

# Confocal Microscopy

## Pinhole principle

Rejection of out-of-focus light

## Optical sectioning

Thin optical slices through sample

## Laser scanning

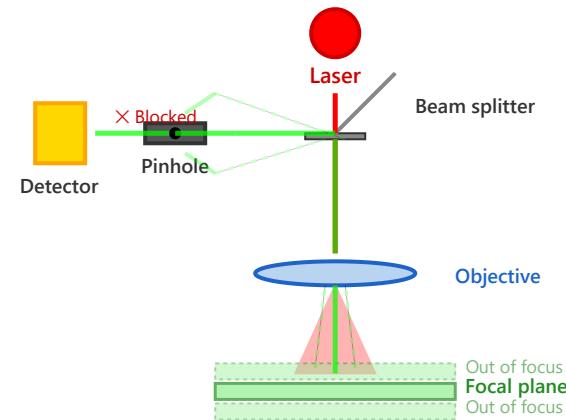
Point-by-point image acquisition

## Z-stack acquisition

Series of optical sections

## 3D rendering

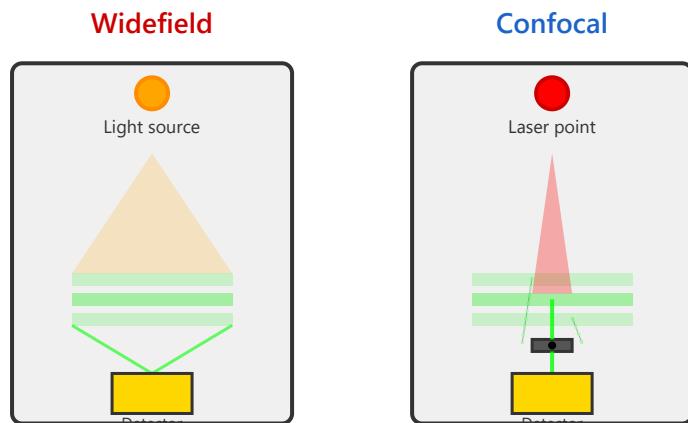
Volumetric visualization from stacks



## Pinhole Principle

- ✓ In-focus light passes through pinhole
- ✗ Out-of-focus light blocked by pinhole
  - High axial resolution (~500 nm)

# 1. Pinhole Principle



## Blurred image

Out-of-focus light  
reduces contrast

## Sharp image

Only in-focus light  
reaches detector

### Key Advantages

- ✓ Eliminates out-of-focus blur
- ✓ Improves contrast and resolution
- ✓ Enables optical sectioning
- ✓ Axial resolution: ~500 nm (vs ~2  $\mu\text{m}$  widefield)

### Mechanism

The pinhole aperture is placed at a conjugate focal plane (confocal) to the specimen focal plane. Light from the in-focus region converges to a point and passes through the pinhole, while out-of-focus light is spatially distributed and blocked.

### Physical Principle

Based on the point spread function (PSF) of the optical system. In-focus light has a tight PSF that fits through the pinhole, while out-of-focus light has a broad PSF that is rejected.

### Pinhole Size

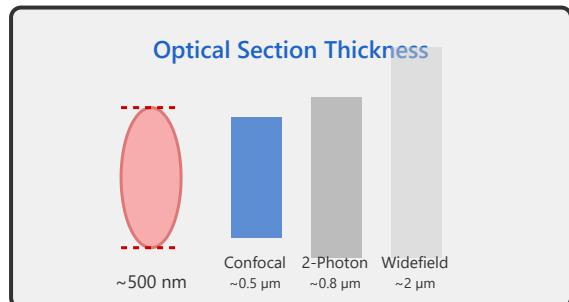
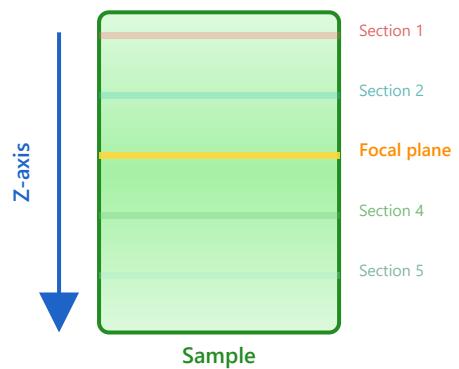
Typical size: 1 Airy Unit (AU)

- Smaller pinhole → Better optical sectioning, lower signal
- Larger pinhole → More signal, reduced sectioning
- Optimal: 0.5-1.5 AU depending on application

### Applications

Essential for imaging thick specimens like tissue sections, embryos, and 3D cell cultures where conventional microscopy suffers from out-of-focus blur.

## 2. Optical Sectioning



### Definition

Optical sectioning is the ability to obtain thin, focused images from different depths within a thick specimen without physical sectioning. Each image represents a single plane of focus.

### Section Thickness

Determined by the numerical aperture (NA) and wavelength:

- Typical thickness: 0.5-1.5  $\mu\text{m}$
- Formula:  $\Delta z \approx 2\lambda / \text{NA}^2$
- Higher NA → Thinner optical sections
- Shorter wavelength → Better resolution

### Advantages

- Non-invasive imaging of thick samples
- Preservation of sample integrity
- Real-time imaging possible
- Sequential sections perfectly aligned

### Applications

Ideal for tissue sections (50-200  $\mu\text{m}$  thick), whole-mount embryos, organoids, and biofilms. Enables visualization of

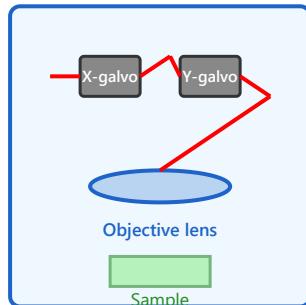
structures at specific depths without interference from other planes.

## 3. Laser Scanning

Raster Scanning Pattern



Scanning System



Scanning Parameters

Speed

- Typical: 1-10 fps
- Fast scan: up to 30 fps
- Trade-off with resolution

Resolution

- Typical:  $512 \times 512$  pixels
- High-res:  $2048 \times 2048$
- Adjustable zoom factor

Dwell Time

- Time laser spends at each pixel: 0.5-10  $\mu$ s
- Longer dwell time → Better signal-to-noise ratio
- Shorter dwell time → Faster imaging, less photobleaching

### Mechanism

A focused laser beam is scanned across the specimen point-by-point using galvanometer mirrors (galvo mirrors). The X-galvo controls horizontal scanning, Y-galvo controls vertical scanning. Fluorescence emission from each point is collected sequentially.

### Scanning Modes

- **Unidirectional:** Scan in one direction, fly back
- **Bidirectional:** Scan both directions (faster)
- **Line scanning:** Rapid scanning along one axis
- **Frame scanning:** Complete 2D image acquisition

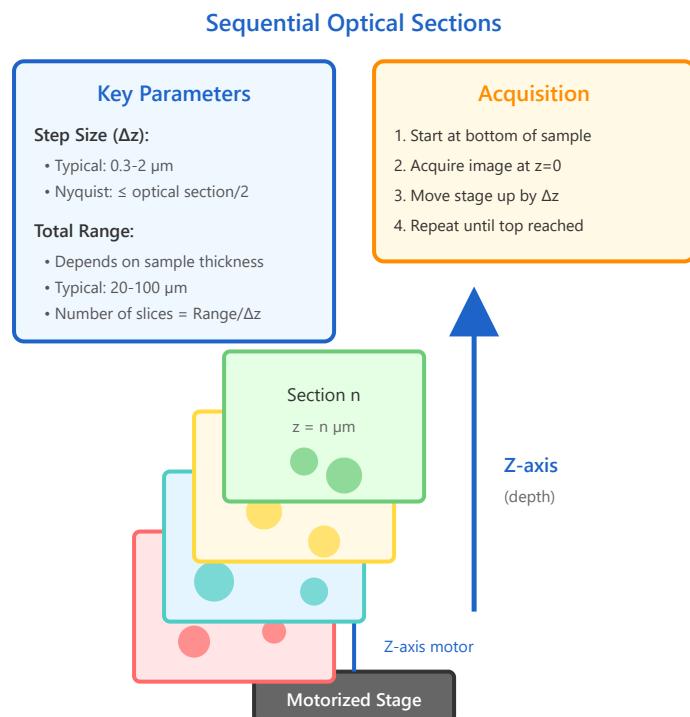
### Advantages

- Precise control of illumination position
- Minimal sample exposure per pixel
- Flexible field of view and zoom
- Compatible with multiple detectors

### Limitations

Sequential acquisition means slower imaging compared to widefield. Speed-resolution trade-off: faster scanning reduces dwell time and signal quality. Resonant scanners can achieve video-rate imaging.

## 4. Z-stack Acquisition



### Definition

A z-stack is a series of optical sections acquired at different focal planes through the depth of a specimen. The microscope stage (or objective) moves in precise increments along the z-axis to capture each plane.

### Step Size Selection

Critical parameter affecting data quality:

- Nyquist criterion:** Step size  $\leq$  optical section thickness / 2
- Too large:** Missing information between slices
- Too small:** Oversampling, more photobleaching, larger files
- Practical:** 0.3-1  $\mu\text{m}$  for most applications

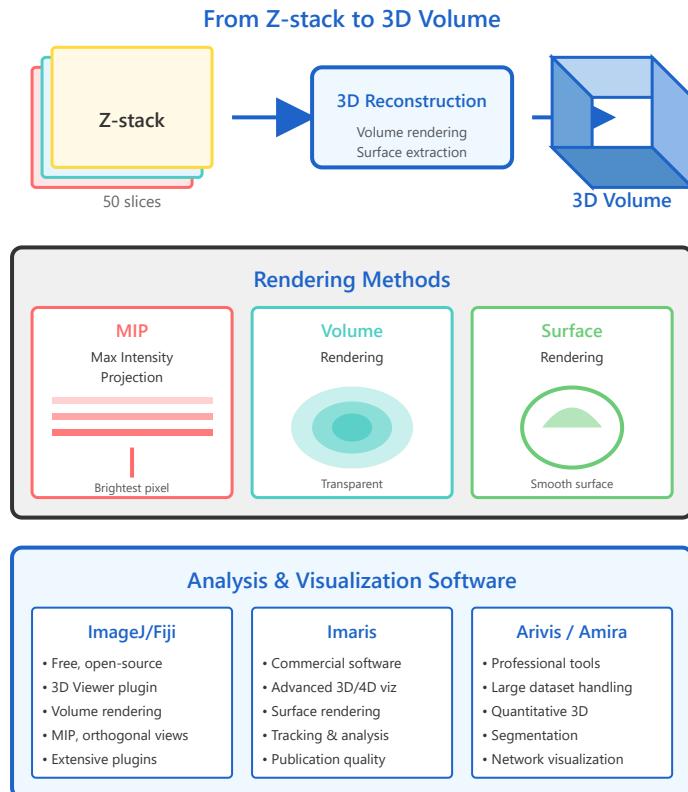
### Considerations

- Total acquisition time increases with number of slices
- Photobleaching accumulates through stack
- Stage drift can affect alignment
- File sizes can be large (GB range)

### Applications

Essential for 3D cell imaging, tissue architecture studies, developmental biology, and neuroscience. Enables volume quantification and 3D reconstruction of cellular structures.

# 5. 3D Rendering & Visualization



## 3D Reconstruction

Z-stack images are compiled into a 3D volume dataset. Each voxel (3D pixel) contains intensity information. The volume can be visualized using various rendering techniques to reveal spatial relationships.

## Visualization Methods

- **MIP:** Projects maximum intensity along viewing axis - good for sparse structures
- **Volume rendering:** Assigns opacity/color based on intensity - shows internal structures
- **Surface rendering:** Creates smooth 3D surface from threshold - ideal for morphology
- **Orthogonal views:** XY, XZ, YZ cross-sections

## Applications

- 3D cell morphology and organelle distribution
- Neuronal network reconstruction
- Vascular architecture mapping
- Volumetric quantification (cell volume, surface area)
- Spatial relationship analysis

## Considerations

Requires adequate sampling (proper step size), sufficient signal-to-noise ratio, and correction for spherical

aberration in deep imaging. Deconvolution can improve 3D resolution.