

Microscopy

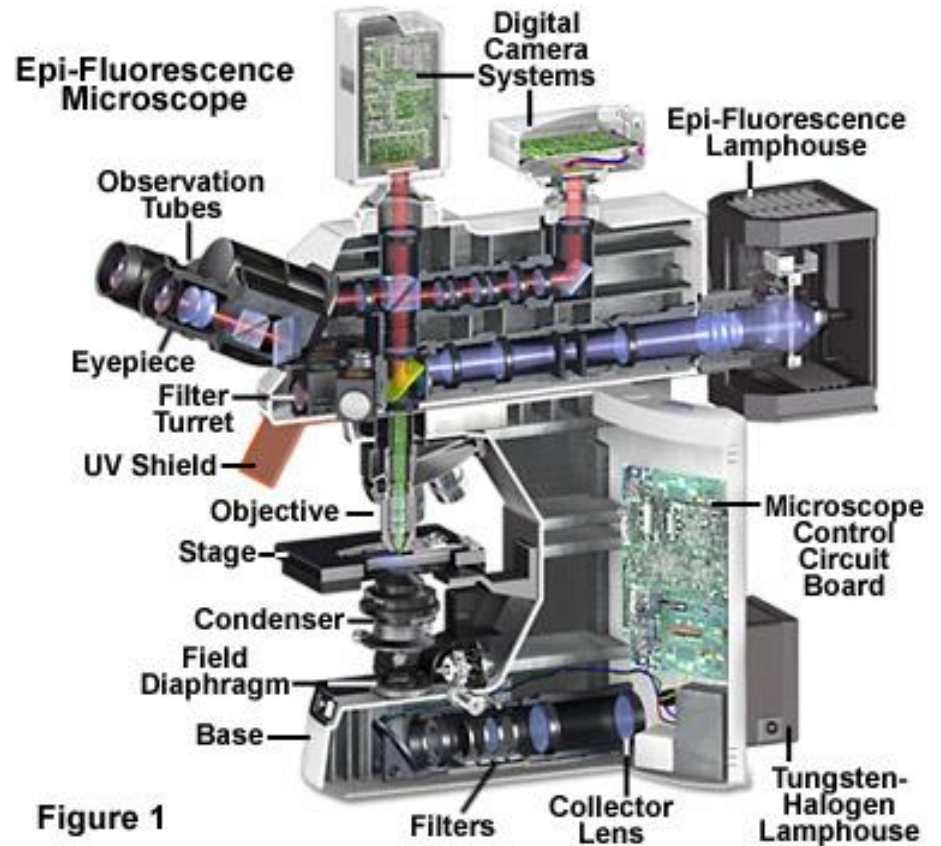


Figure 1

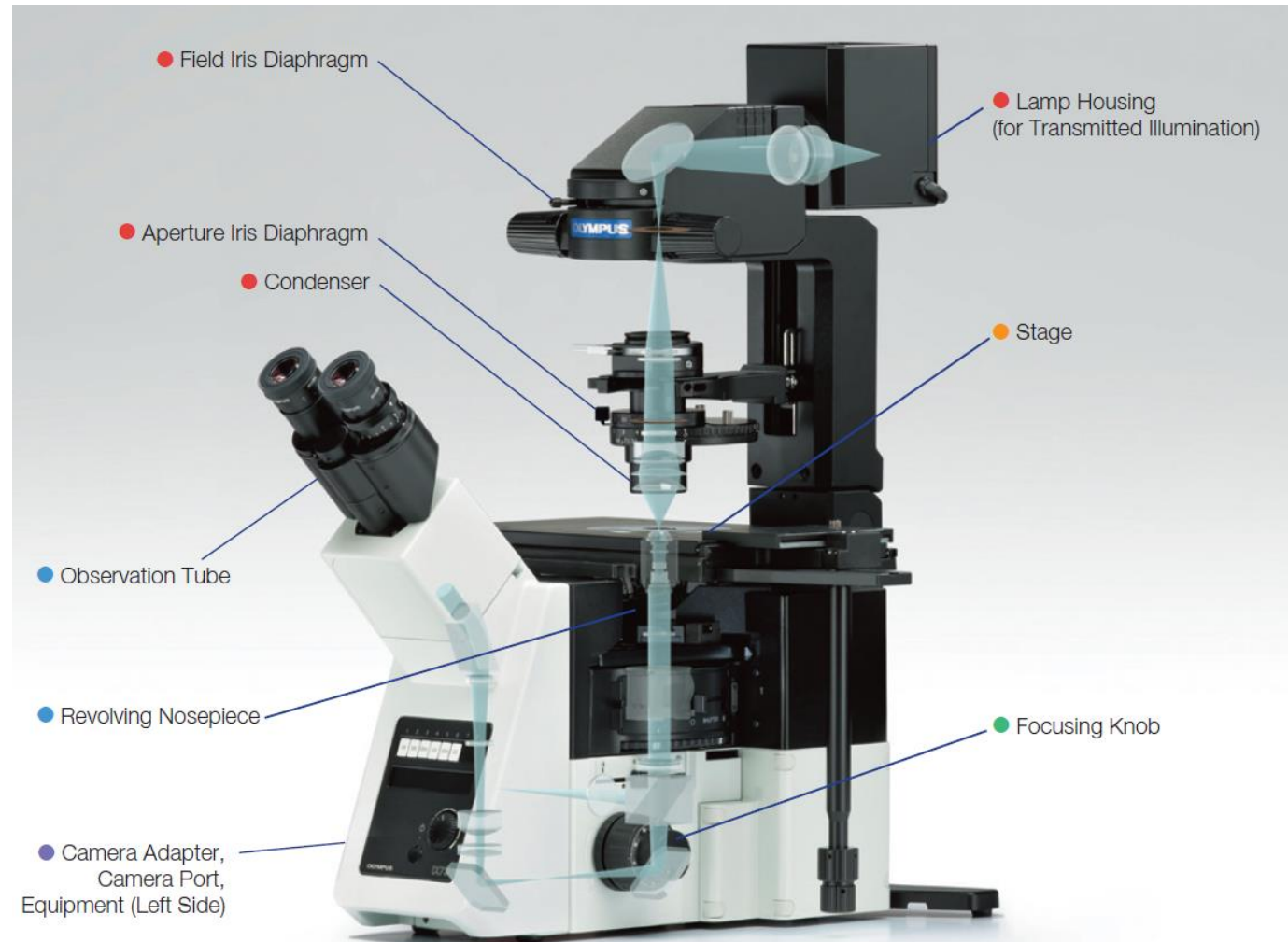
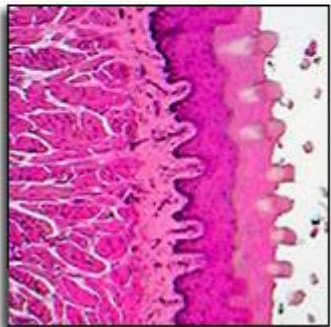
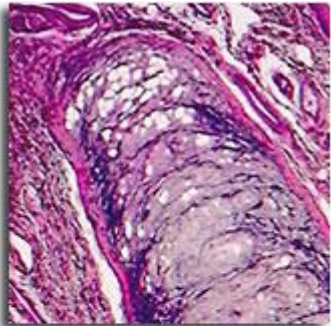
Light microscopy resources

- www.microscopyu.com
- [https://www.olympus-lifescience.com/en/microscope-resource/\(click on "Microscopy Resource Center"\)](https://www.olympus-lifescience.com/en/microscope-resource/(click%20on%20%E2%80%9C%20Microscopy%20Resource%20Center%E2%80%9D))
- <https://www.leicabiosystems.com/knowledge-pathway/>
- <https://www.zeiss.com/microscopy/us/home.html>

Transmission / brightfield microscopy



Brightfield



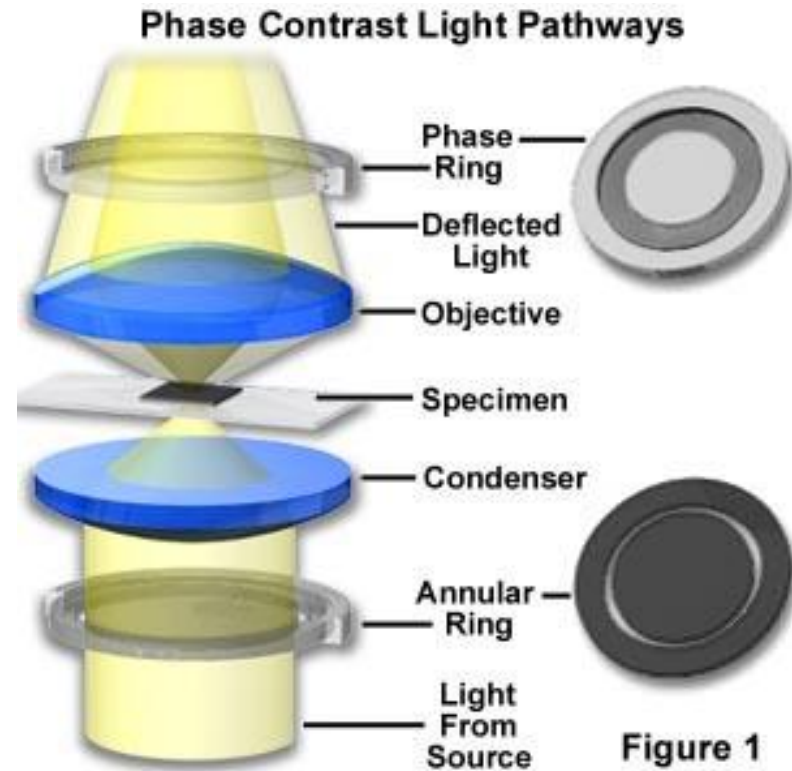
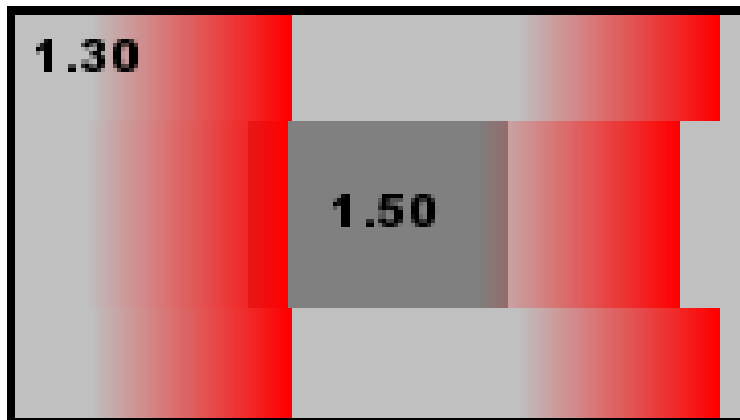
Phase contrast microscopy



Brightfield



Phase contrast



Phase contrast microscopy



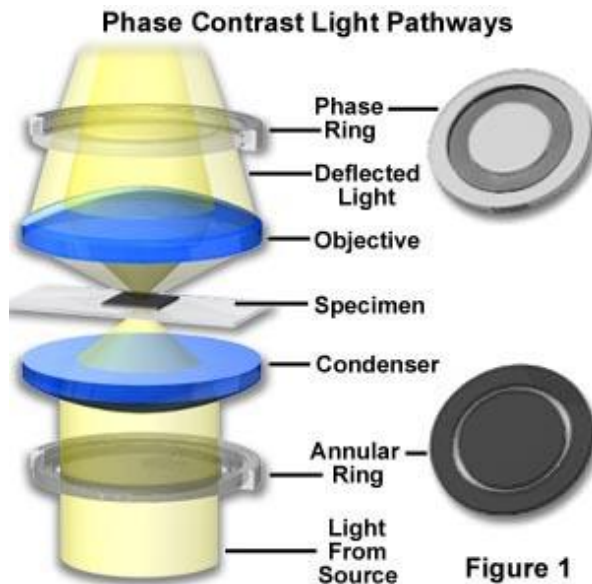
Brightfield



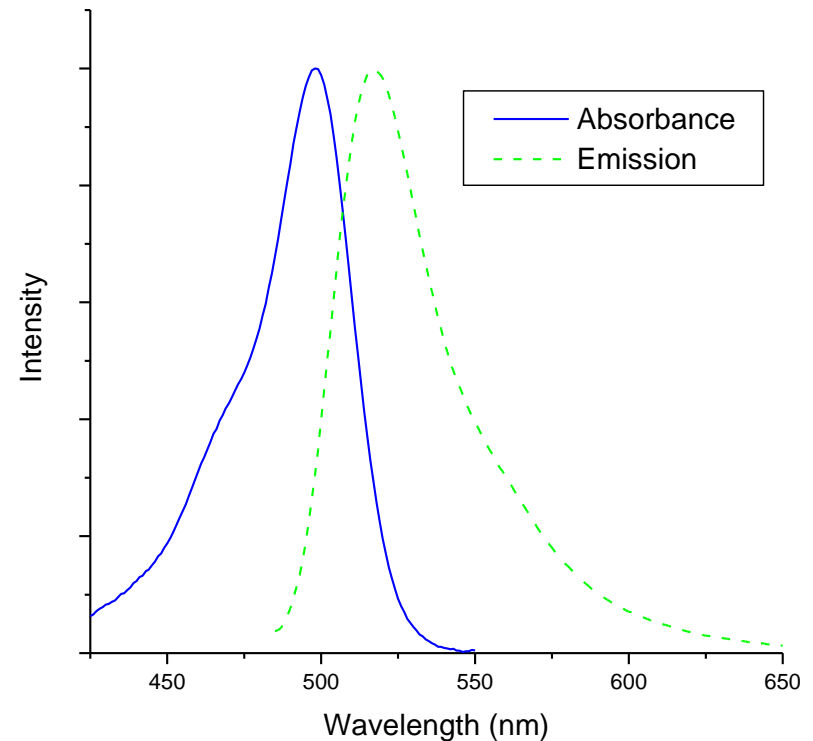
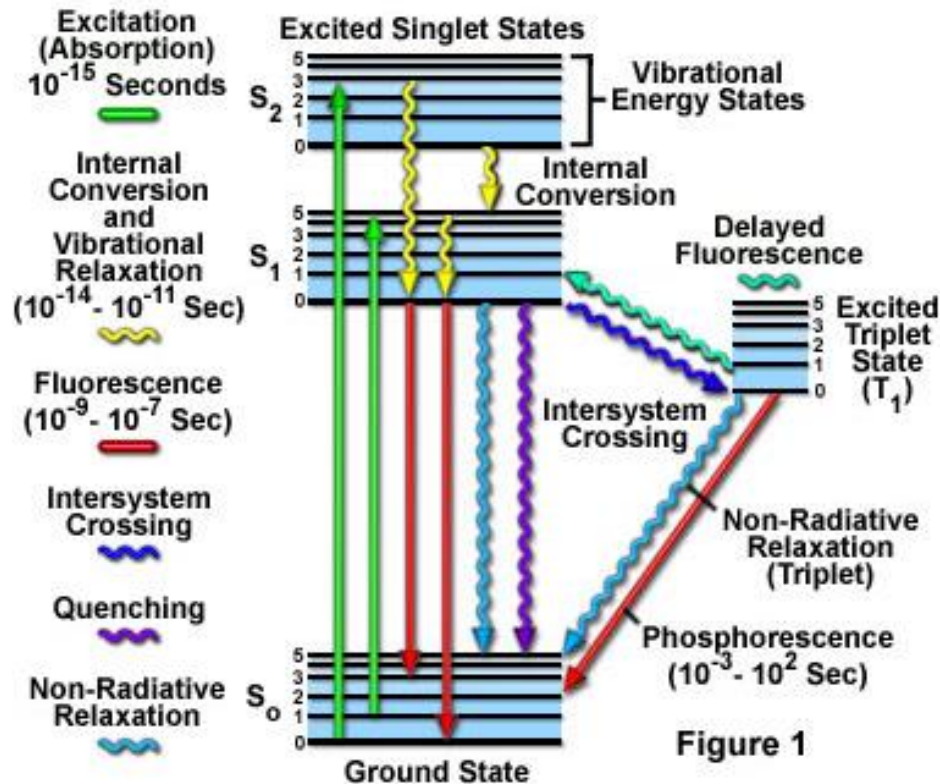
Phase contrast



Differential
Interference
Contrast
(DIC)
(beyond the scope here)

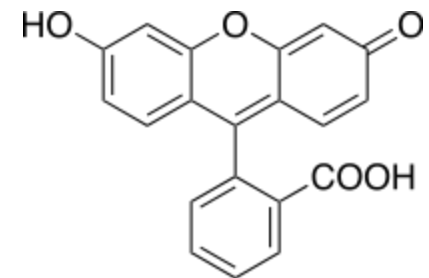


Fluorescence

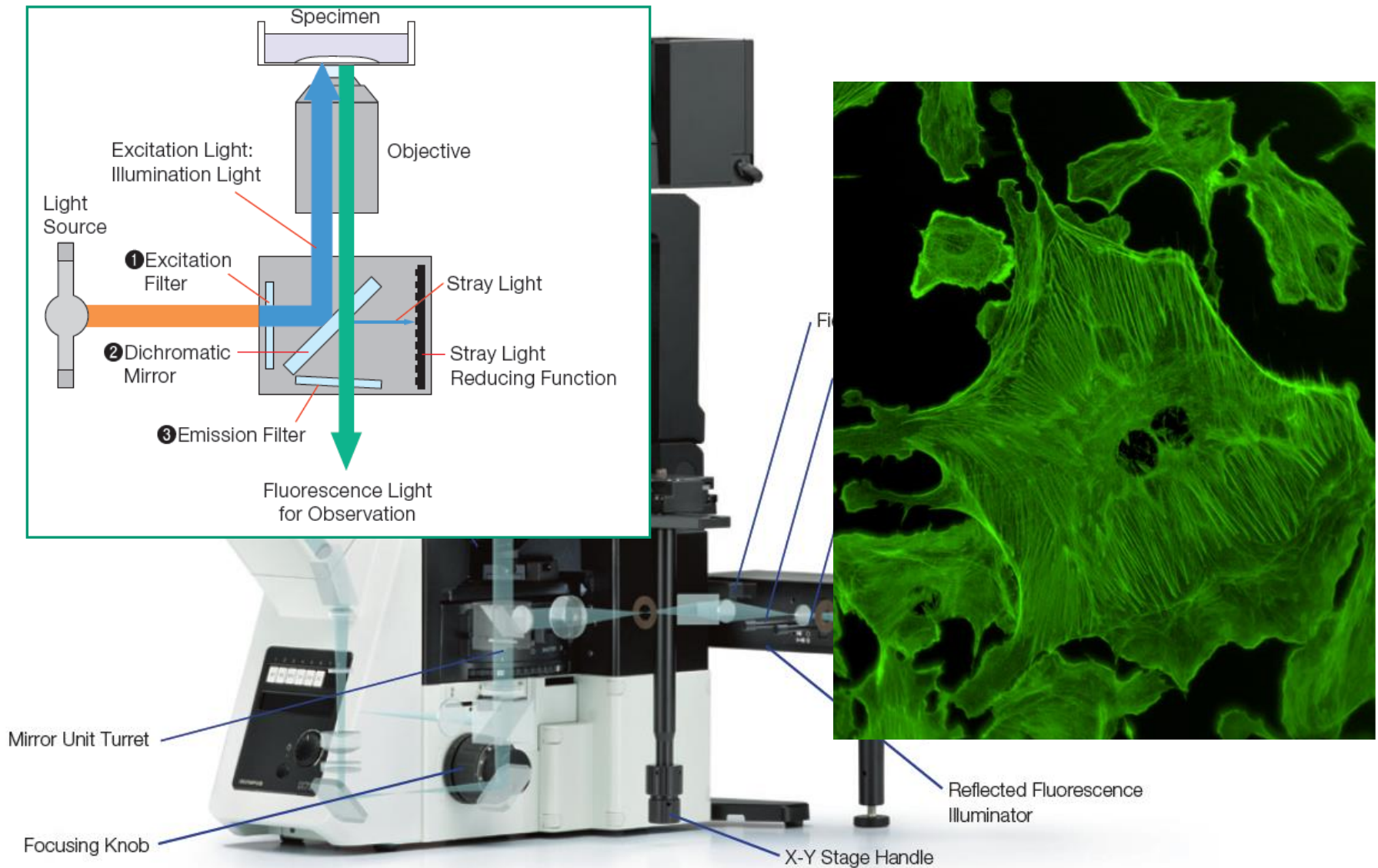


Wide range of fluorescent molecules

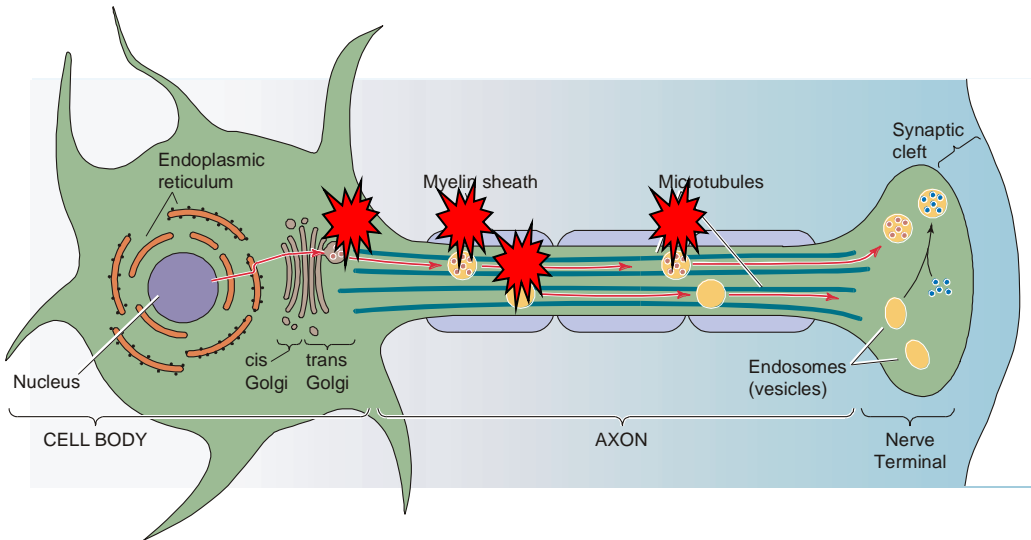
- Early examples: fluorescein / rhodamine
- Modified versions: Texas Red, Alexa
- Semiconductor / quantum dots
- Hybrids: Brilliant™ series from BD



Fluorescence microscopy

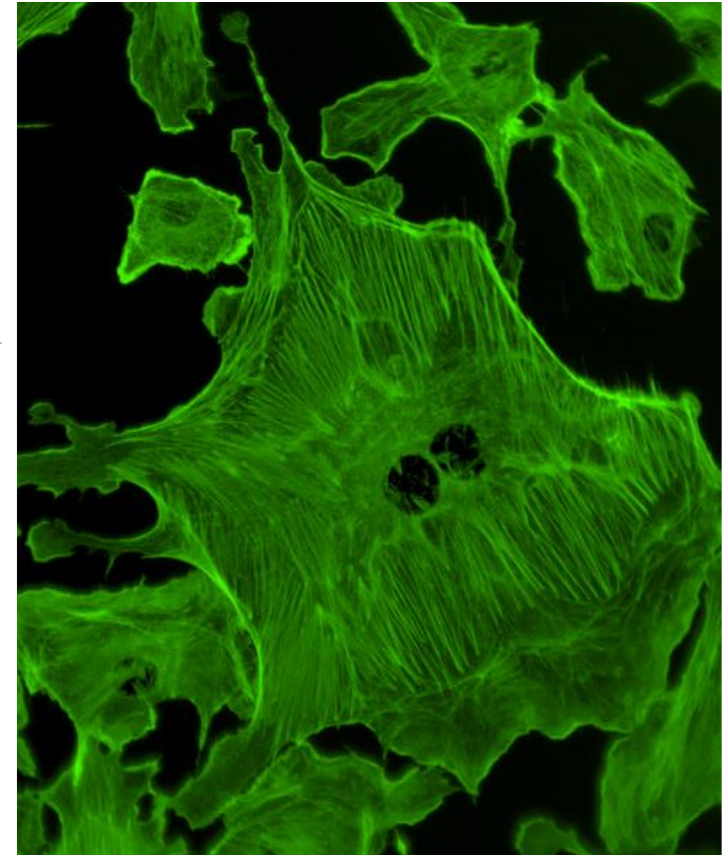


Fluorescence microscopy / Immunohistochemistry



Biomolecular identity provided by tagging with fluorescent molecules

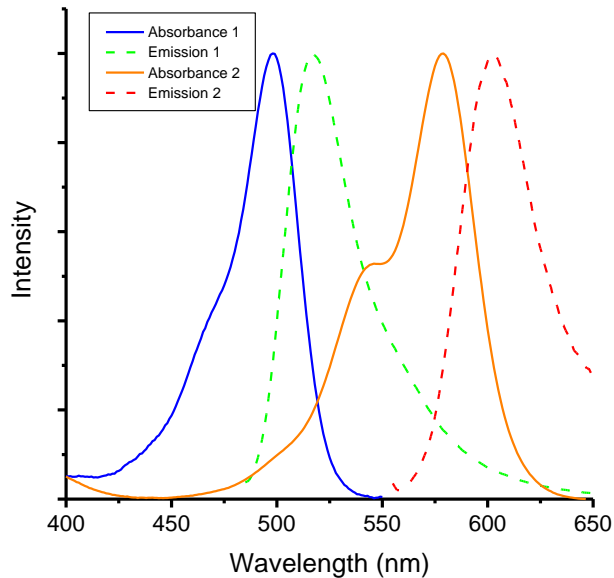
- antibodies; the most robust, molecule specific system around
- specialized molecules; phalloidin binds f-actin
- these require fixation (killing) and staining of cells



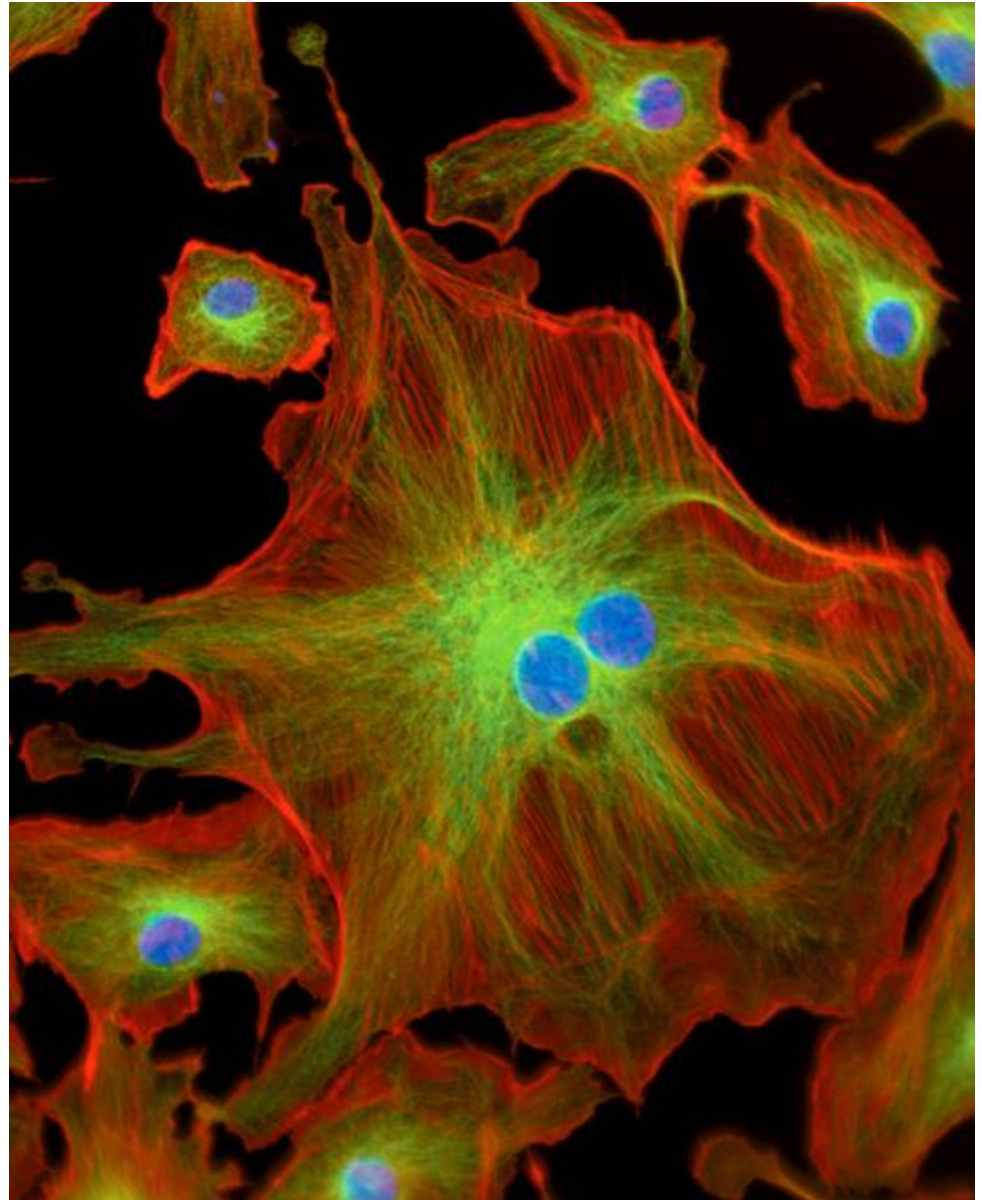
Vital dyes

- compounds that can enter live cells

Contemporary fluorescence microscopy



- Molecular identity
- Molecular conformation / activity / state
- High sensitivity – single molecule
- Multicolor
- However, limits on resolution



Green Fluorescent Protein

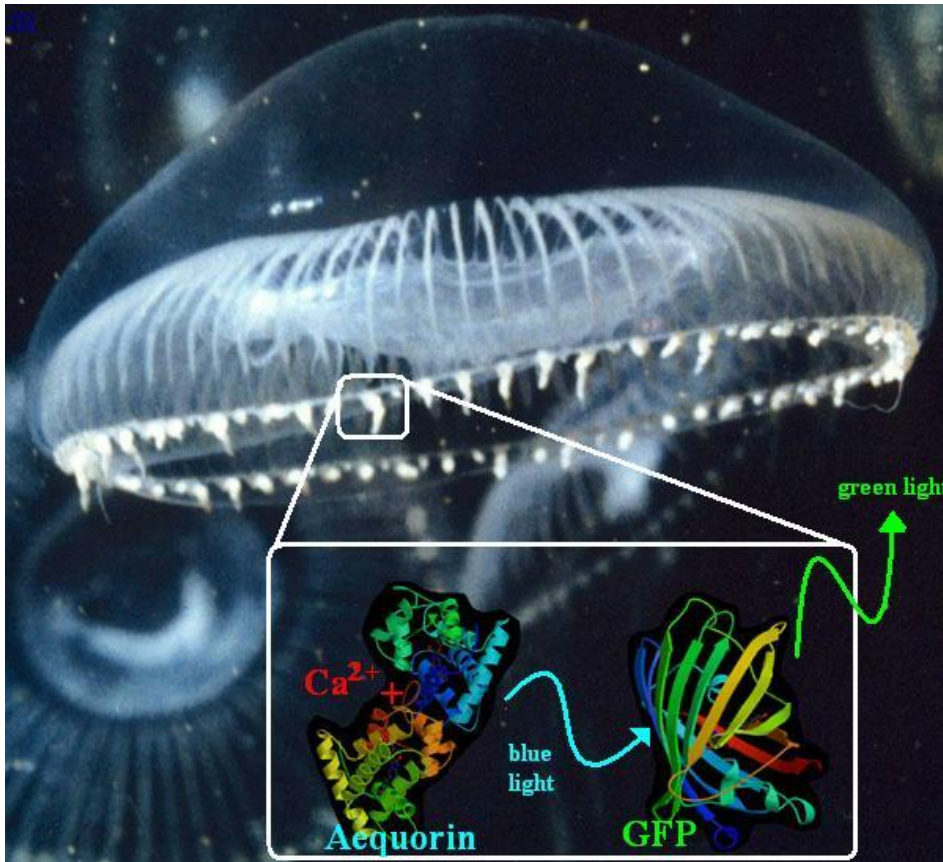
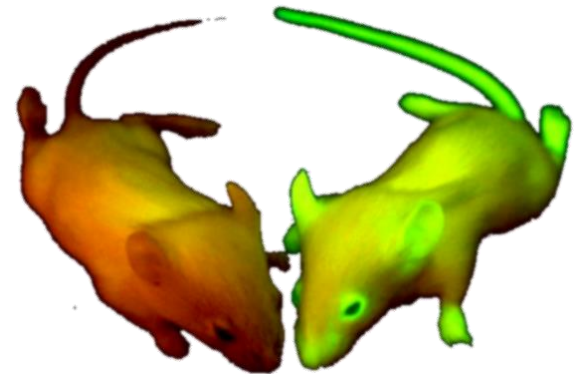
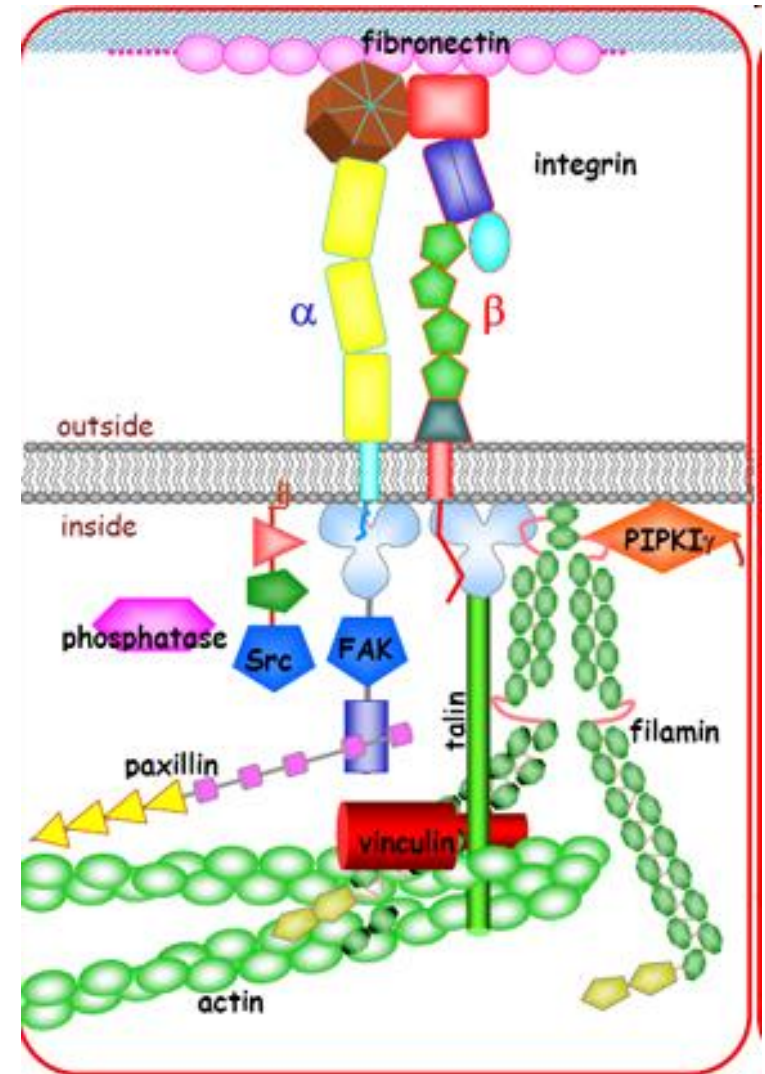
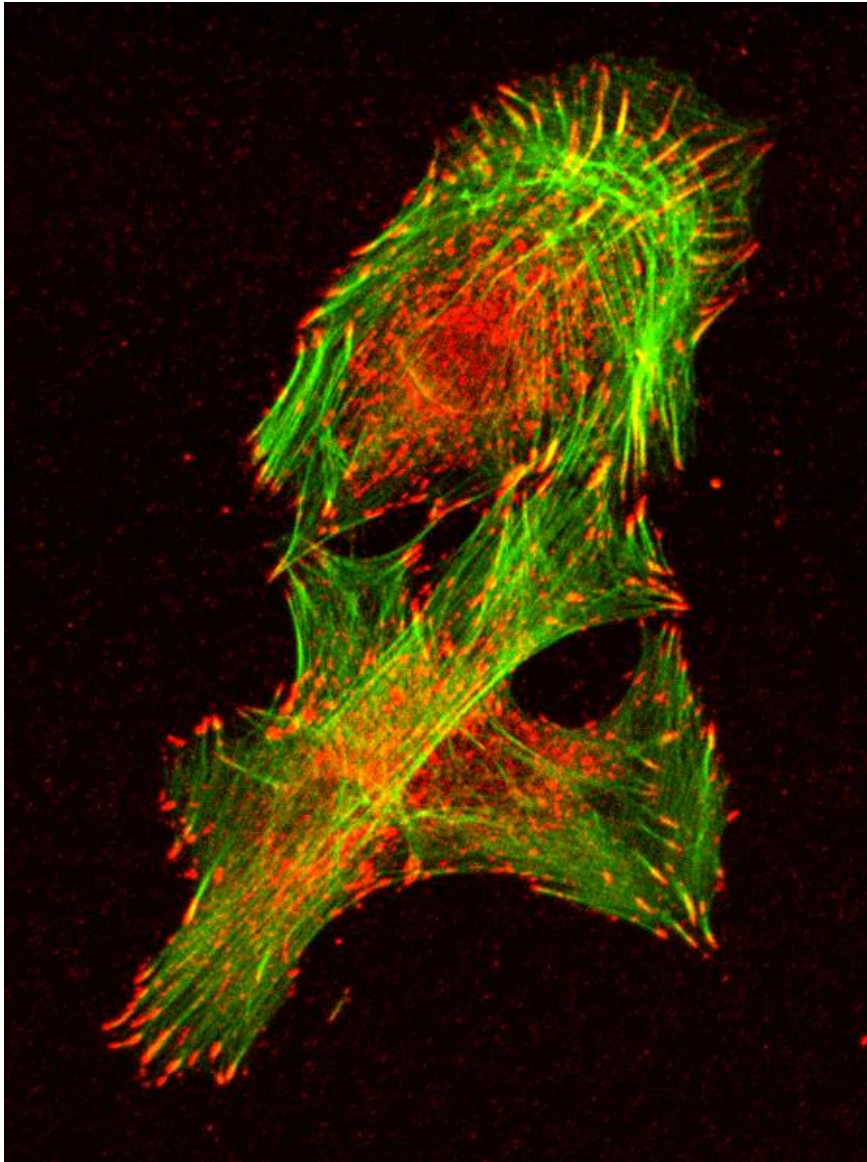


photo attributed to Osamu Shimomura

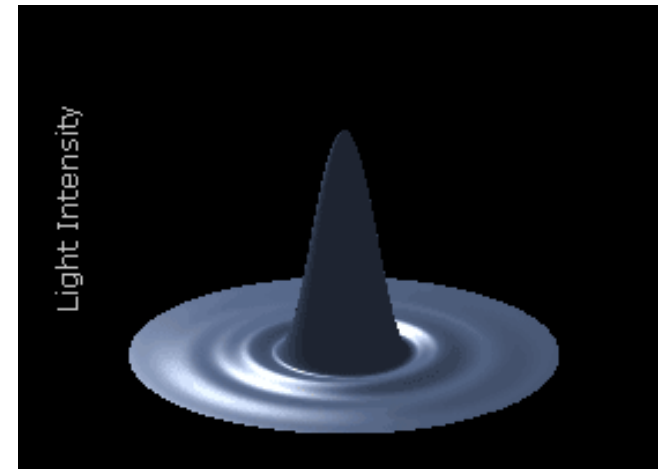
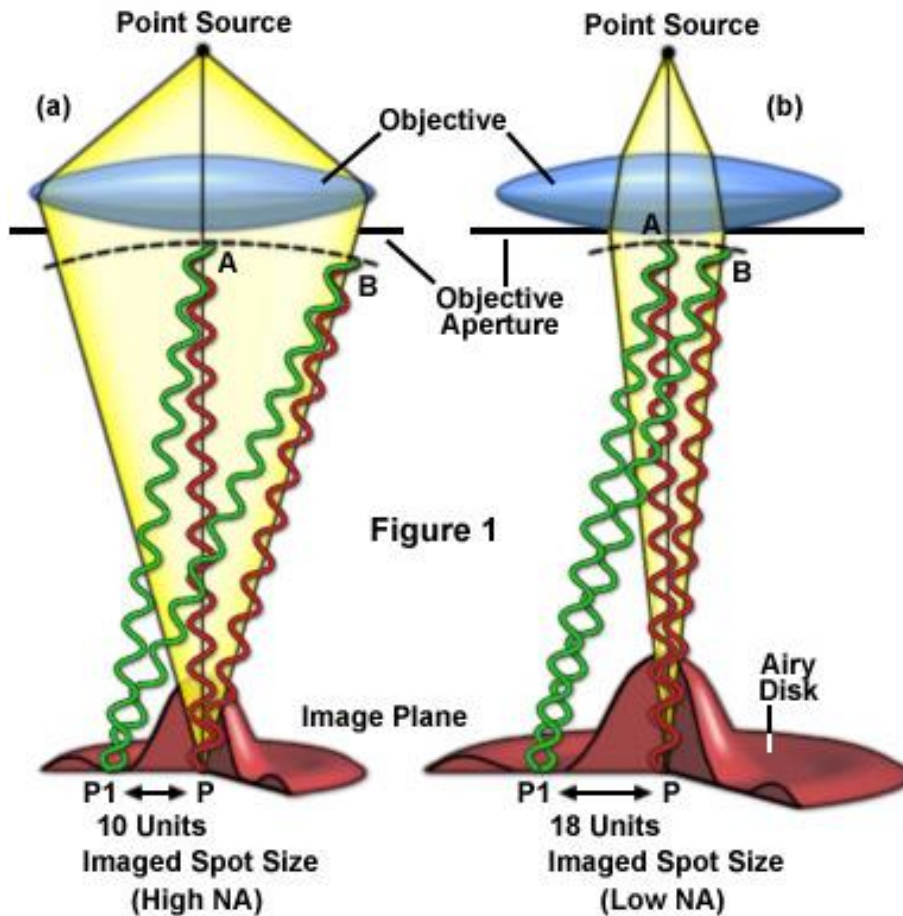


Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., Prasher, D.
Science 263(5148):802-5 (1994)

Cellular structures are small

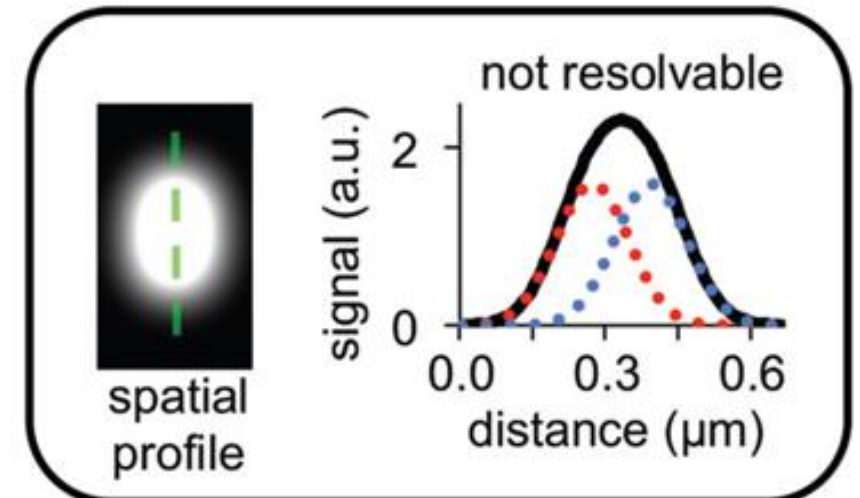
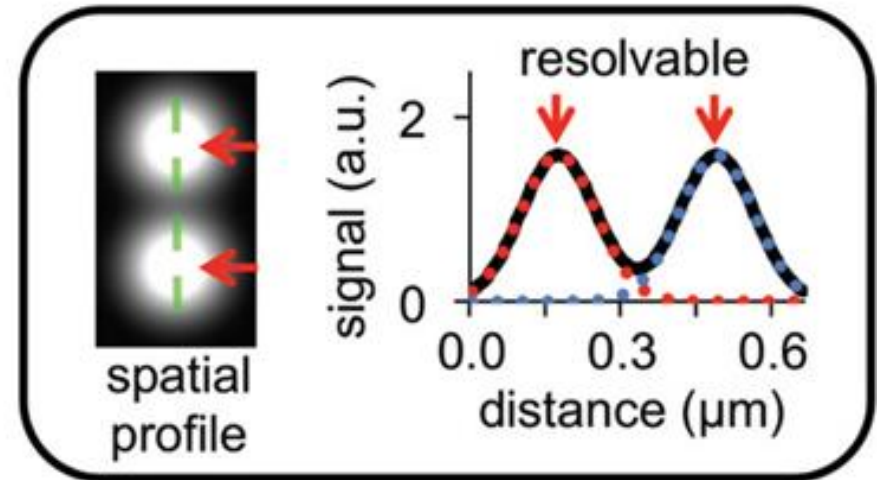
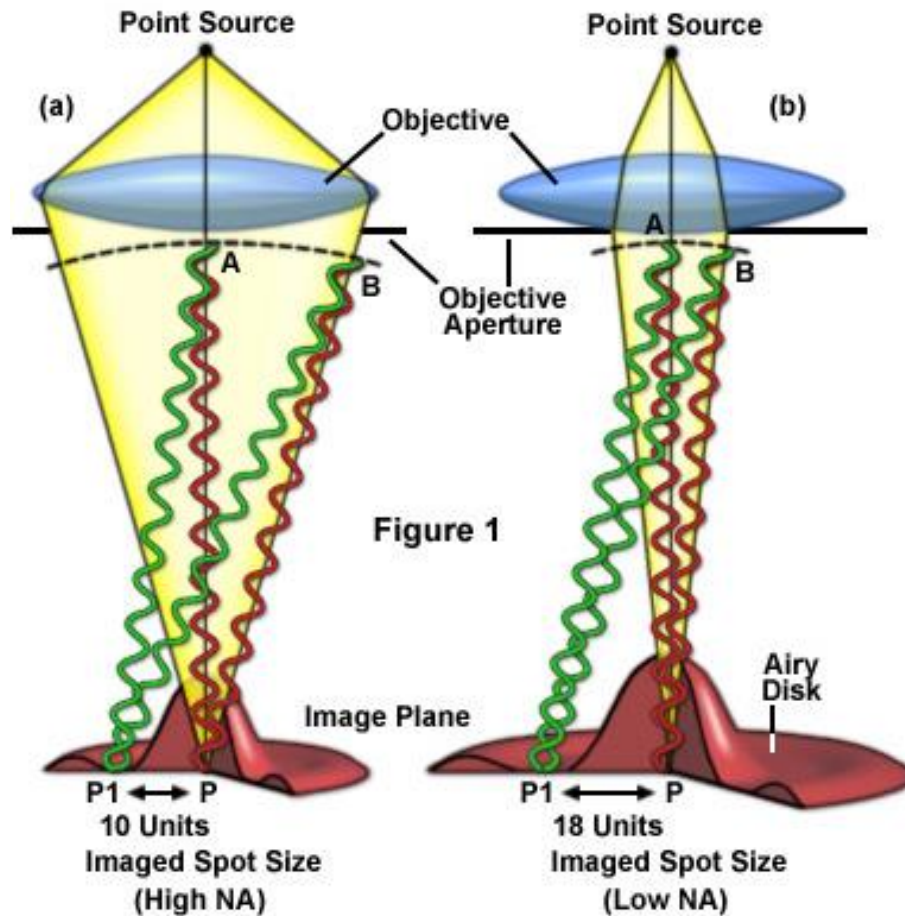


Spatial limits to light

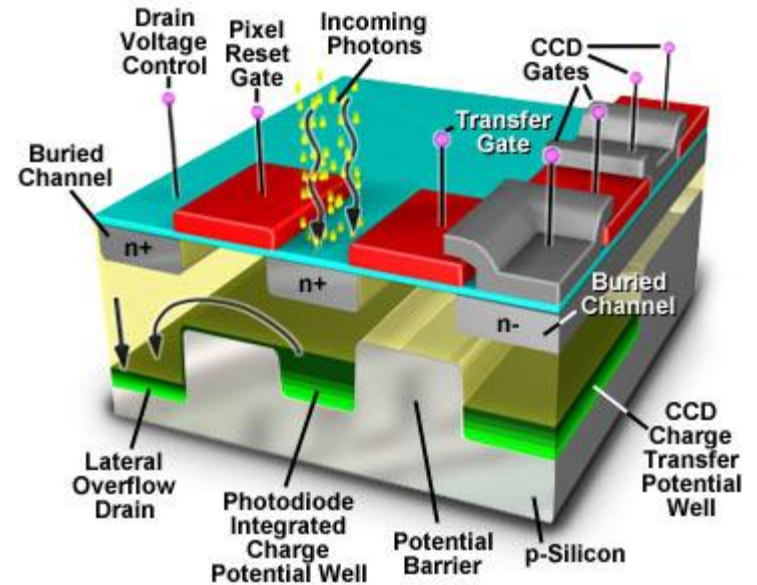
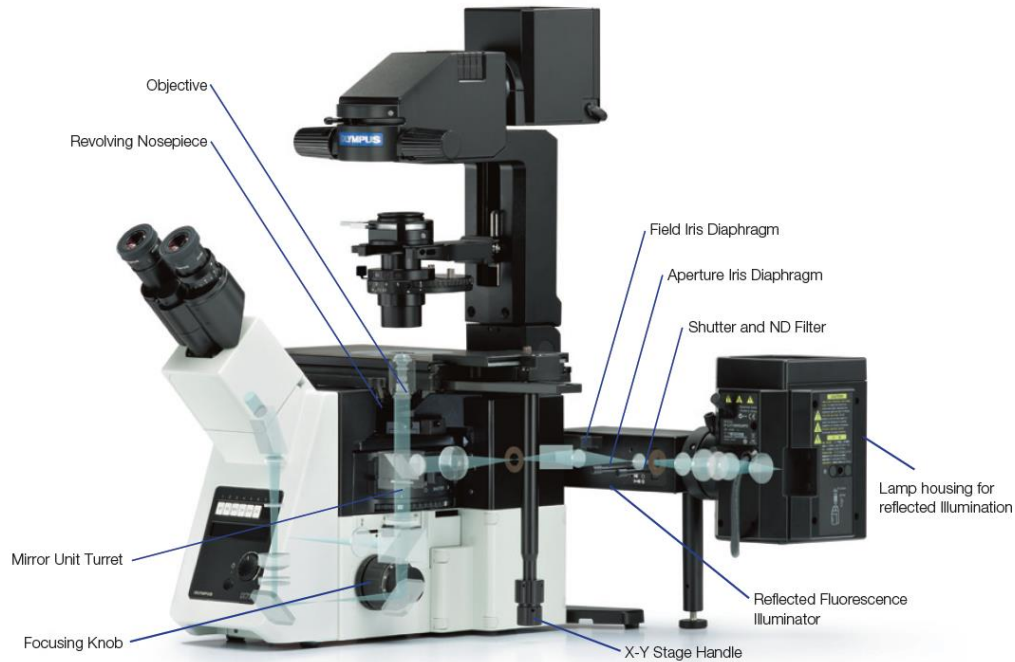
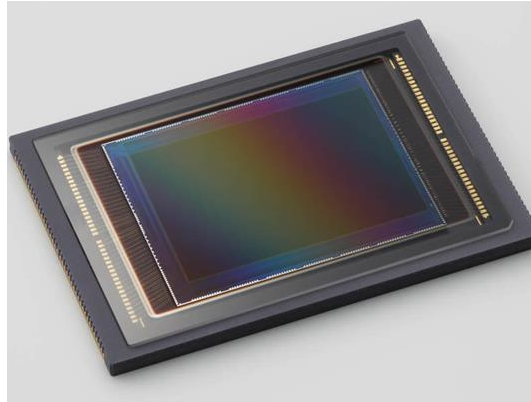


- Airy disk
 - spread of point function on imaging through finite lens.
 - dependent on wavelength of light (λ) and numerical aperture ($NA = n \cdot \sin(\theta)$).
- Resolution = $\lambda / (2 \cdot NA)$
 - NA in range of 0.9 – 1.45
 - limit of ~200 nm.

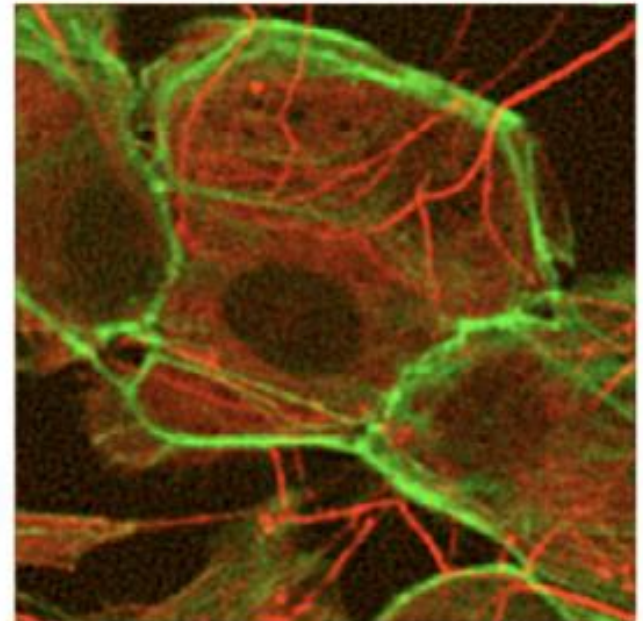
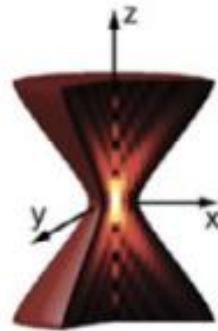
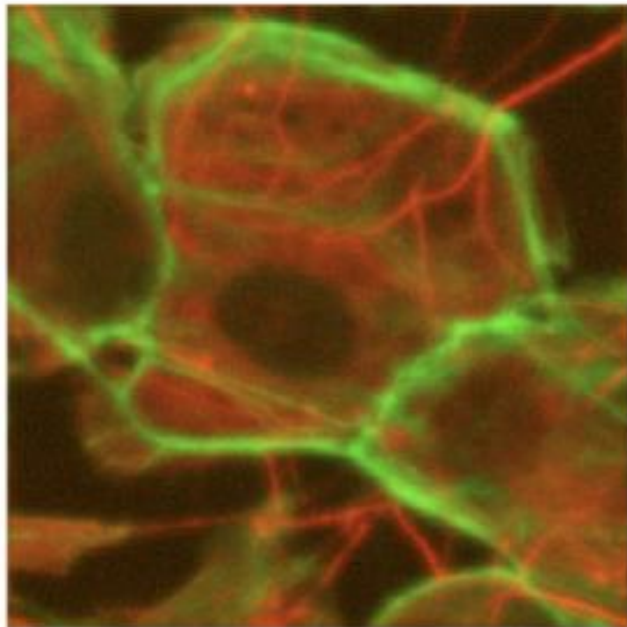
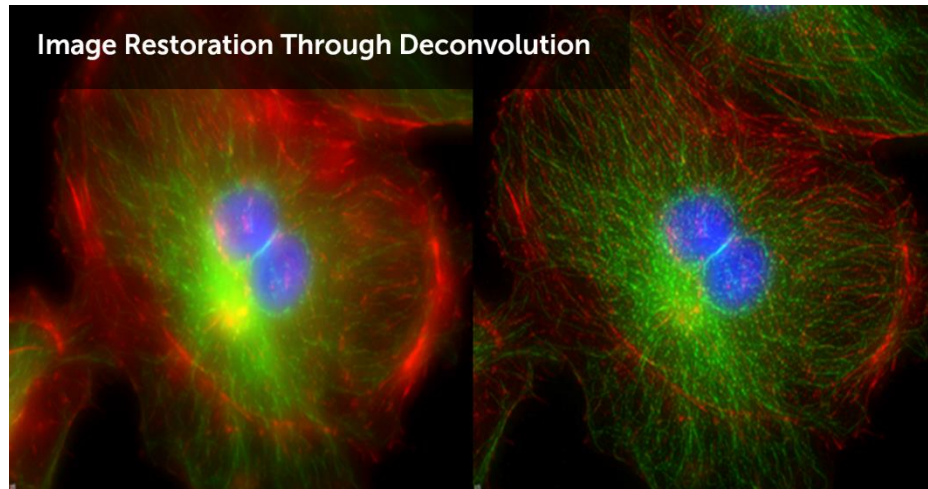
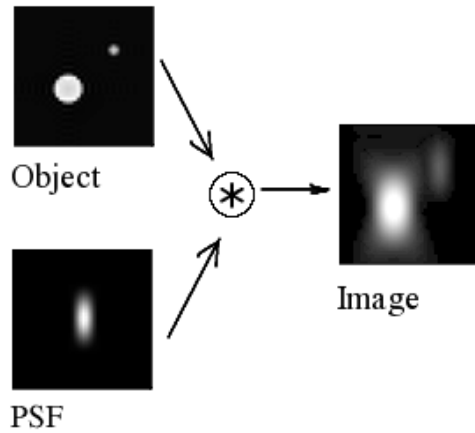
Diffraction limit



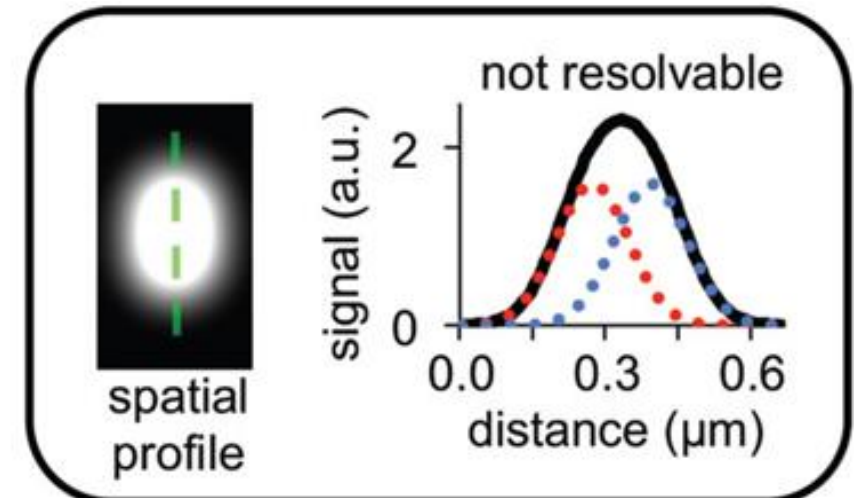
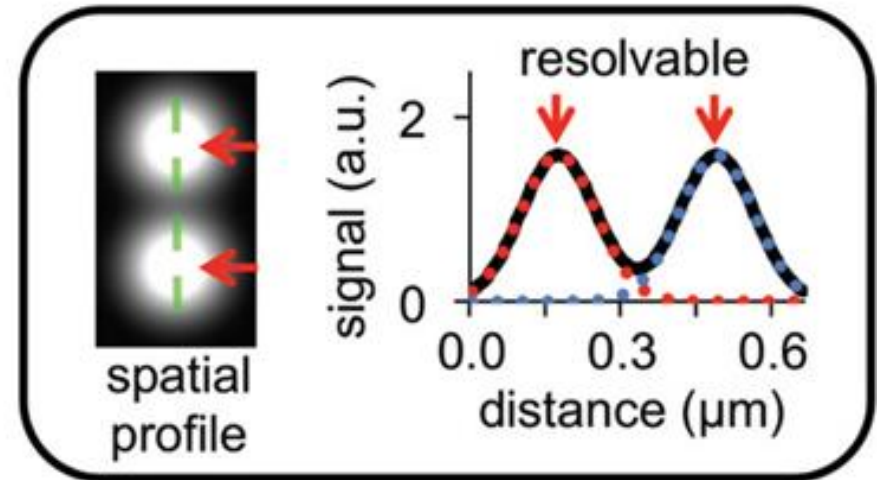
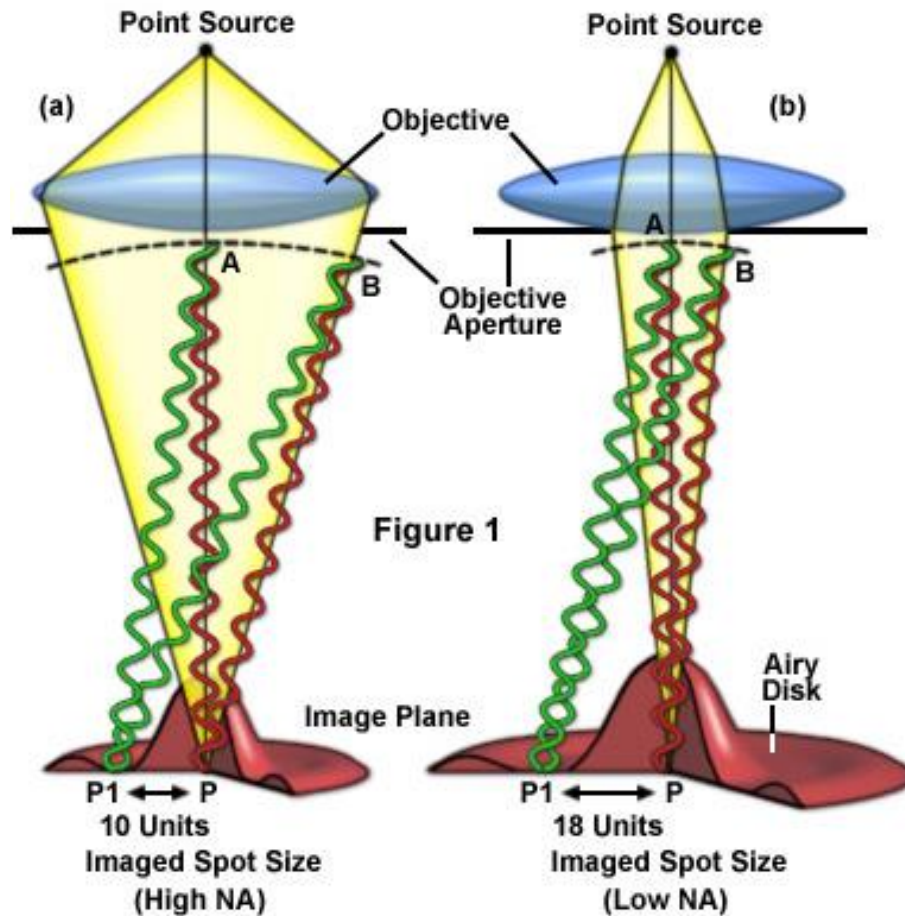
Fluorescence microscopy – digital imaging



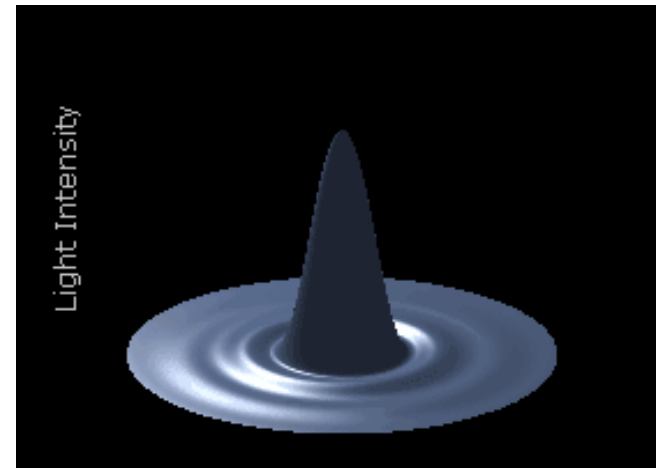
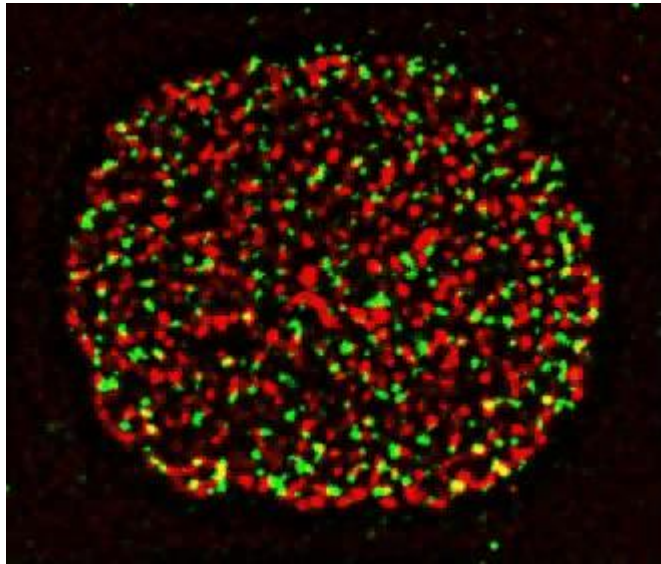
Deconvolution



Diffraction limit



Single molecule imaging



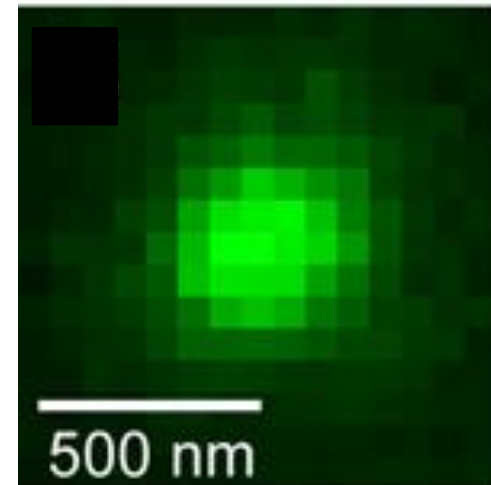
- Least square fit to Gaussian

$$I(x, y) = I_0 e^{-2[(x-x_0)^2 + (y-y_0)^2]/r_0^2} + \text{offset}$$

- Localization precision

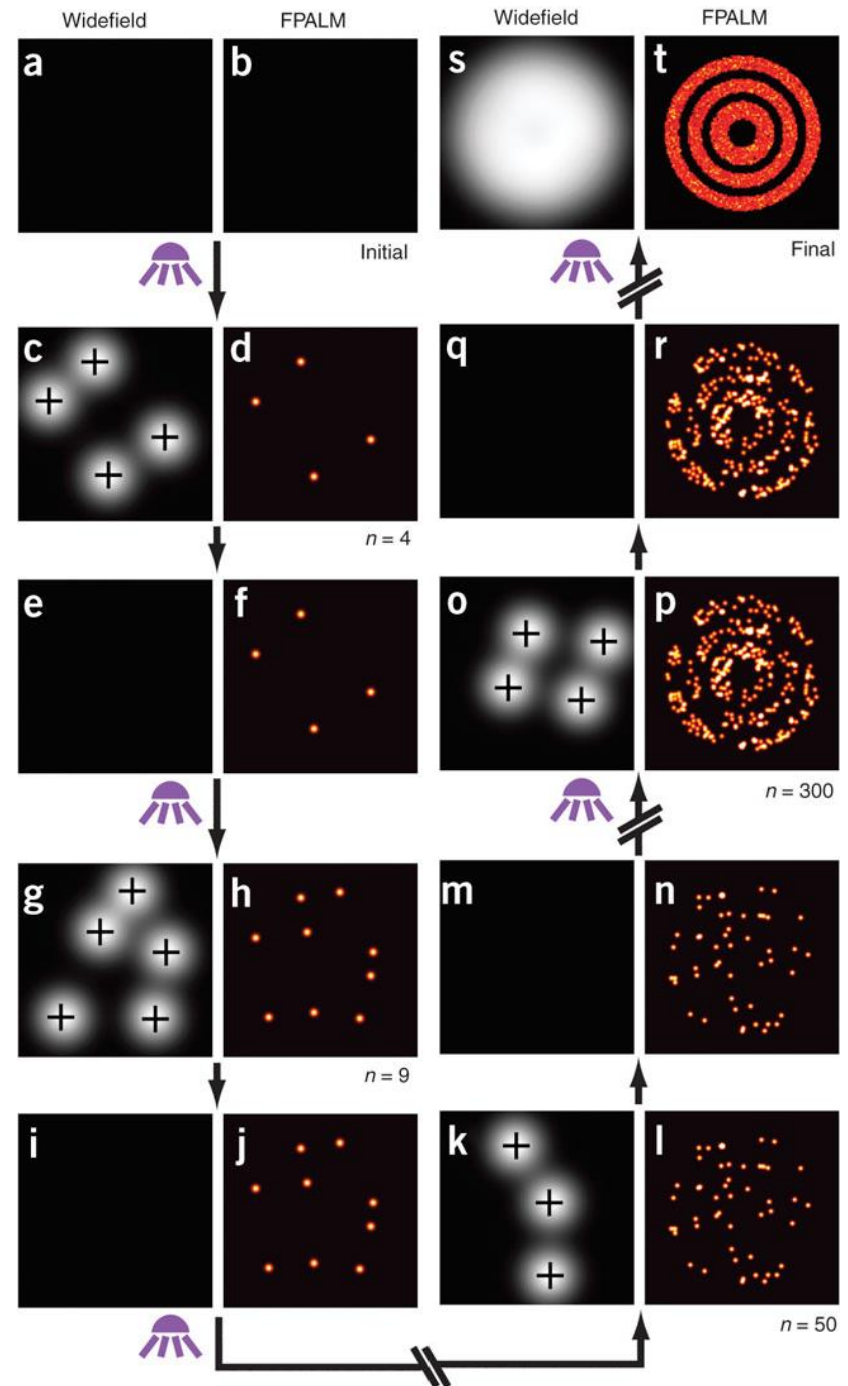
$$\sigma_{xy}^2 = \frac{s^2 + q^2/12}{N} + \frac{8\pi s^4 b^2}{q^2 N^2}$$

- q = size of pixel; N = # of photons
- s = stdev of dist; b = stdev of background



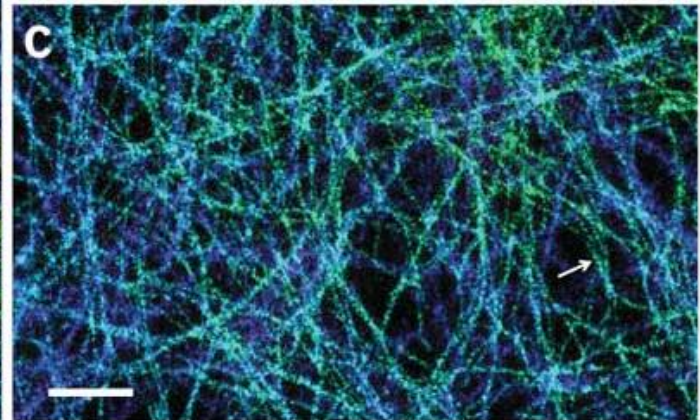
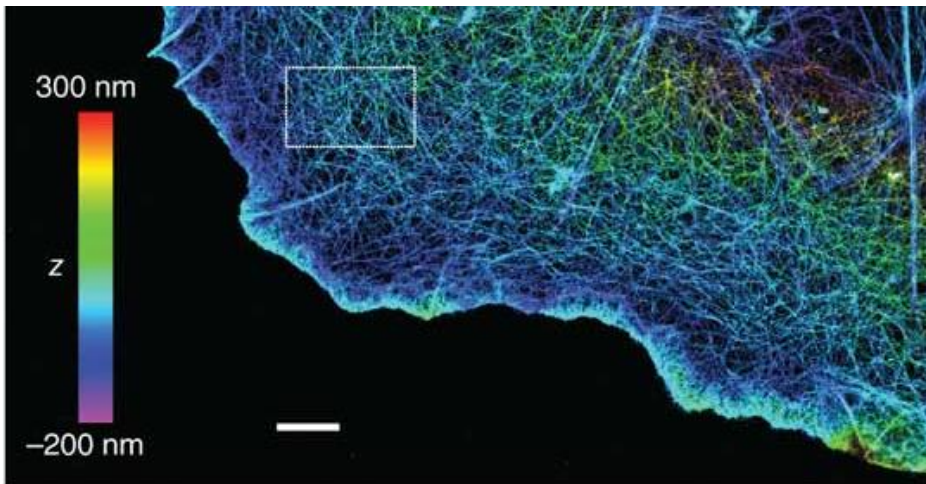
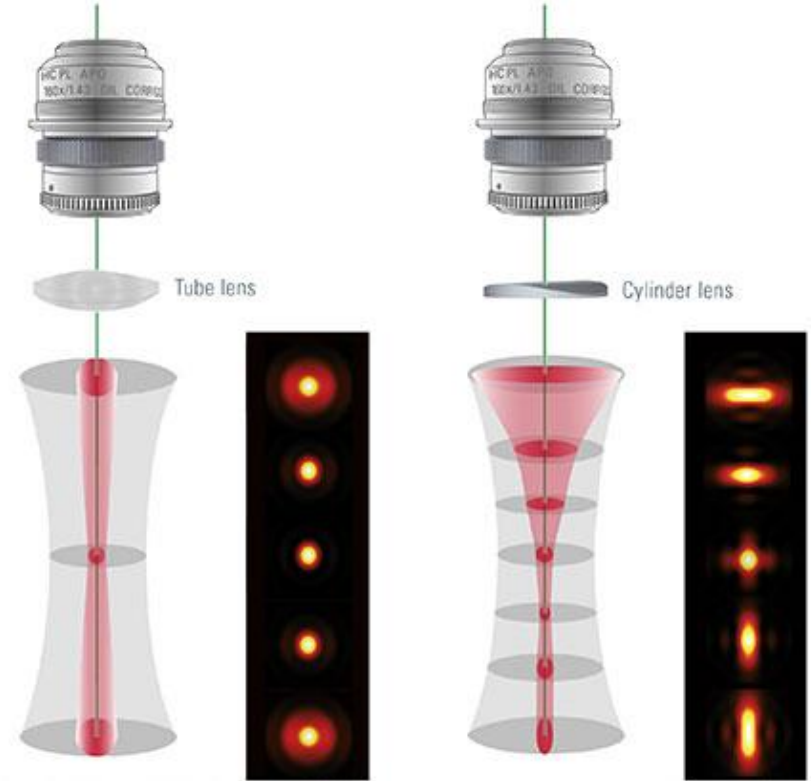
Photoactivated Localization Microscopy (PALM / FPALM)

- Work around spatial overlap of PSF by turning on fluorophores sequentially.
 - Activate subset of fluorophores (photoconversion)
 - Image (fluorophores bleach)
 - Compute locations, add to emerging image
 - Repeat
- Requires high sensitivity sensor, fine control of activation light and balance of fluorophore concentration.



3D – manipulation of point spread function

- The PSF is sharpest when fluorophore is at focal plane, gets wider above or below that plane.
- Additional optics can use this blurring to determine z-position.
- Cylindrical lens is the simplest

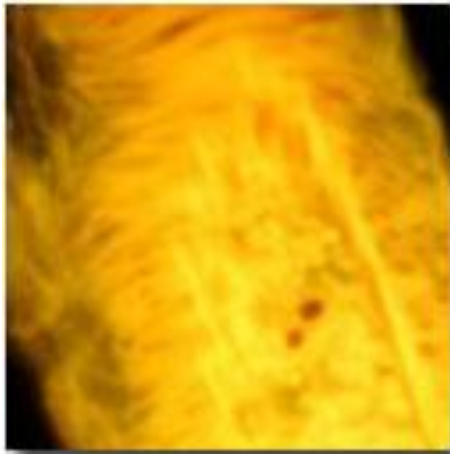


Out of plane light is problematic

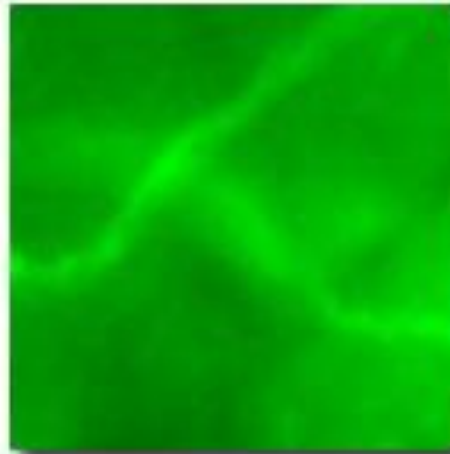


Out of plane light is problematic

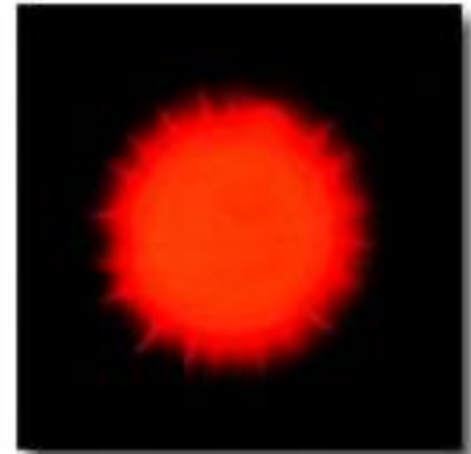
widefield
(epifluor)



(a)

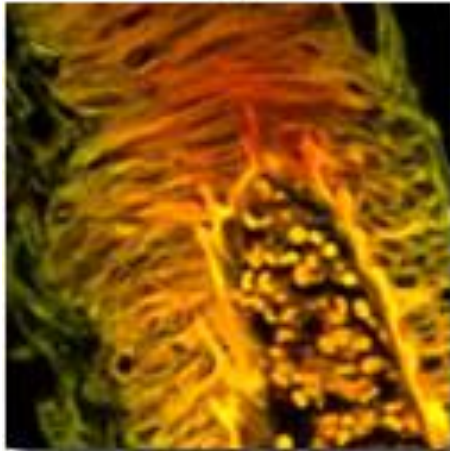


(b)

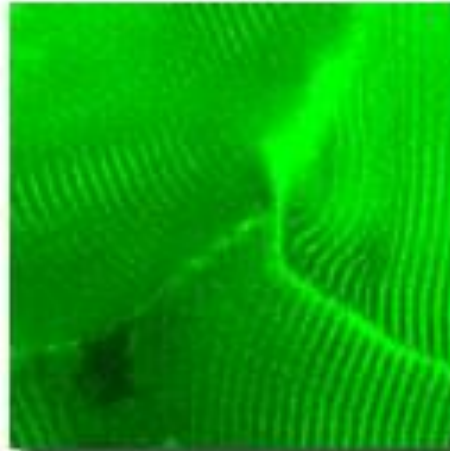


(c)

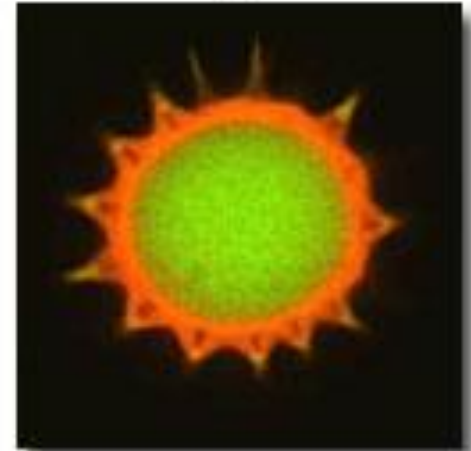
confocal



tissue
section

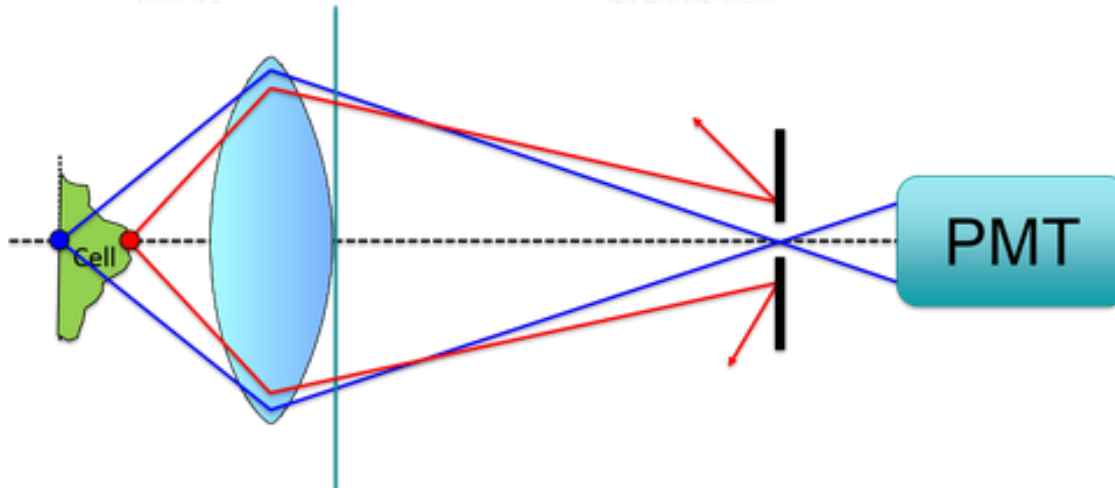
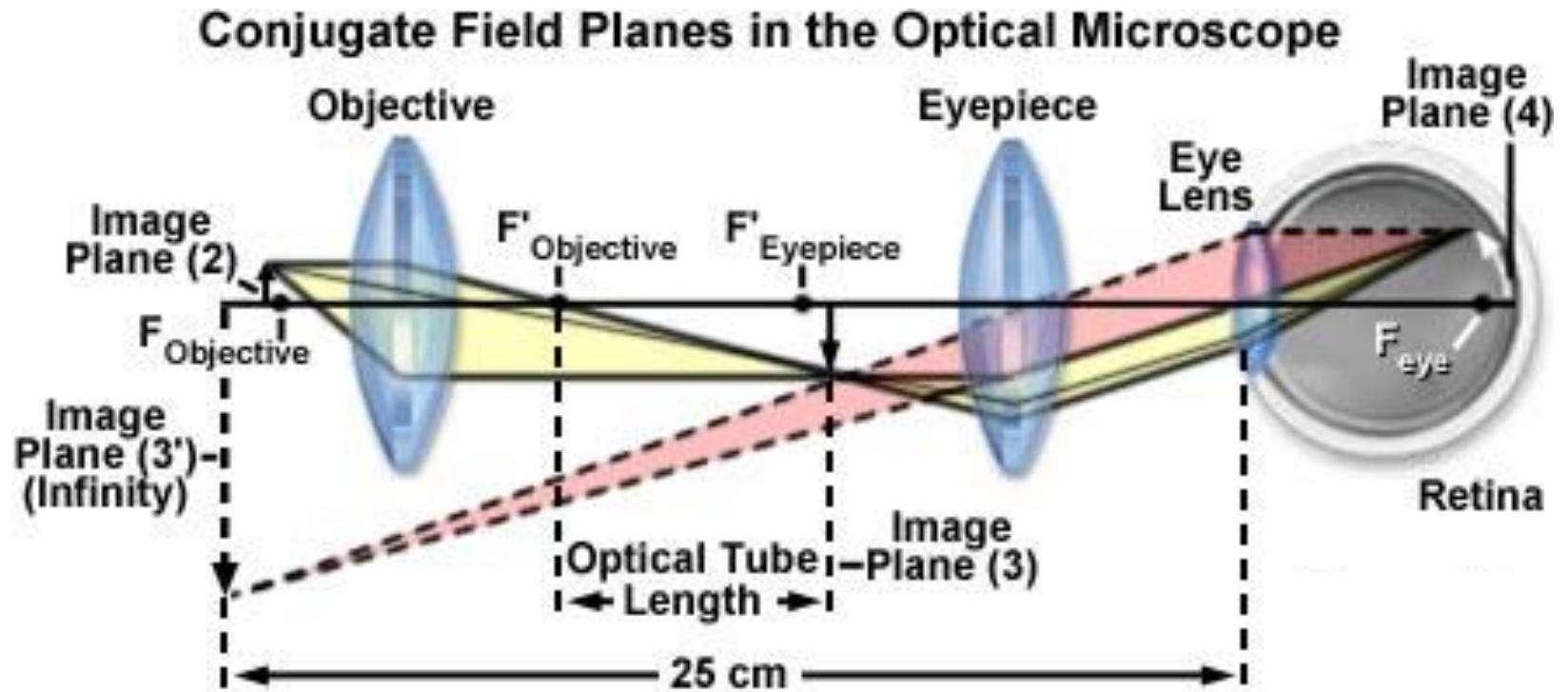


muscle fibers

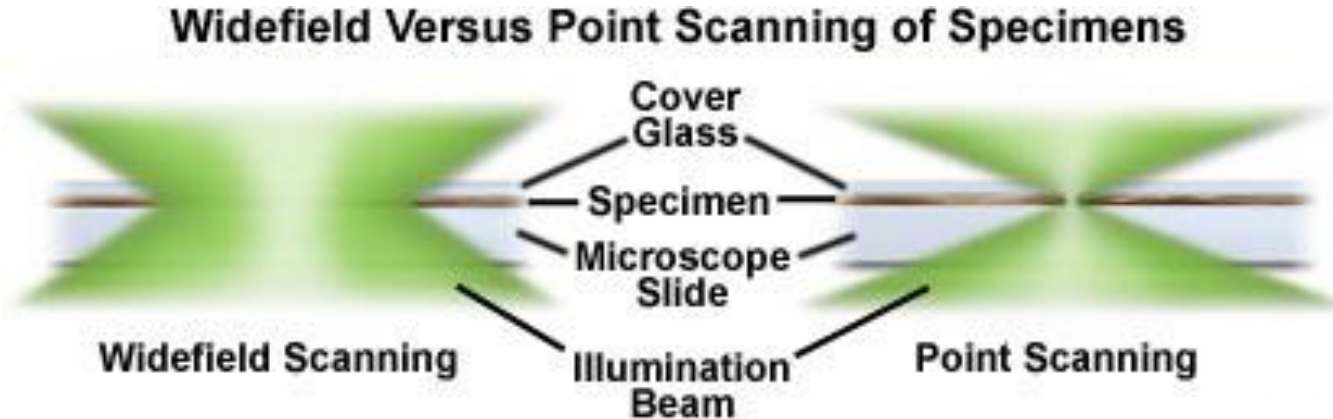


pollen grain

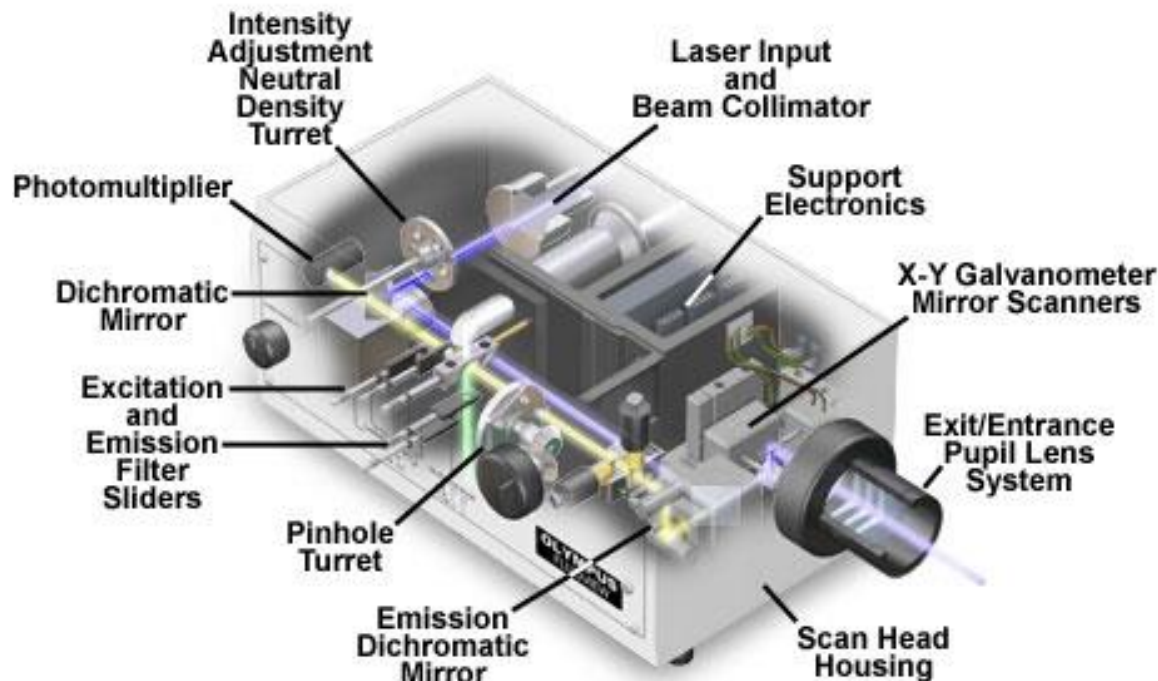
Confocal – rejection of out-of-plane



Scanning confocal – confinement of illumination



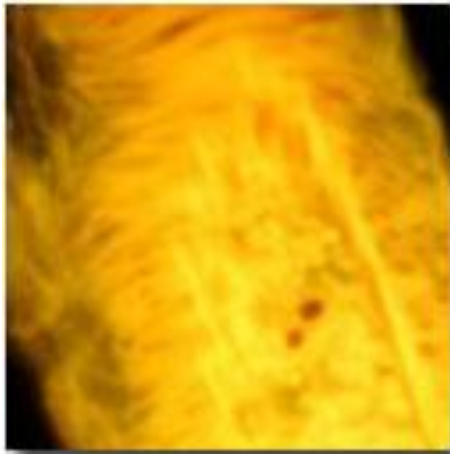
Confocal Microscopy Scanning Unit



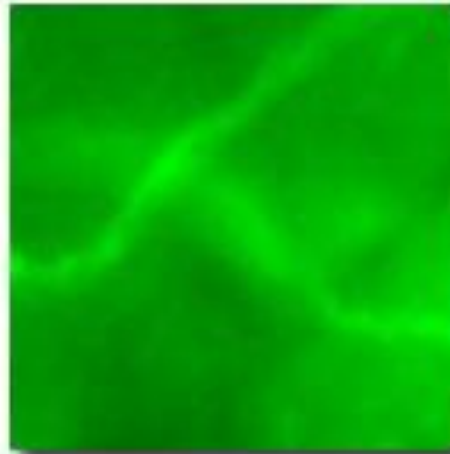
- Smallest spot achieved with laser
- Serial process
- Slow
- Intense light
- Resonant techniques help

Out of plane light is problematic

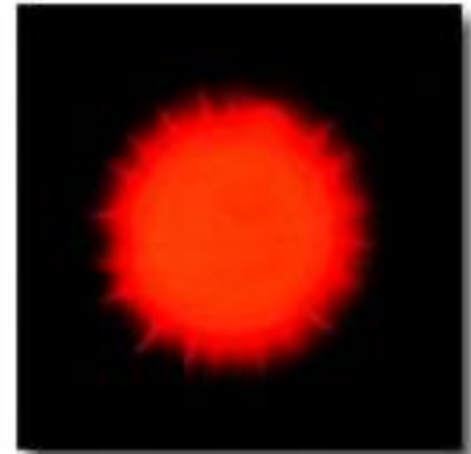
widefield
(epifluor)



(a)

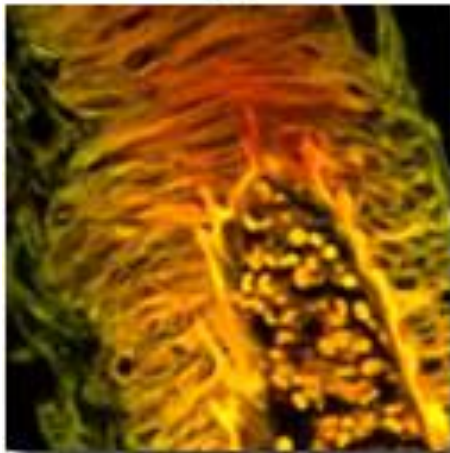


(b)

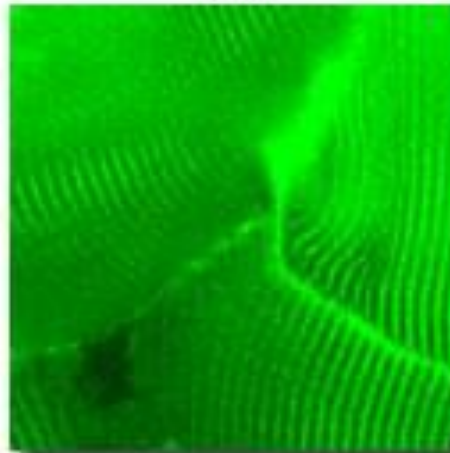


(c)

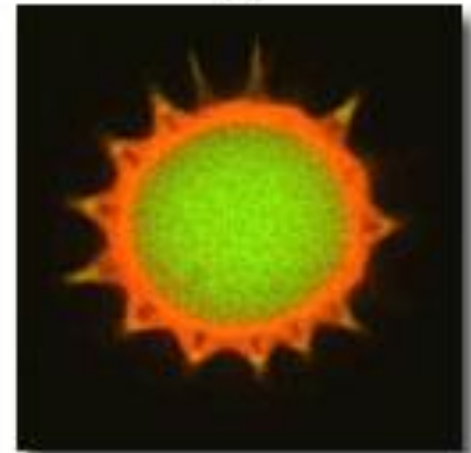
confocal



tissue
section

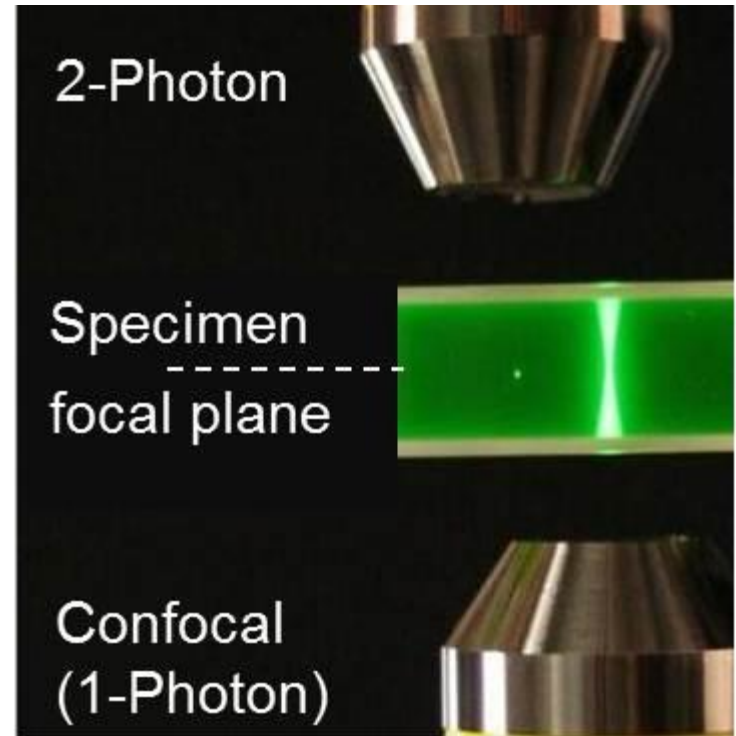
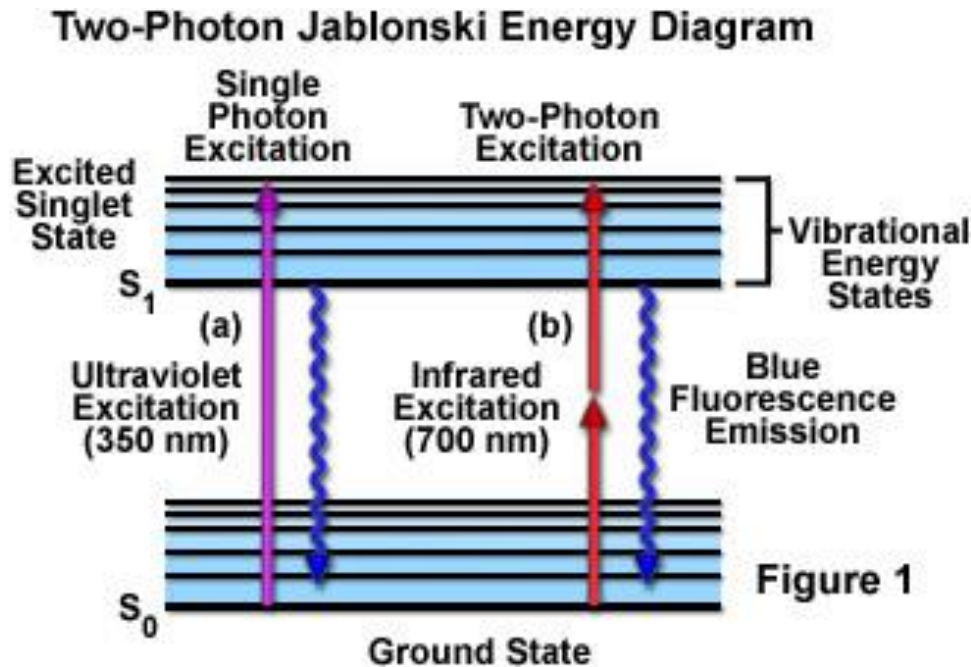


muscle fibers



pollen grain

Multiphoton



Photography: Ciceron Yanez, University of Central Florida

- Certain dyes respond to multiphoton excitation
- Excitation often goes as intensity^2
- Less photobleaching
- Longer wavelength excitation (IR)
 - better penetration into tissues, mm
 - larger focal point than shorter wavelength

hhmi | janelia

Research Campus

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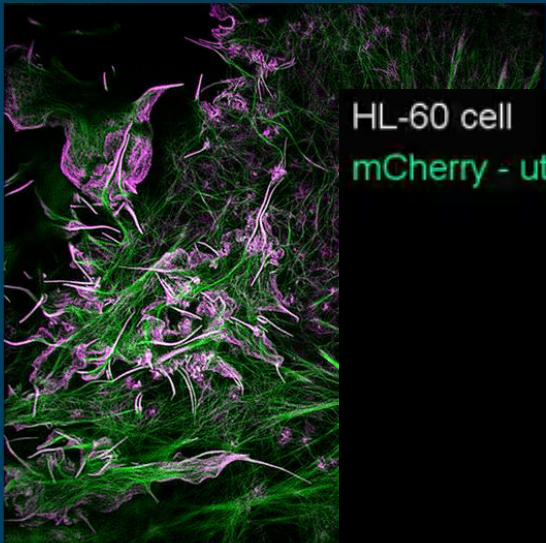
Our Research

Support Teams

Open Science

You + Janelia

About Us



HL-60 cell

mCherry - utrophin

FITC - collagen

Lab Home

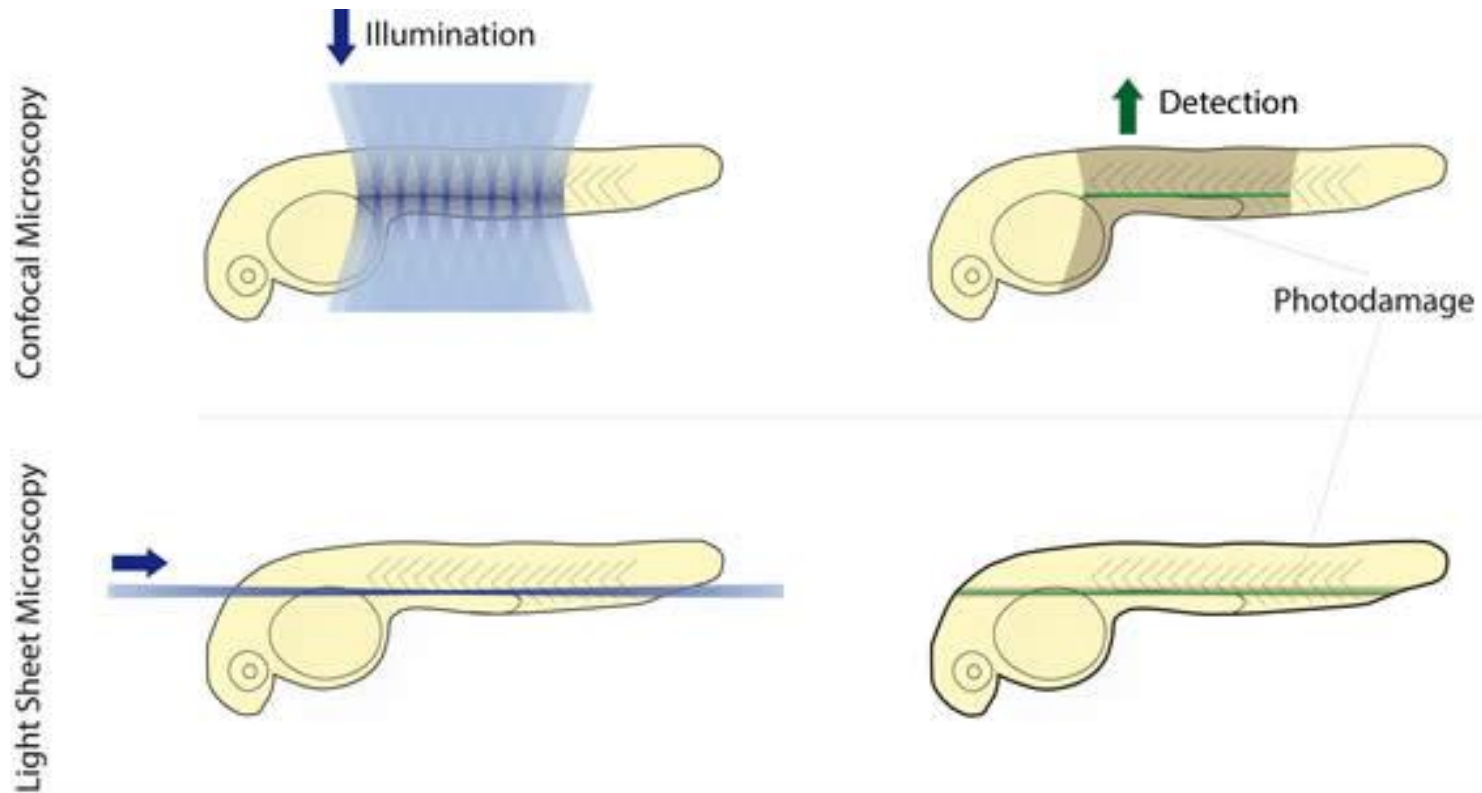
Lab Members

Pub

gging tools in an

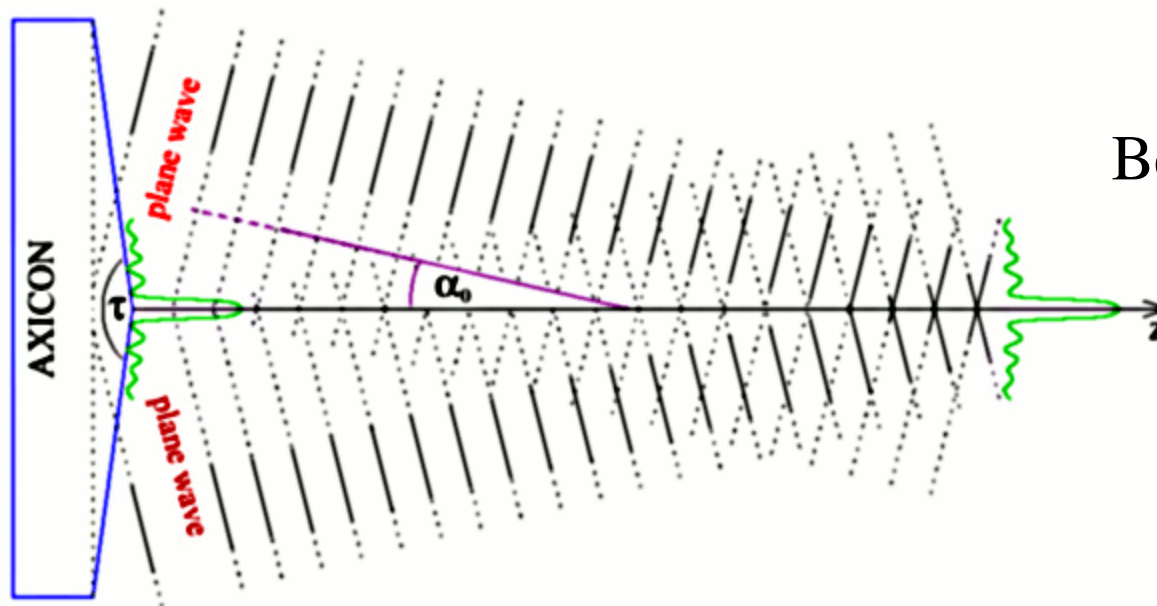
ar, and neurobiology.

Light sheet microscopy



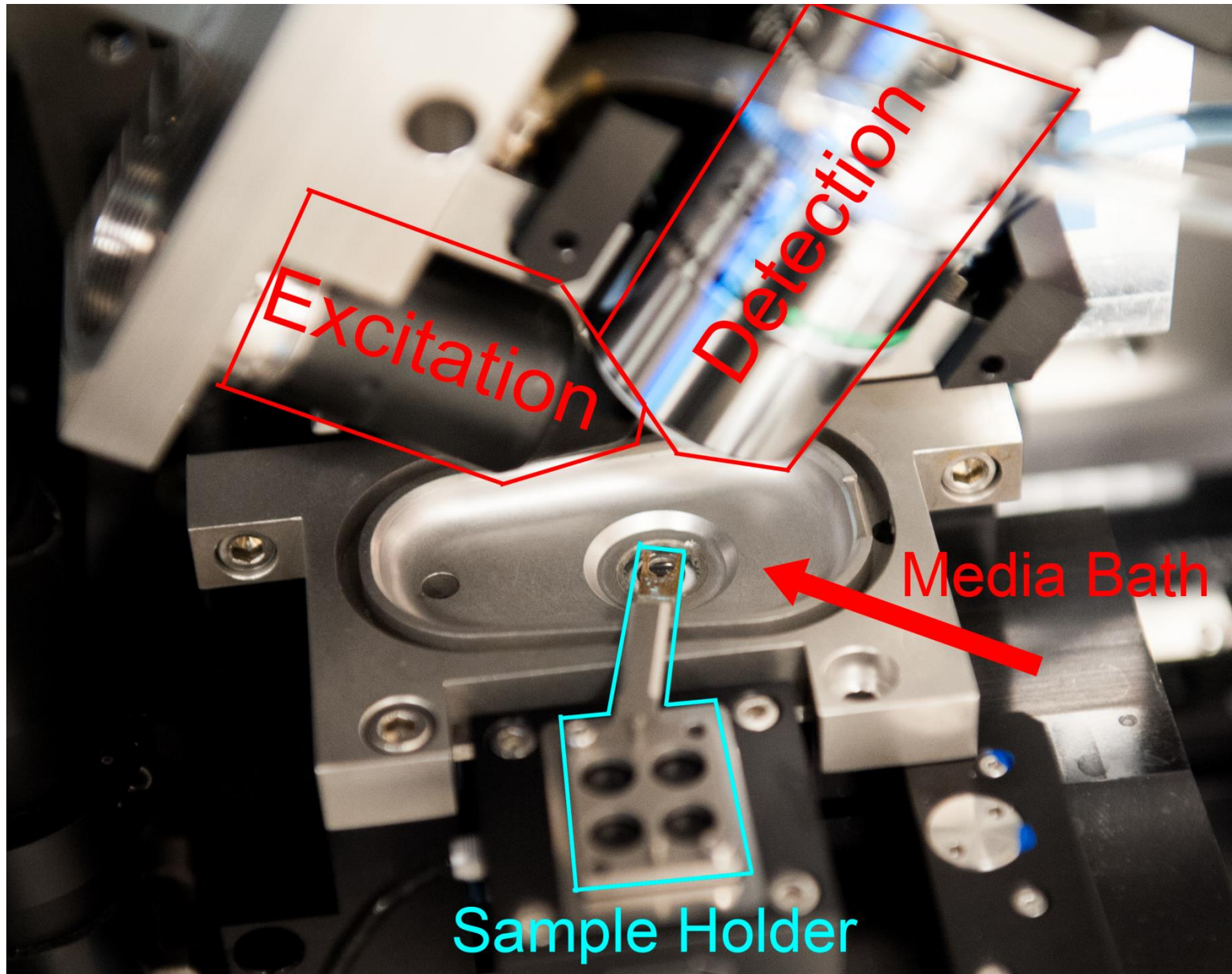
- Exciting only a plane of a sample takes advantage of modern 2D imaging sensors (CCD, sCMOS) while minimizing exposure.
- Sample is stepped or rotated relative to illumination

Generating a light sheet

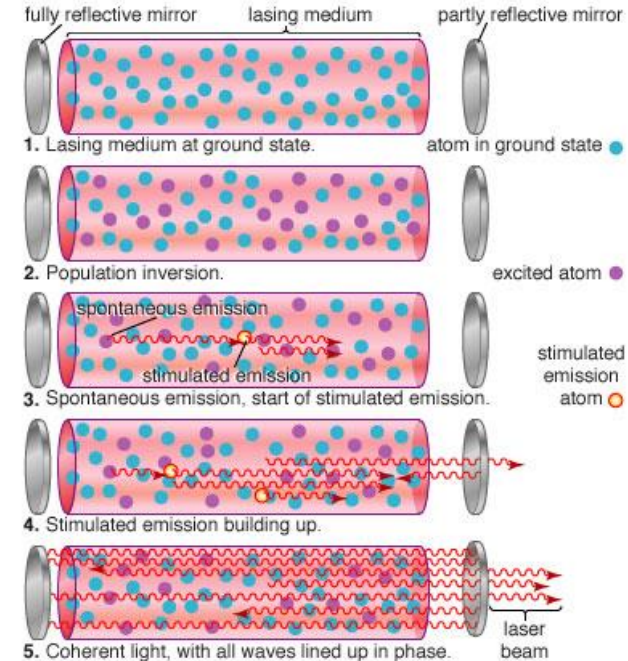
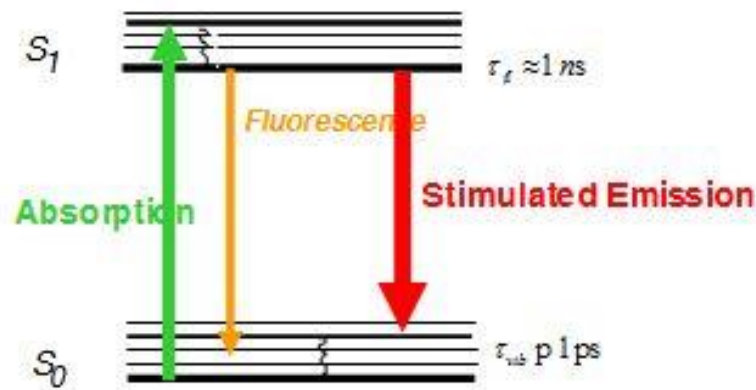
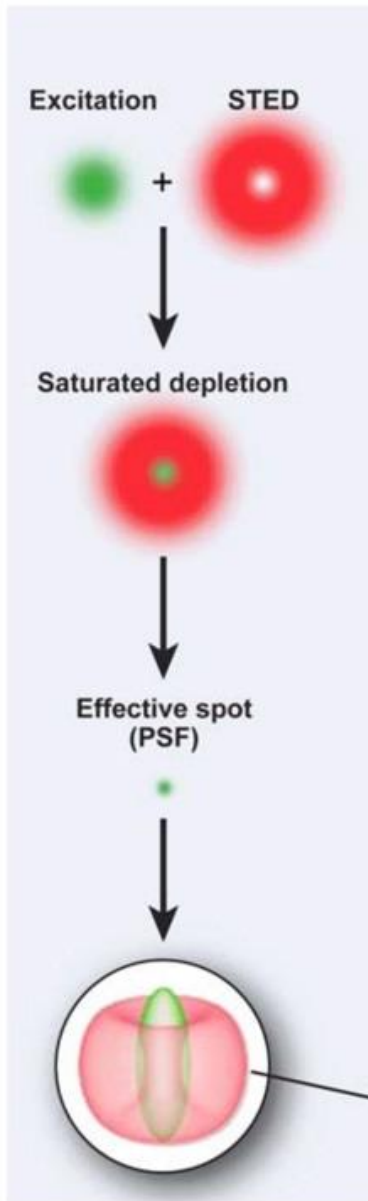


Bessel beam

Unusual configuration

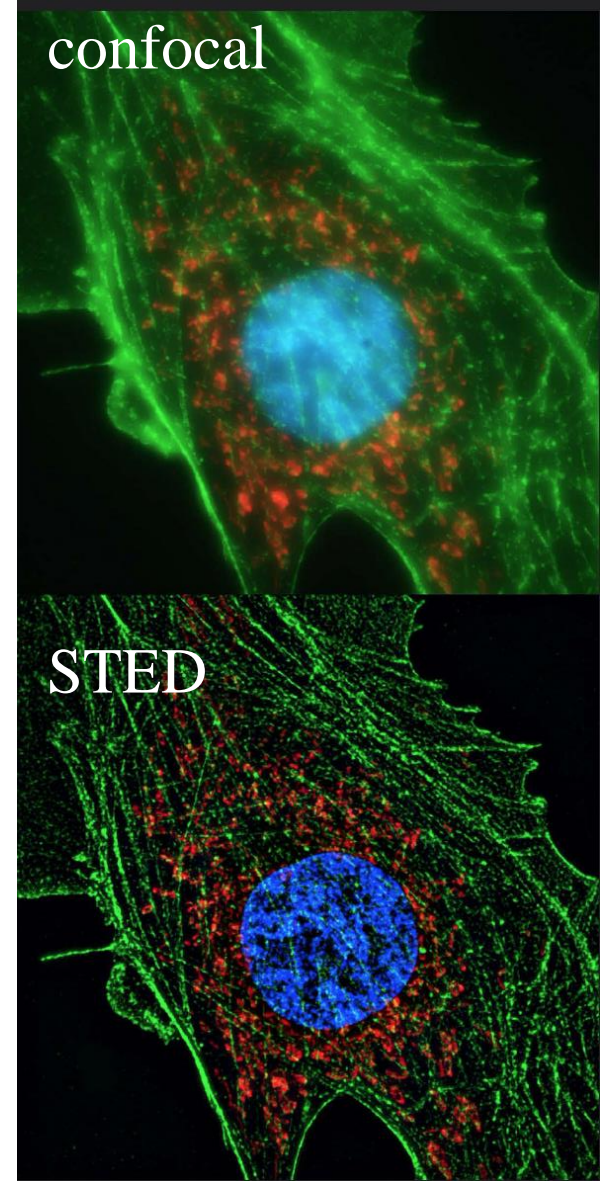
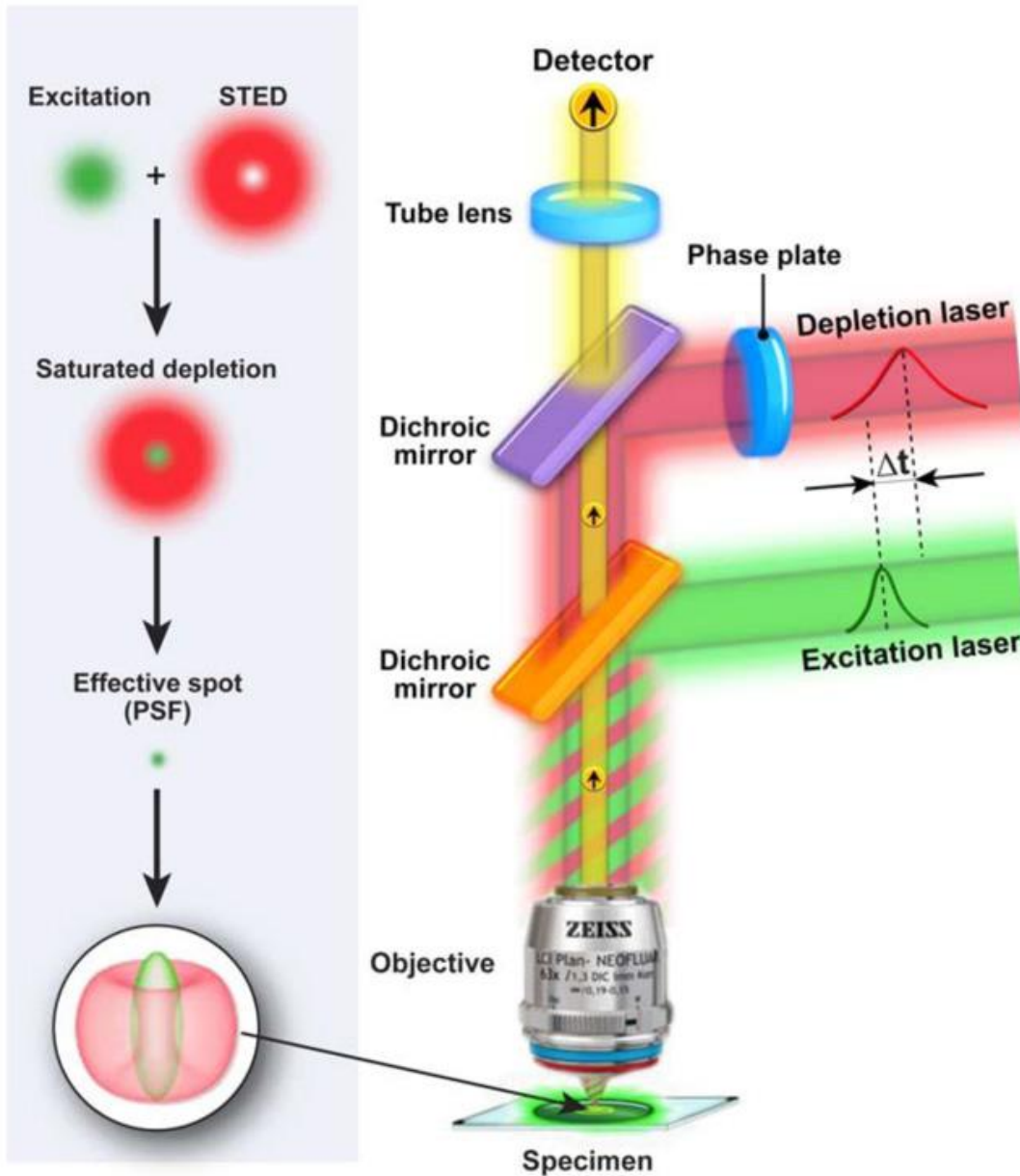


Stimulated emission depletion: STED

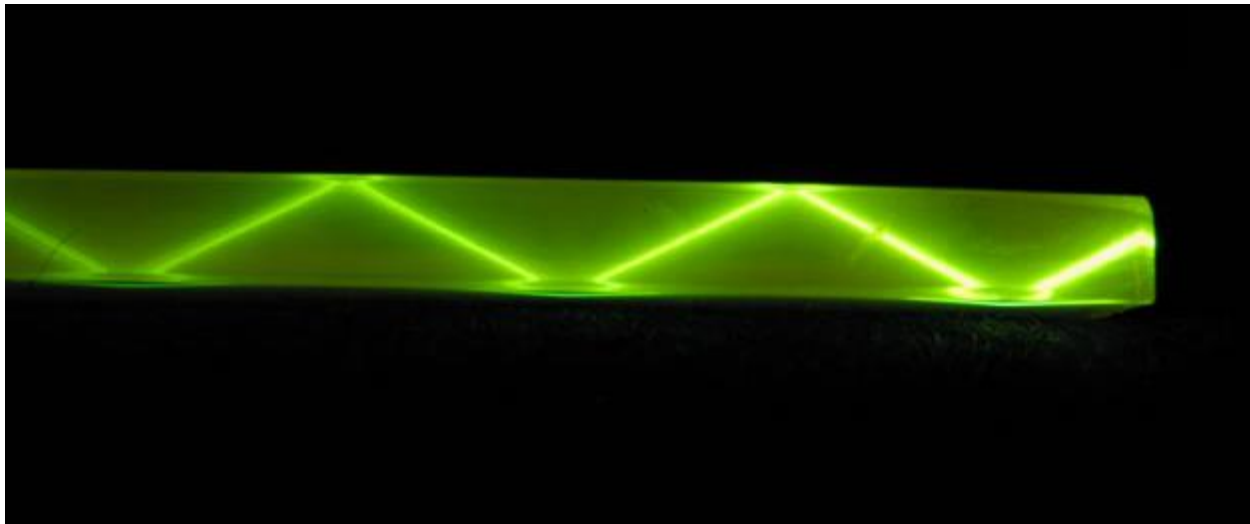
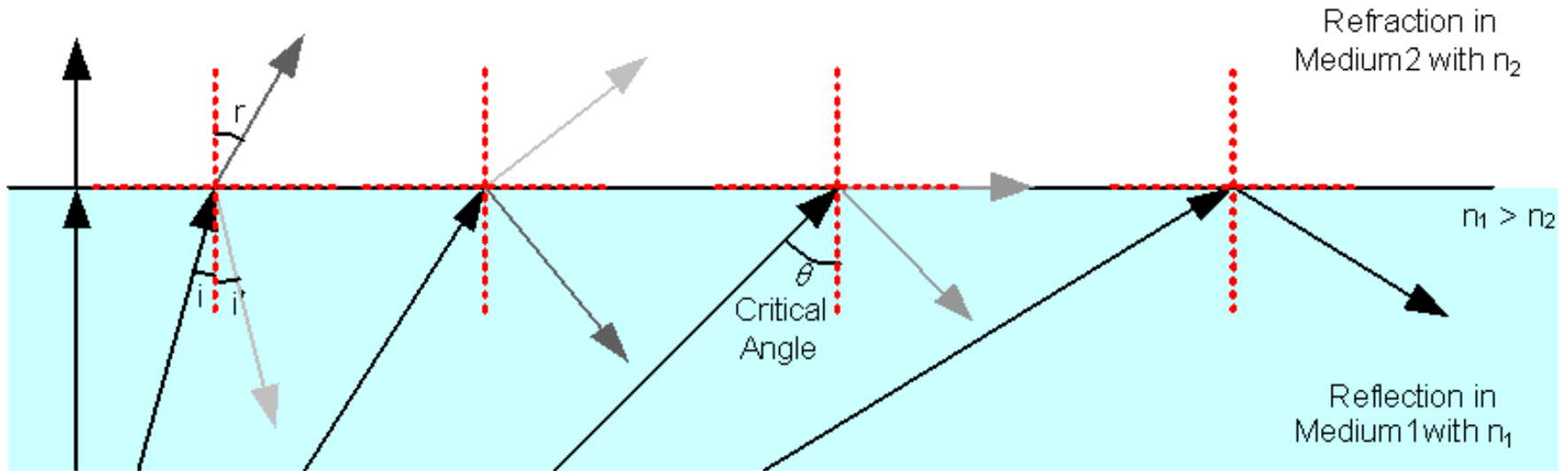


- Laser with wavelength in emission of fluorophore “deactivates”
- Concentric lasers, scanning configuration
- Resolution down to 50 nm (x-y)
- 3D confinement also possible (100 nm)

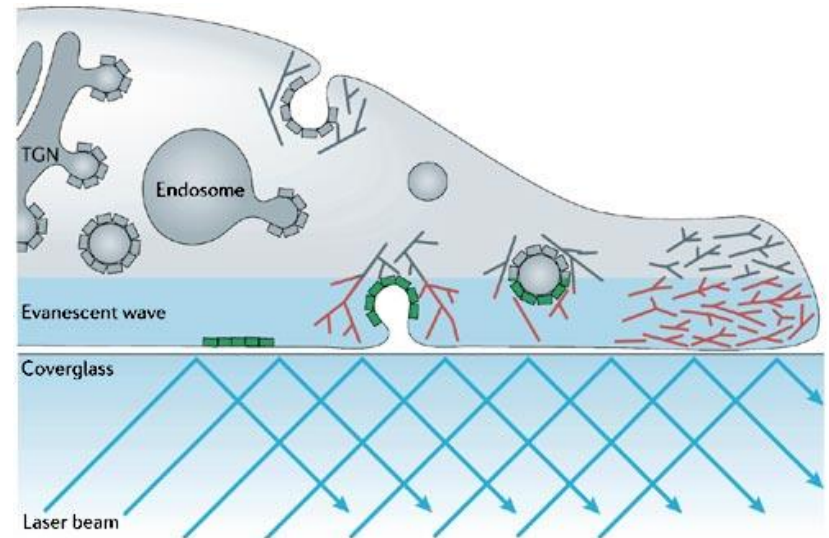
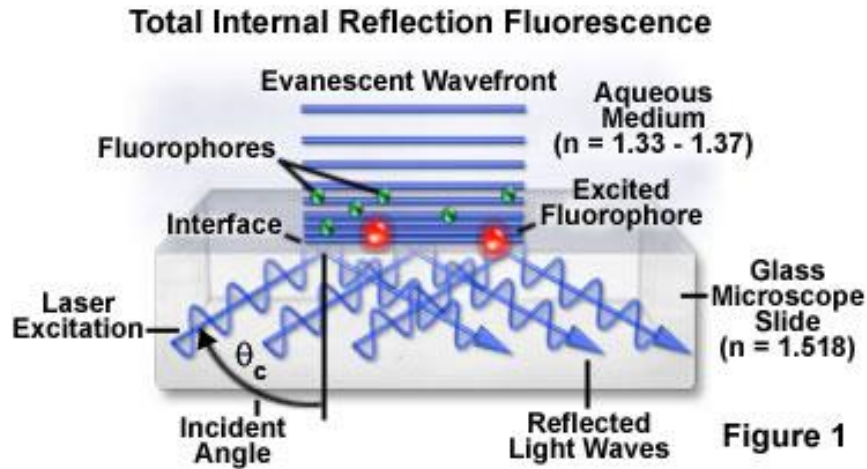
Stimulated emission depletion: STED



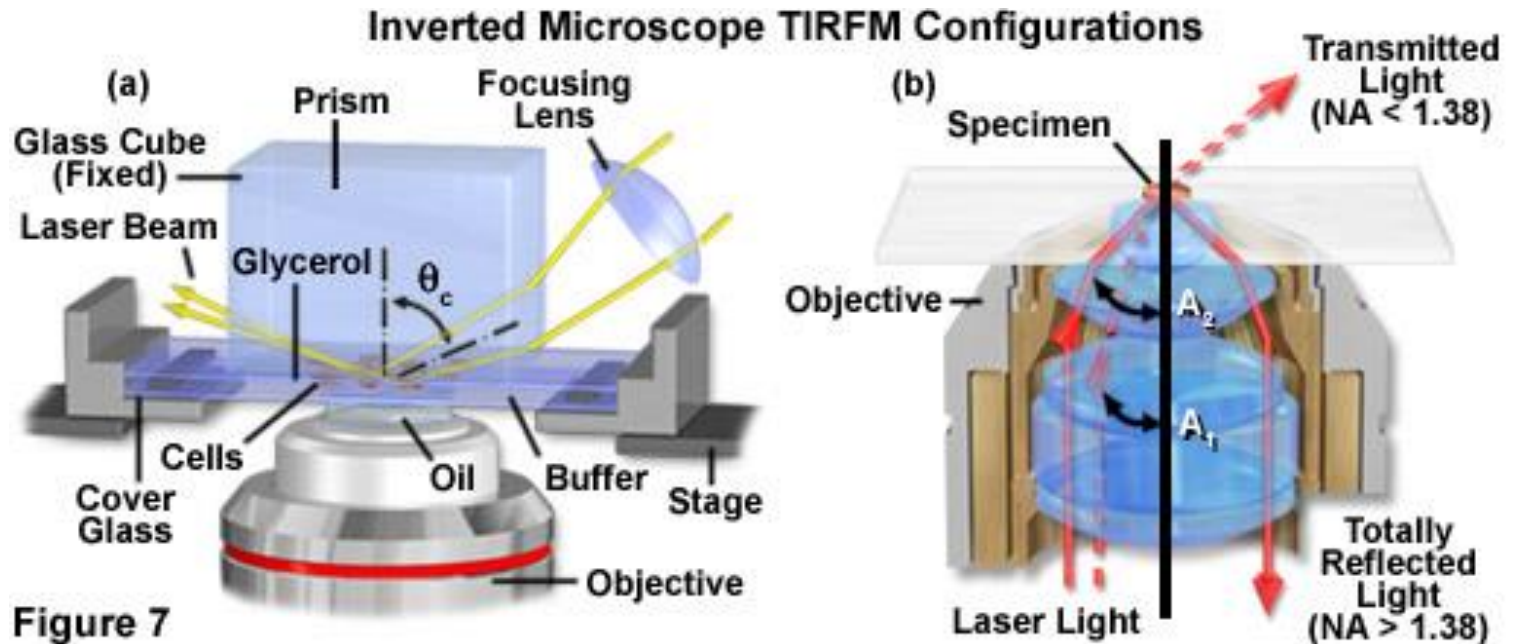
Total Internal Reflection microscopy



Total Internal Reflection microscopy



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Total Internal Reflection microscopy

