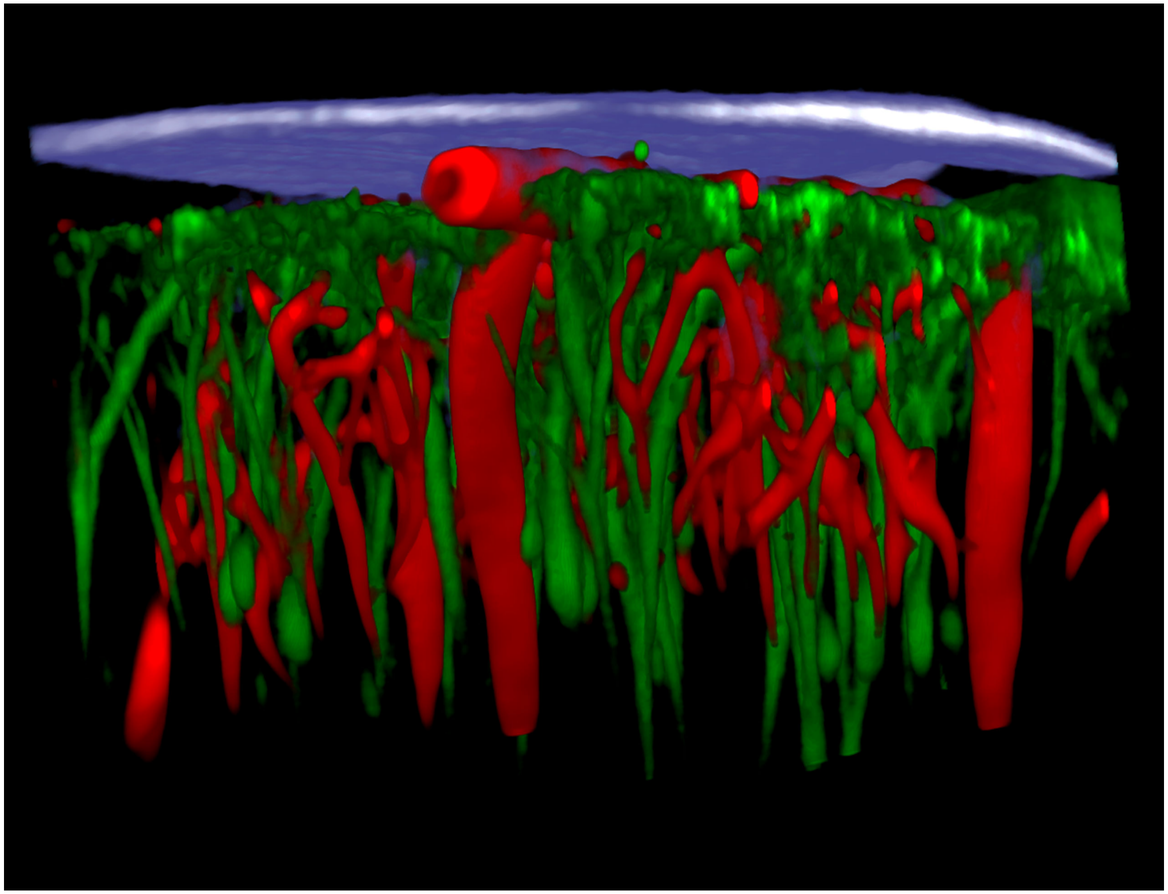
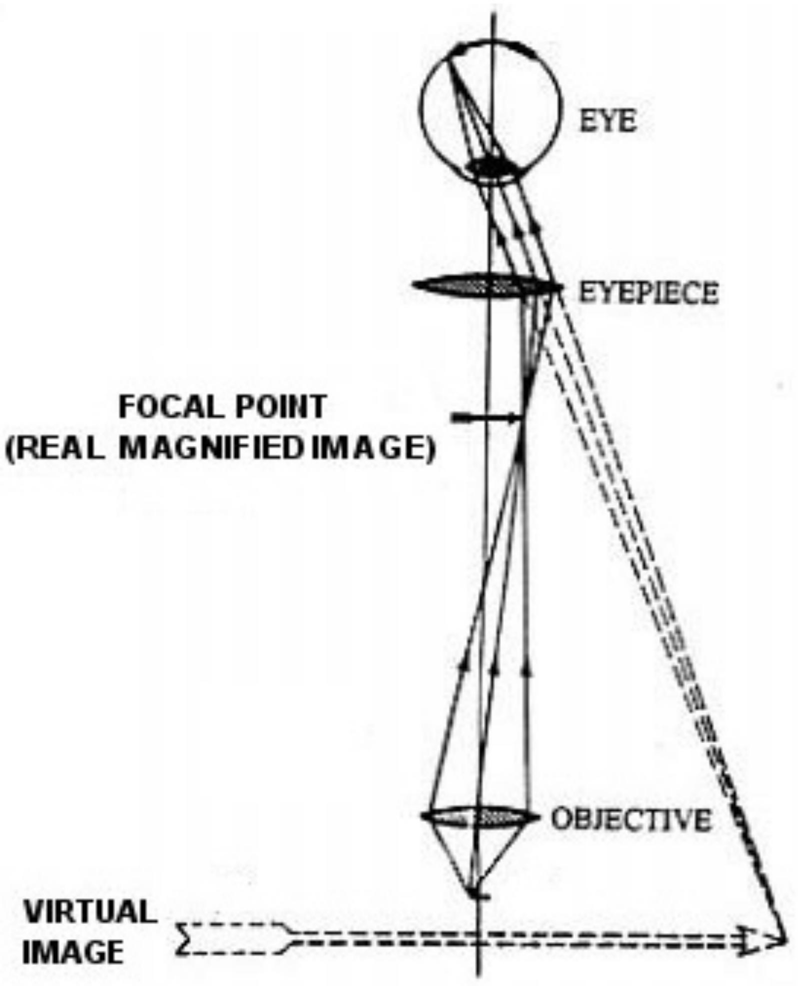
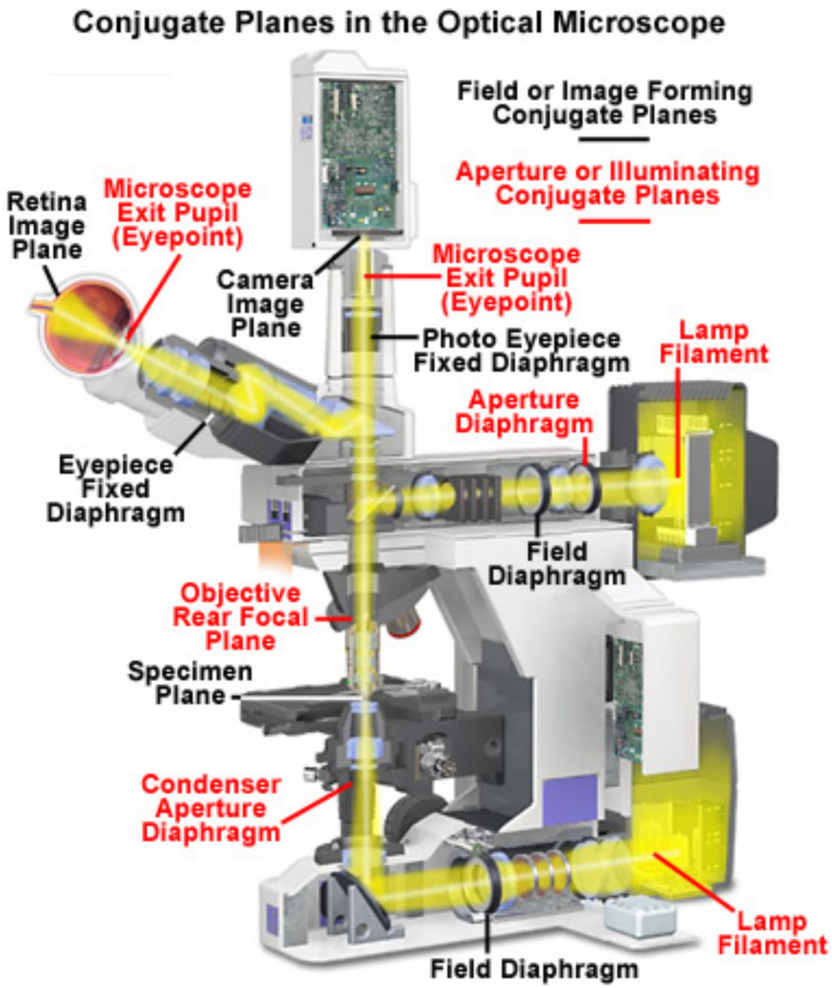
Idiosynchratic primer on principles of light microscopy for neuroscience

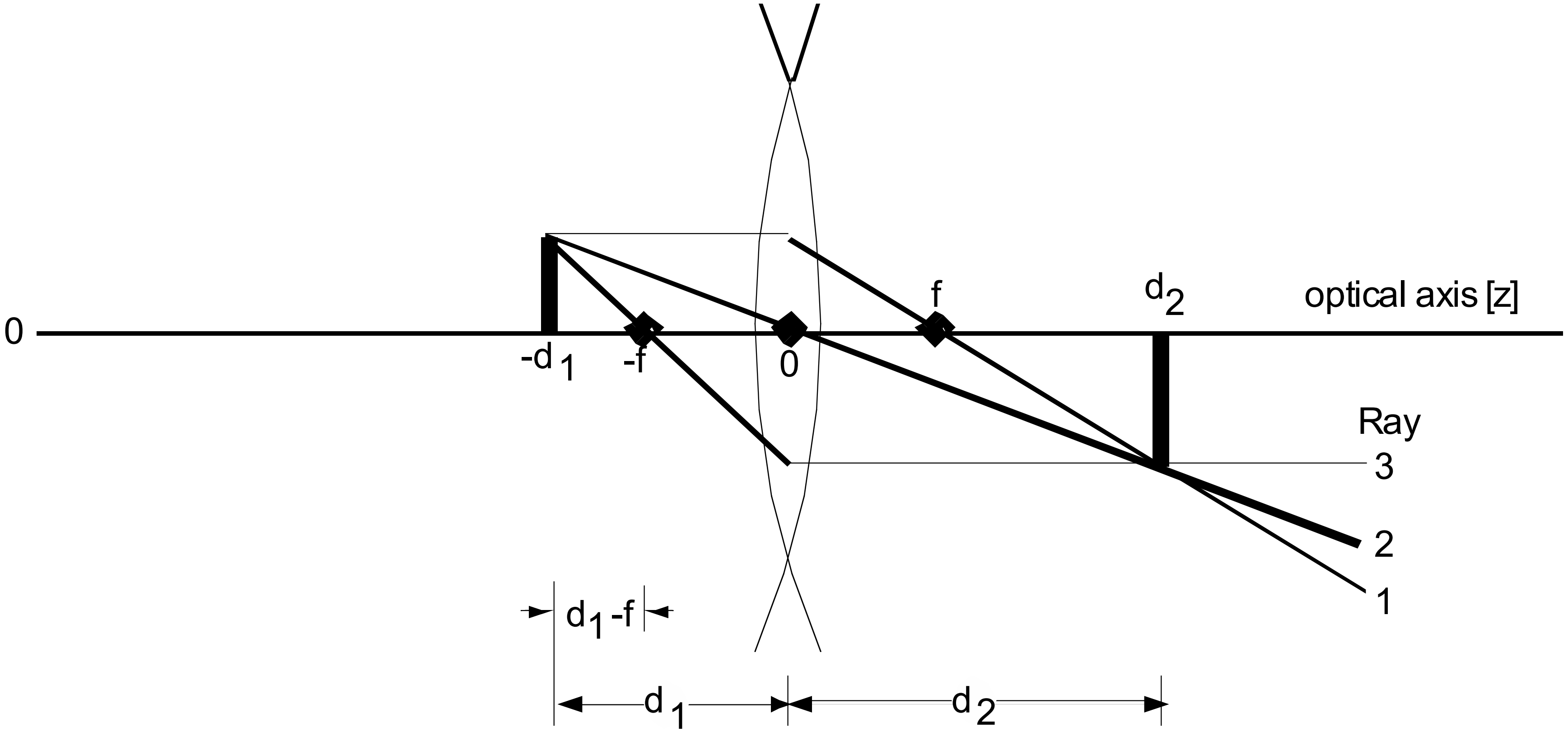


Basic features of a microscope for transmission and fluorescence





The lens maker's formula (thin lens, paraxial beam, …): A good start to understand imaging

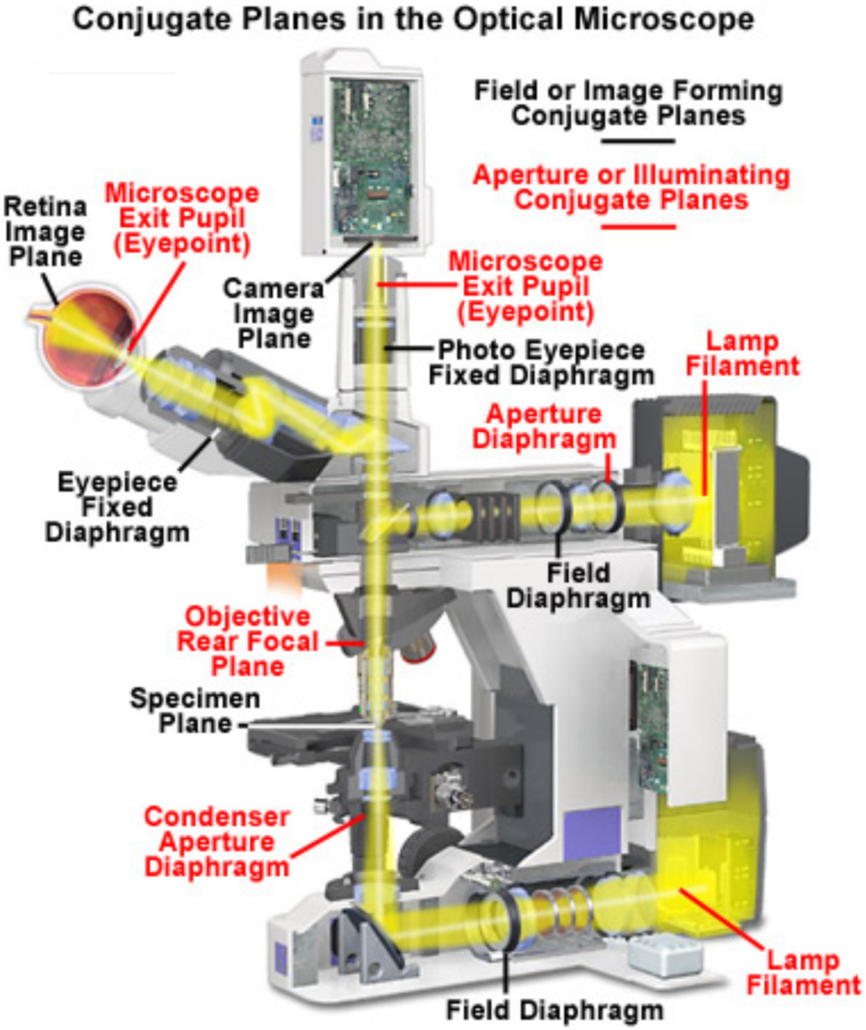


$$(d_1 - f)\,(d_2 - f) = f^2$$

Infinity corrected microscopes: Two pathways in brighfield imaging

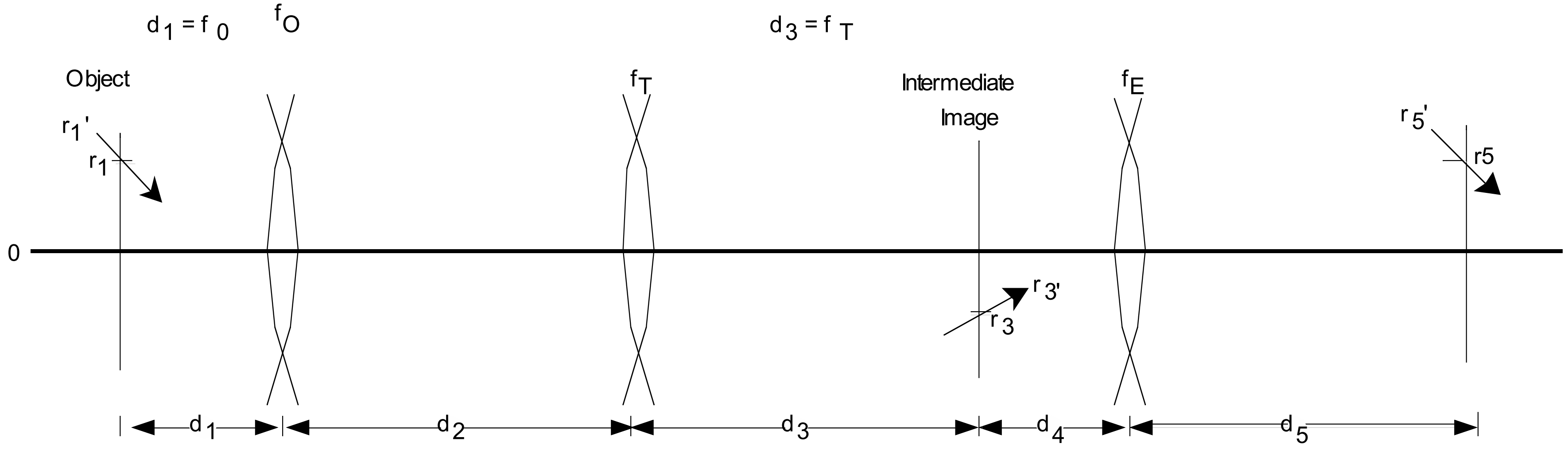
(1) Imaging path

(2) Illumination path



Imaging path: Three essential lenses to magnify object

- **An objective with focal length f_O**
- **A tube lens with focal length f_T (typically f_T = 160 mm).**
- **An eyepiece with focal length f_E.**

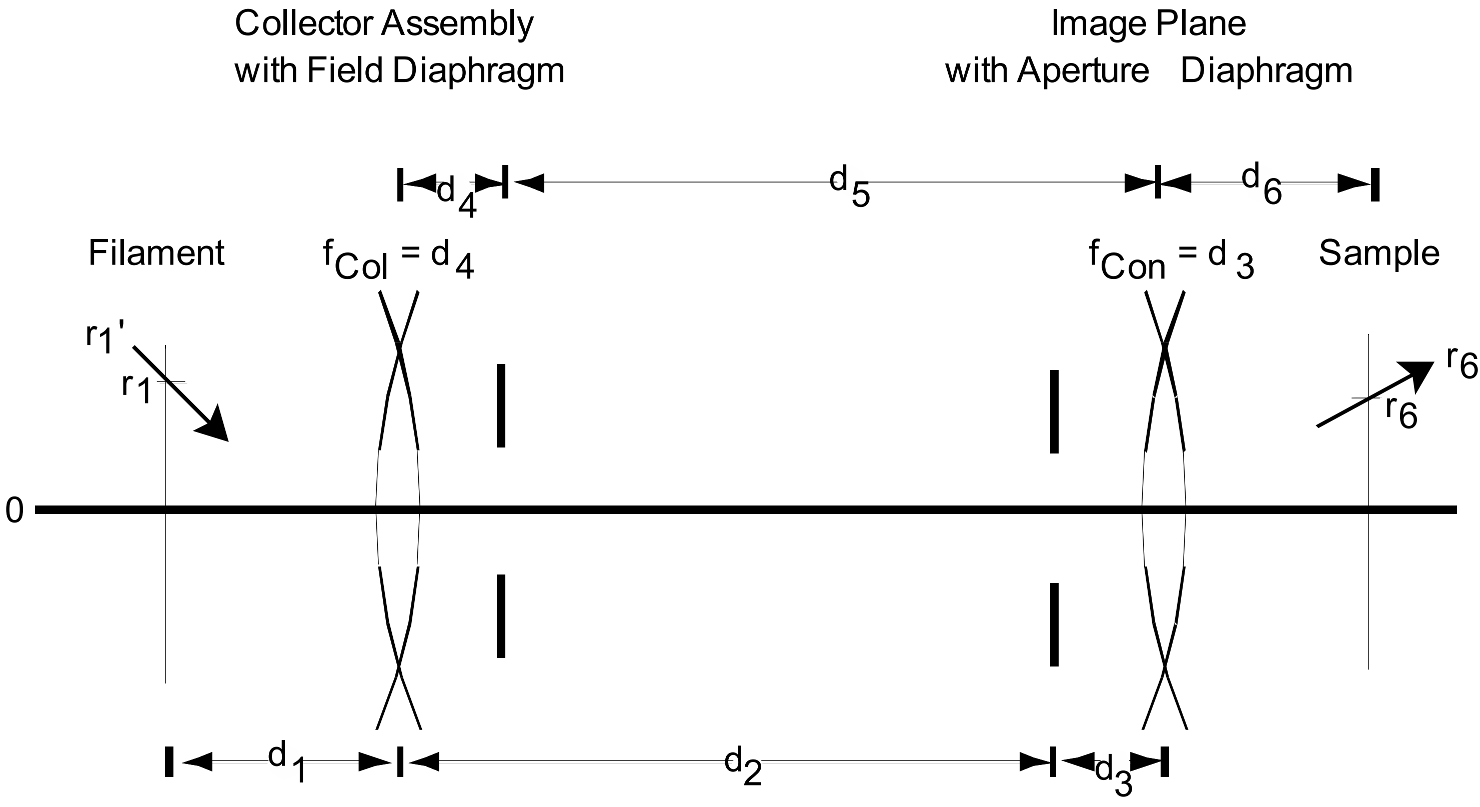


Magnification at intermediate plane, set by objective and tube lens, is $\frac{r_3}{r_1} = -\frac{f_T}{f_O}$

Magnification at eye, set by eyepiece, is $\frac{r_5}{r_3} = -\frac{d_5}{d_4}$

Total magnification of object is $\frac{r_5}{r_1} = \frac{f_T}{f_O}\frac{d_5}{d_4}$

Extended source illumination: Two essential lenses for illumination



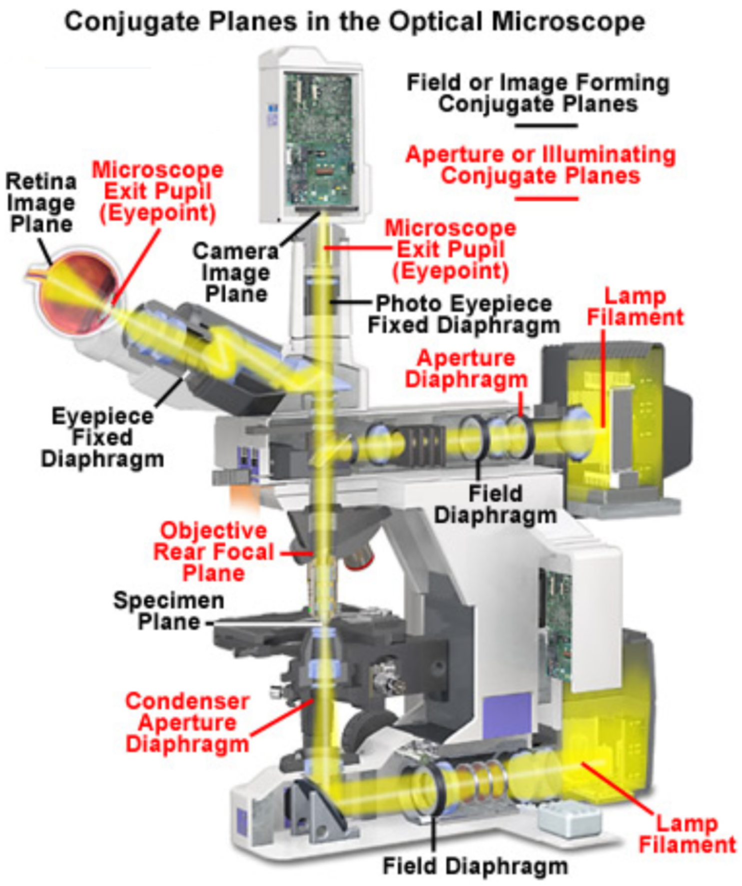
Range of angles at the sample depends on the height of the source, with slope

$$r_6' = \frac{r_1}{d_6 - f_{Con}} \frac{f_{Con}}{f_{Col}}$$

Infinity corrected microscopes: Two pathways in brighfield imaging

(1) Imaging path

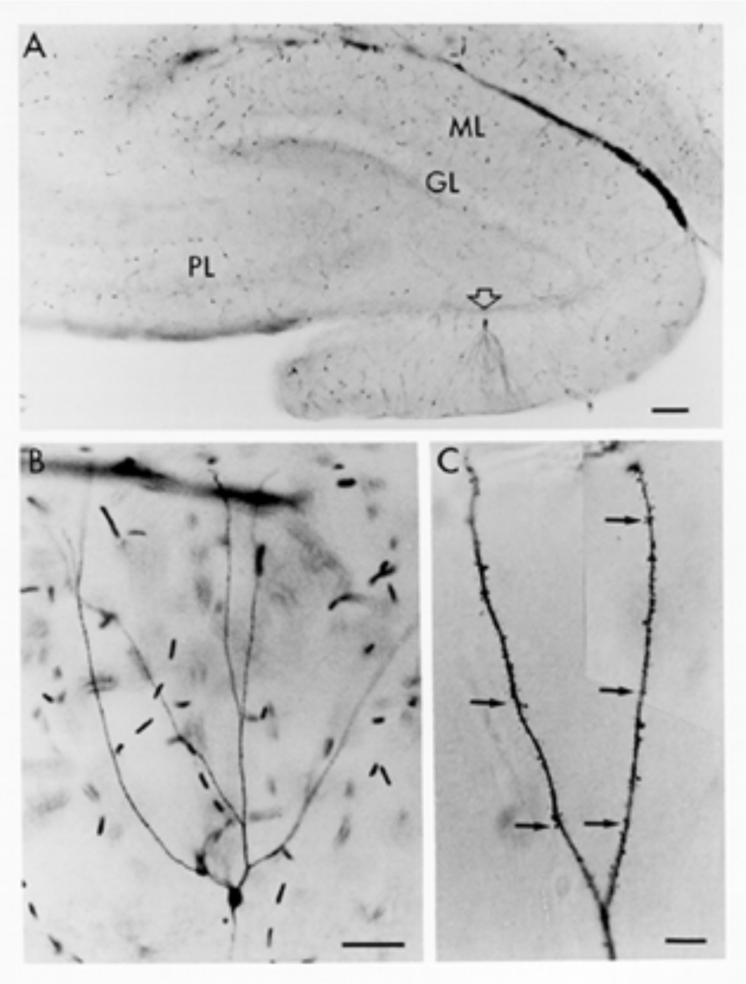
(2) Illumination path

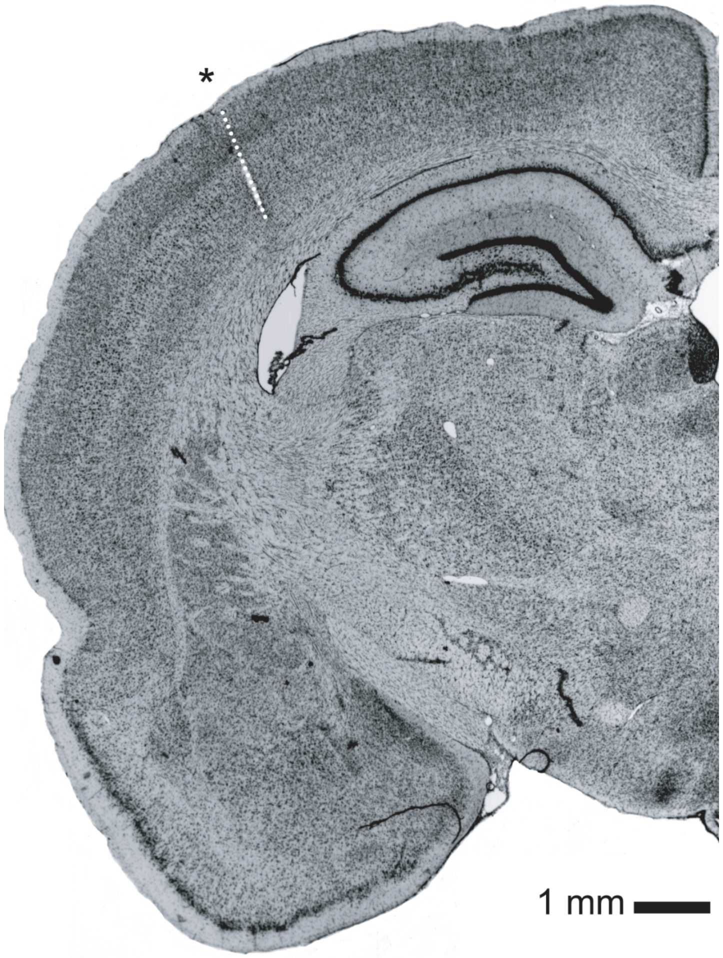


- Aperture diaphragm controls the angular spread of the light and thus depth-of-field.
- Field diaphragm controls the size of the illumination spot

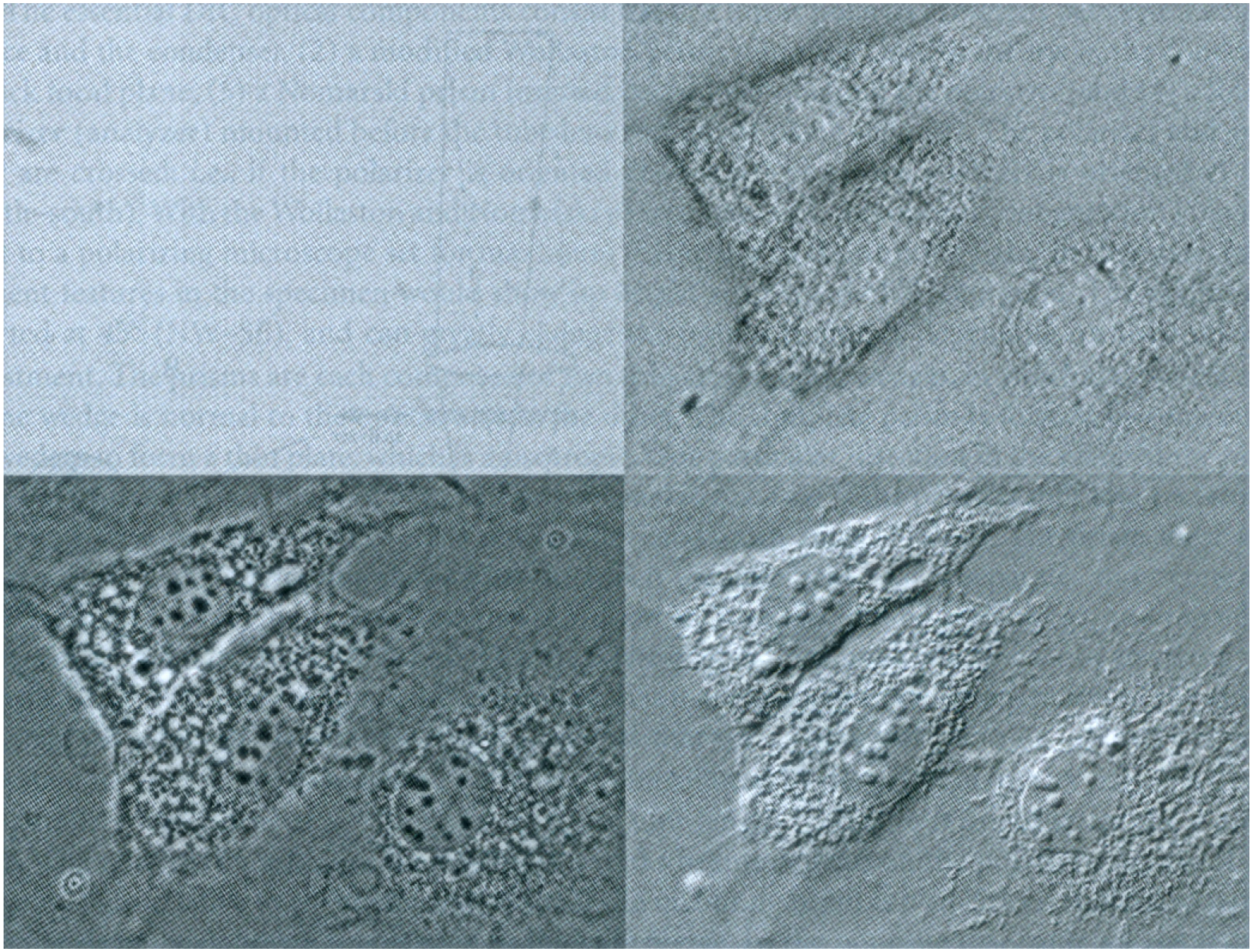
Modes of imaging

Transmission: Contrast based on absorption and scattering



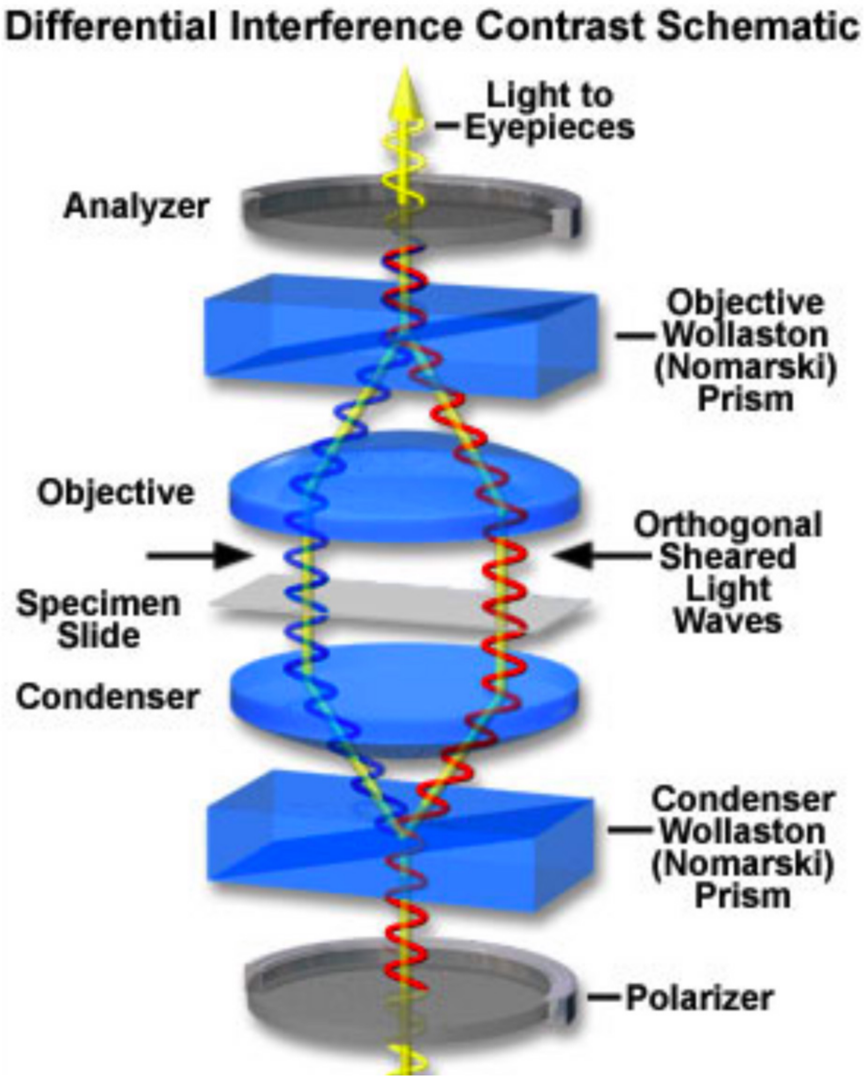


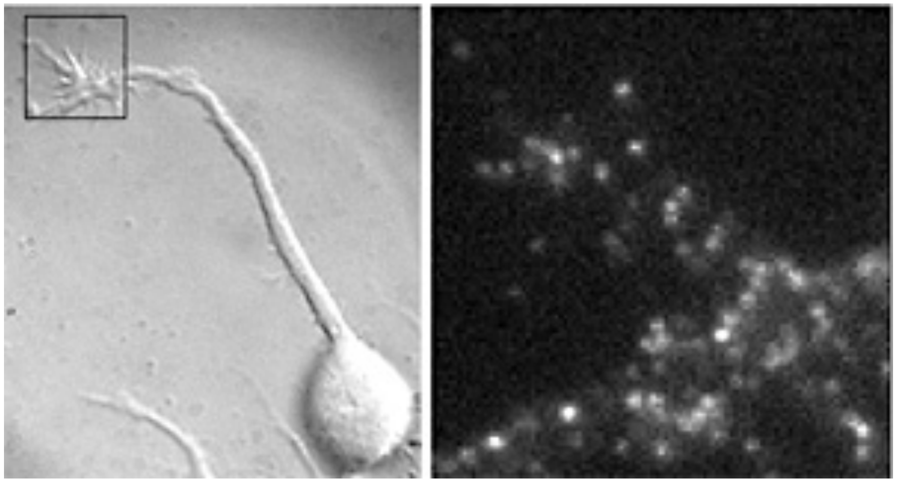
Transmission: Contrast based on refraction (change in optical path length)

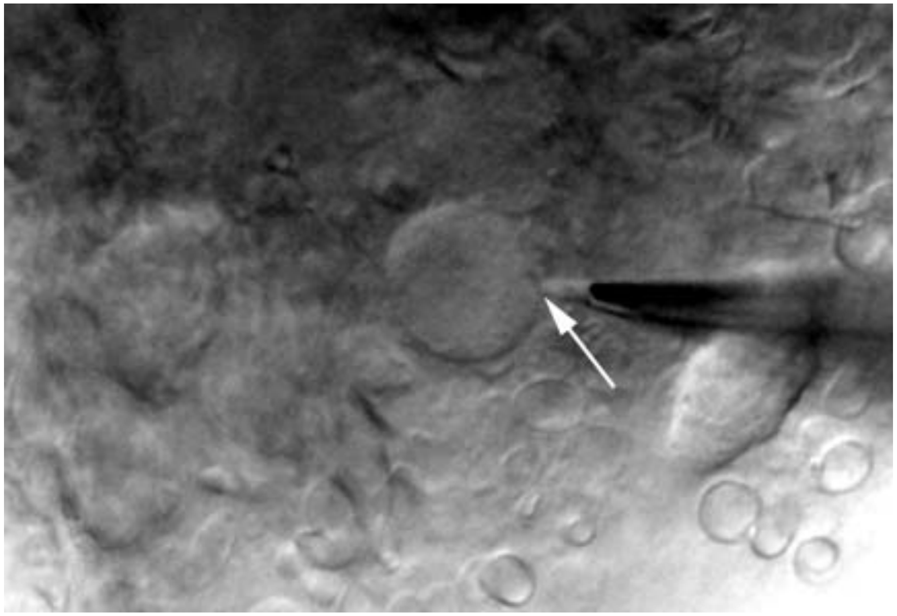


CW from 9 o’clock: bright field; enhanced bright field; differential interference contrast (DIC); phase

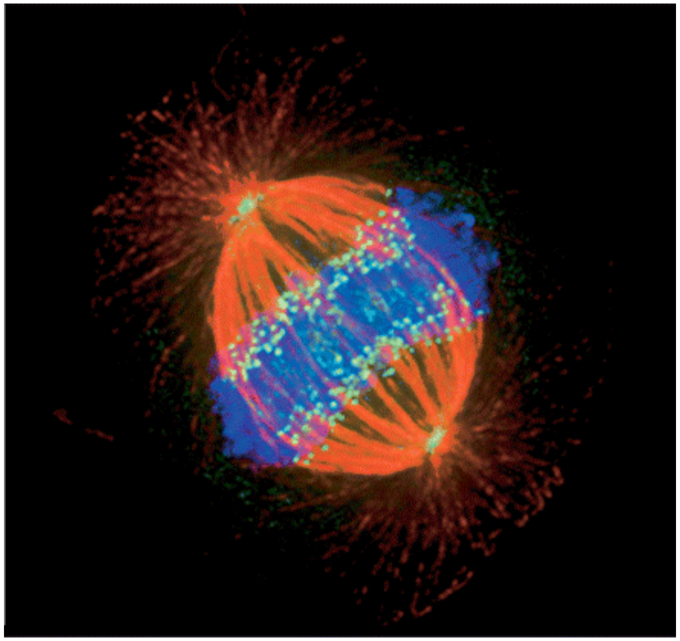
Example: DIC microscopy to image $\nabla_{\text{optical path}}$

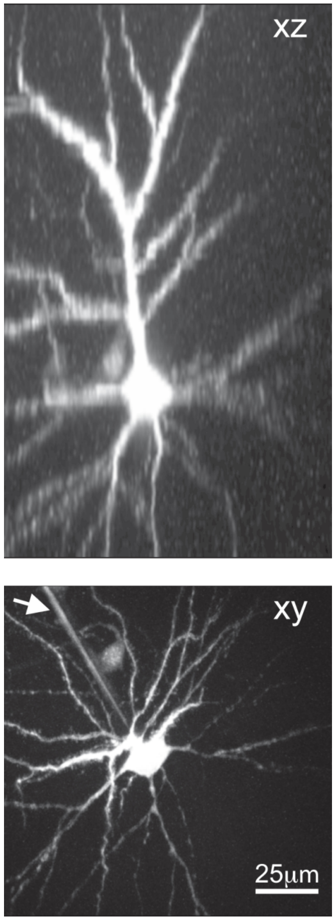


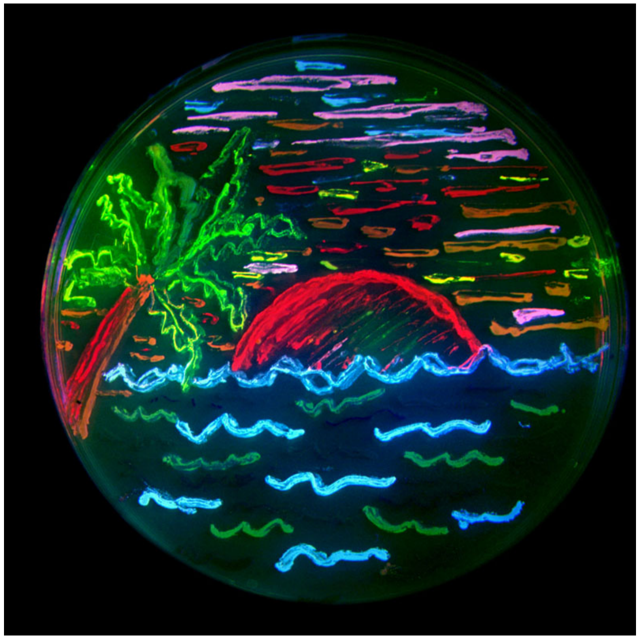




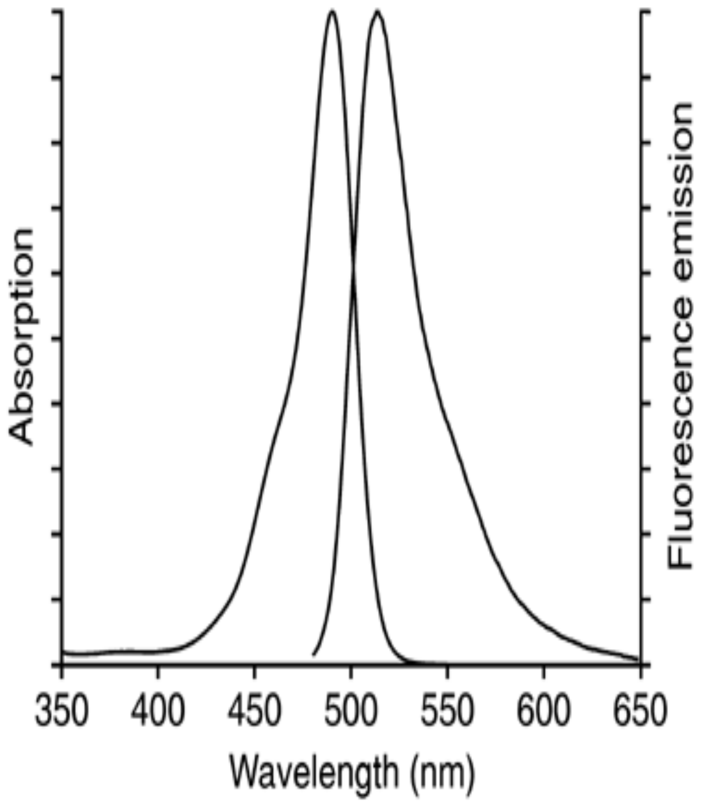
Fluorescence: Contrast based on absorption and incoherent emission

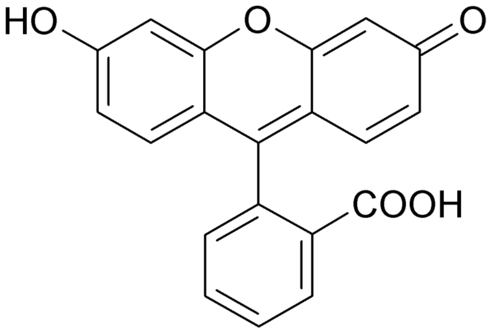






Fluorescein – Large-scale labeling in biology and the Windy City





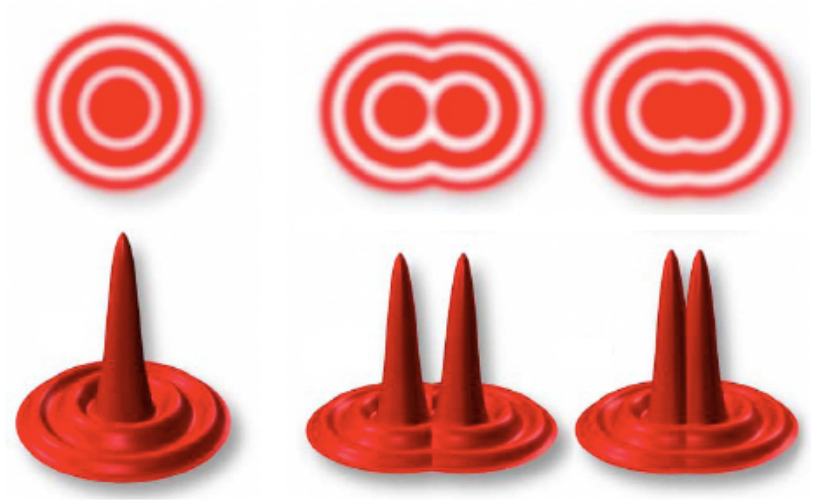
$\varepsilon = 9\text{x}10^4\ M^{-1}cm^{-1}$ at $\lambda = 500$ nm

Rule of thumb: $\varepsilon_{maximum} \sim$ 20,000 x (number of rings)

Example: $\varepsilon = 90{,}000\ M^{-1}cm^{-1}$ at $\lambda = 500$ nm for fluorescein

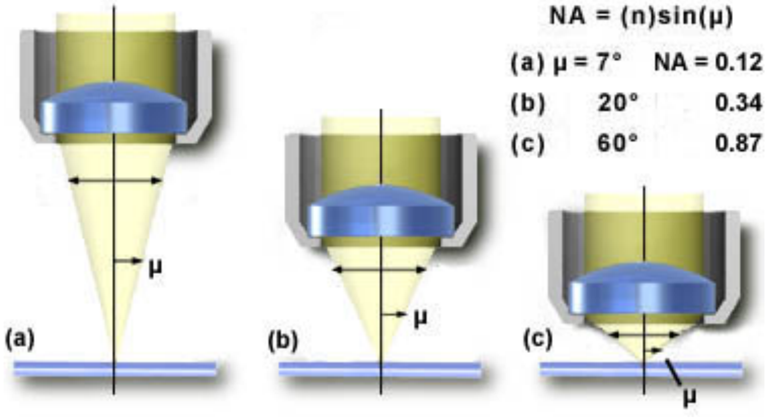
Spatial resolution: Set by the wavelength and geometry

What does a point object look like? Expect to visualize it as a dot of order one wavelength



First zero of pattern, J(r)/r, at r = λ x (0.6 / NA).

What is numerical aperture (NA)?



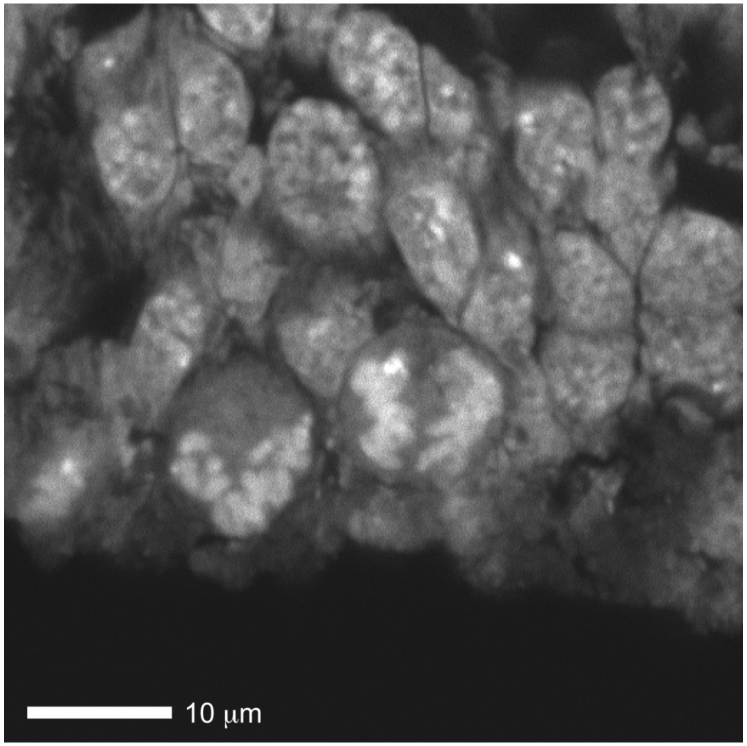
NA = (n)sin(μ)

(a)	μ = 7°	NA = 0.12
(b)	20°	0.34
(c)	60°	0.87

At the very best, NA = 1 and r = 0.3 µm for green light.

Better resolution for samples embedded in high index media.

Sampling theorem requires ≥ 2 samples per resolution unit, so we need to sample at 0.15 µm.



For the example CCD with 7200 pixels on edge, the maximum field is 7200 x 0.15 µm = 1.1 mm.

In practice, most lenses do not have such a large fields

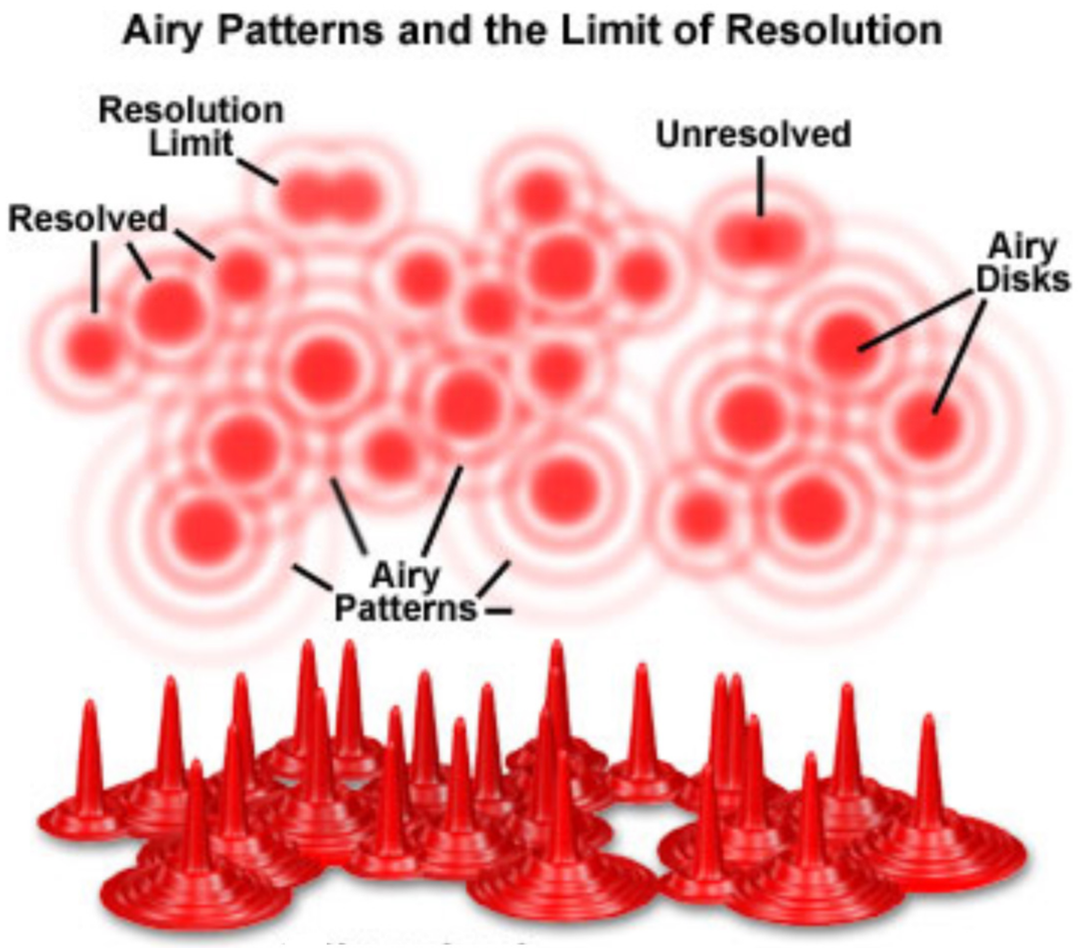
***e.g.*, Zeiss 40X 1.2 NA water objective has 500 µm field and r = 0.25 µm.**

This implies a CCD resolution of $(500/0.25/2)^2 = (4000)^2$ ~ 16 Mpixels; quite reasonable.

Detection versus discrimination

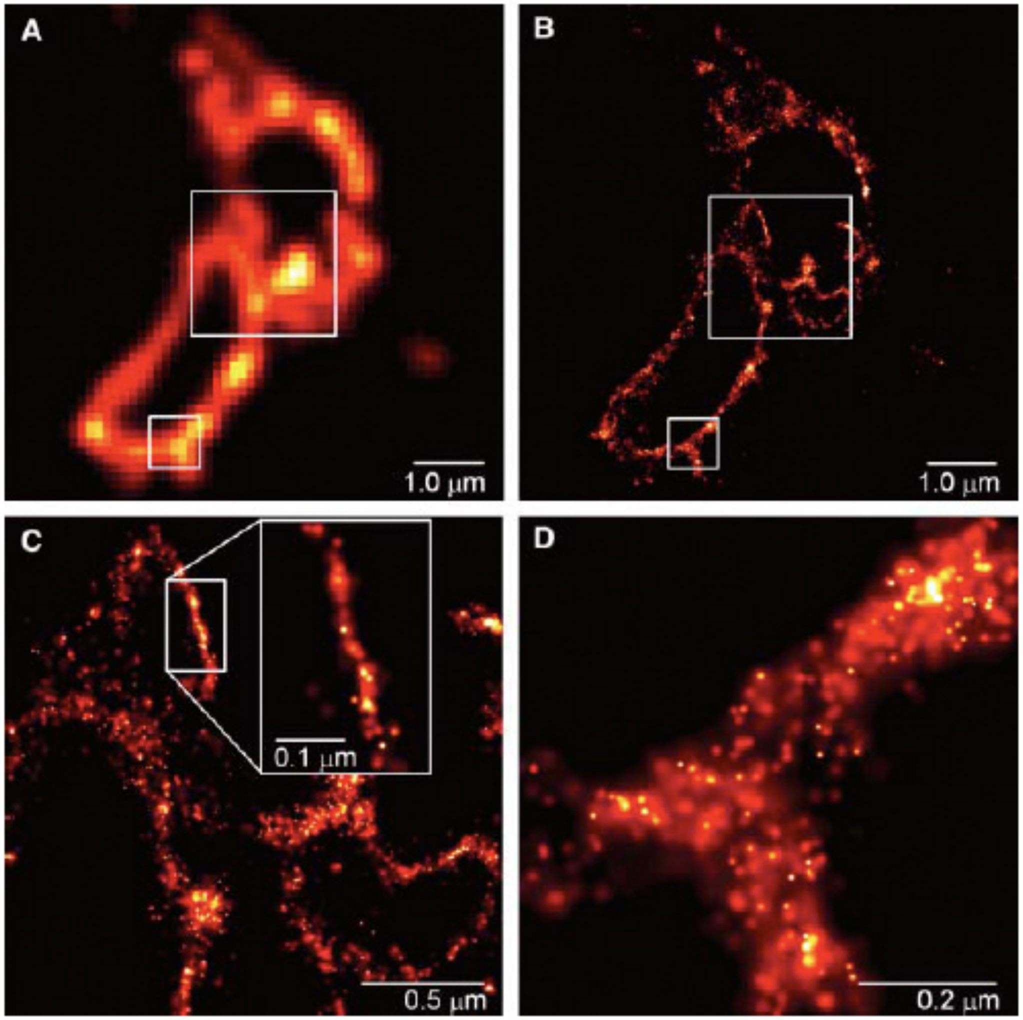
Detection has arbitrary accuracy.

Resolution implies a minimum distance between identical objects



PALM

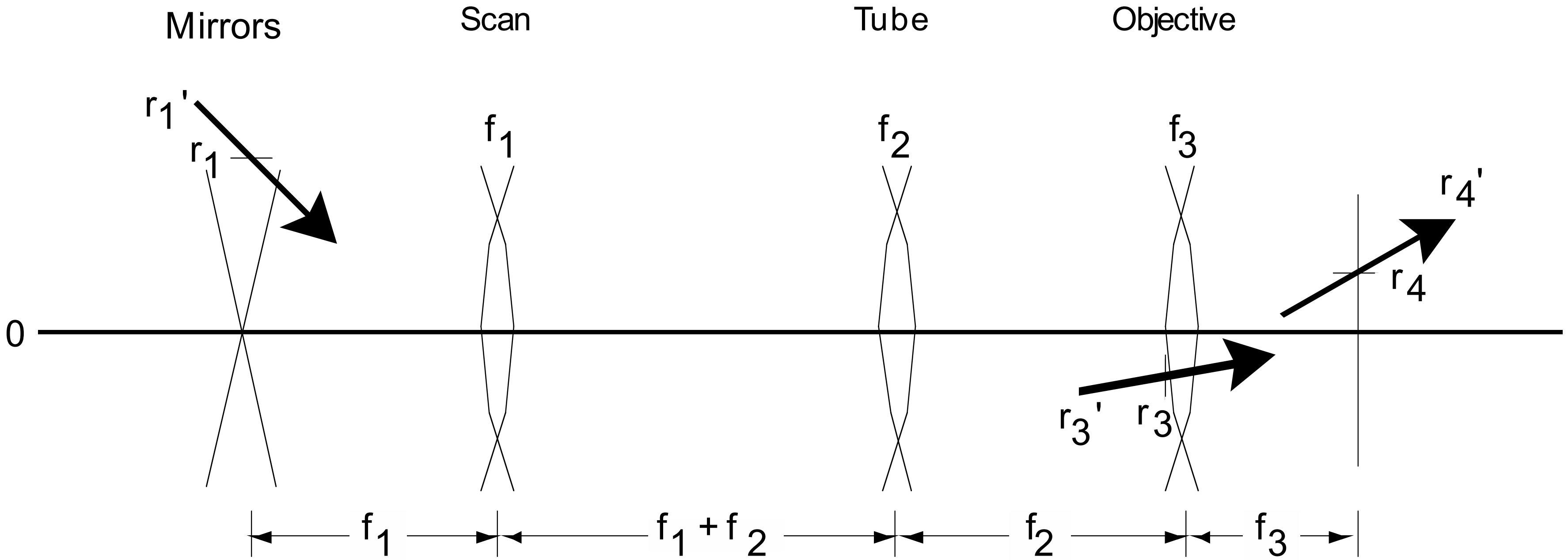
Imaging of overlapping, identical fluorophores that switched on at low density



Betzig, Patterson, Sougrat, Lindwasser, Olenych, Bonifacino, Davidson, Lippincott-Schwartz & Hess (Science 2006)
Rust, Bates & Zhuang (Nature Methods 2006)

Scanning microscopy for optical sectioning

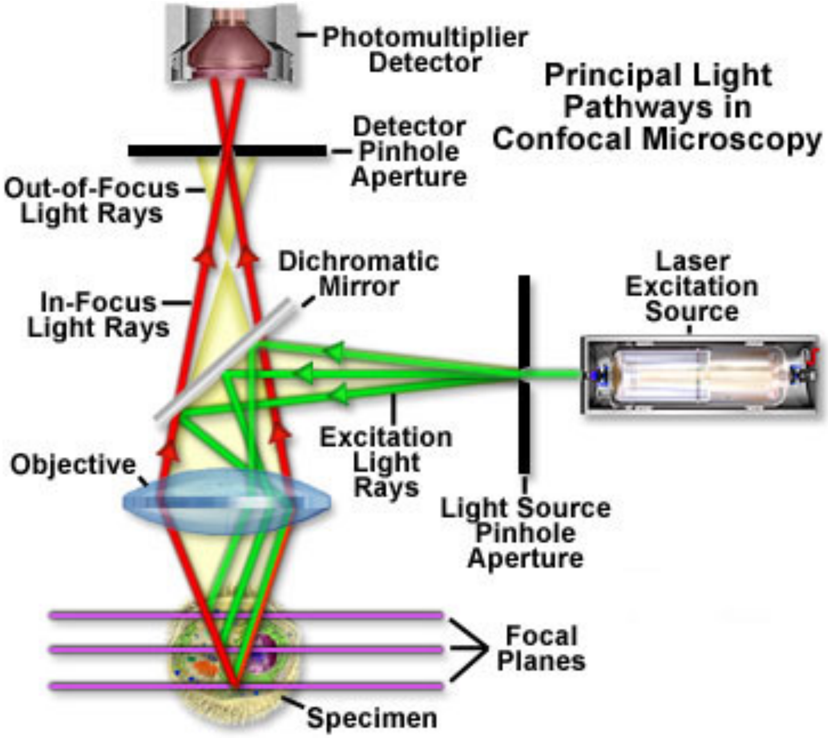
Pixel-by-pixel excitation for confocal, multiphoton, harmonic, STED, … , microscopy

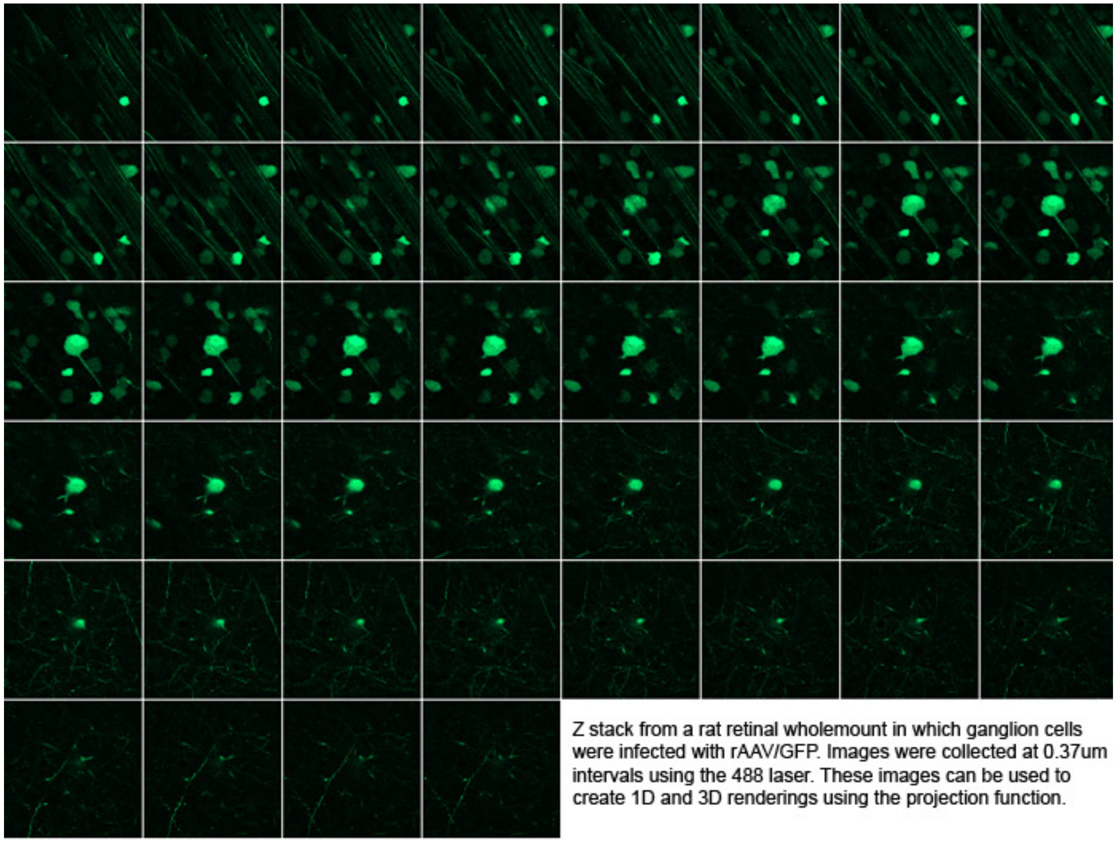


Change in slope of the mirror, r_1', is turned into a change in position in the focal plane, r_4,

with $r_4 = -f_3 \frac{f_1}{f_2} r_1'$

Confocal scanning microscopy (Minsky, Amos, …., *ça* 1960)

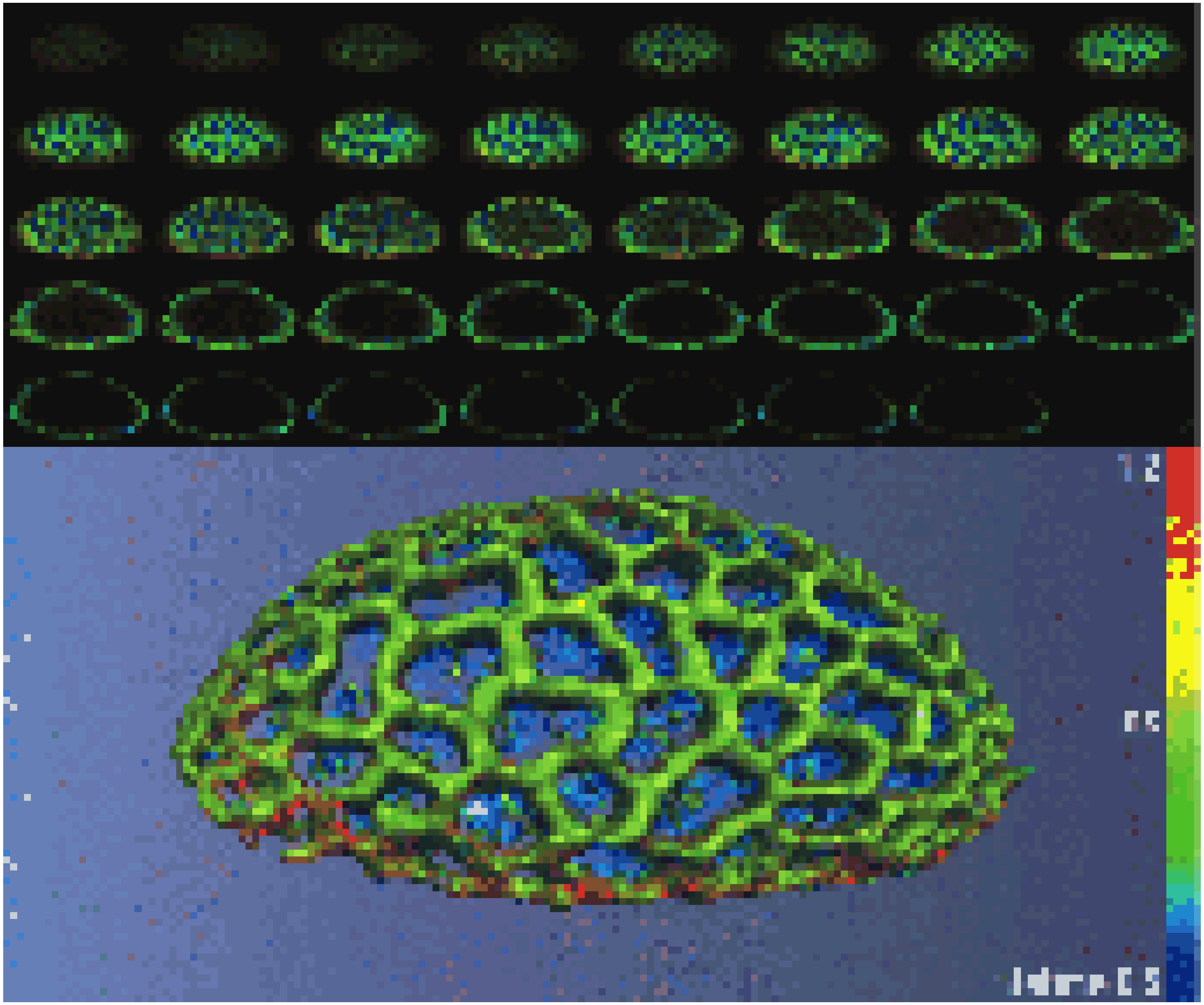




Z stack from a rat retinal wholemount in which ganglion cells were infected with rAAV/GFP. Images were collected at 0.37um intervals using the 488 laser. These images can be used to create 1D and 3D renderings using the projection function.

Each image has a depth of field that is given by $\Delta z \sim 2\,\lambda\,/\,(NA)^2$

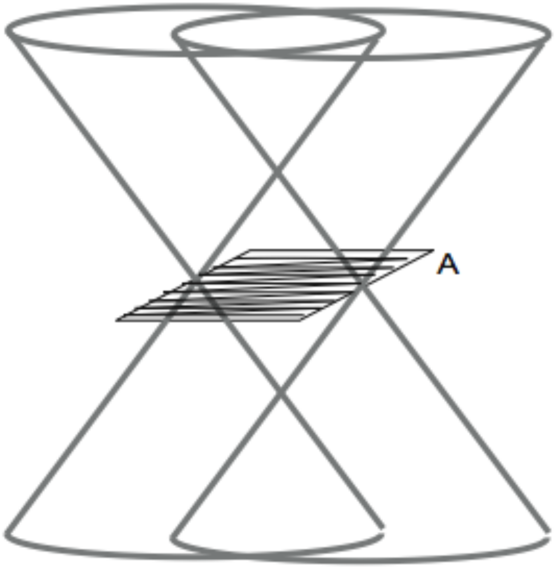
Images must be reassembled to form a 3-D view

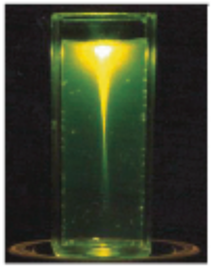
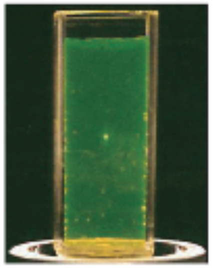

1.2
0.5

Two-photon laser scanning microscopy (Denk *et al*. Science 1990)

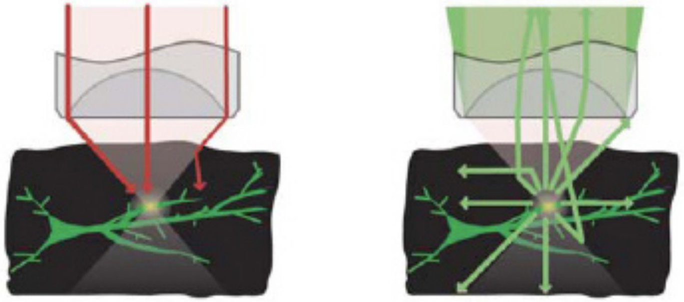
Integrated Absorption Probability

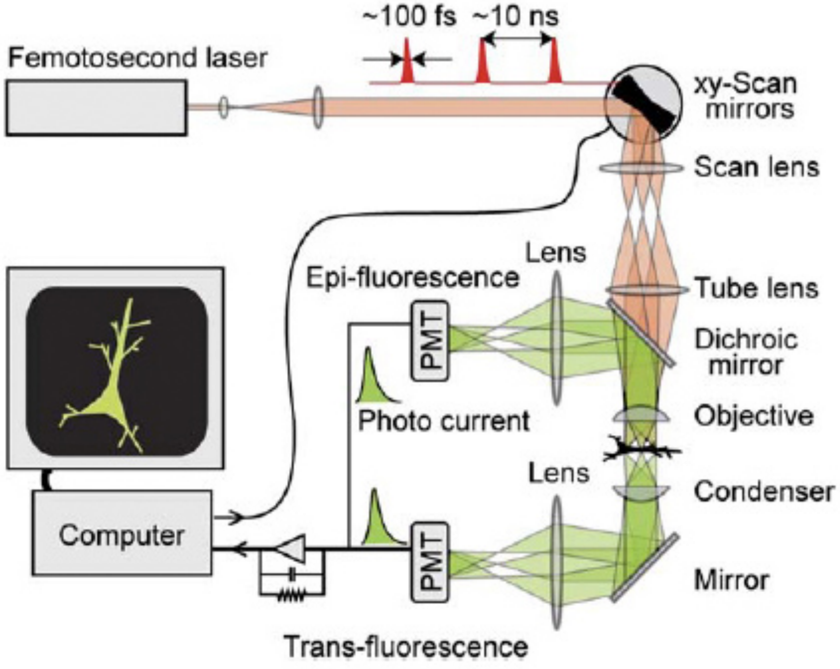
Area A is scanned in a time t with power P

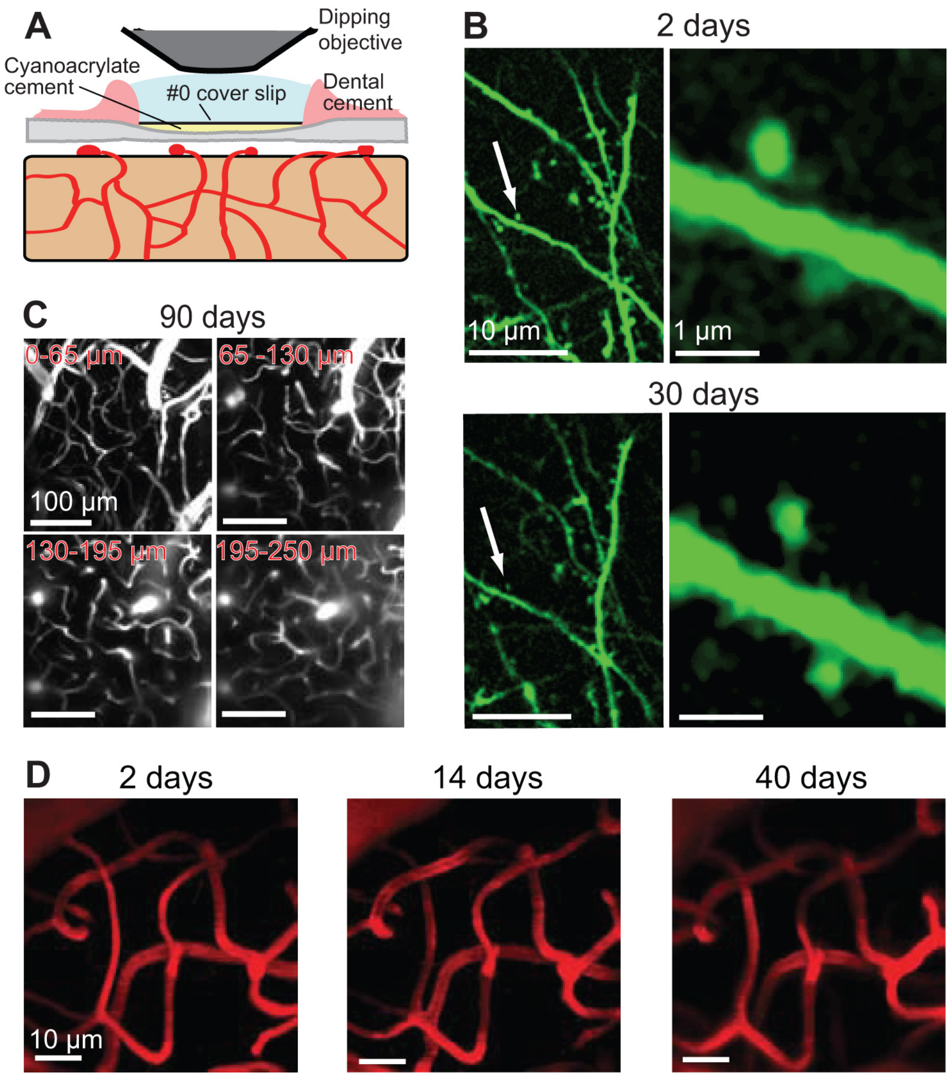


	One photon ($N_{absorption} \sim P\ t$)	Two photon ($N_{absorption} \sim P^2\ t$)
Out of focus	$\frac{P_o}{A} A = P_o$	$\left(\frac{P_o}{A}\right)^2 A = \frac{P_o^2}{A}$
In focus	P_o	P_o^2
In focus Out of focus	~ Constant 	$\frac{1}{A}$ 

In vitro and *in vivo* two-photon microscopy for structural studies

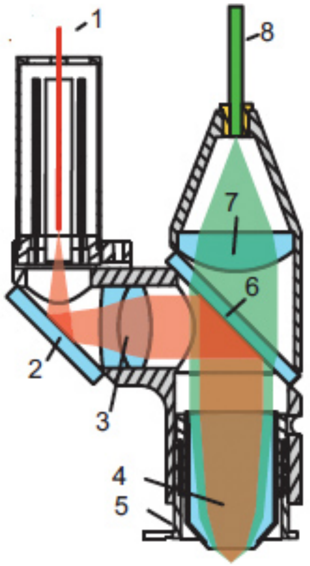


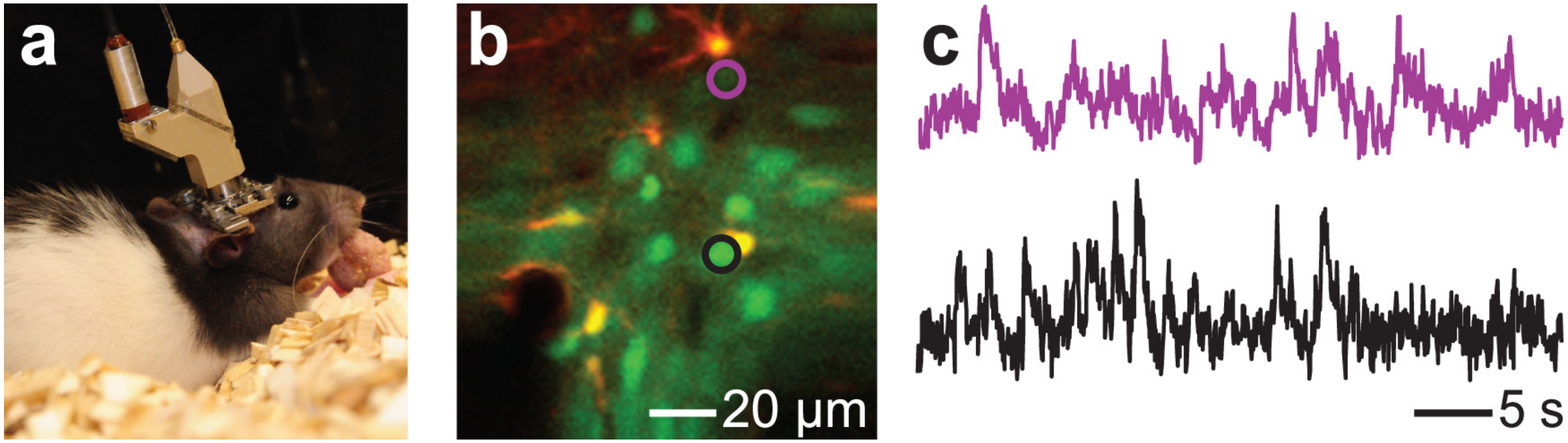




Currently, one typically images 500 µm deep and can image to 1000 µm with special equipment.

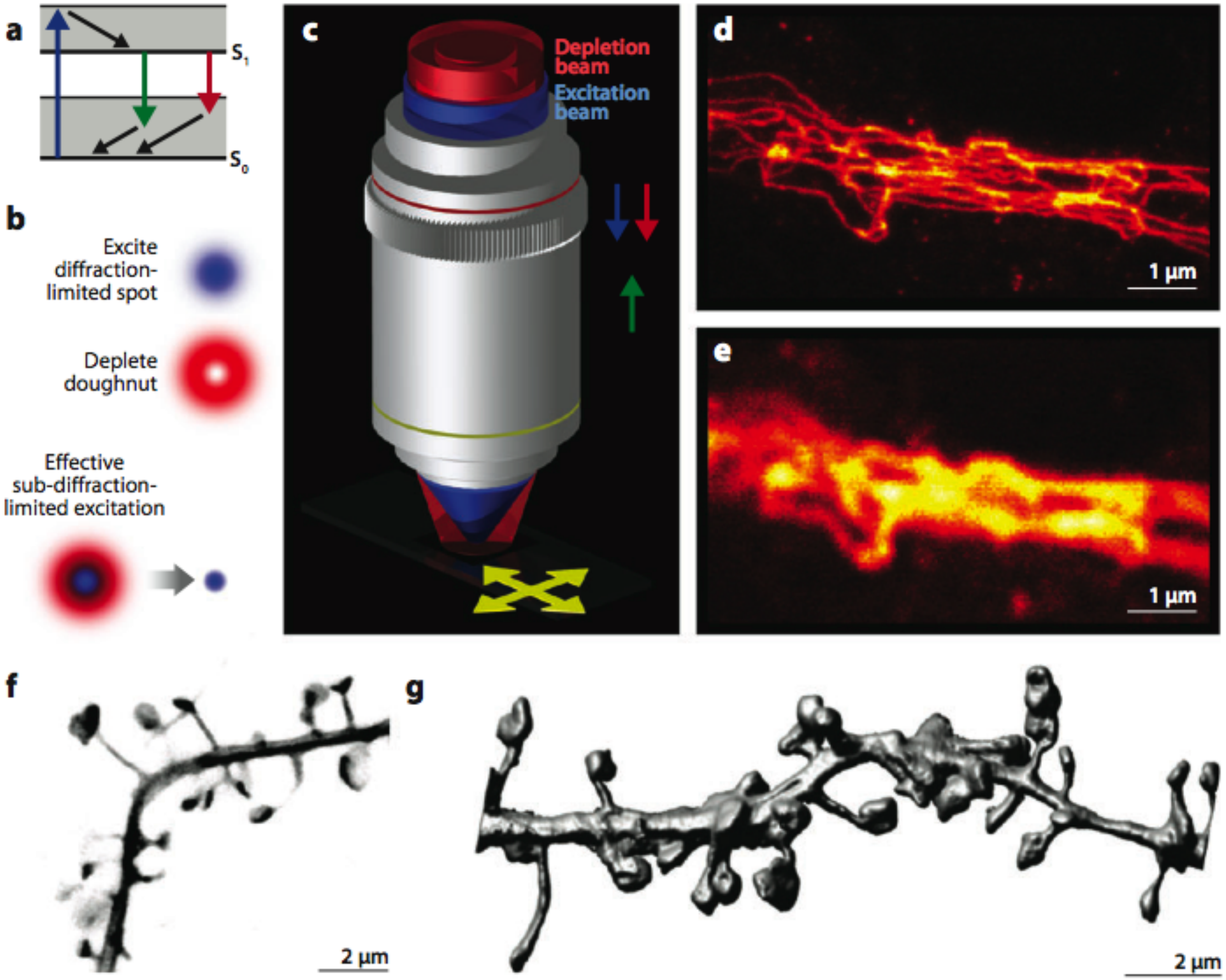
*T*wo-photon microscopy with free-ranging animals for cell function





Sawinski, Wallace, Greenberg, Grossmann, Denk & Kerr (2009 PNAS)

Super-resolution scanning microscopy through stimulated emission: STED (Hell & coworkers 1994)



The goal is science, not (only) pretty pictures: Vectorization of data