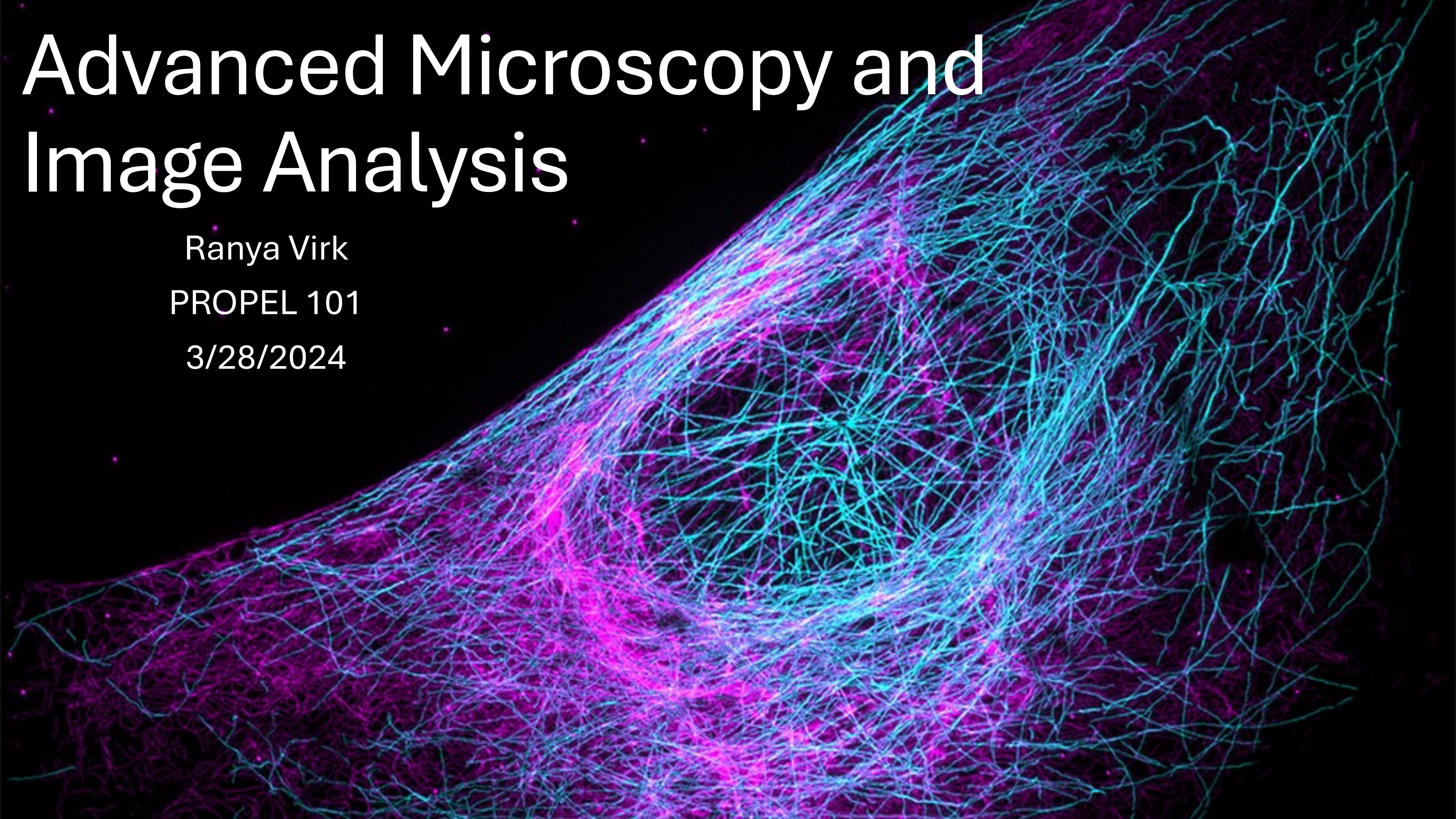
Advanced Microscopy and Image Analysis

Ranya Virk

PROPEL 101

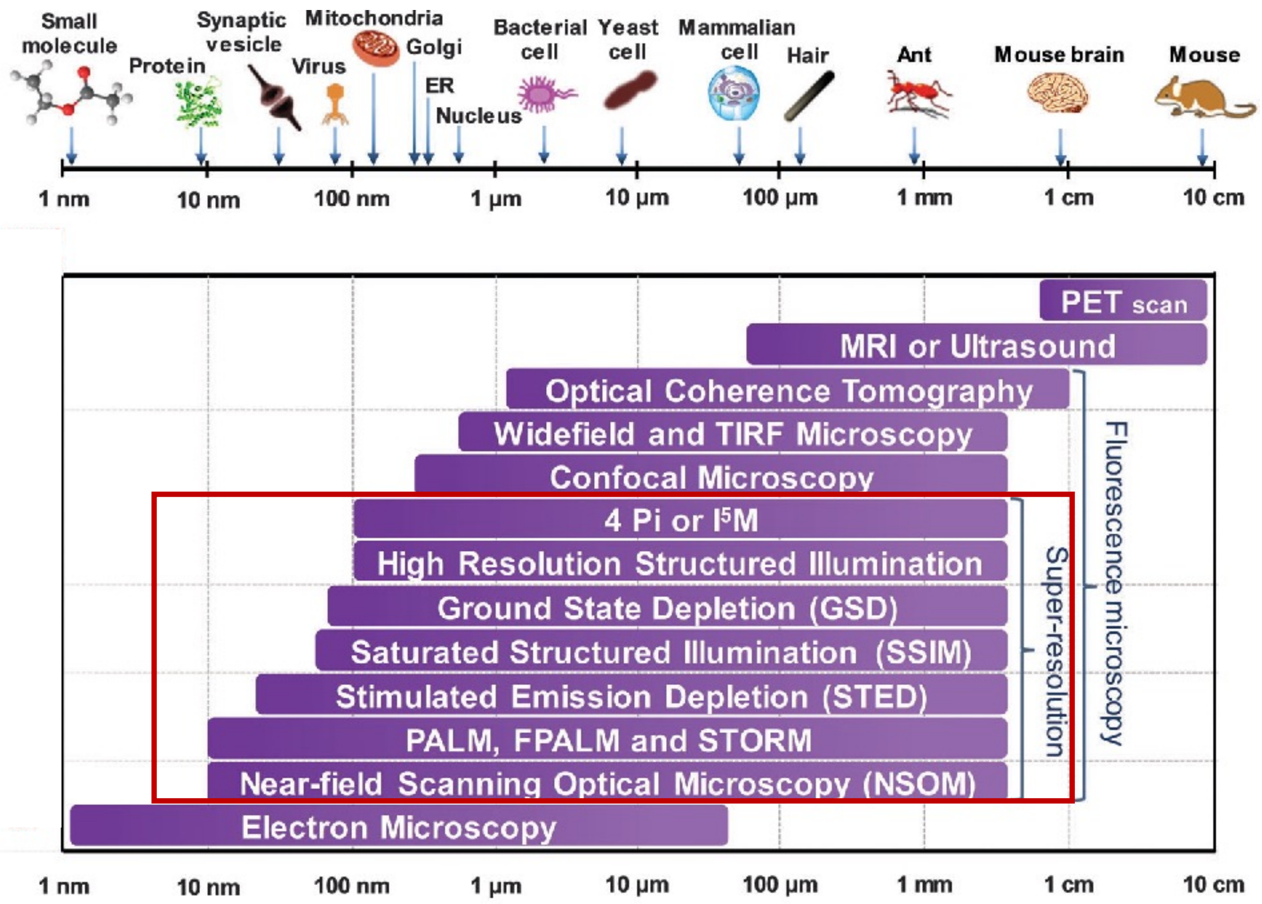
3/28/2024



Learning Objectives

1. Define basic terminology for fluorescence microscopy
2. Define diffraction-limited resolution
3. Understand how super-resolution microscopy beats the diffraction limit of light (in 2D)
4. Understand how lattice light-sheet microscopy is more effective at acquiring 3D images than traditional confocal microscopy

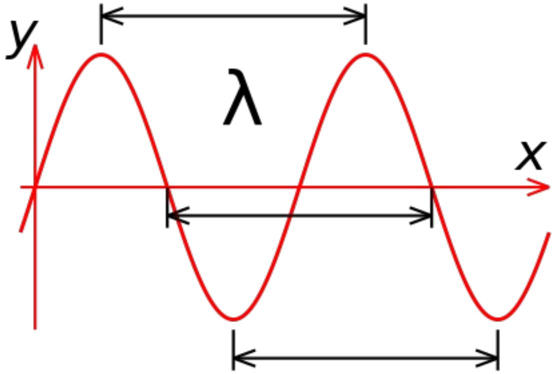
Super-resolution microscopy enables more precise imaging of molecular structures

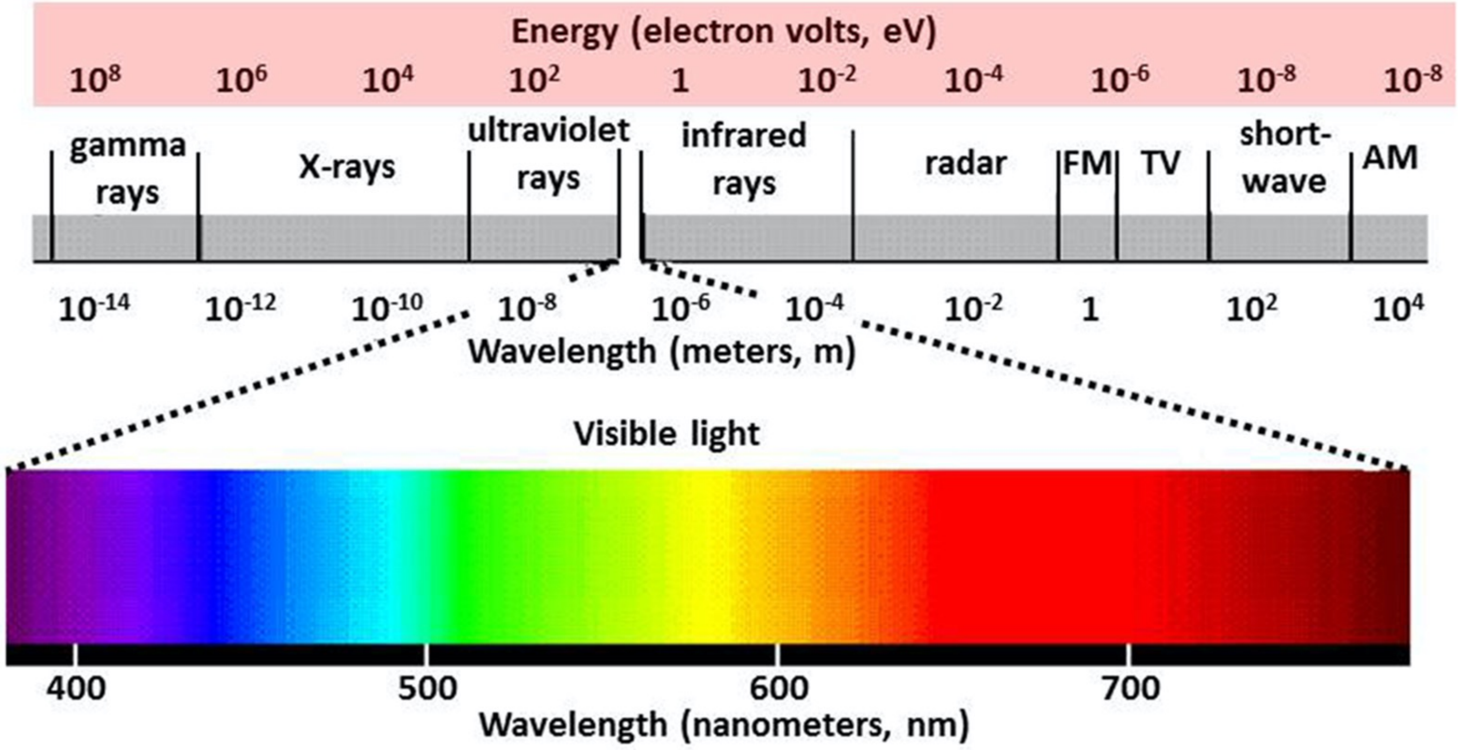


Yang et al., Chemical Society Reviews (2016)

Optical microscopy uses visible light

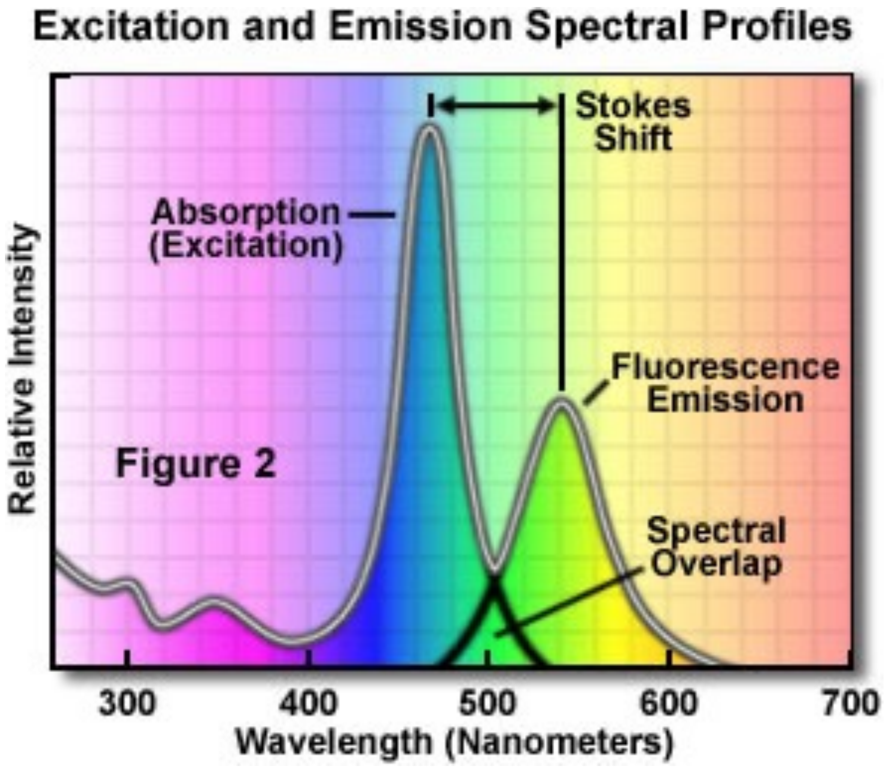
Light behaves as a wave that can be described by **wavelength** λ





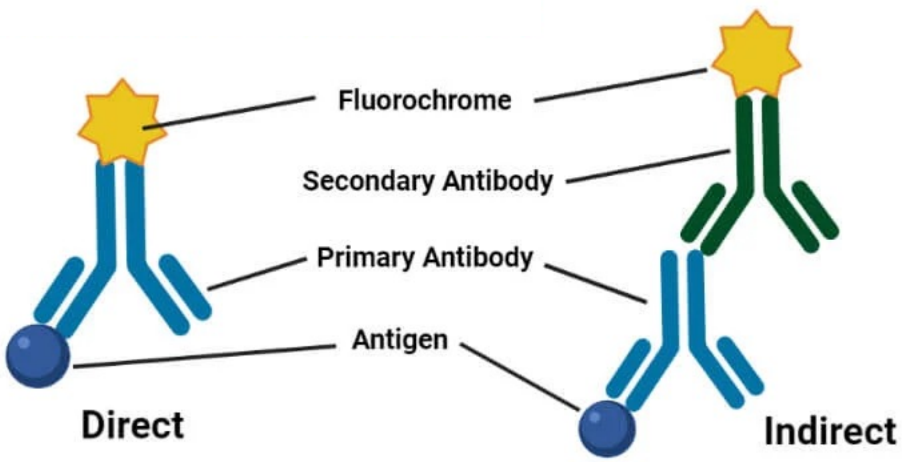
Fluorescence microscopy basics

Fluorophores absorbs light energy of a specific wavelength and re-emits it at a longer wavelength



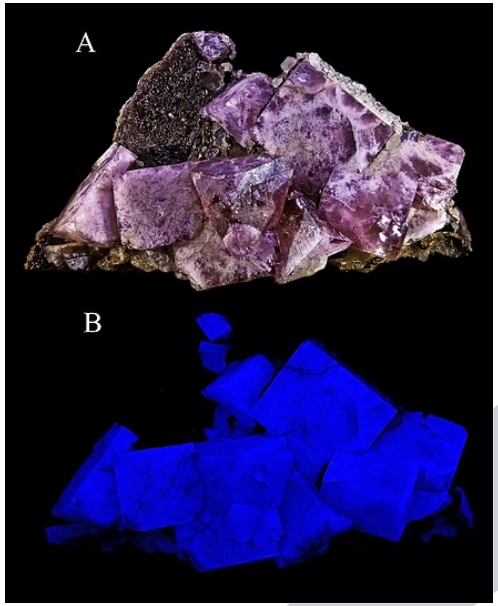
Source: Olympus

Immunofluorescence: combines specific antibodies targeting cellular component of interest with fluorophore



Source: Microbe Notes

A brief history of fluorophores



1852: Term "fluorescence" is coined to describe fluorite exposed to UV

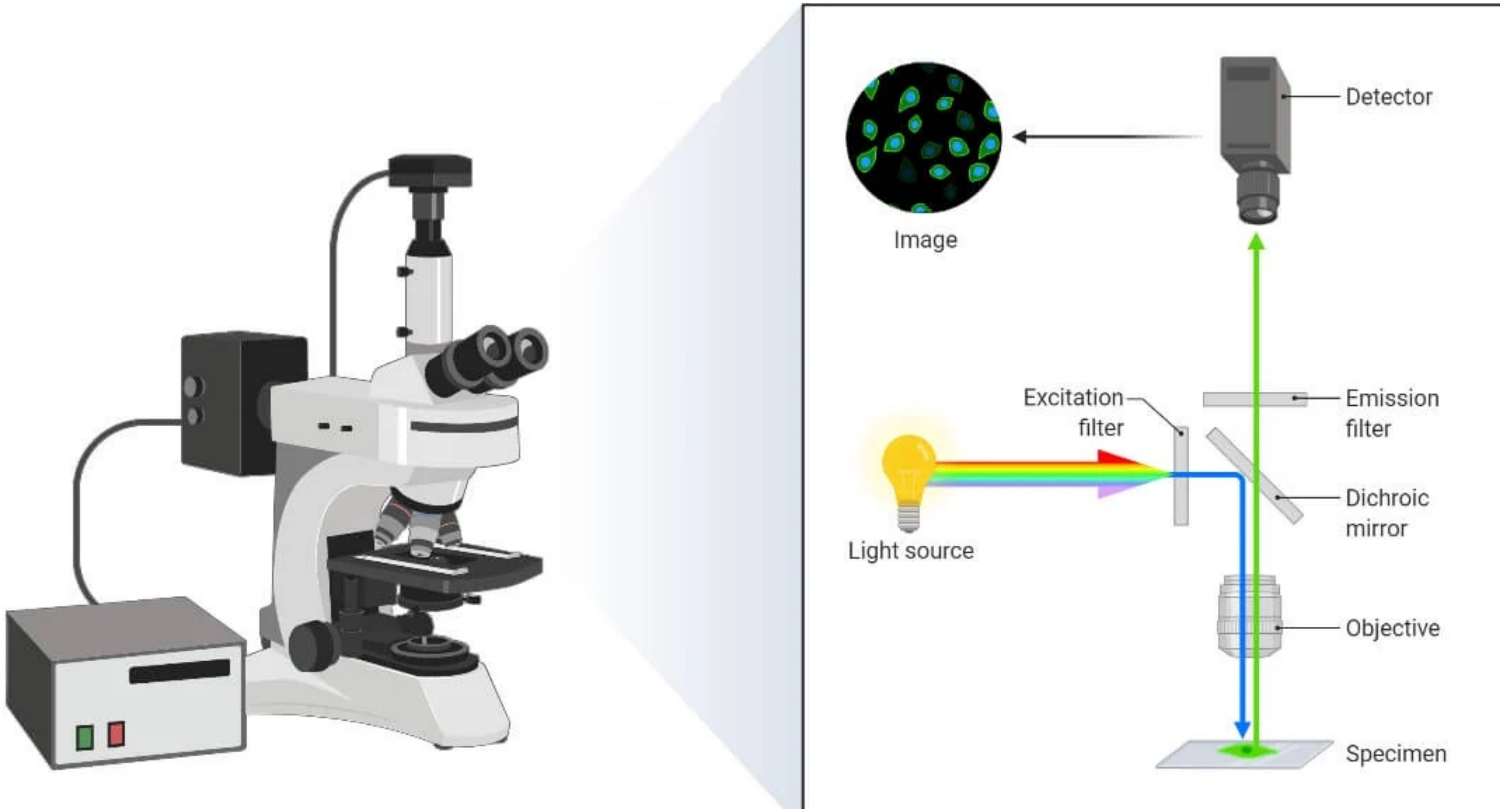
1871: One of earliest synthetic dyes fluorescein is synthesized

1960s: Green Fluorescent Protein (GFP) first isolated in jellyfish

1994: GFP first used as reporter of gene expression in C. elegans

Credit: UPenn

Fluorescence microscope setup



Source: Microbe Notes

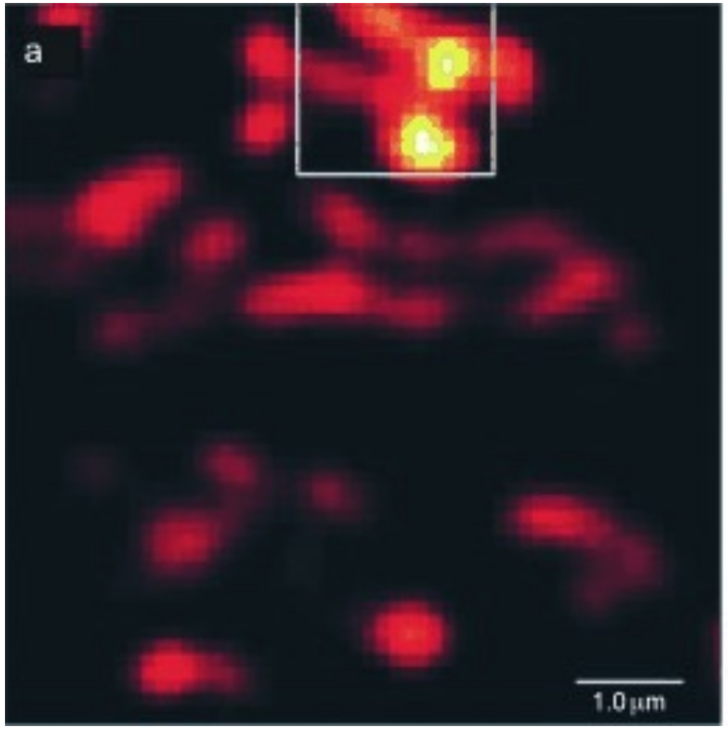
Filters: selectively transmit or block specific wavelengths of light

Objective: gathers and magnifies light from specimen to produce an enlarged and focused image

Dichroic mirrors: facilitate separation of excitation and emission light

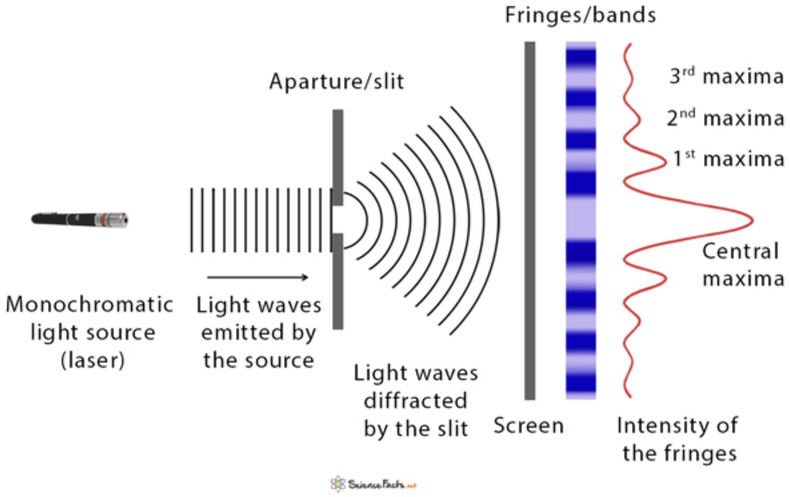
Diffraction-limited microscopy prevents precise imaging of molecular structures

Example: Mitochondria



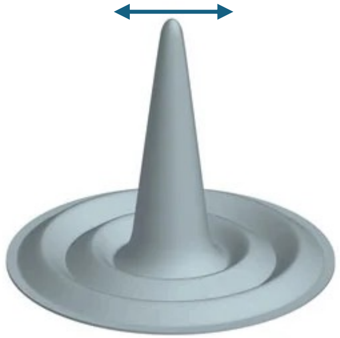
Bertzig, et al., Science 2006

Diffraction: interference or bending of waves (e.g., light) around an obstacle (e.g., specimen)



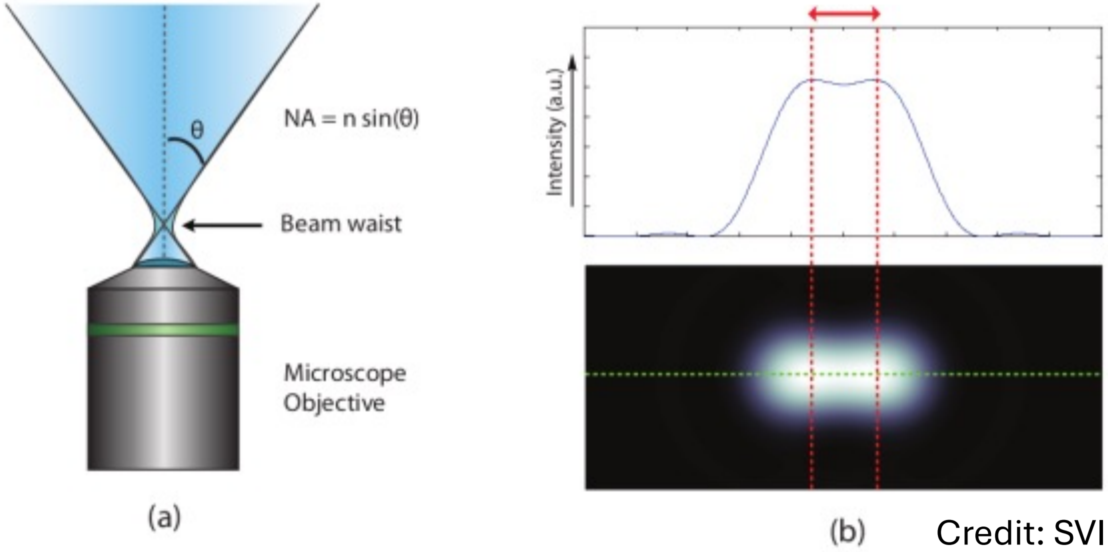
Point spread function (PSF): describes how point source of light is spread out or blurred in an image due to diffraction

Central peak: 250-300 nm



Credit: Leica

Diffraction-limited resolution in optical microscopy



Credit: SVI

NA = **Numerical aperture**

n = **Refractive index**
(speed of light in medium/speed of light in vacuum)

θ= **Half-angle** of cone of light entering optical system
(max angle at which light can enter objective and still be collected efficiently)

Abbe **diffraction limit** for lateral (XY) resolution

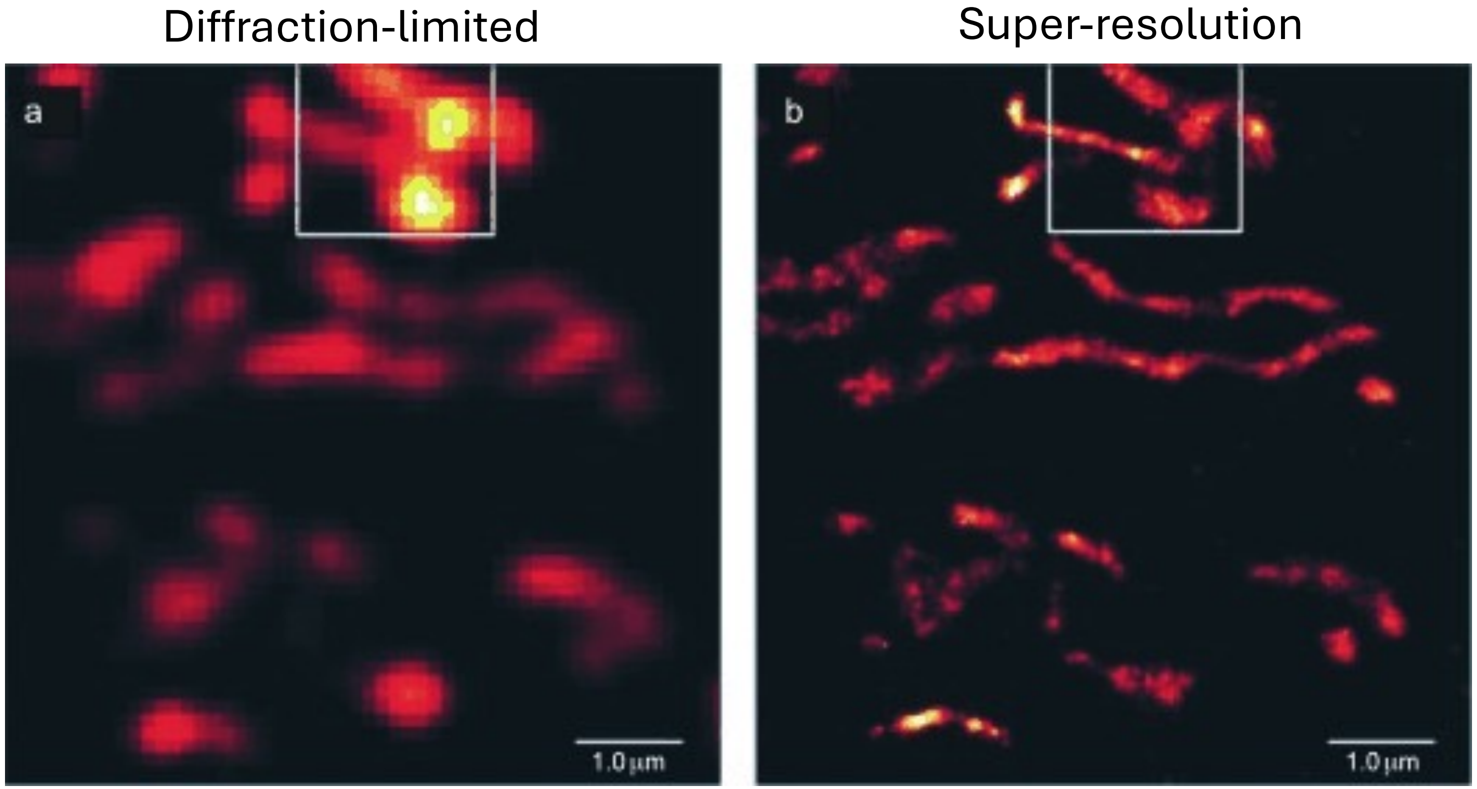
$$d = \frac{\lambda}{2NA}$$

How to increase diffraction-limited resolution:

1. Increase numerical aperture
   (e.g., using immersion oil instead of air w/ microscope objective)
2. Use fluorophore with lower wavelength of emission
   (e.g., purple part of visible light spectrum)

Super-resolution microscopy enables more precise imaging of molecular structures

Example: Mitochondria

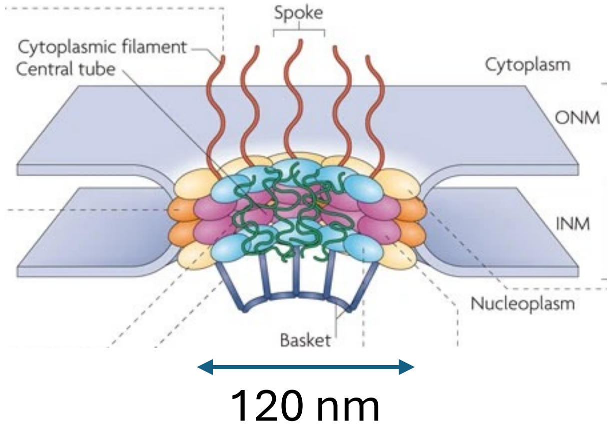


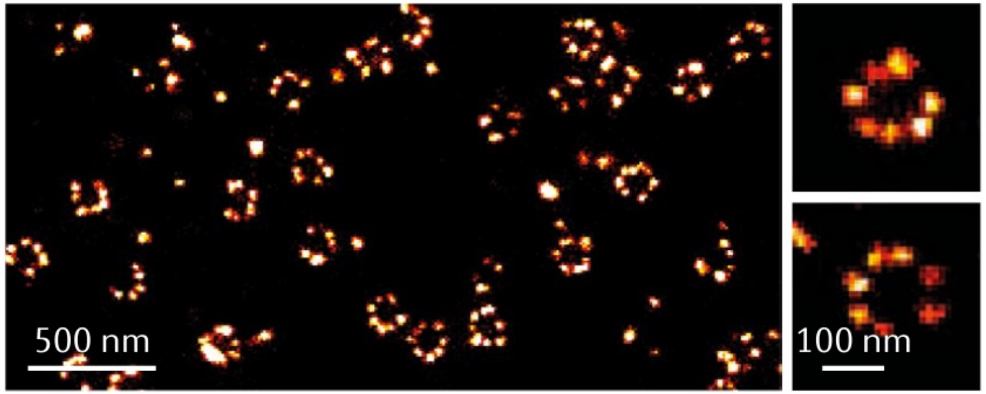
Bertzig, et al., Science 2006

Eric Bertzig, Stefan Hell, and William Moerner win Nobel Prize in Chemistry in 2014 for the development of super-resolved fluorescence microscopy

Other applications of super-resolution microscopy

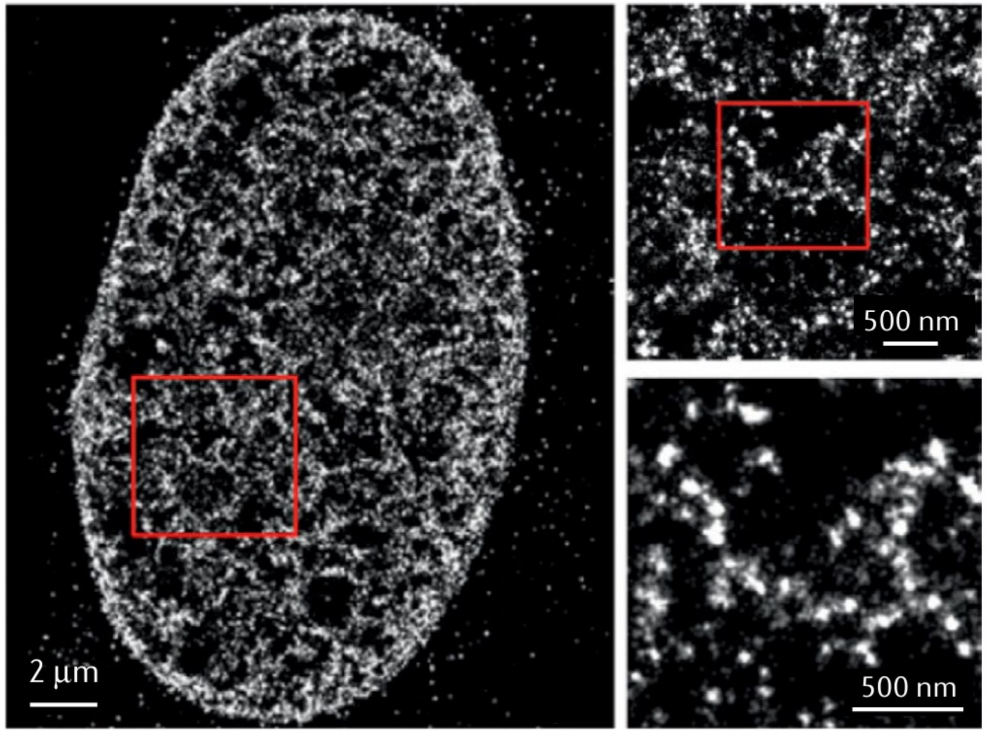
Nuclear pore complex





Thevathasan et al., Nature Methods (2019)

DNA + Chromatin

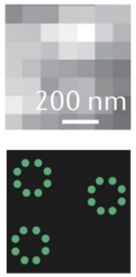


Ricci et al., Cell (2015)

Single-molecule localization microscopy (SMLM) involves random activation, localization, and deactivation of individual fluorescent molecules

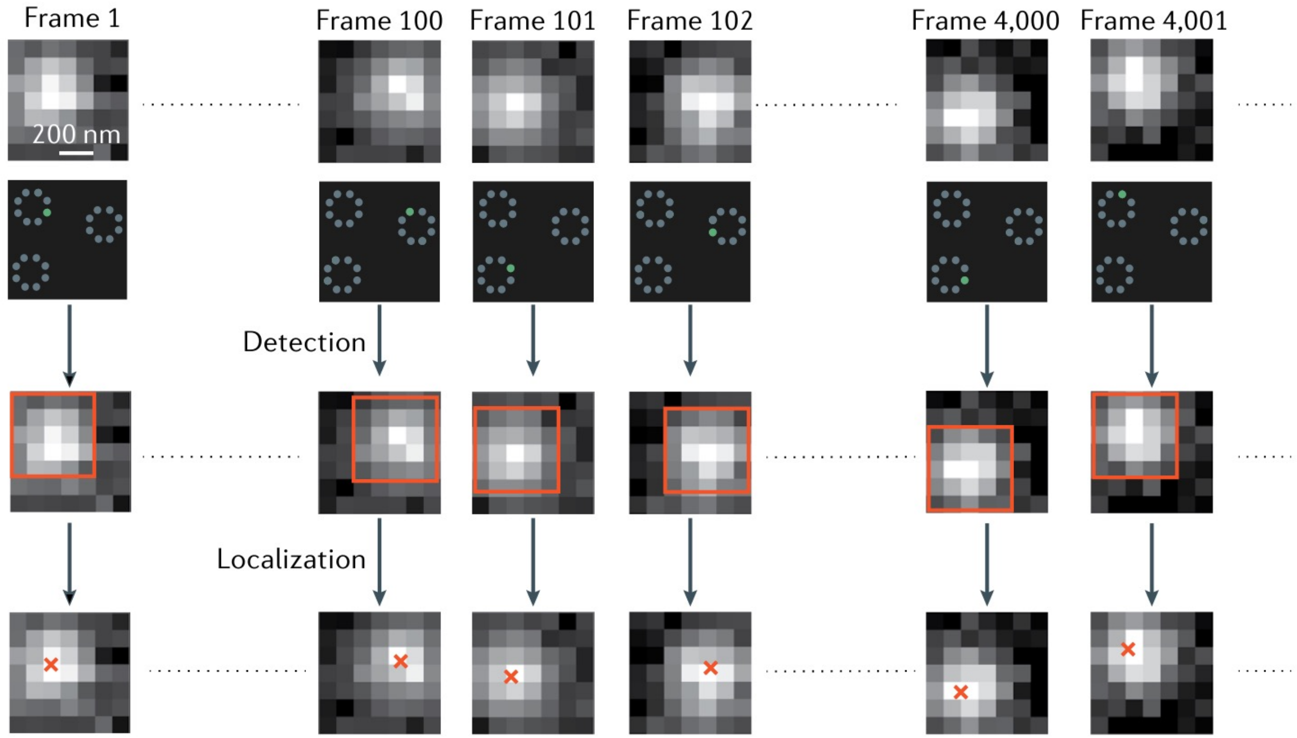
Diffraction-limited

f



Nuclear pore complex

SMLM



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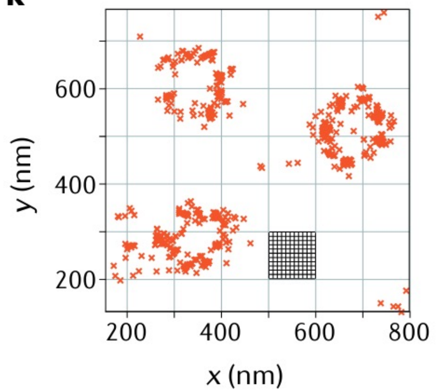
Frame	*x* (nm)	*y* (nm)	*N*	...
1	293.7	569.6	632	...
2	673.2	442.6	780	...
3	363.3	229.2	530	...
...	...	...	...	...

k



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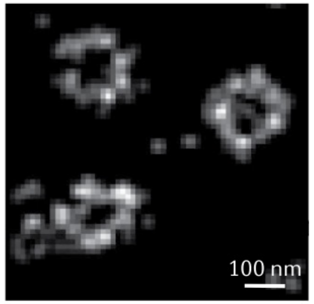
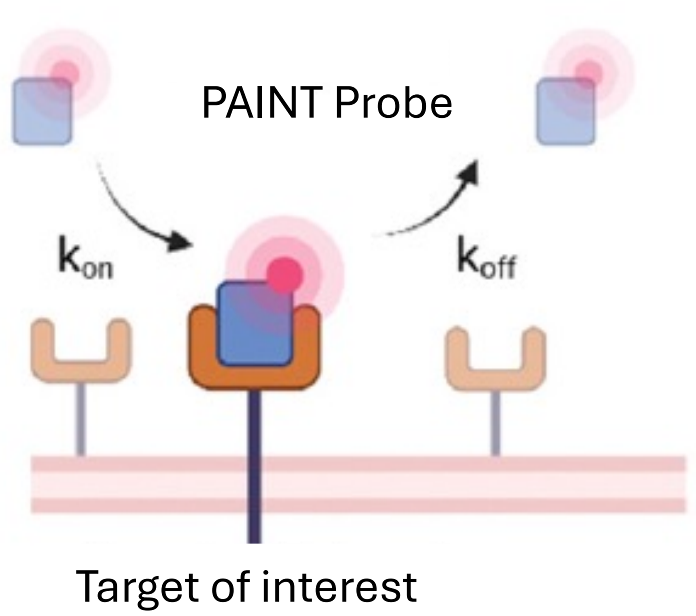


Lelek et al., Nature Reviews 2021

Point Accumulation for Imaging in Nanoscale Topography (PAINT): low density of fluorophores transiently bind to their specific targets

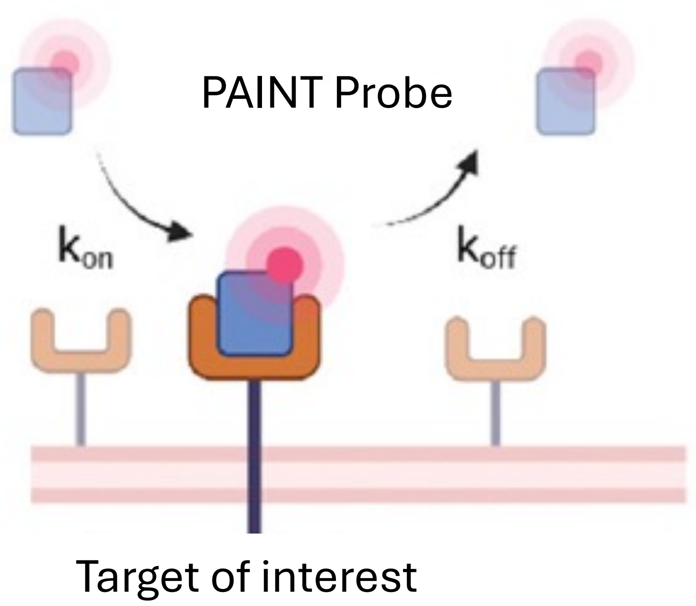


Tholen, et al., Chem Comm (2023)

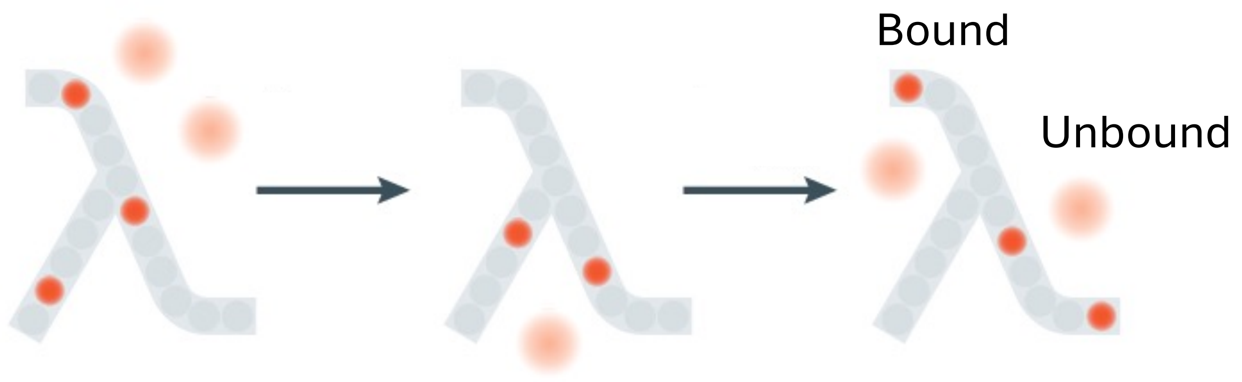
Specificity: ability of antibody to recognize and specifically bind target antigen

Affinity: strength of binding (k_{off})

Point Accumulation for Imaging in Nanoscale Topography (PAINT): low density of fluorophores transiently bind to their specific targets



Tholen, et al., Chem Comm (2023)



Lelek et al., Nature Reviews 2021

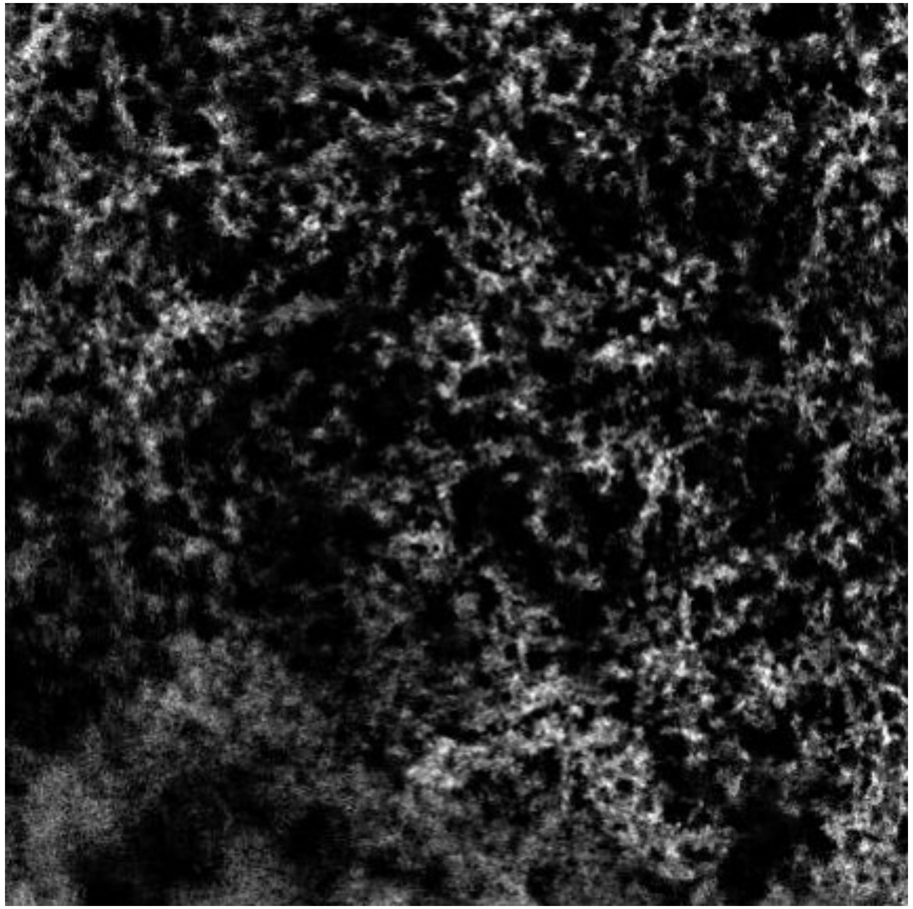
Specificity: ability of antibody to recognize and specifically bind target antigen

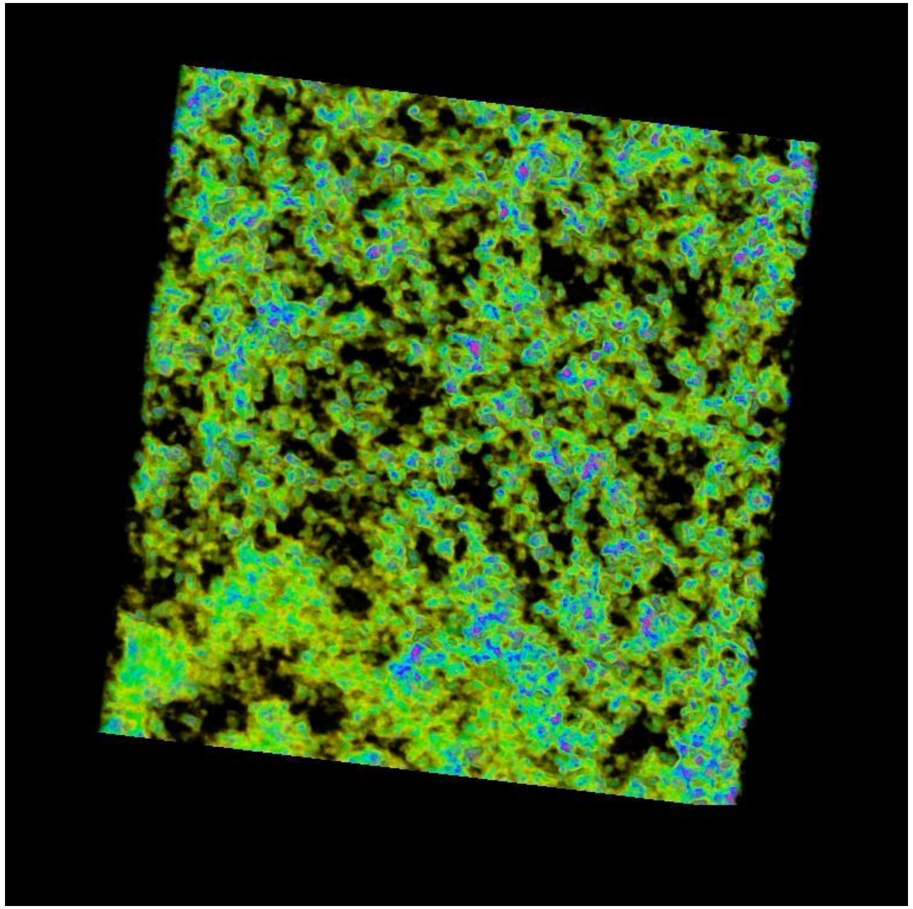
Affinity: strength of binding (k_{off})

Signal to noise ratio (SNR): ratio of bound fluorophores to background/unbound fluorophores

3D Imaging: Reconstruction of consecutive 2D slices

Example: Electron microscopy imaging of chromatin density

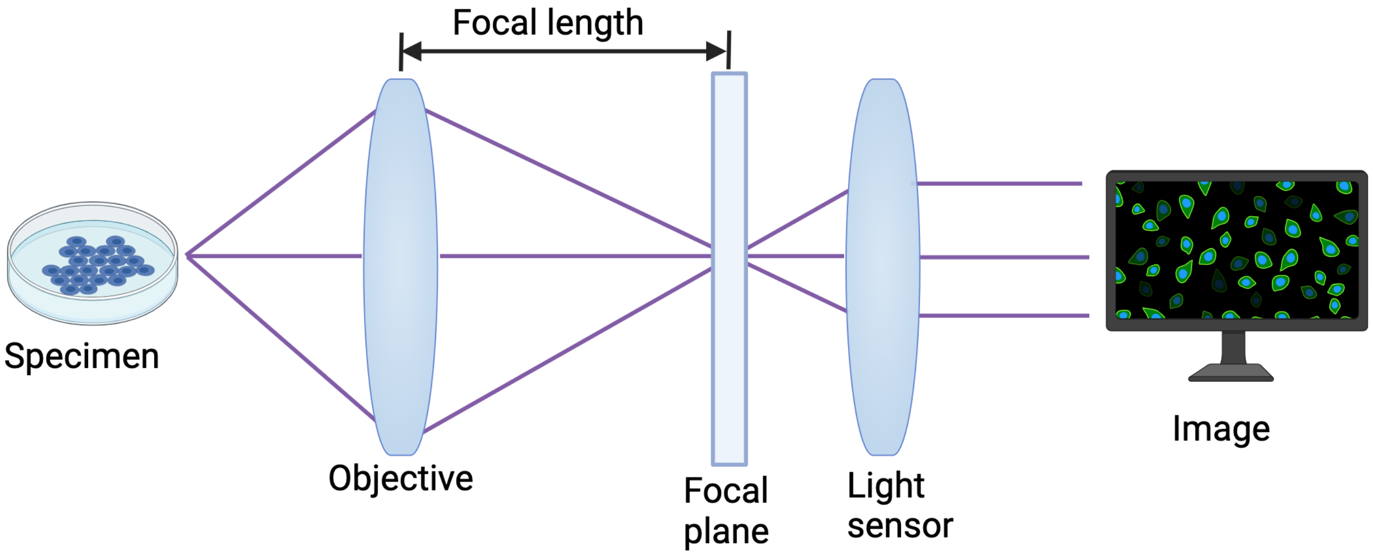




Li, Agrawal, Virk, Scientific Reports (2022)

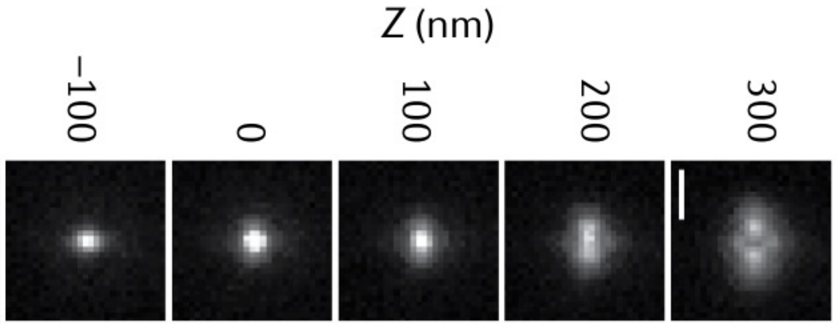
How to focus in the z plane

Focal plane: specific plane where light rays converge to form a focused image



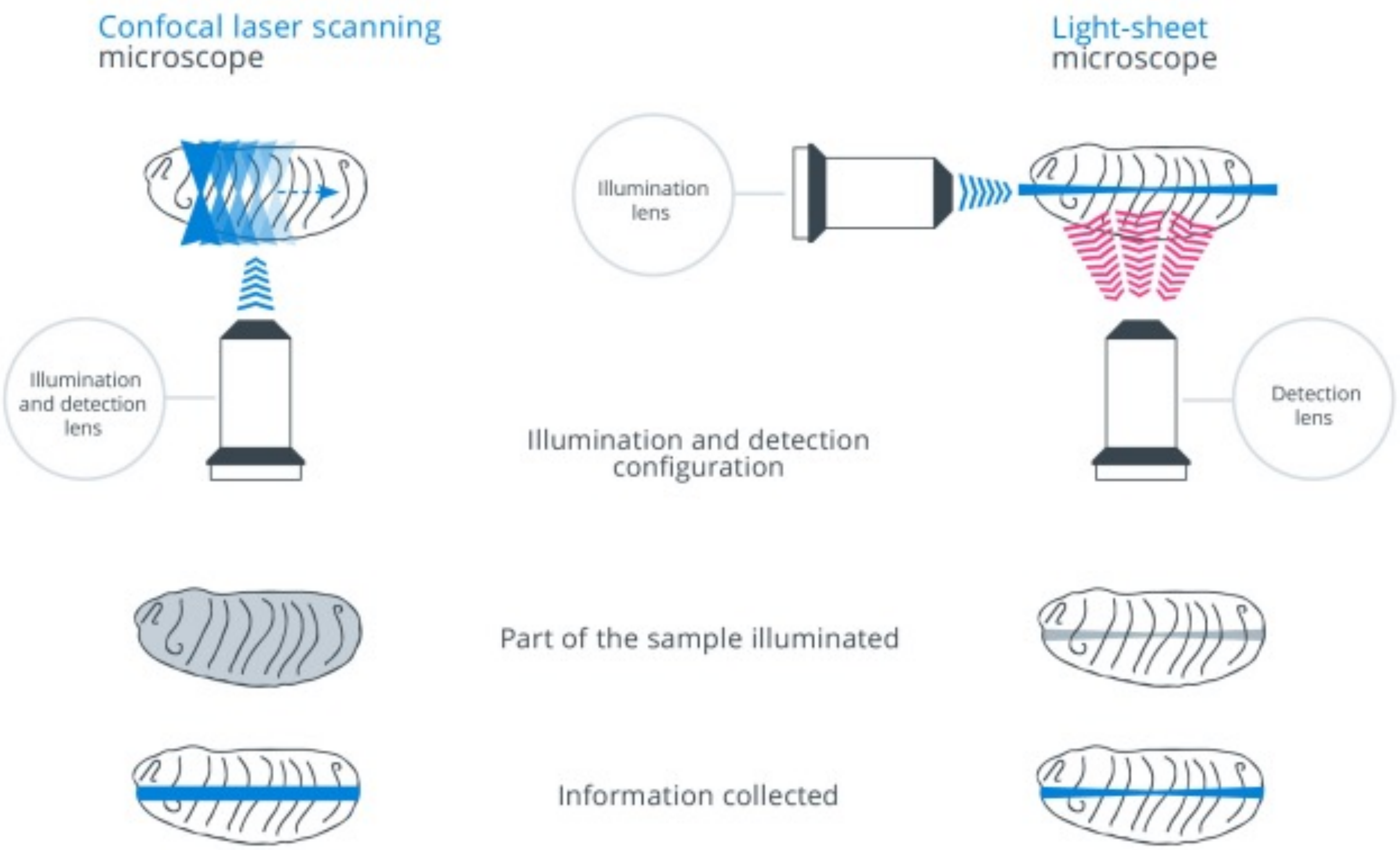
Signal is sharpest when specimen/sample is centered at the focal plane

Depth of focus: range of distances (z positions) that are acceptably in focus



Lelek et al., Nature Reviews 2021

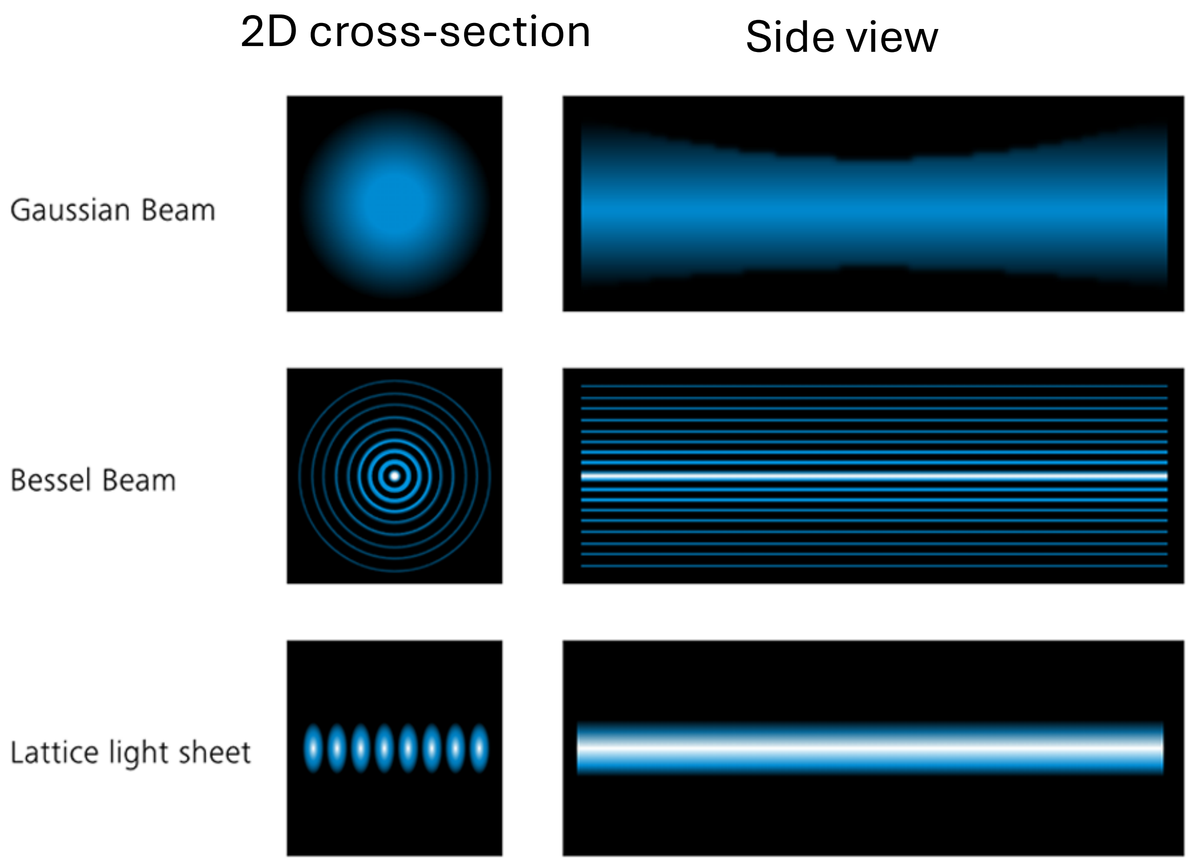
Light-sheet microscopy uses thin sheets of light to illuminate only the part of the specimen that is in the focal plane

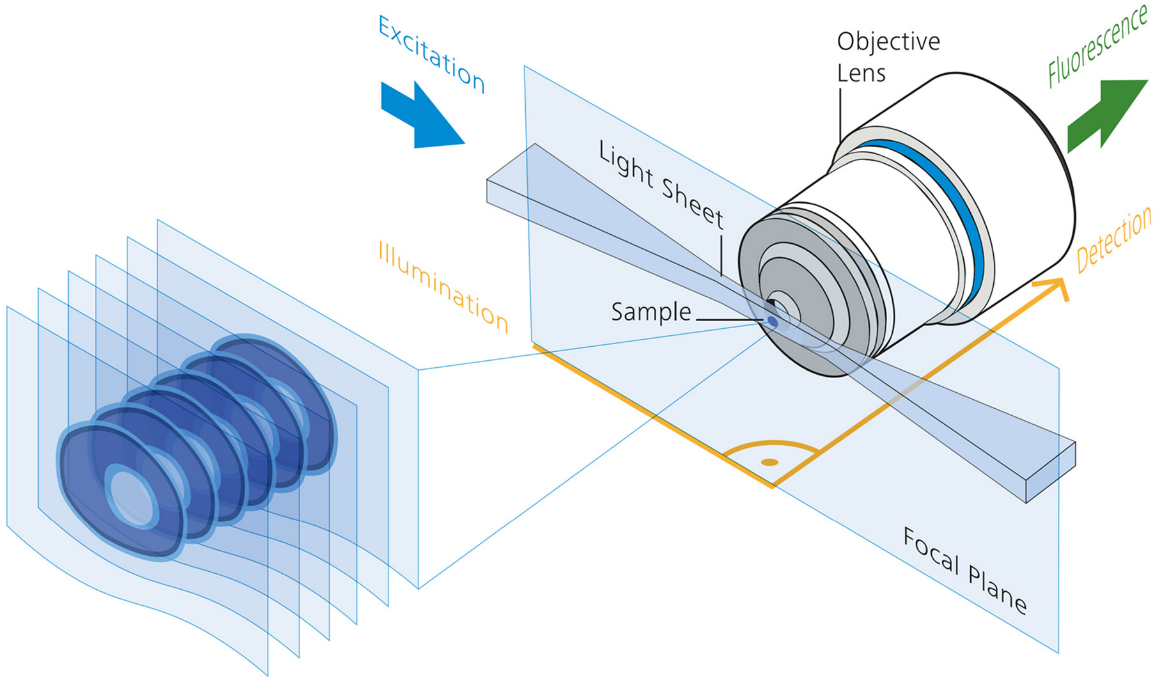


Credit: Bruker

Excitation volume: 3D region within sample where fluorophores are excited by incoming light

Lattice light-sheet microscopy





Credit: Zeiss

Benefits of lattice light-sheet microscopy

1) <u>Minimizes **photobleaching:**</u> when fluorescence emitted by fluorophore diminishes and eventually disappears upon prolonged light exposure

Mouse intestine

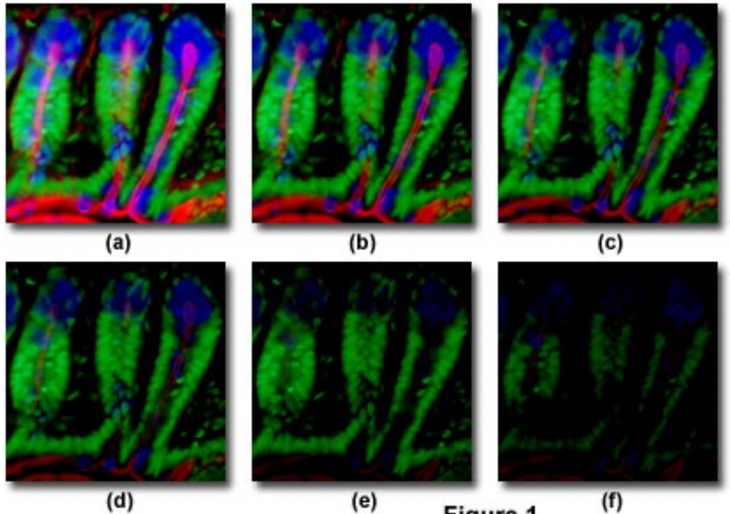
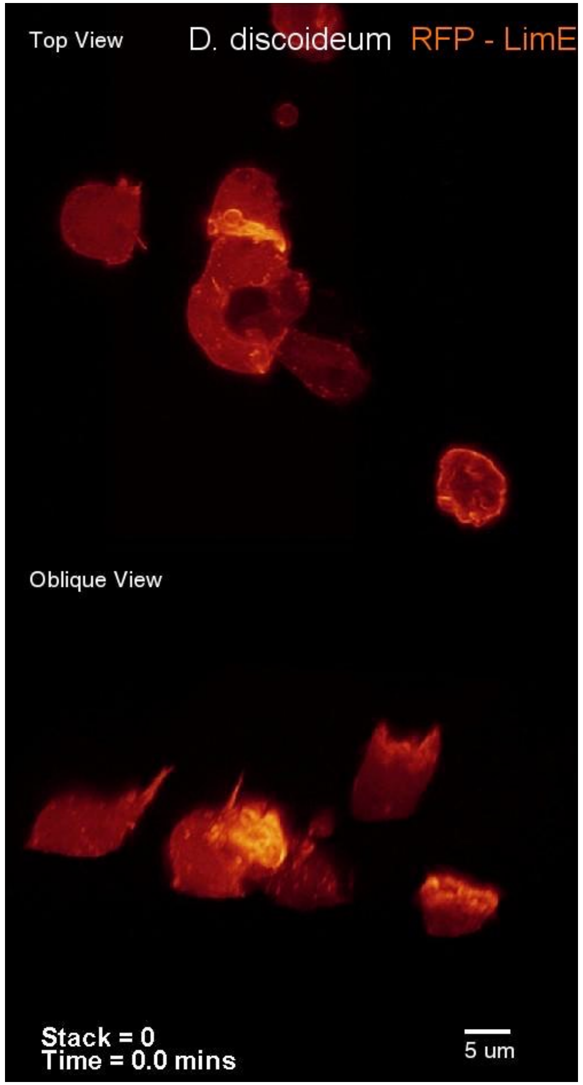


Figure 1

Credit: Molecular Expressions

2) <u>High **axial resolution:**</u> ability of a microscope to distinguish between two closely spaced objects along z-axis

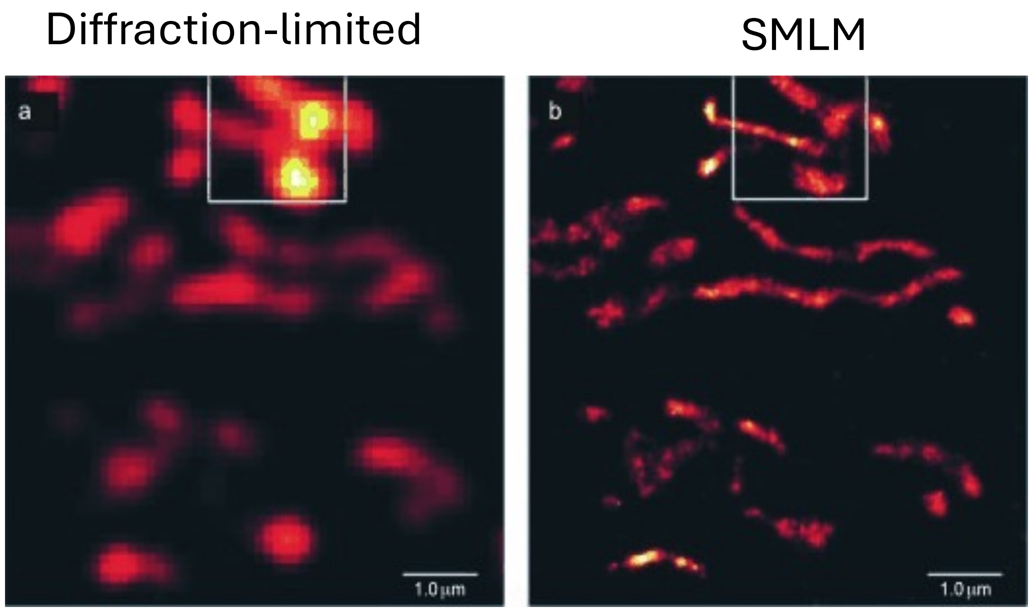
3) <u>High-speed 3D imaging:</u> images can be acquired at different focal planes simultaneously



Chen et al., Science (2014)

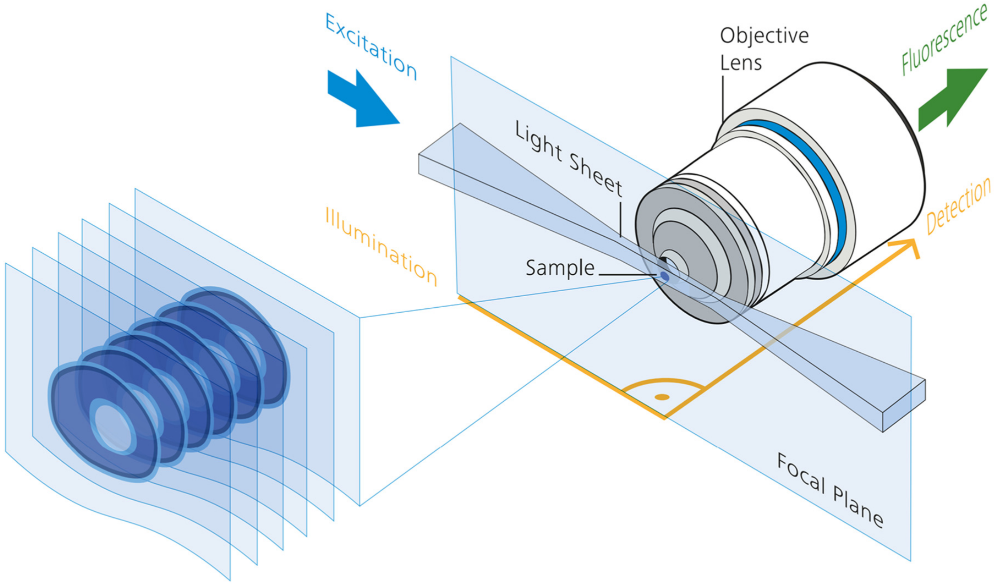
Summary

- **Super-resolution microscopy** beats the diffraction limit for optical fluorescence microscopy
- **Single-molecule localization microscopy (SMLM)** involves random activation, localization, and deactivation of individual fluorescent molecules



Bertzig, et al., Science 2006

- **Light sheet microscopy** only illuminates part of the specimen that is in the focal plane
- **Lattice light sheet microscopy** illuminates specimen w/ series of thin light sheets arranges in lattice pattern enabling high-resolution, high-speed 3D imaging



Credit: Zeiss