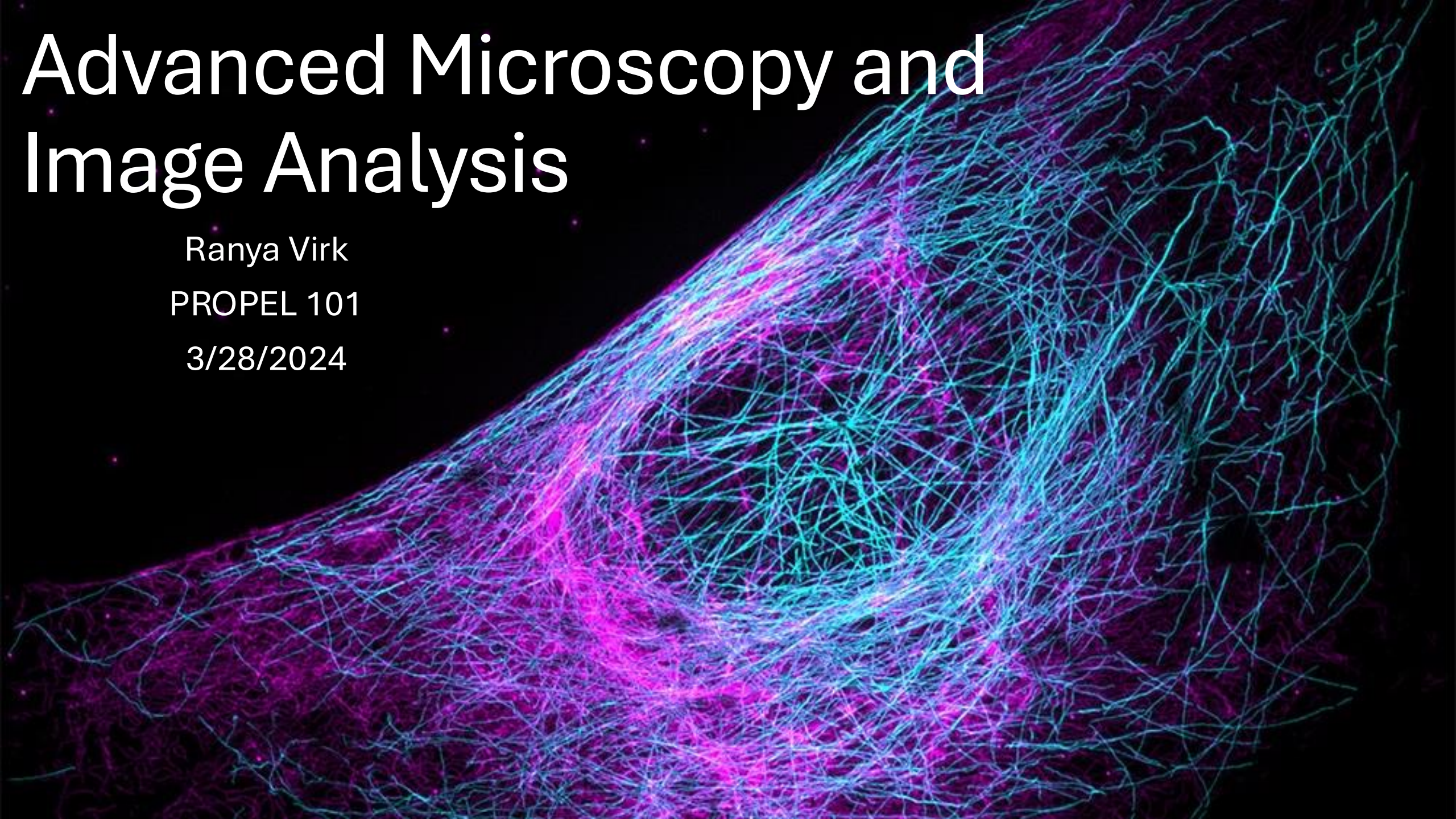


Advanced Microscopy and Image Analysis

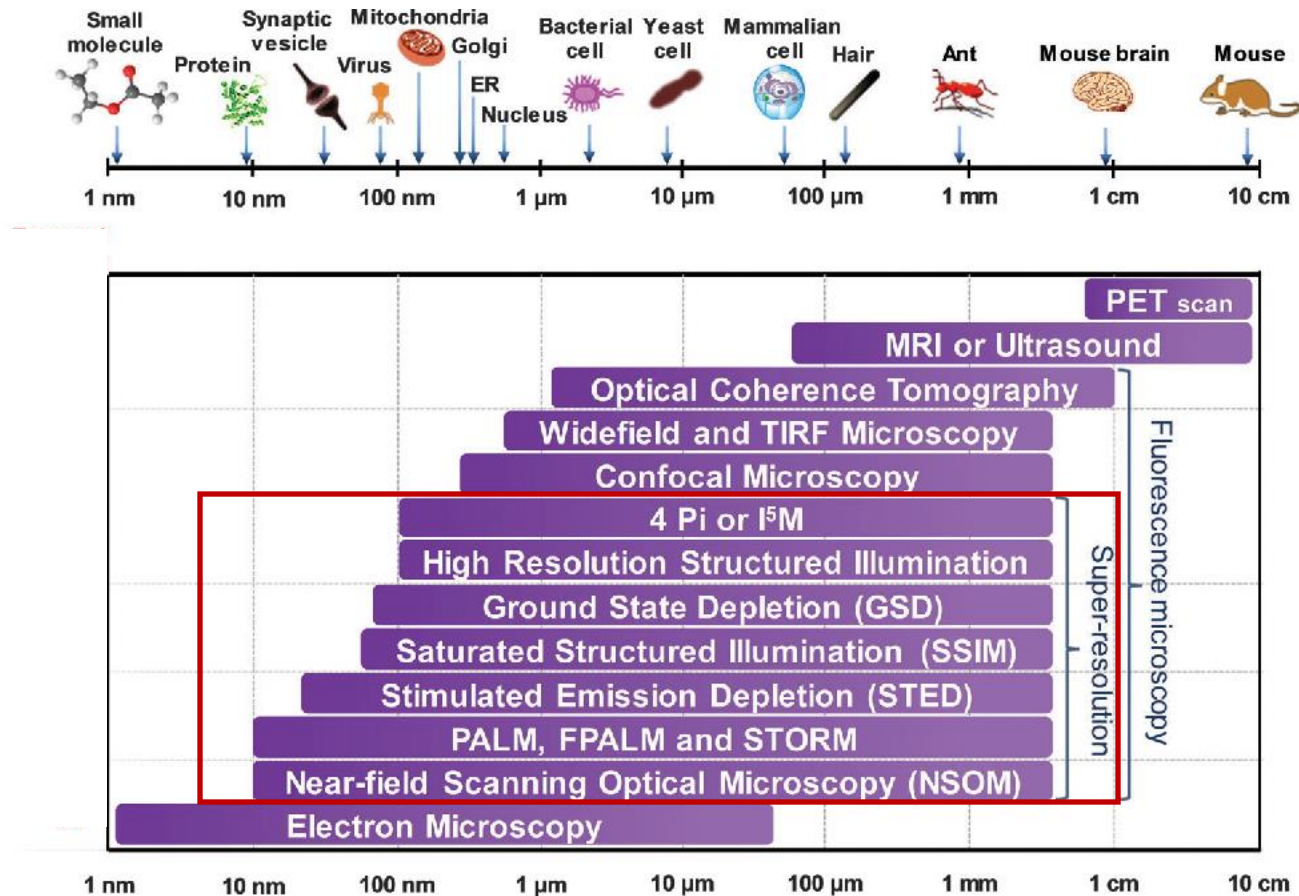
Ranya Virk
PROPEL 101
3/28/2024



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

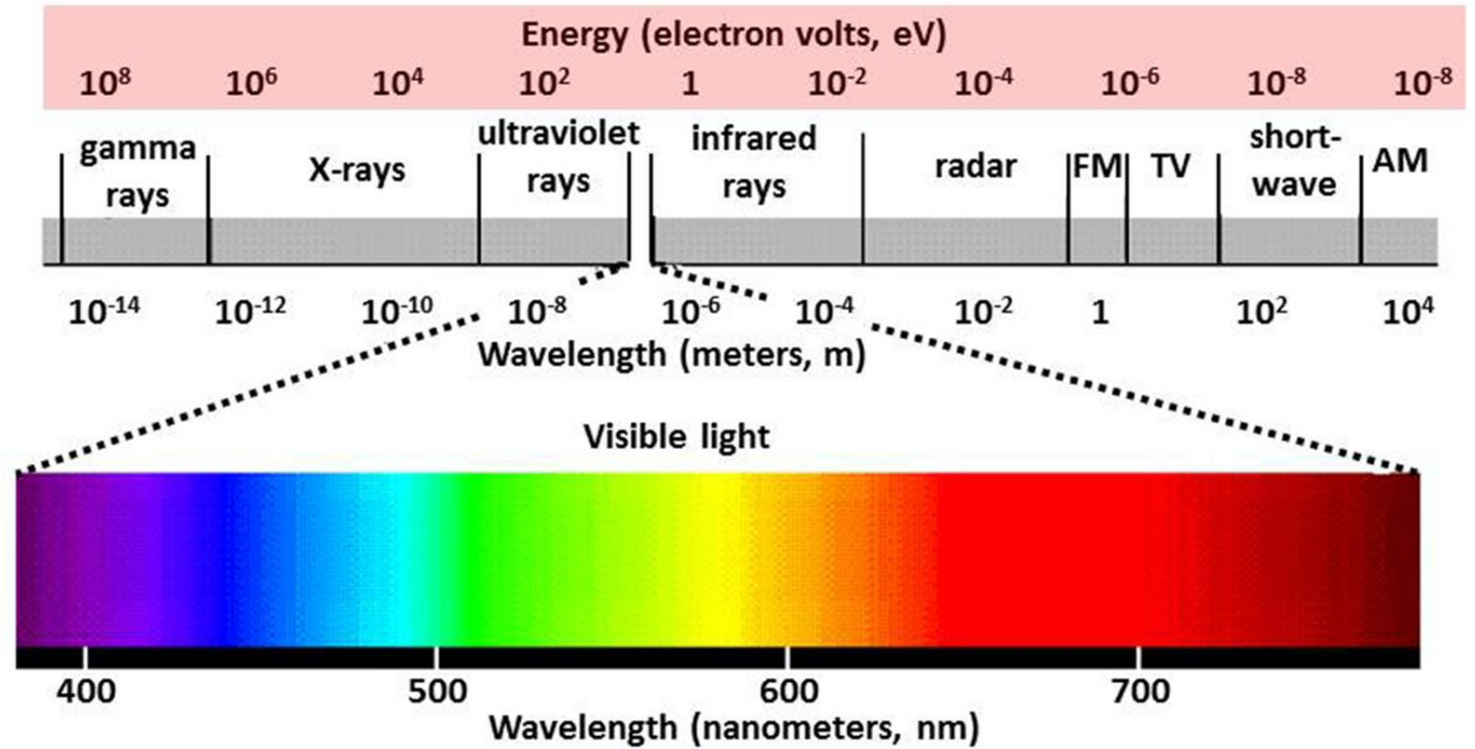
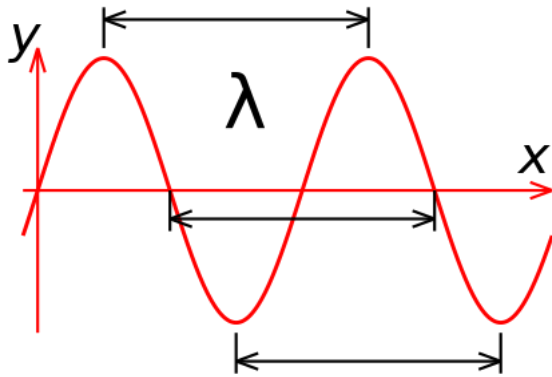
Super-resolution microscopy enables more precise imaging of molecular structures



Yang et al., Chemical Society Reviews (2016)

Optical microscopy uses visible light

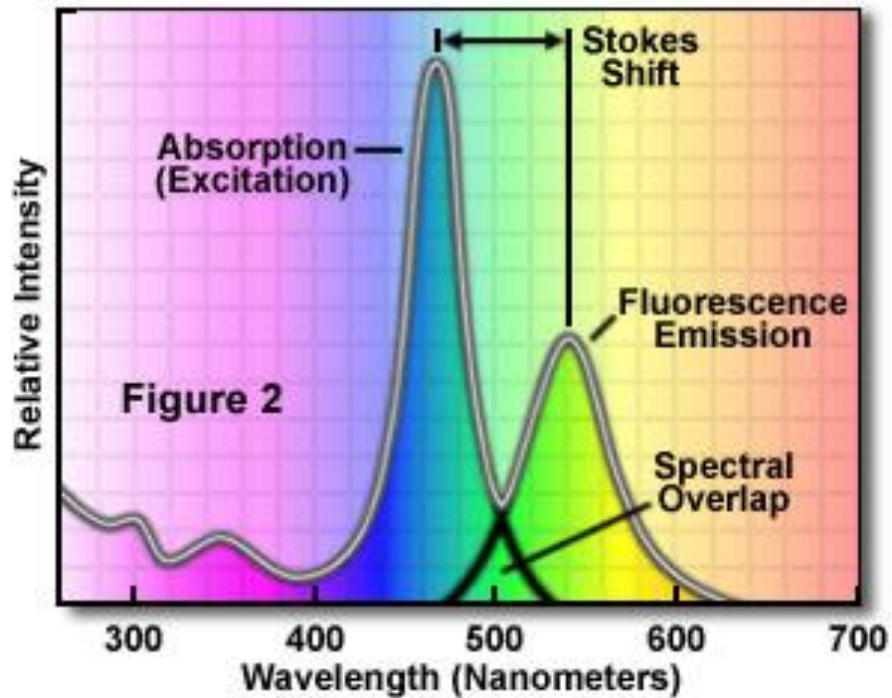
Light behaves as a wave that can be described by **wavelength** λ



Fluorescence microscopy basics

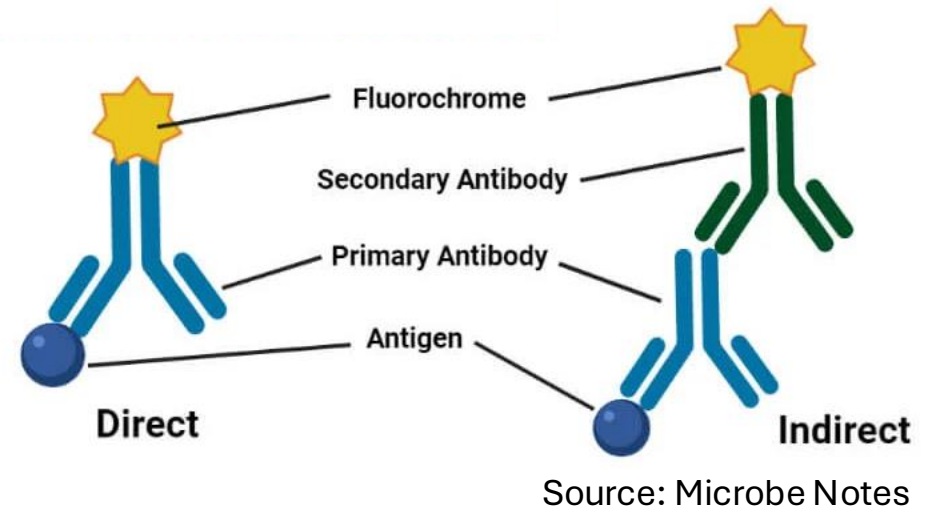
Fluorophores absorb light energy of a specific wavelength and re-emit it at a longer wavelength

Excitation and Emission Spectral Profiles

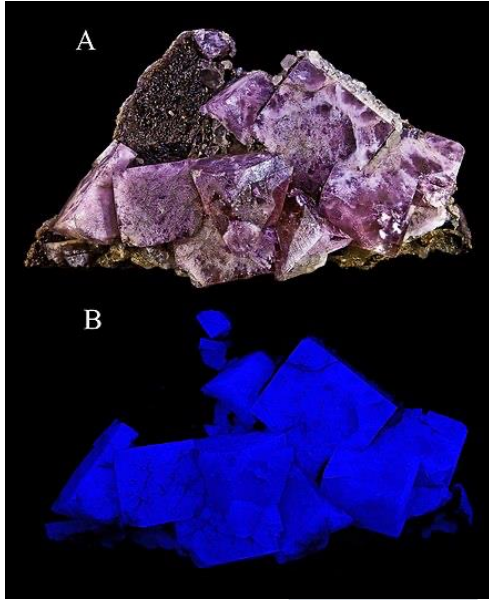


Source: Olympus

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



A brief history of fluorophores



1852: Term “fluorescence” is coined to describe fluorite exposed to UV



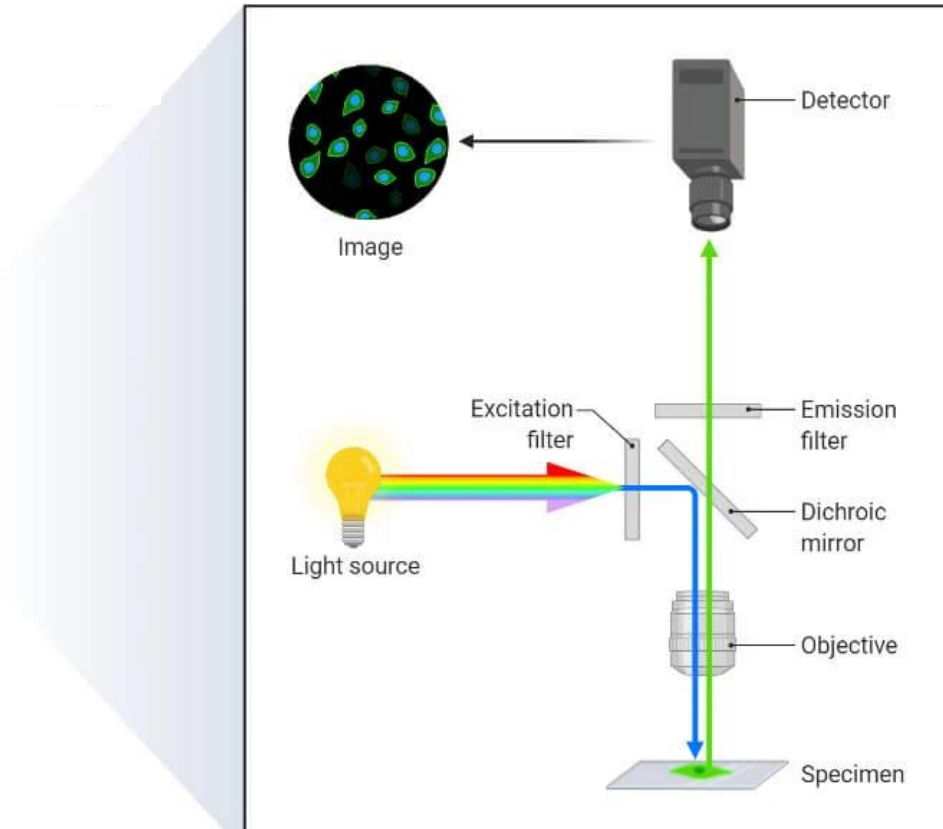
1960s: Green Fluorescent Protein (GFP) first isolated in jellyfish

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



Credit: UPenn

Fluorescence microscope setup



Source: Microbe Notes

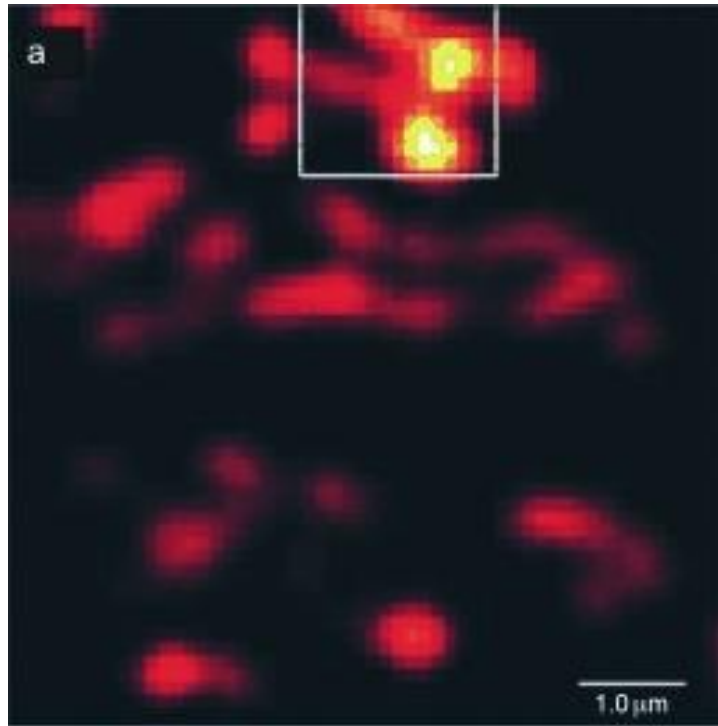
Filters: selectively transmit or block specific wavelengths of light

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

Dichroic mirrors: facilitate separation of excitation and emission light

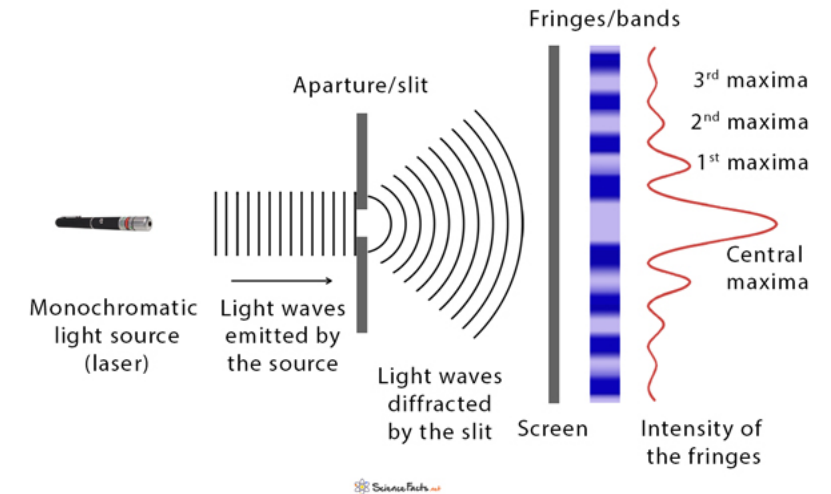
Diffraction-limited microscopy prevents precise imaging of molecular structures

Example: Mitochondria

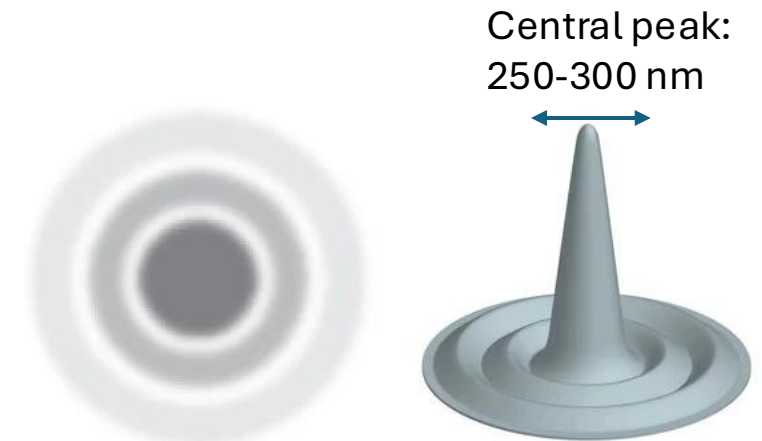


Bertzig, et al., Science 2006

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

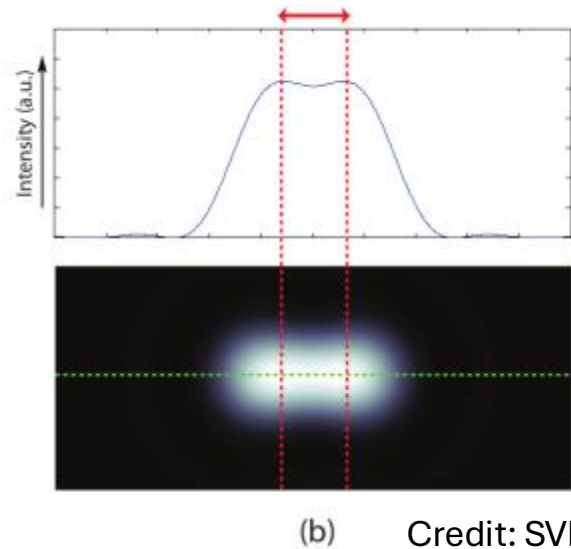
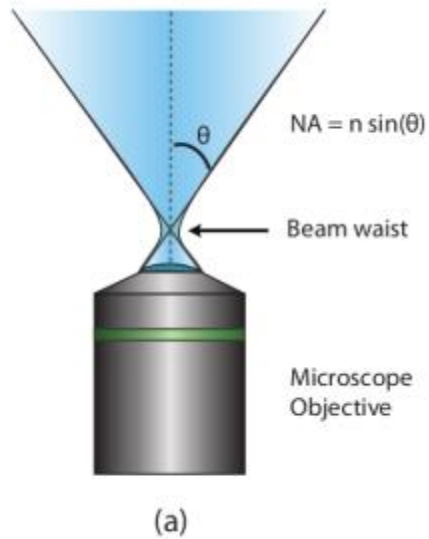


Point spread function (PSF): describes how point source of light is spread out or blurred in an image due to diffraction



Credit: Leica

Diffraction-limited resolution in optical microscopy



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

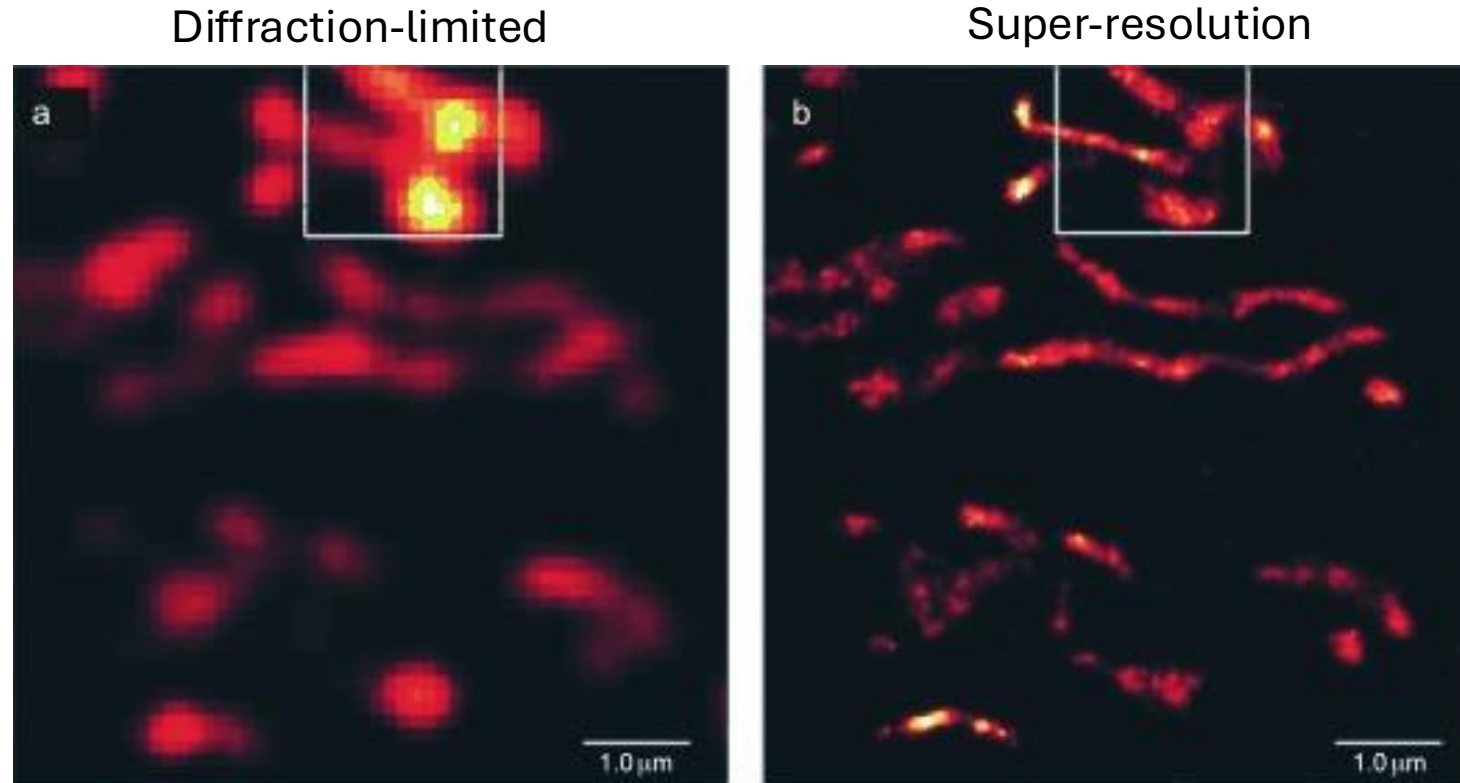
$$d = \frac{\lambda}{2NA}$$

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



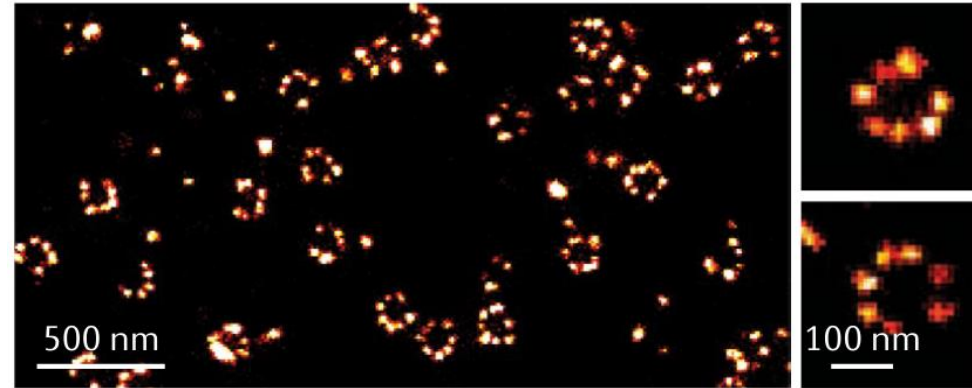
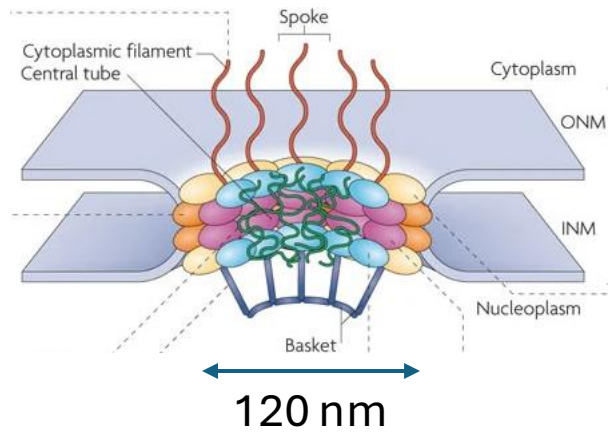
Bertzig, et al., Science 2006



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

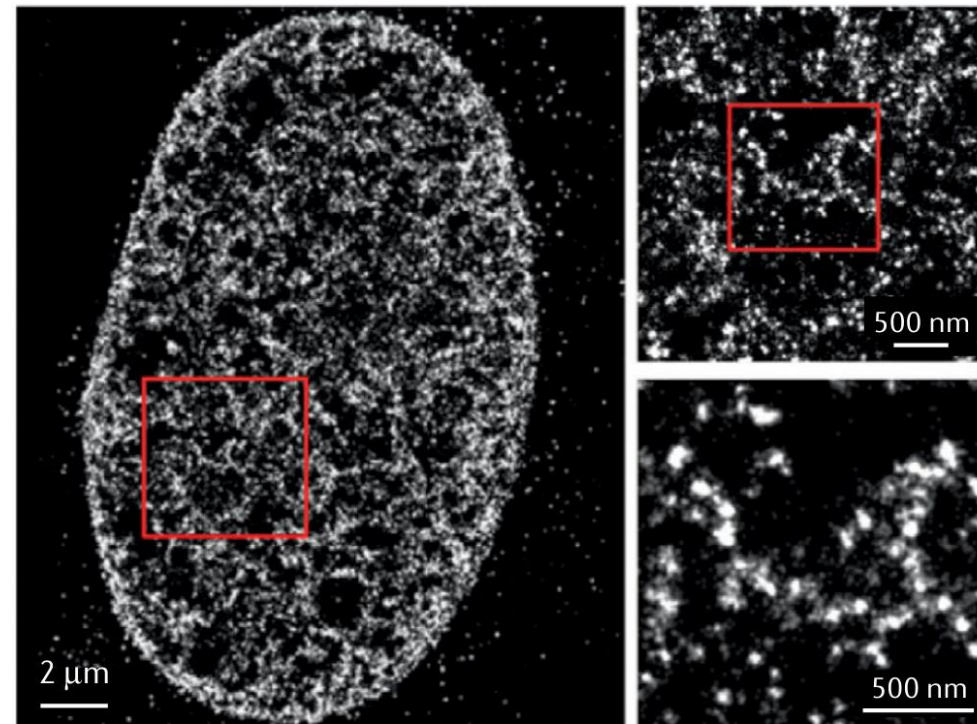
Other applications of super-resolution microscopy

Nuclear pore complex



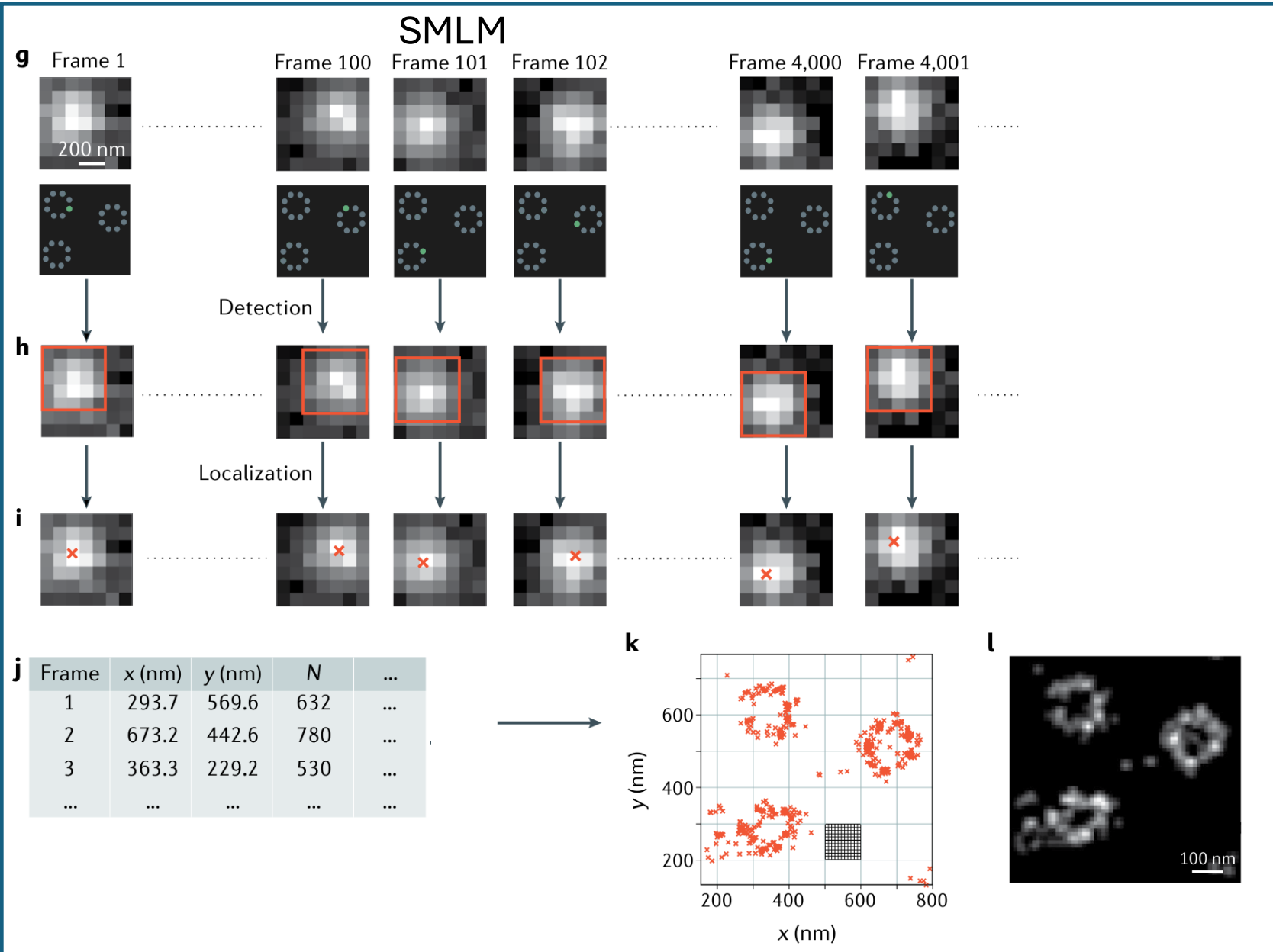
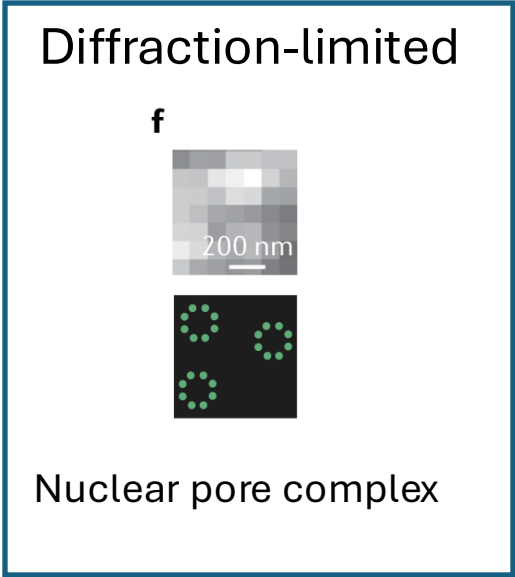
Thevathasan et al., Nature Methods (2019)

DNA + Chromatin

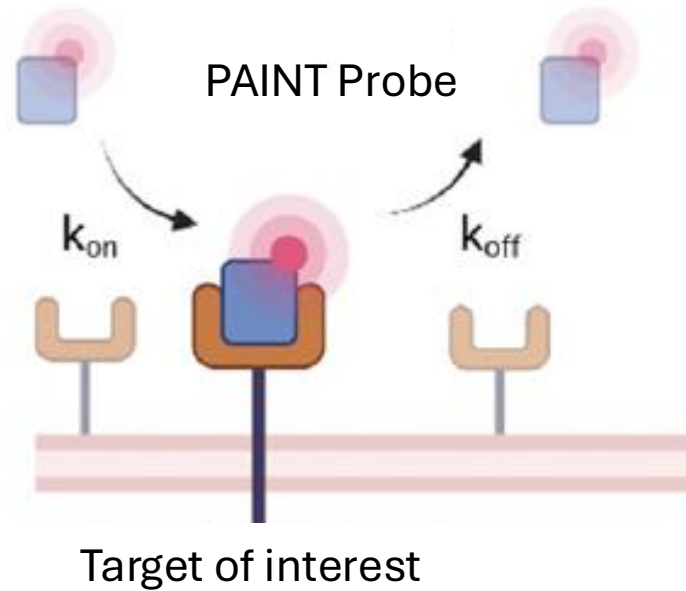


Ricci et al., Cell (2015)

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



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

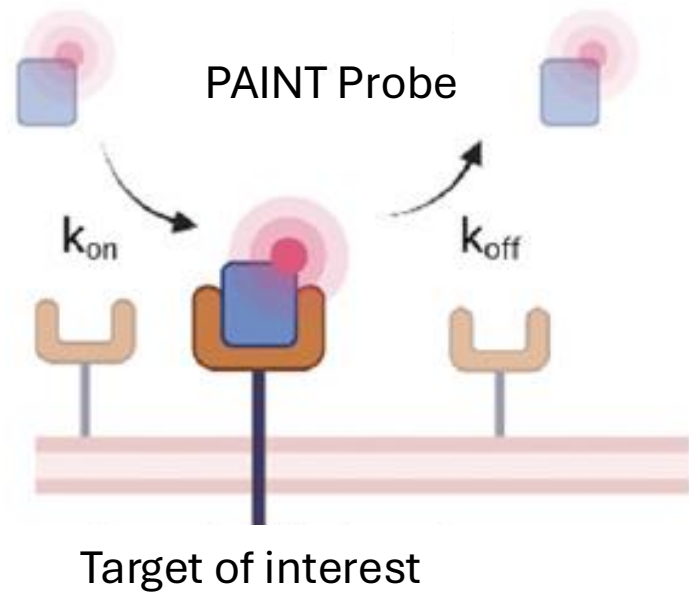


Tholen, et al., Chem Comm (2023)

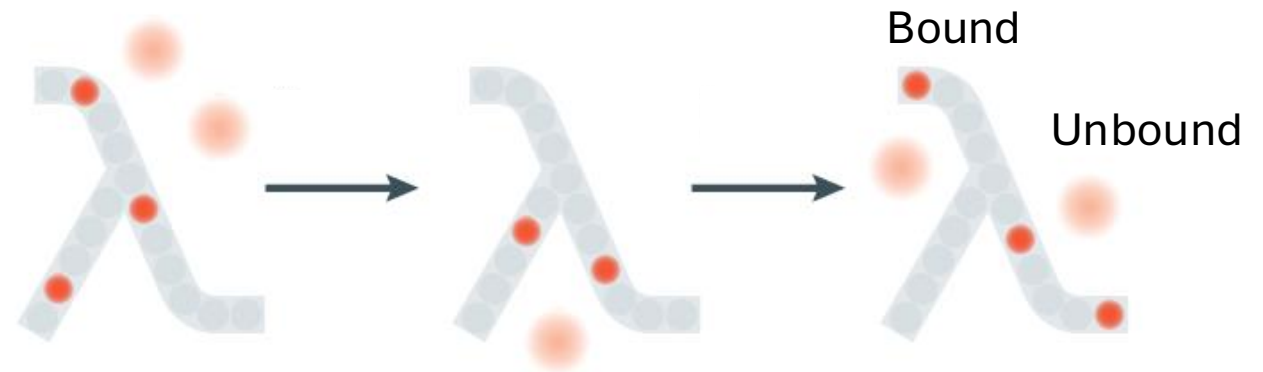
Specificity: ability of antibody to recognize and specifically bind target antigen

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

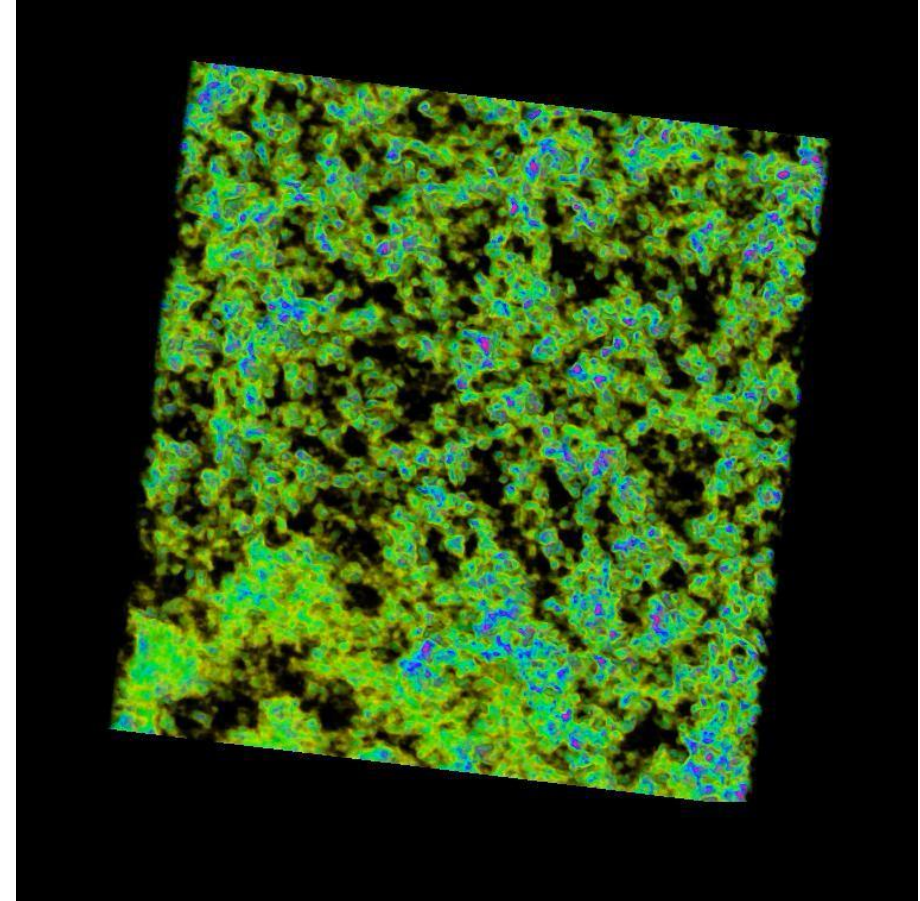
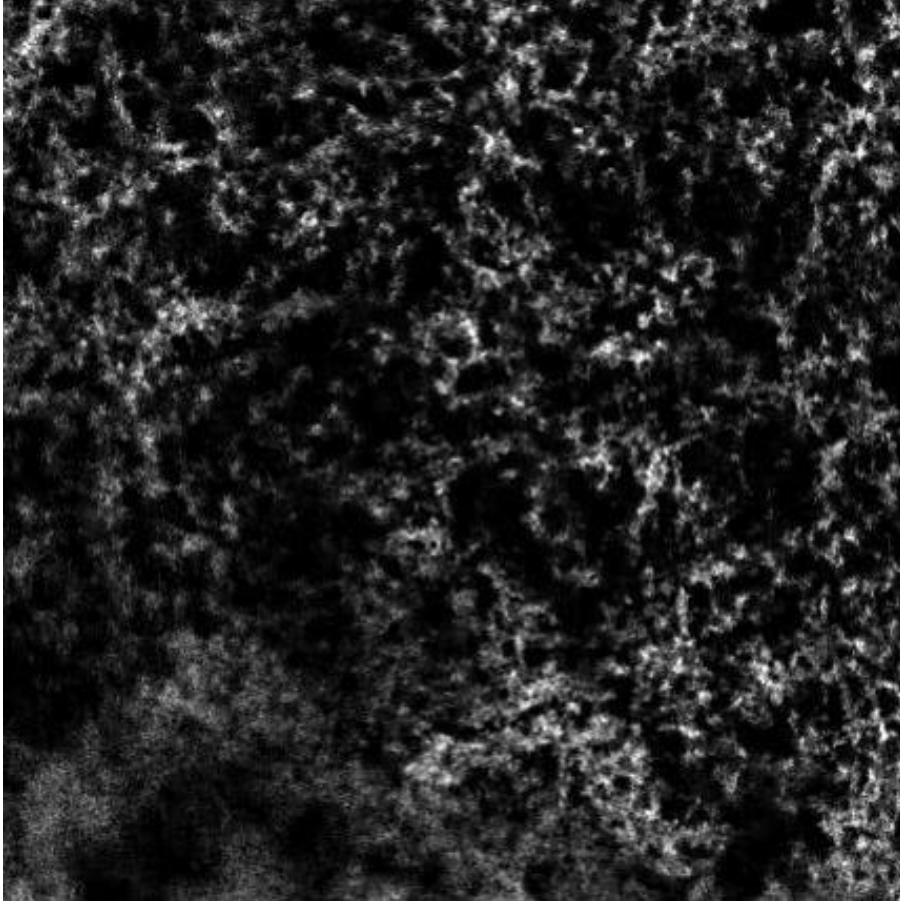
Specificity: ability of antibody to recognize and specifically bind target antigen

Affinity: strength of binding (k_{off})

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

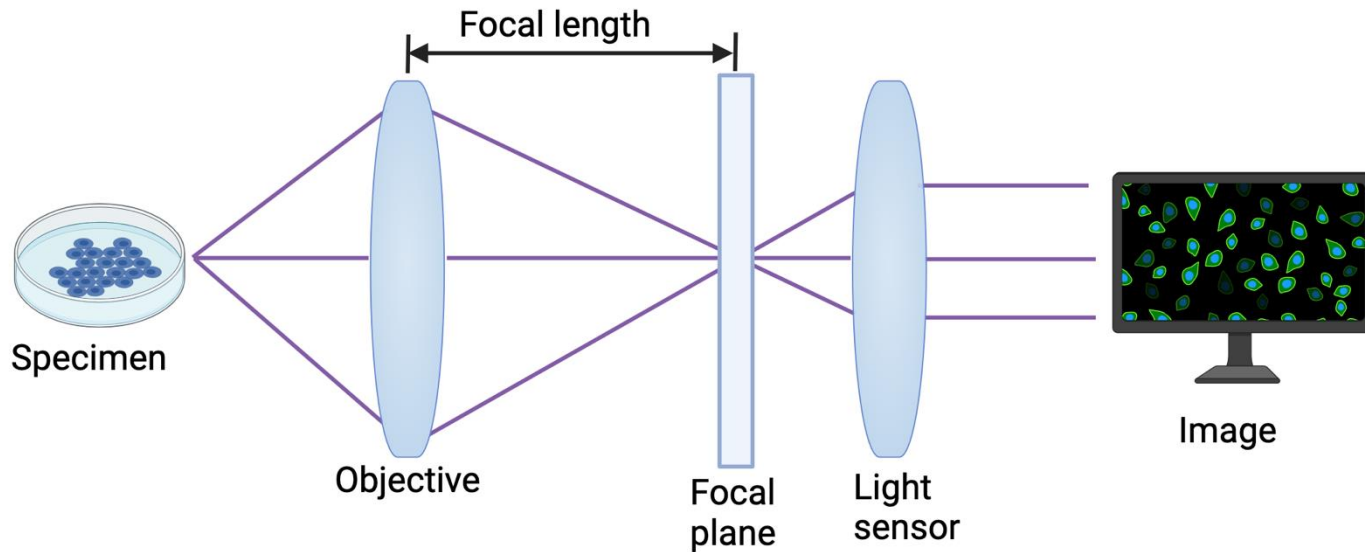
3D Imaging: Reconstruction of consecutive 2D slices

Example: Electron microscopy imaging of chromatin density



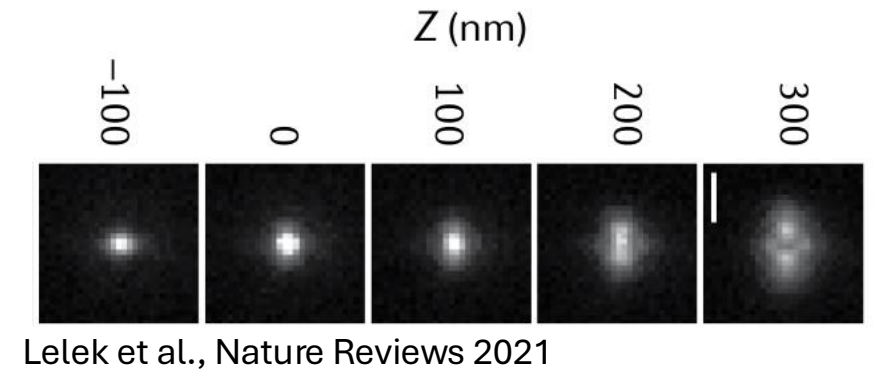
How to focus in the z plane

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

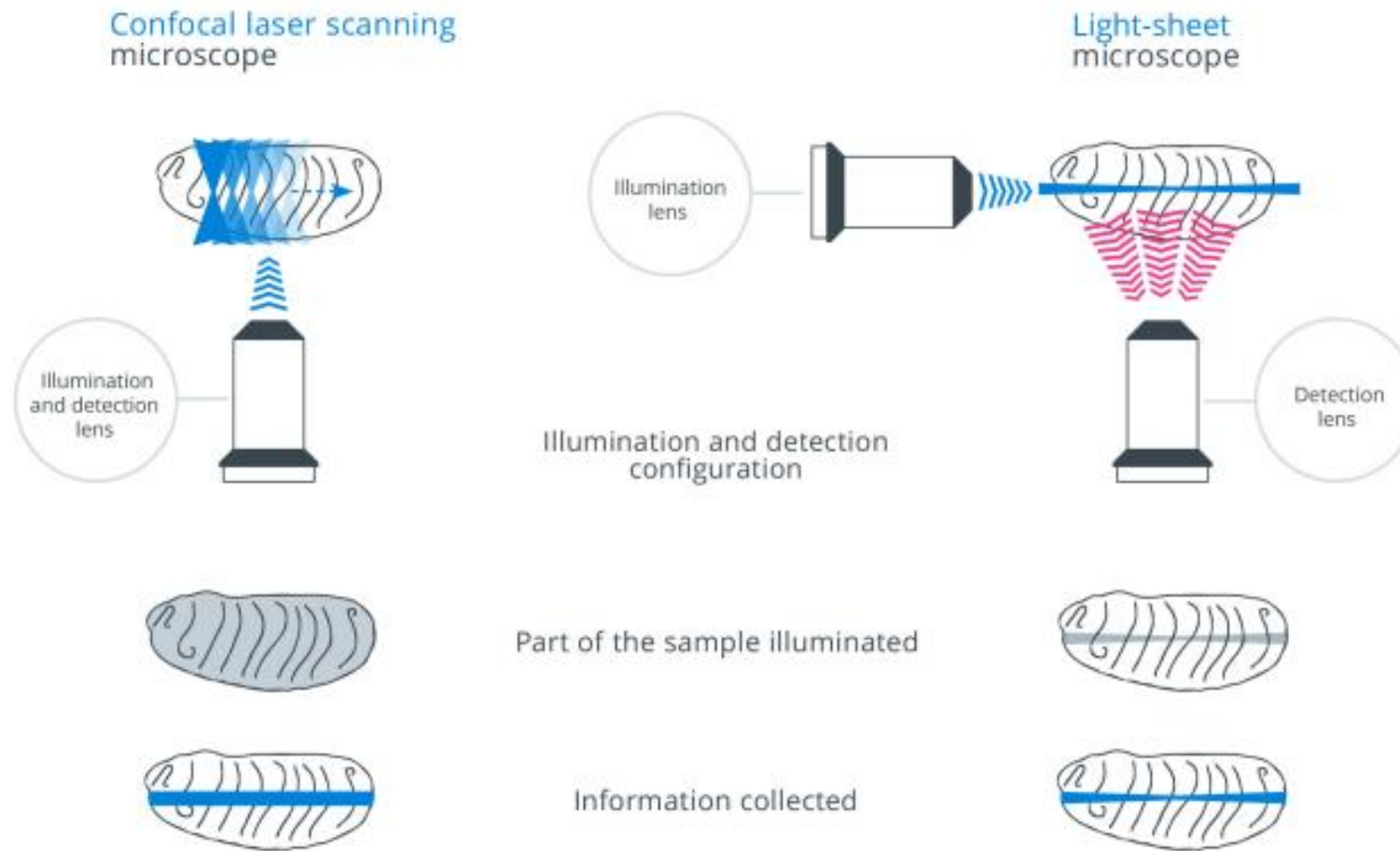


Signal is sharpest when specimen/sample is centered at the focal plane

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



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



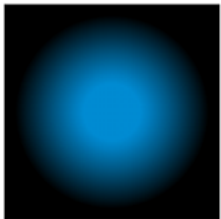
Credit: Bruker

Lattice light-sheet microscopy

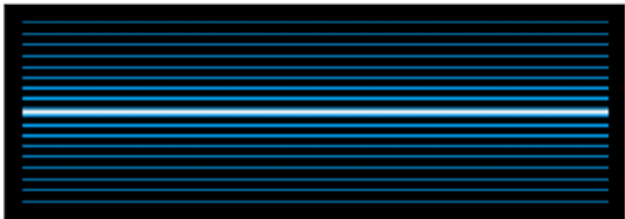
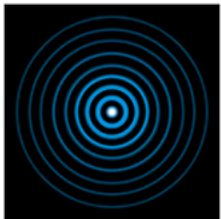
2D cross-section

Side view

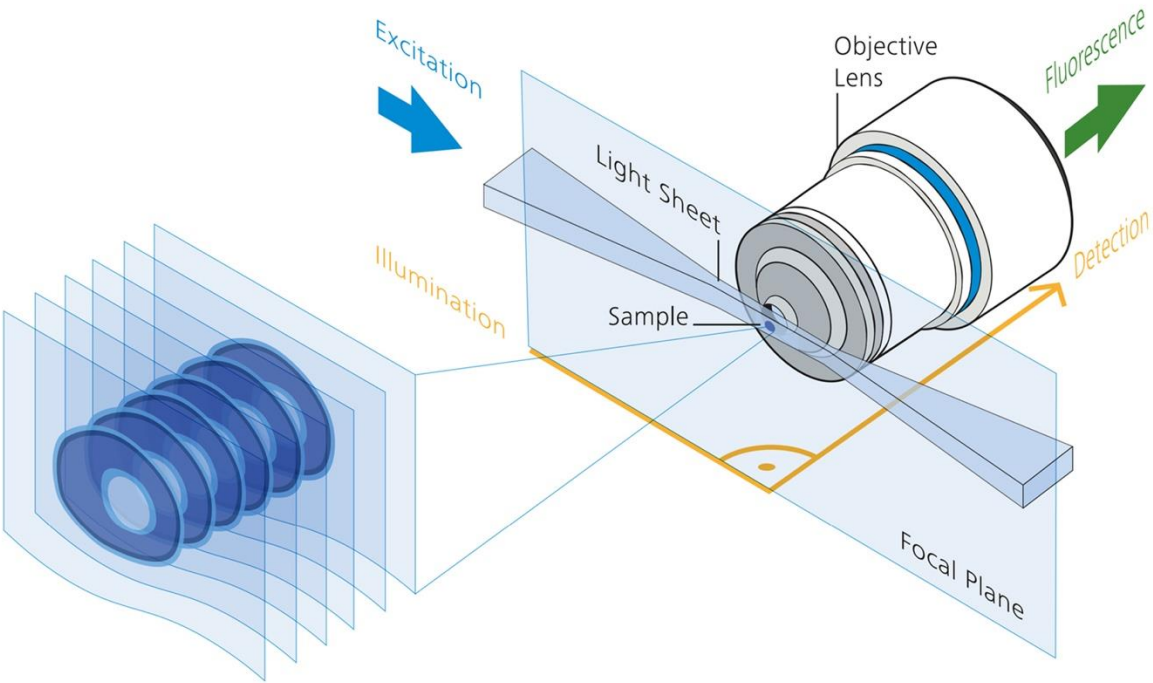
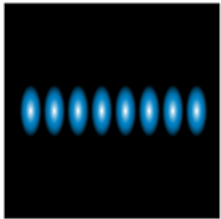
Gaussian Beam



Bessel Beam



Lattice light sheet

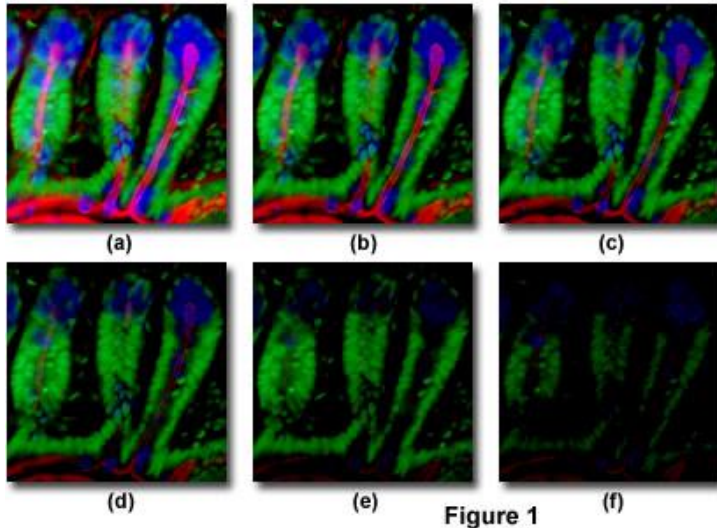


Credit: Zeiss

Benefits of lattice light-sheet microscopy

1) Minimizes photobleaching: when fluorescence emitted by fluorophore diminishes and eventually disappears upon prolonged light exposure

Mouse intestine



Credit: Molecular Expressions

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

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

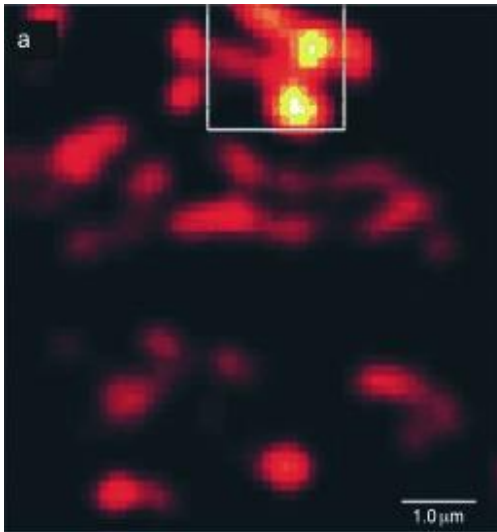


Chen et al., Science (2014)

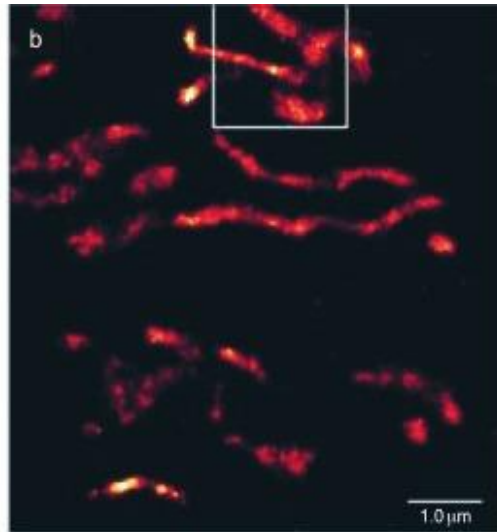
Summary

- **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

Diffraction-limited

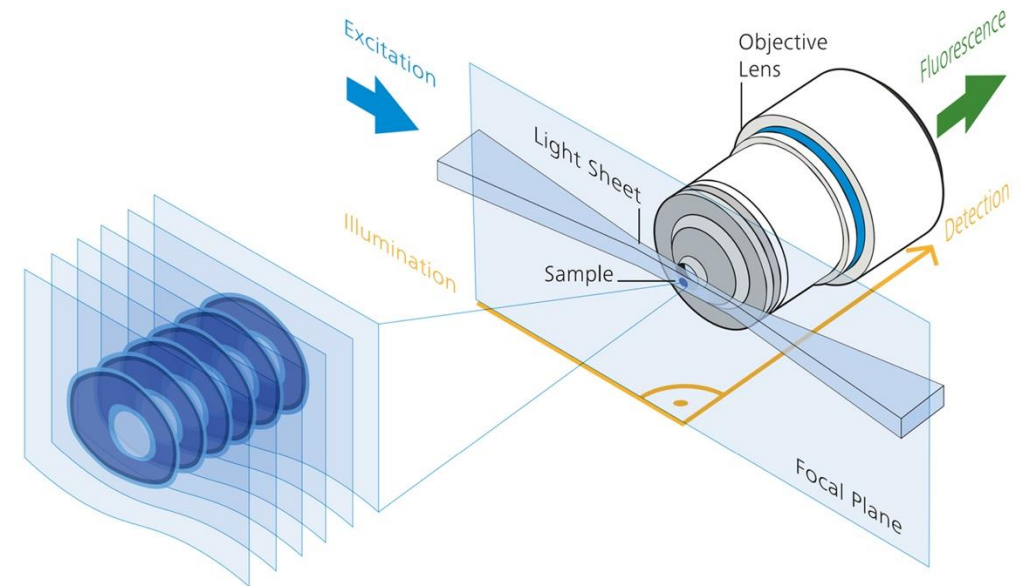


SMLM



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 arranged in lattice pattern enabling high-resolution, high-speed 3D imaging



Credit: Zeiss