diffraction pattern of light emitted from an infinitely small point source in the specimen and transmitted to the image plane through a high numerical aperture (NA) objective.

❖ It is considered to be the fundamental unit of an image in theoretical models of image formation.

❖ When light is emitted from such a point object, a fraction of it is collected by the objective and focused at a corresponding point in the image plane.

❖ However, the objective lens does not focus the emitted light to an infinitely small point in the image plane. Rather, light waves converge and interfere at the focal point to produce a diffraction pattern of concentric rings of light surrounding a central, bright disk, when viewed in the x-y plane.



❖The radius of disk is determined by the NA, thus the resolving power of an objective lens can be evaluated by measuring the size of the Airy disk (named after George Biddell Airy).

❖ The size of the confocal pinhole needs to be matched to the size of the Airy disk. Any smaller, and you are throwing out useful light. Any larger, and you see more out of focus light.

THANKS