Modern Optical Microscopy

Chau-Hwang Lee（李超煌）

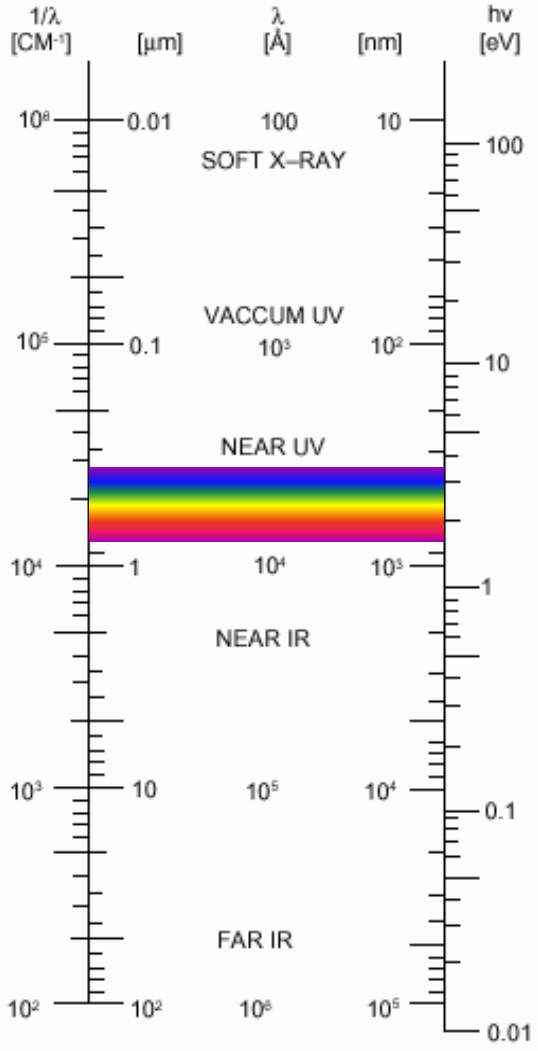
Associate Research Fellow

Research Center for Applied Sciences, Academia Sinica
128 Sec. 2, Academia Rd., Nankang, Taipei 11529, Taiwan

E-mail: clee@gate.sinica.edu.tw

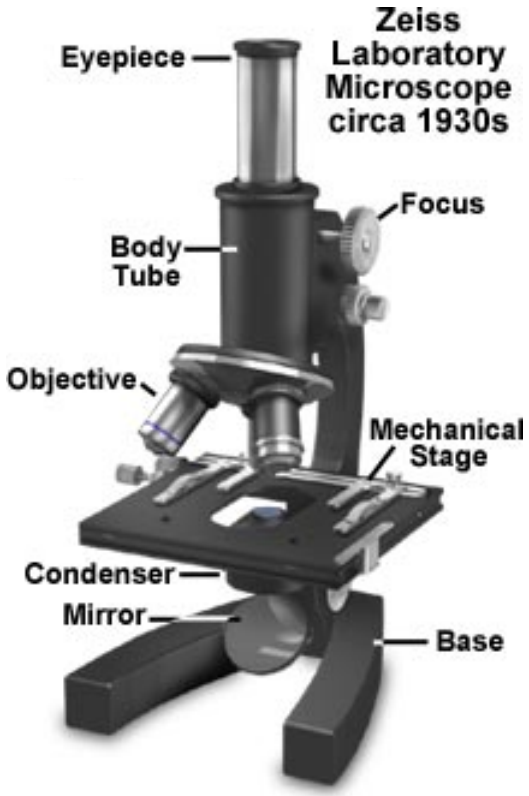
Principles

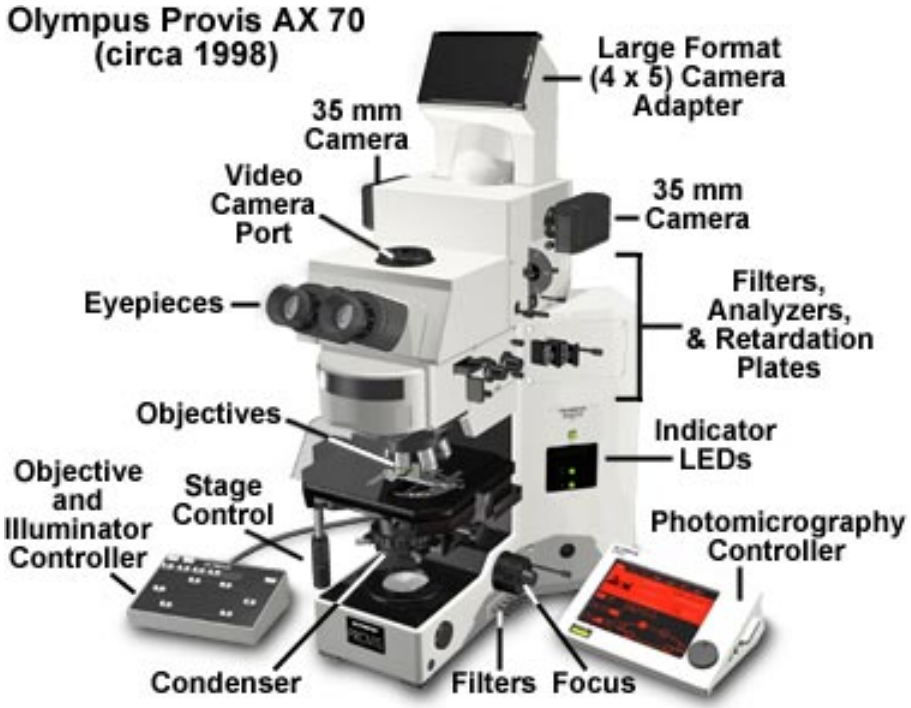
Optical spectrum



25–200 nm	vacuum ultraviolet	VUV
200–400 nm	ultraviolet	UV
400–700 nm	visible	VIS
700–1000 nm	near-infrared	NIR
1–3 μm	short-wavelength infrared	SWIR
3–5 μm	medium-wavelength infrared	MWIR
5–14 μm	long-wavelength infrared	LWIR
14–30 μm	very long wavelength infrared	VLWIR
30–100 μm	far-infrared	FIR
100–1000 μm	submillimeter	SubMM

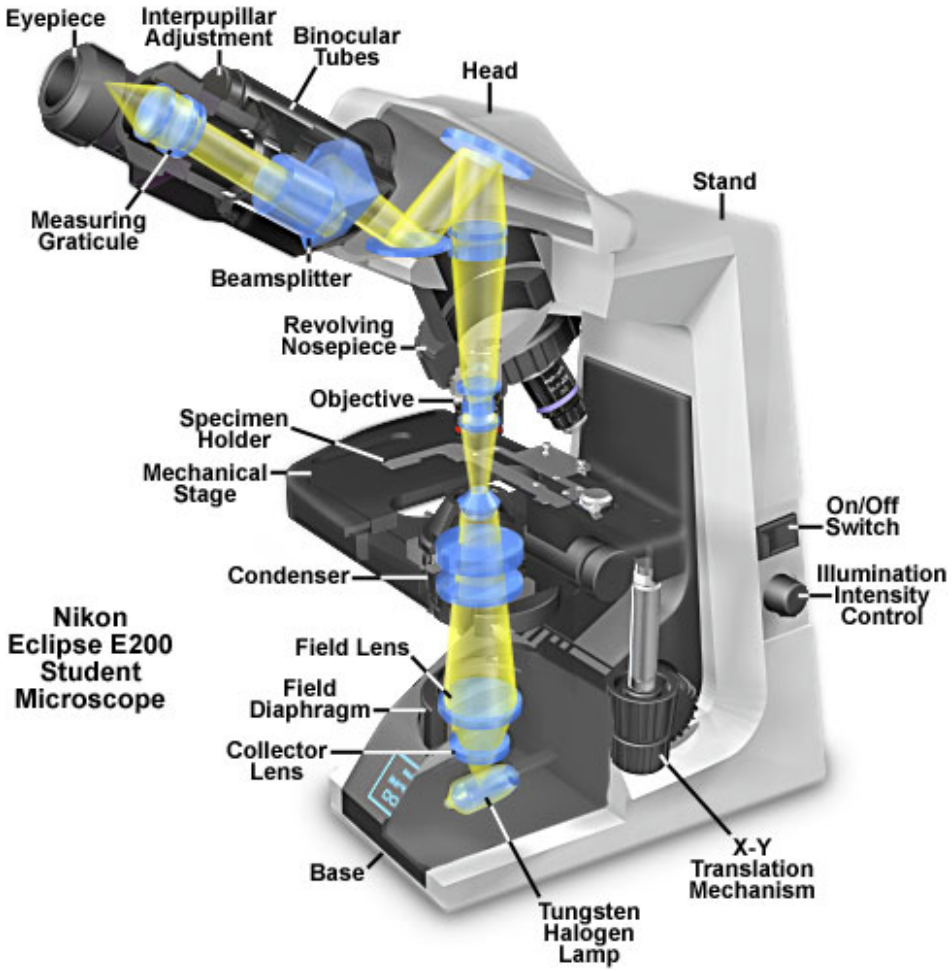
Optical microscope





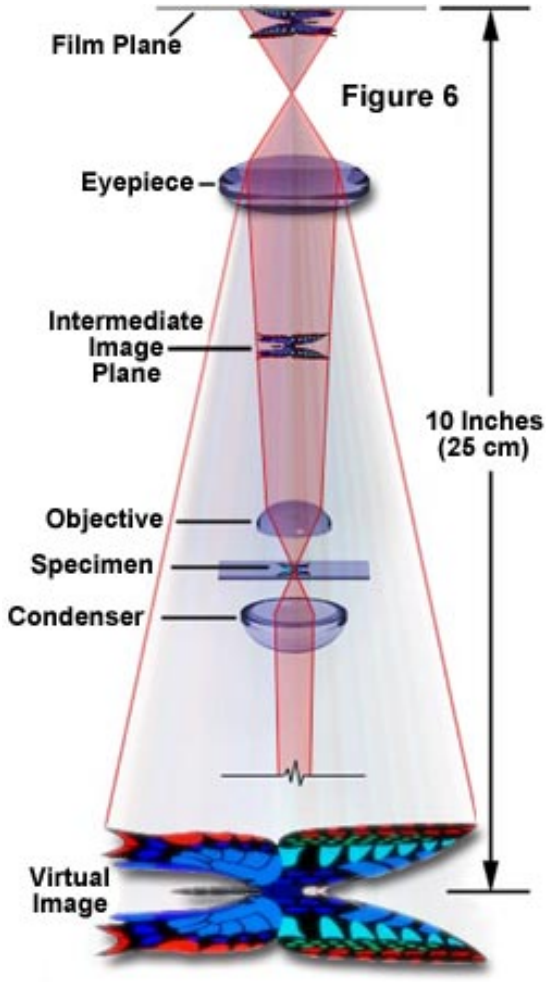
Images are from http://micro.magnet.fsu.edu/

Light path in an optical microscope



Images are from http://micro.magnet.fsu.edu/

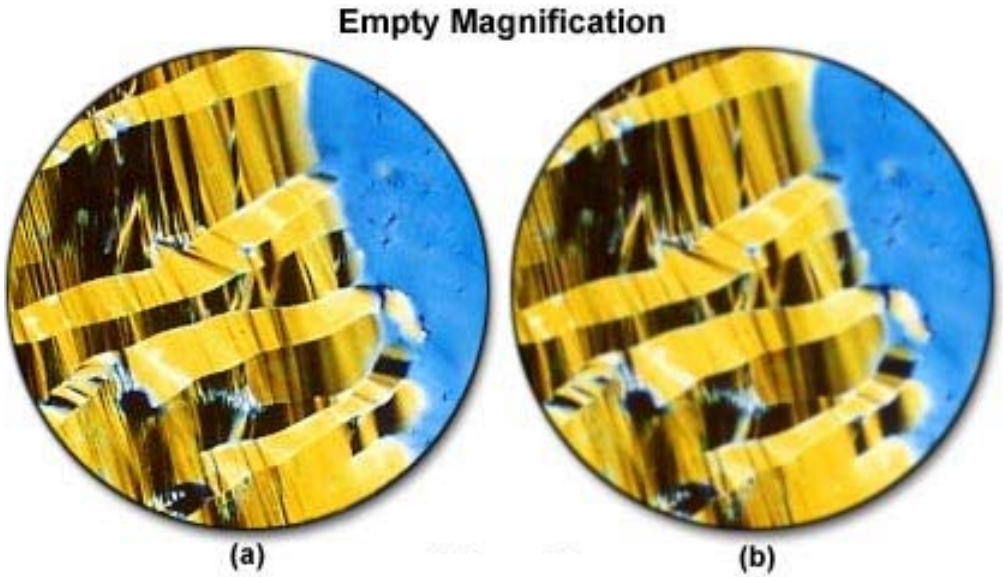
Magnification of an optical microscope



Objective	Eyepieces				
(NA)	10x	12.5x	15x	20x	25x
2.5X (0.08)	---	---	---	x	x
4X (0.12)	---	---	x	x	x
10X (0.35)	x	x	x	x	x
25X (0.55)	x	x	x	x	---
40X (0.65)	x	x	x	---	---
60X (0.85)	x	x	x	---	---
100X 1.4	x	x	---	---	---
x = good combination					

Images are from http://micro.magnet.fsu.edu/

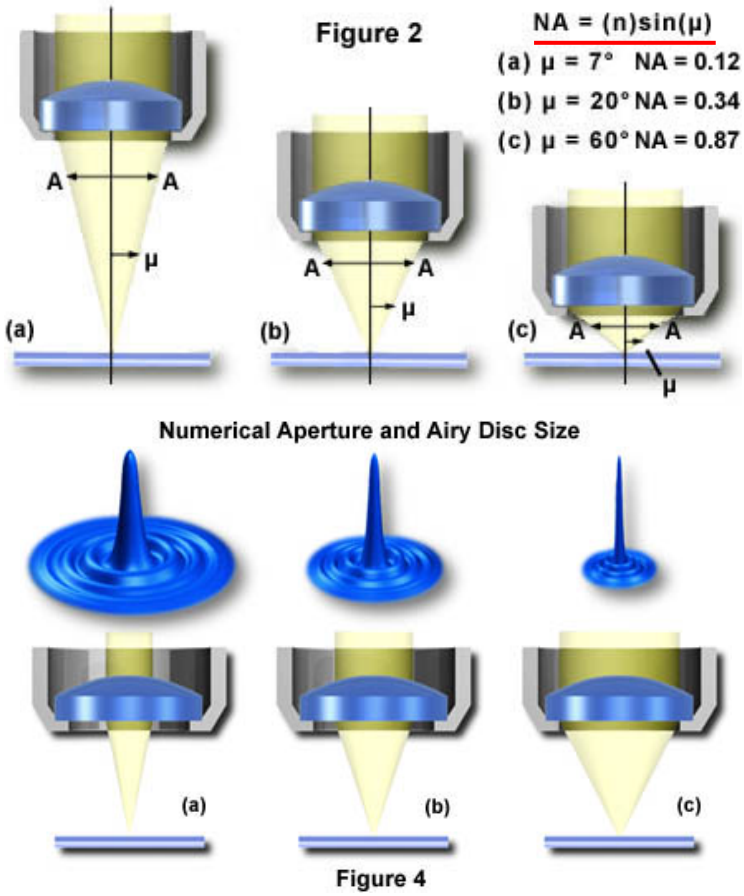
Resolution



Without resolution, magnified images cannot provide detailed information.

Images are from http://micro.magnet.fsu.edu/

Numerical aperture



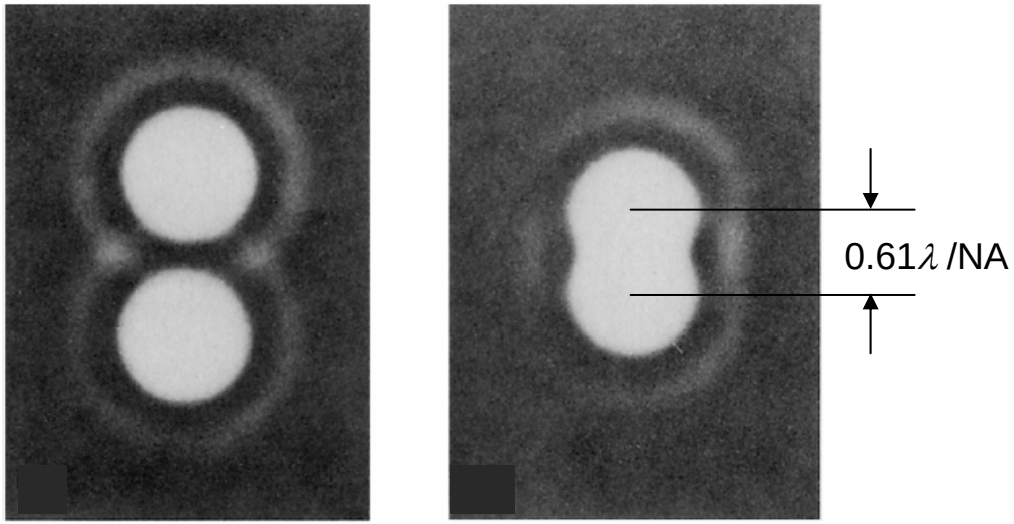
Images are from http://micro.magnet.fsu.edu/

Numerical aperture and resolution

Rayleigh criterion:

resolution ~ 0.61λ /NA

For dry samples, NA < 1.0

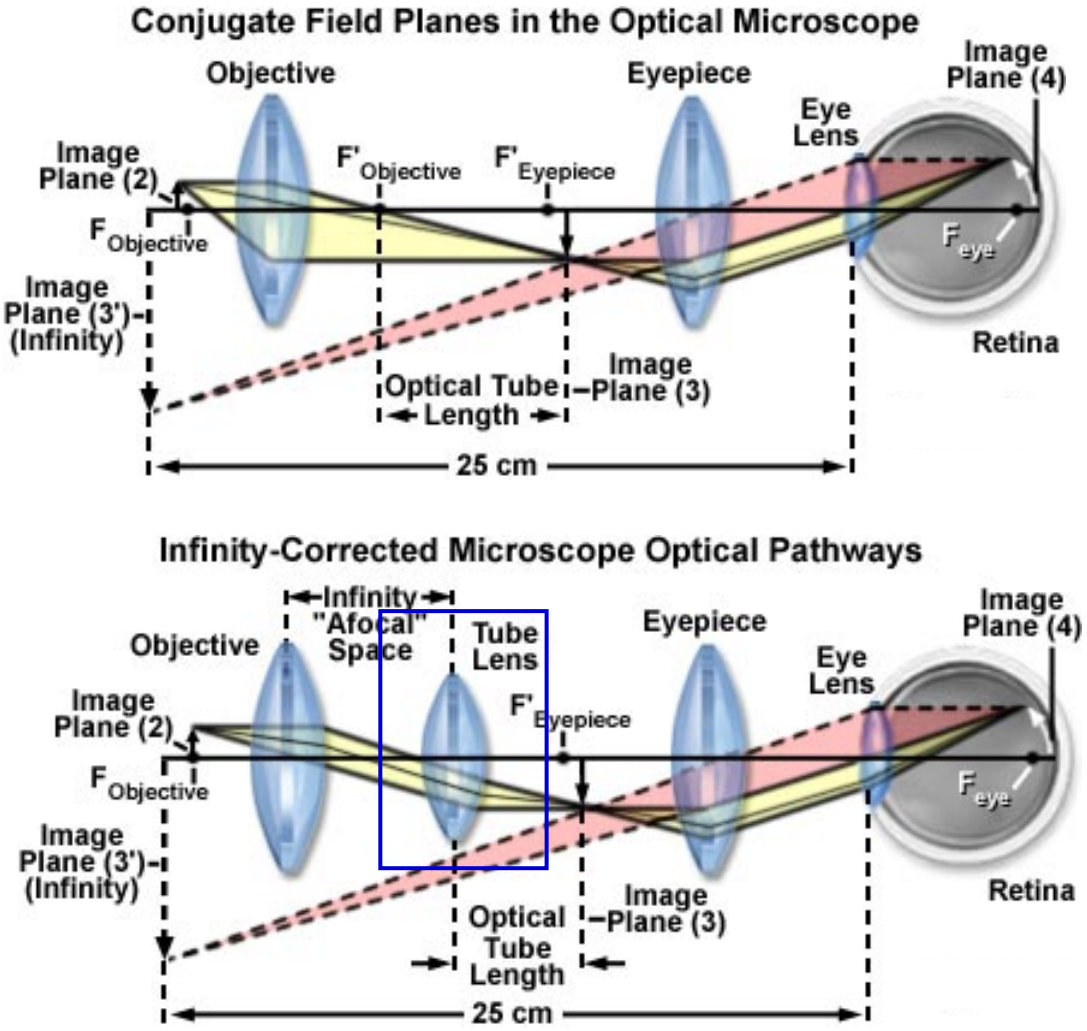


clearly resolved

resolution limit

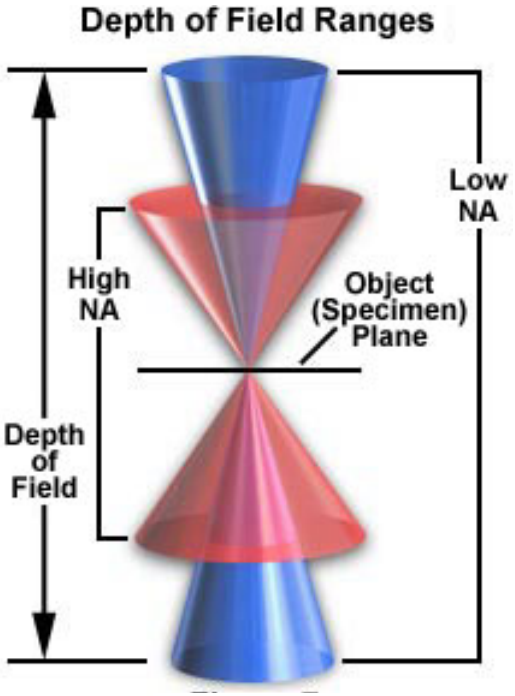
Ref: M. Born and E.Wolf, *Principles of Optics*, 6th ed. (Pergamon, Oxford, 1980), Chap. 8.

Image formation



Images are from http://micro.magnet.fsu.edu/

Depth of field



$$d = \lambda n/(\mathrm{NA})^2$$

Images are from http://micro.magnet.fsu.edu/

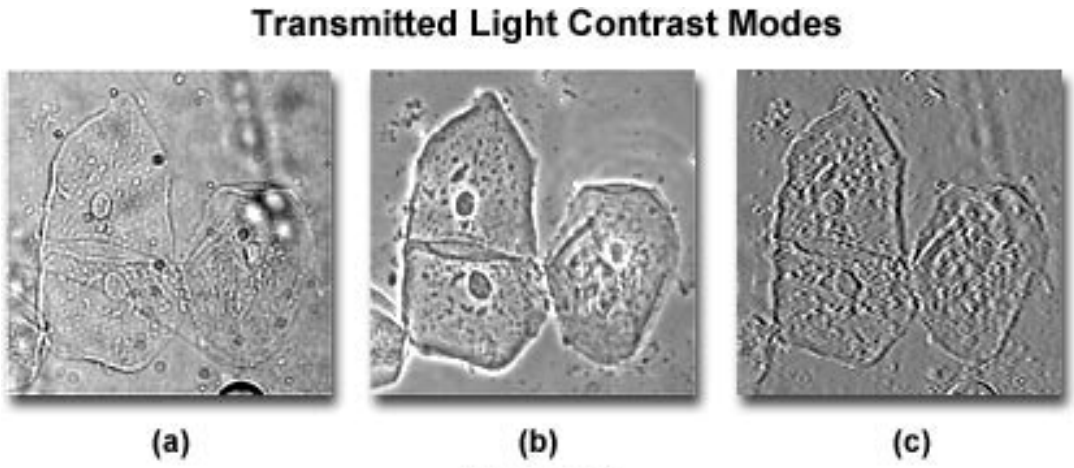
Specifications of an objective

Figure 1

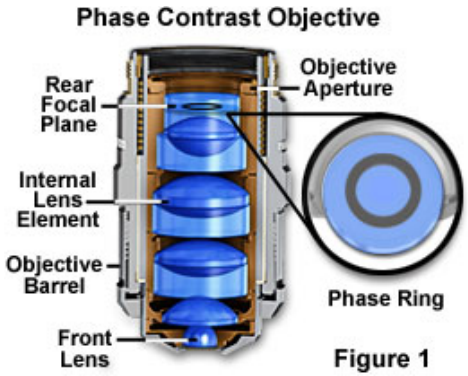
Images are from http://micro.magnet.fsu.edu/

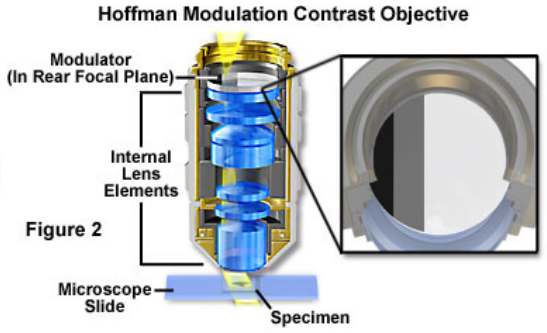
Contrast

Types of contrast

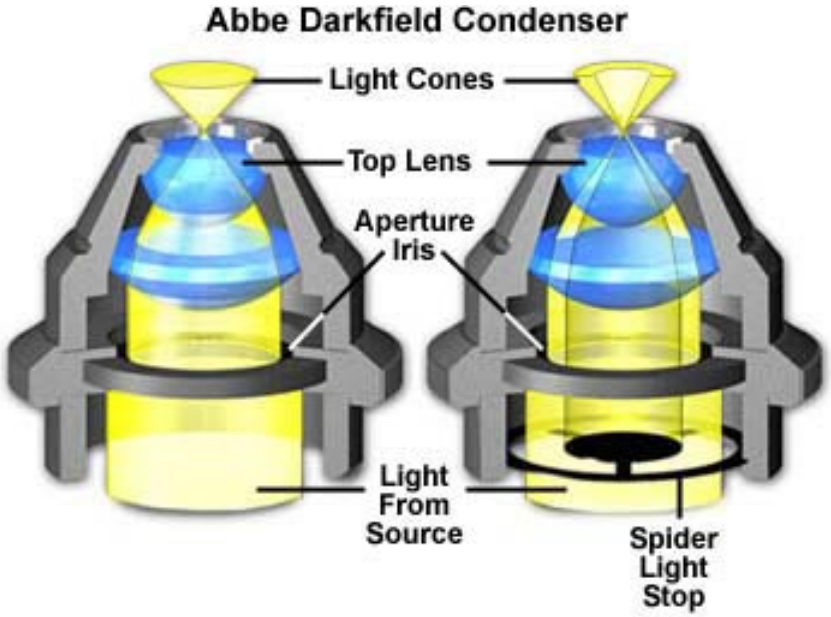


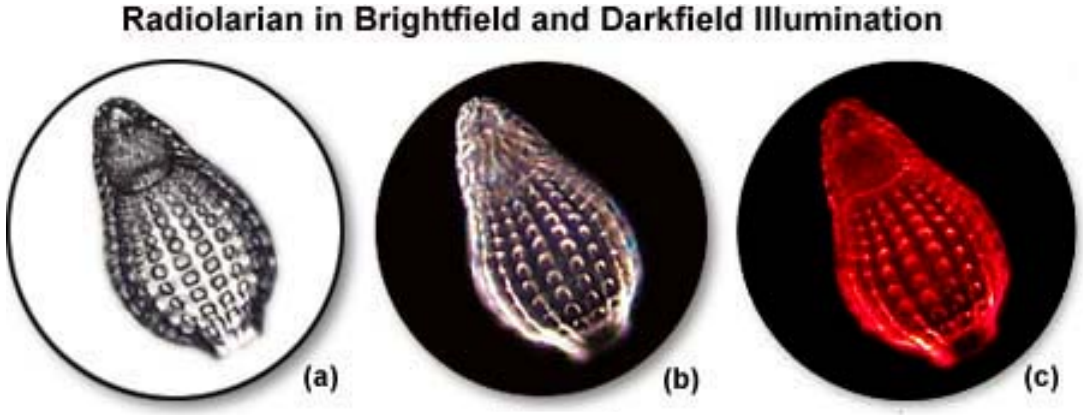
bright field phase contrast Hoffman modulation contrast



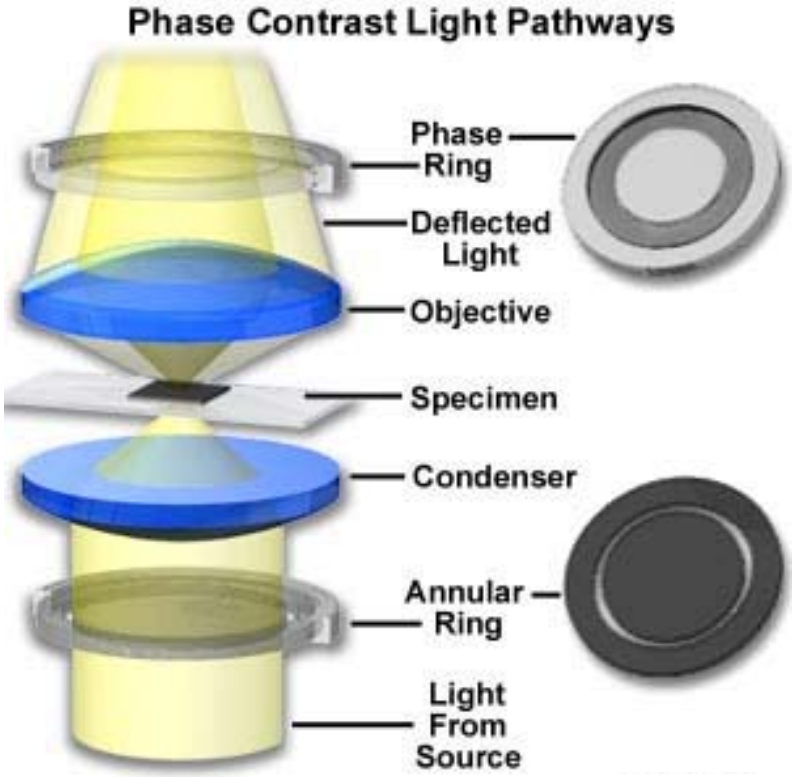


Darkfield illumination





Phase contrast



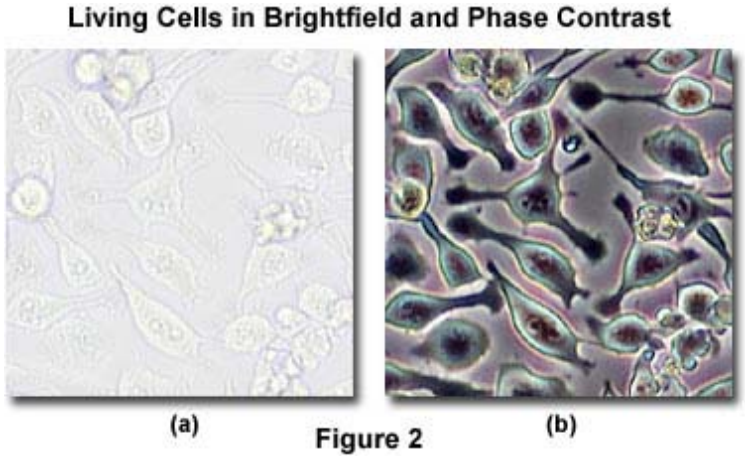
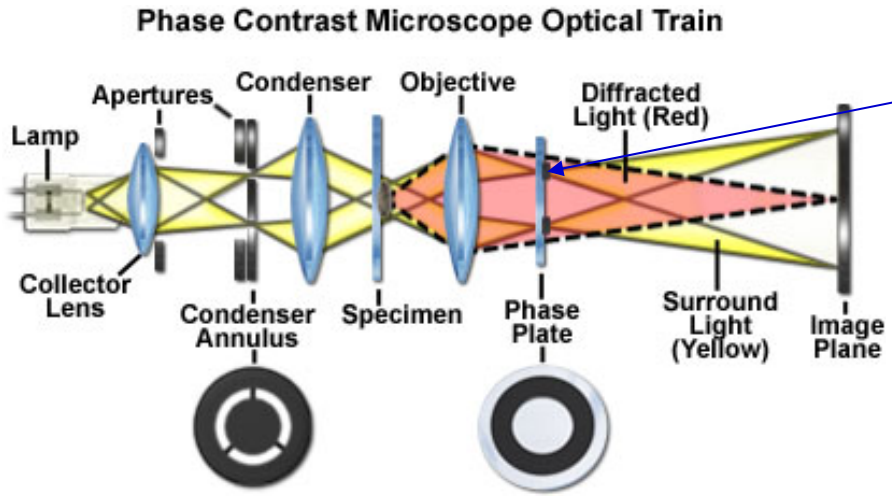


Figure 2

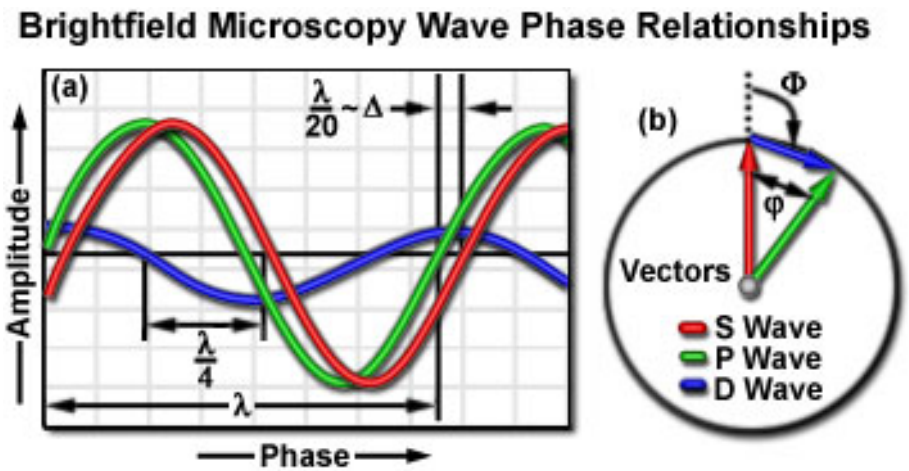
The contrast is from optical path (phase) difference.

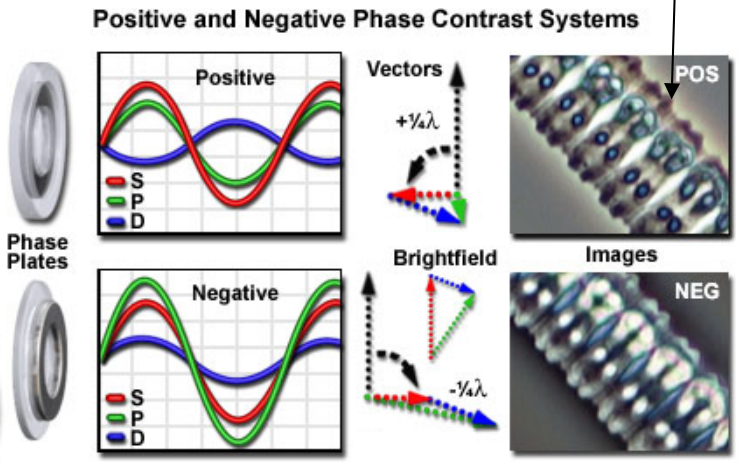
Images are from http://micro.magnet.fsu.edu/

Phase contrast images



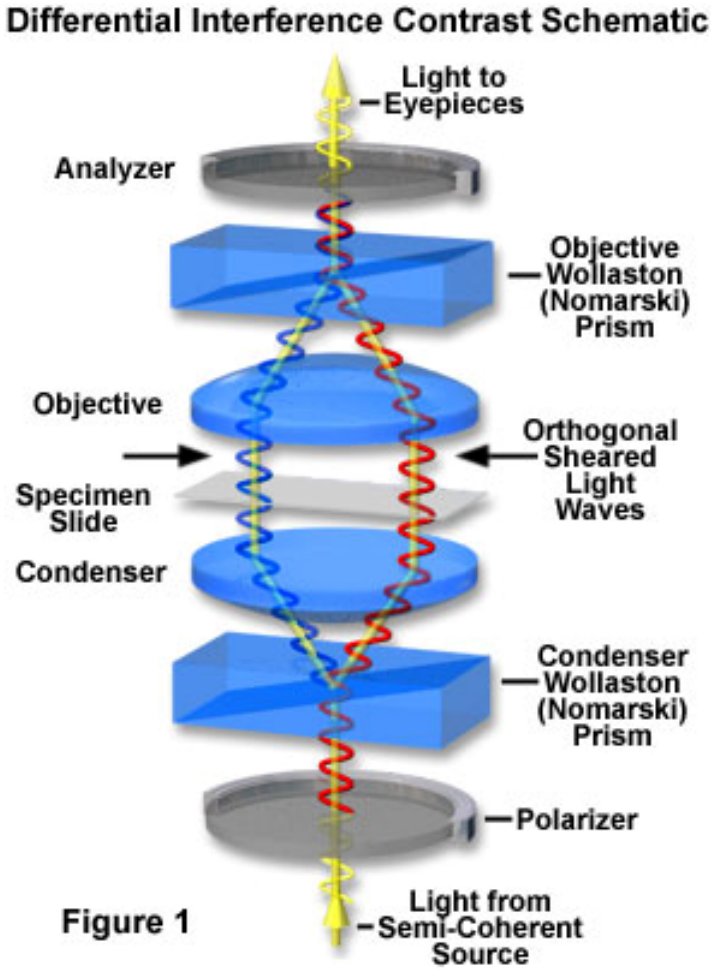
Halos occurs because the ring on the phase plate also receives light diffracted by the specimen. This part of light is of low spatial frequency, and because there is no additional phase difference to the 0-th order light, the intensity of this part becomes larger than the background.



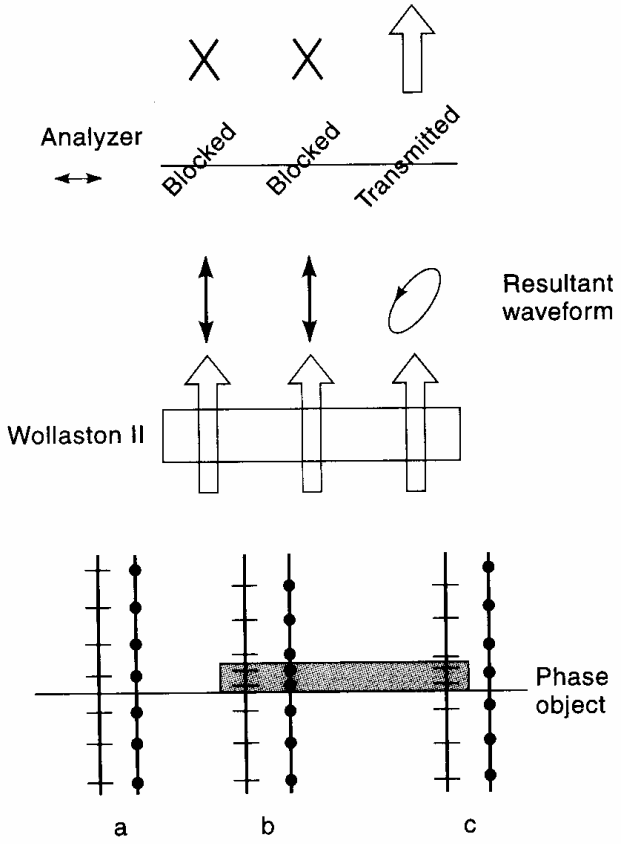


S: background, D: diffracted, P: image

Differential interference contrast (DIC)



The contrast is from the **gradient** of the optical paths, not the optical paths.



Orientation in DIC

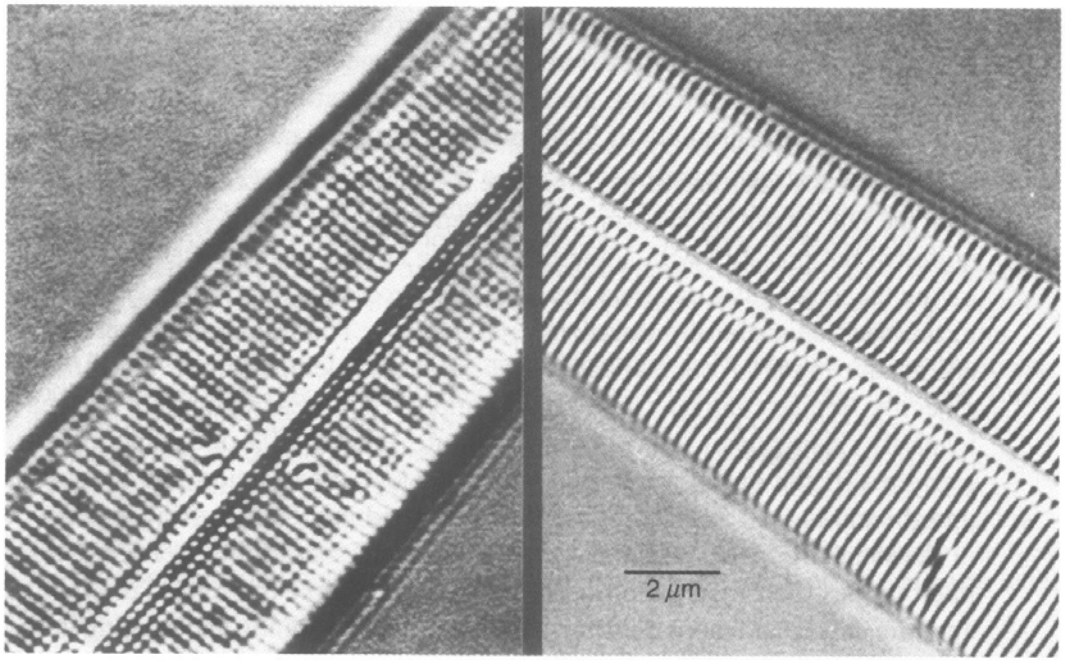


Figure 10-8

Effect of specimen orientation in DIC microscopy. Since the shear axis is fixed in DIC optics, the specimen itself must be rotated to highlight different features. Notice the differential emphasis of pores and striae in the shell of a diatom, *Amphipleura,* using video-enhanced DIC optics.

Ref: D. B. Murphy, *Fundamentals of Light Microscopy and Electronic Imaging* (Wiley-Liss, New York, 2001).

Comparison between phase contrast and DIC

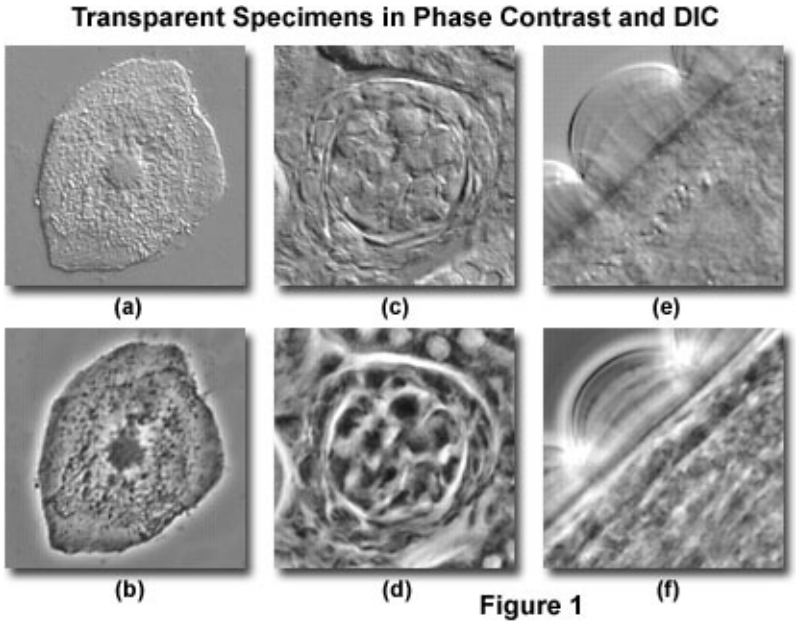


Figure 1

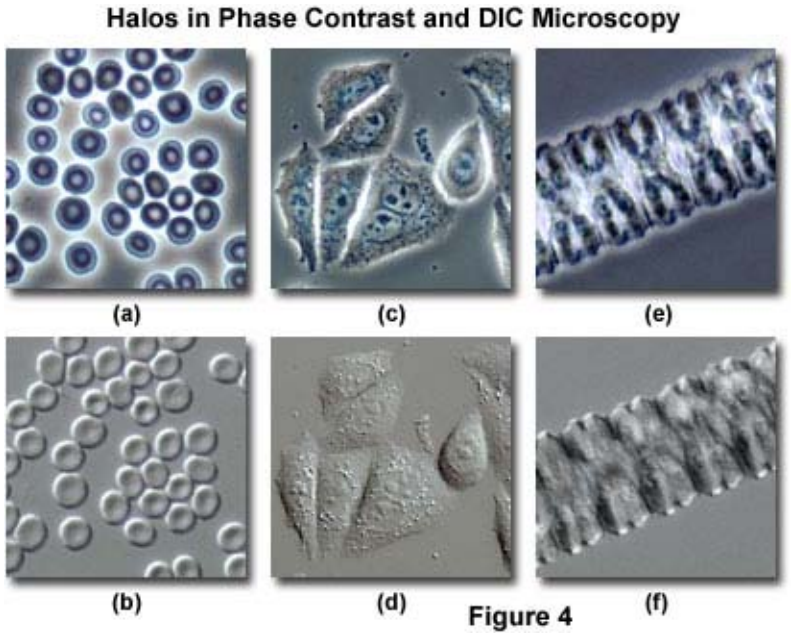
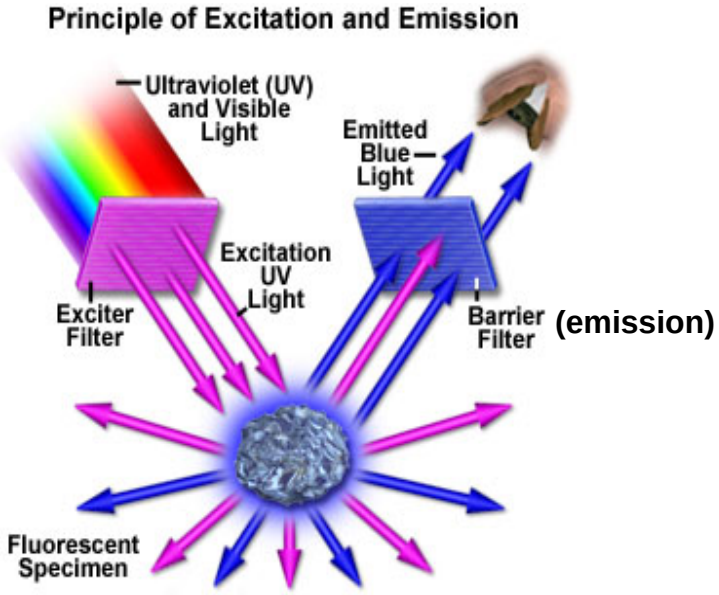


Figure 4

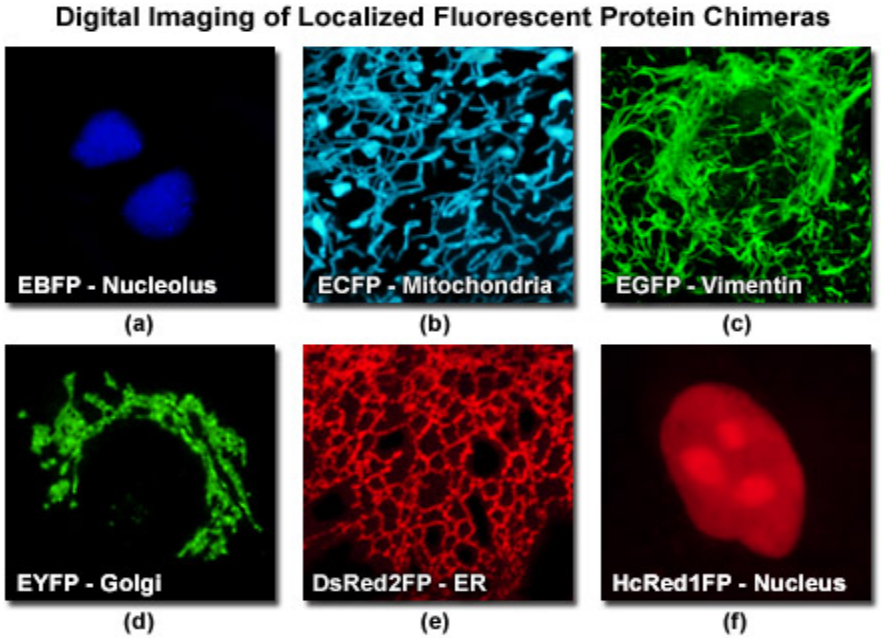
Comparison between phase contrast and DIC

Characteristic	Phase Contrast	DIC
Image Brightness (Brightfield = 100 Percent)	1.3 Percent	0.36 - 2.3 Percent
Epi-Fluorescence Light Loss (Brightfield = 0 Percent)	28 Percent	73 Percent
Lateral Resolution	Condenser Annulus Restricted	Superior
Axial Resolution (Depth Discrimination)	Poor	Superior
Illuminating Aperture	10 Percent of Objective NA	Variable
Phase Shift Detection Limit	$< \lambda/100$	$< \lambda/100$
Utility at High Phase Shifts	Not Useful	Useful
Azimuthal Effects	No	Yes
Halos and Shade-Off	Yes	No
Stained Specimens	Not Useful	Useful
Birefringent Specimens	Useful	Not Useful
Birefringent Specimen Containers	Yes	No
Brightfield Image Deterioration	Slight	None
Cost	Moderate	High

Fluorescence microscopy



False color images. Usually a monochrome camera is used to capture the images, and color is added in the digital image files.



Images are from http://micro.magnet.fsu.edu/

Fluorescence microscopy

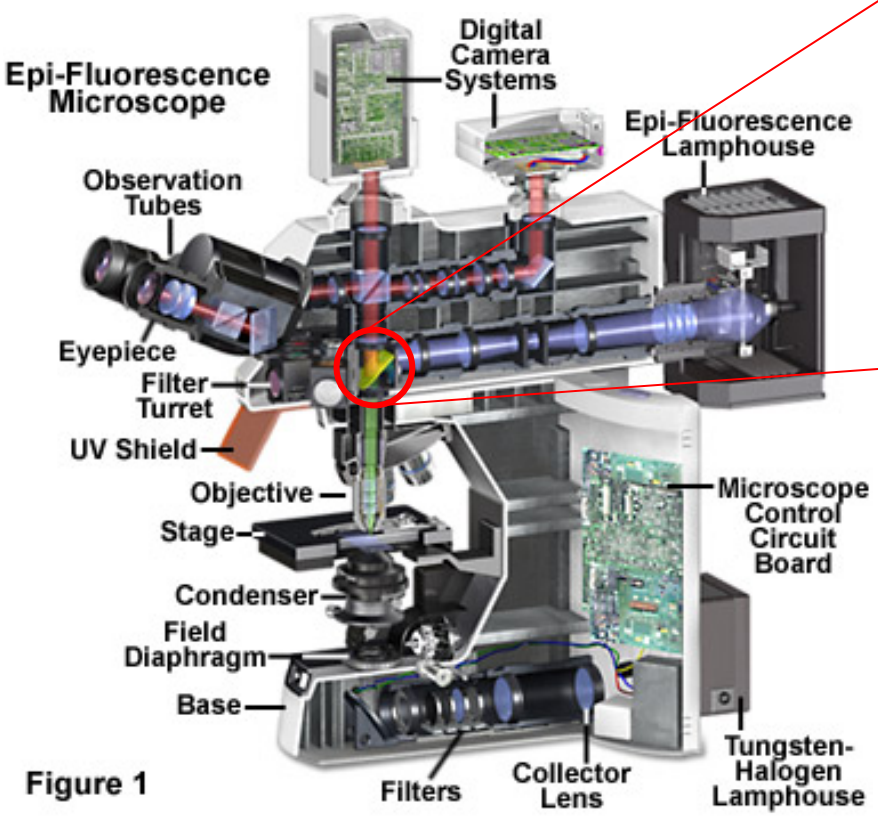


Figure 1

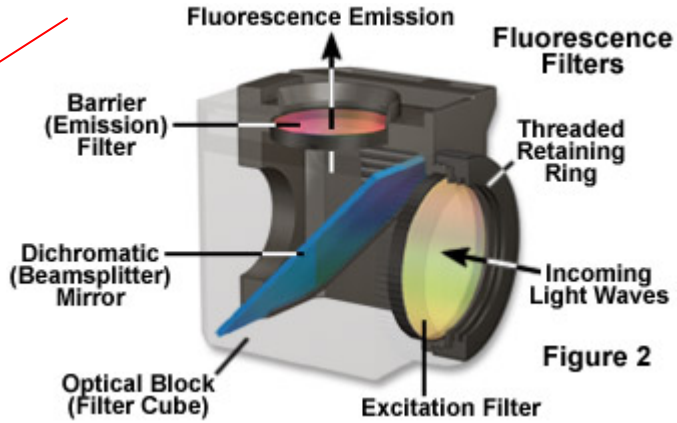


Figure 2

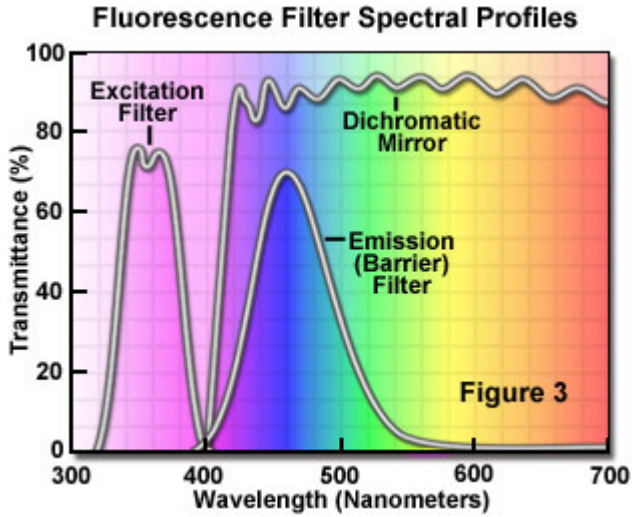
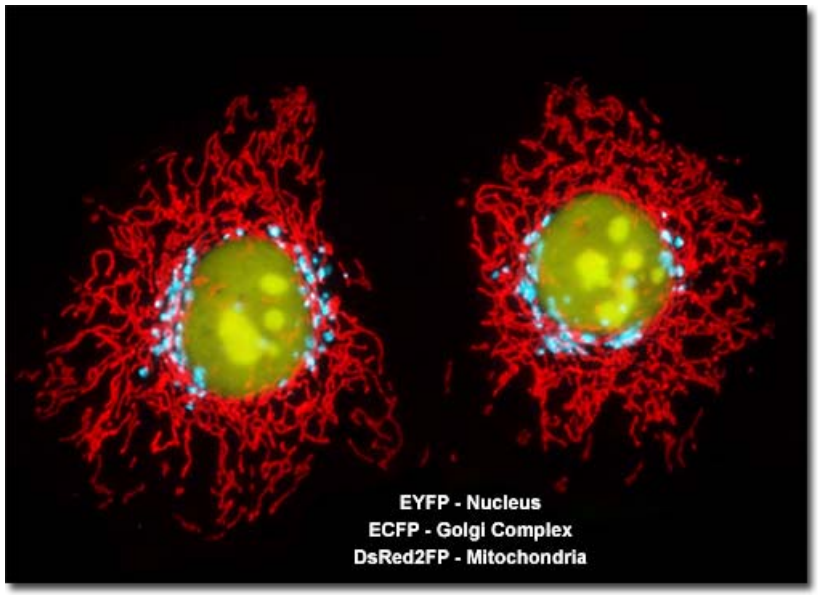


Figure 3

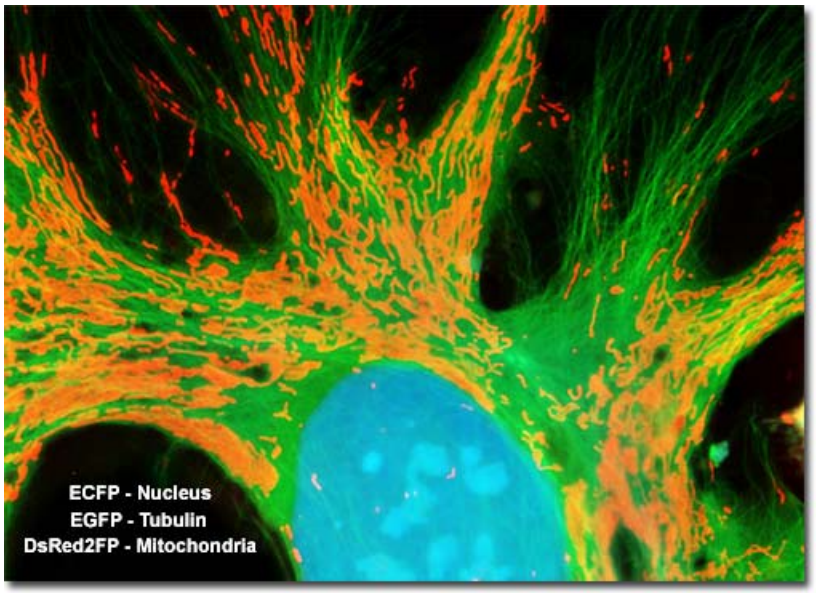
Images are from http://micro.magnet.fsu.edu/

Fluorescence proteins

Human Cervical Adenocarcinoma Cells



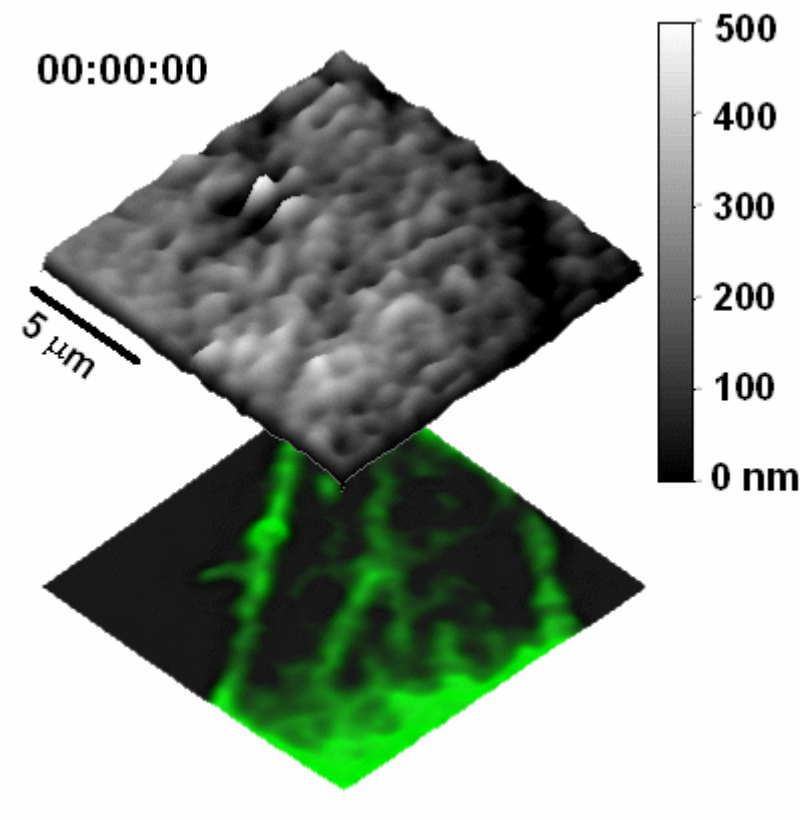
Opossum Kidney Cortex Epithelial Cells



Fluorescence proteins can be transfected into living cells, enabling dynamic observations of intracellular structures.

Images are from http://micro.magnet.fsu.edu/

Dynamics of cell membranes and cytoskeletons

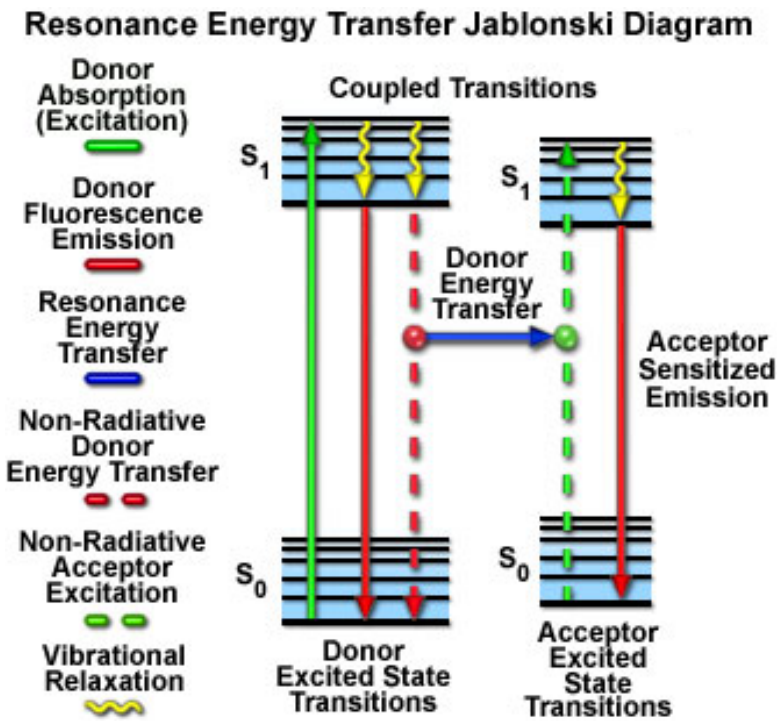


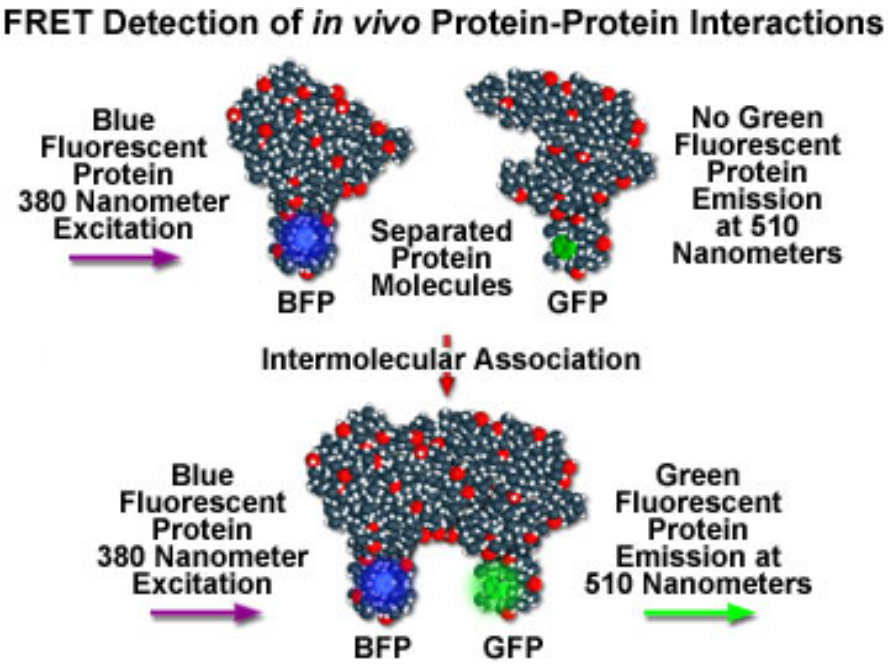
The 1-μm beads are coated with fibronectin, which links to actin filaments (GFP labeled) through integrins.

From the motion of the bead we calculated that the treadmill speed of the actin on the filament is ~ 17 nm/min, while the elongation rate of the actin branch is ~ 8 nm/min.

Ref: C.-C. Wang, J.-Y. Lin, H.-C. Chen, and C.-H. Lee, *Opt. Lett*. **31,** in press (2006).

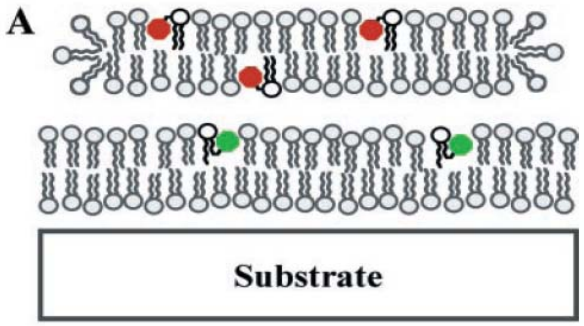
Fluorescence resonance energy transfer (FRET) Microscopy





Images are from http://micro.magnet.fsu.edu/

Membrane topography by FRET



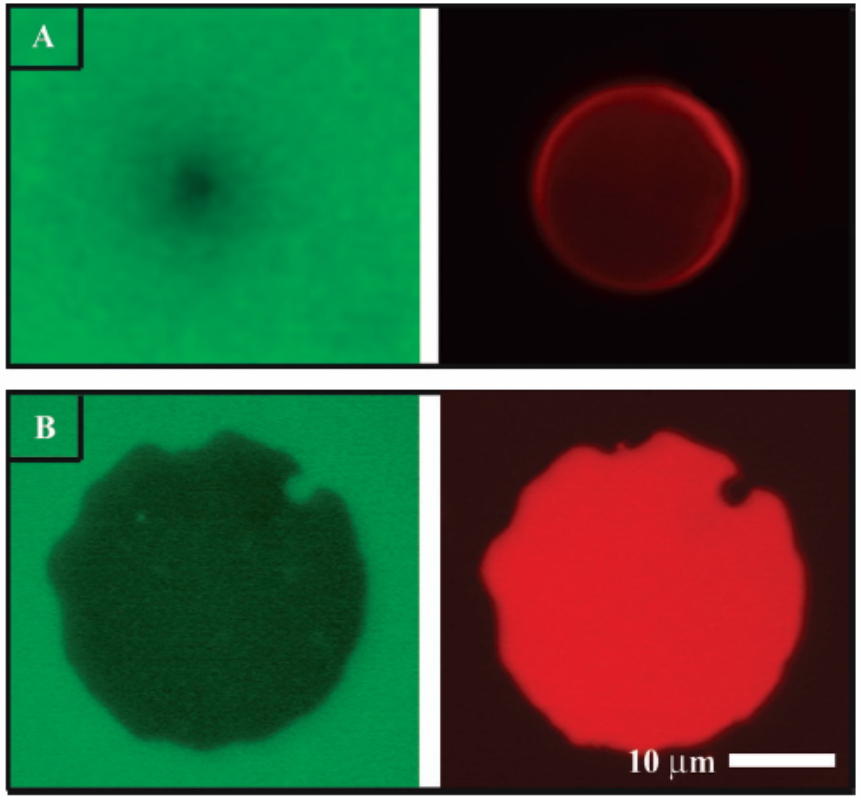
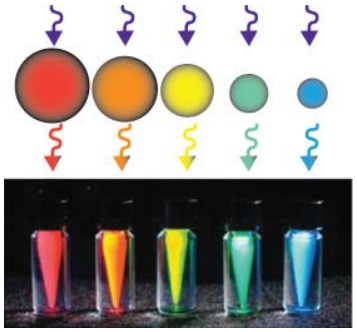


Fig. 2. (*A*) Fluorescence image of the lower (green, *Left*) and upper (red, *Right*) membranes of an unruptured GUV on a supported membrane. (*B*) Image of a supported membrane junction formed after rupture of a GUV. In both cases, patterns seen in the lower membranes result from intermembrane FRET to acceptors in the upper membrane.

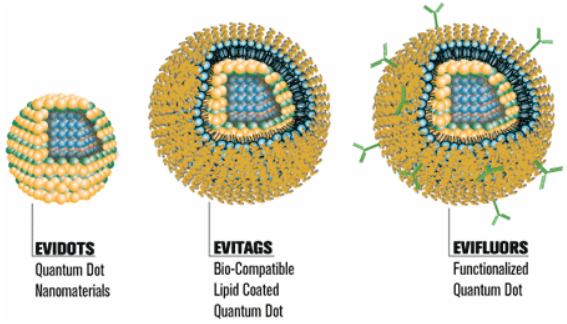
Ref: A. P. Wong and J. T. Groves, *Proc. Natl. Acad. Sci. USA* **99,** 14147 (2002).

Functionalized quantum dots

With lower cellular toxicity, quantum dots are becoming common fluorescent labels.

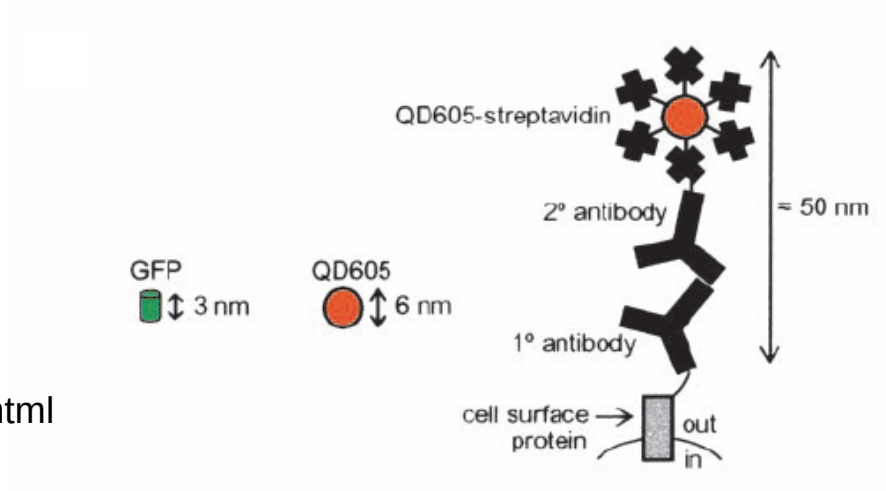


http://probes.invitrogen.com/products/qdot/overview.html



http://www.evidenttech.com

Targeting to membrane proteins



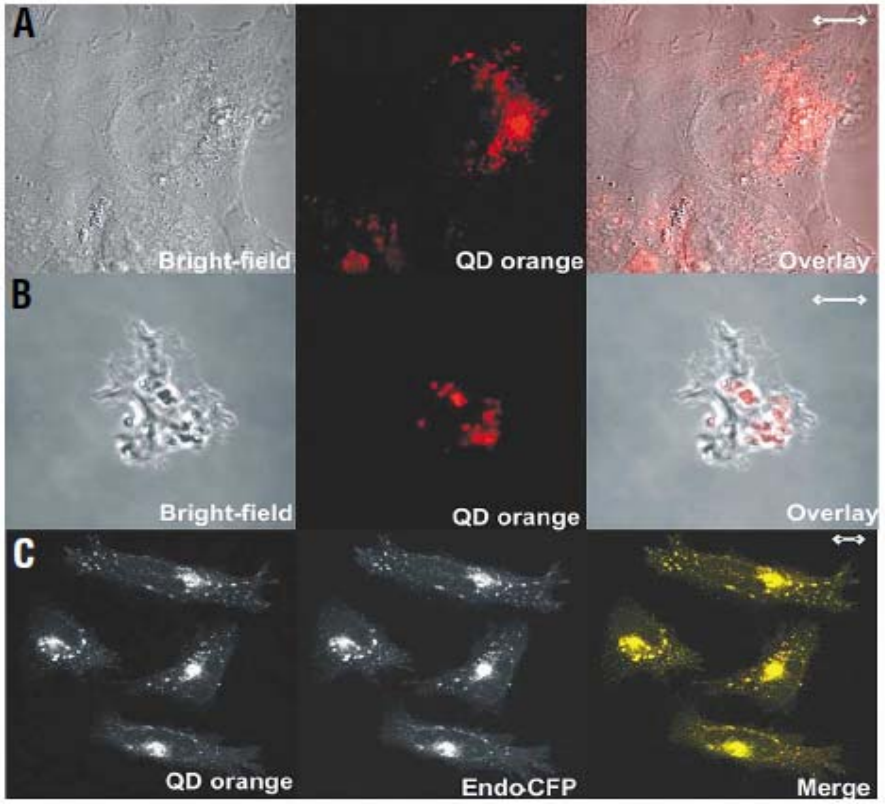
Ref: M. Howarth, K. Takao, Y. Hayashi, and A. Y. Ting, *Proc. Natl. Acad. Sci. USA* **102,** 7583 (2005).

Living cells labeled by quantum dots

Confocal images

HeLa cell

D. discoideum cell



Ref: J. K. Jaiswal, H. Mattoussi, J. M. Mauro, and S. M. Simon, *Nature Biotech.* **21,** 47 (2002).

Confocal Microscopy

Confocal microscopy

Improved depth resolution

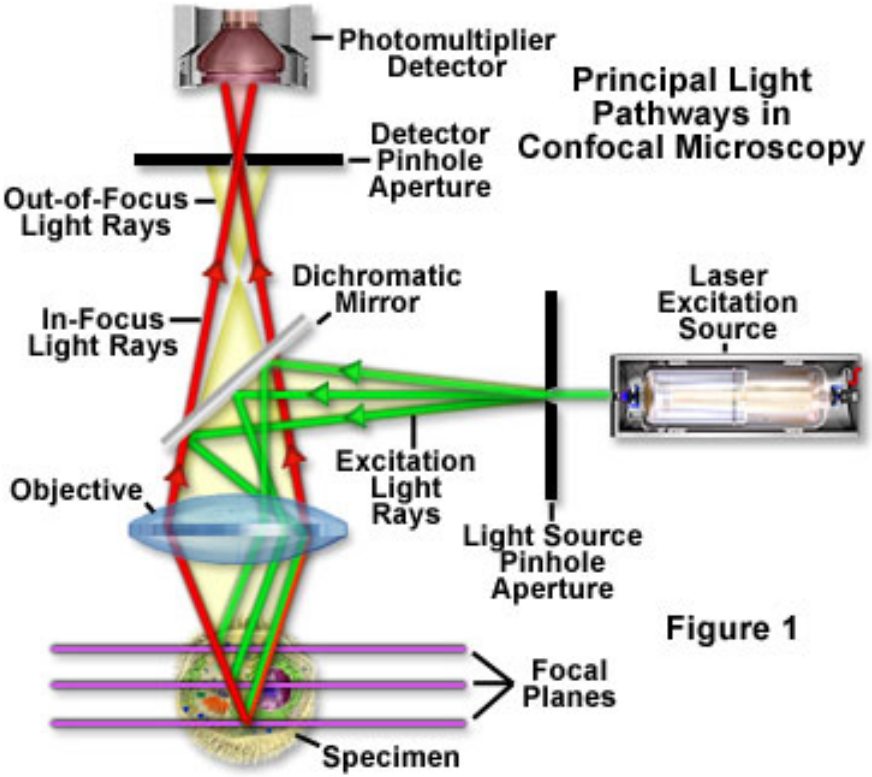
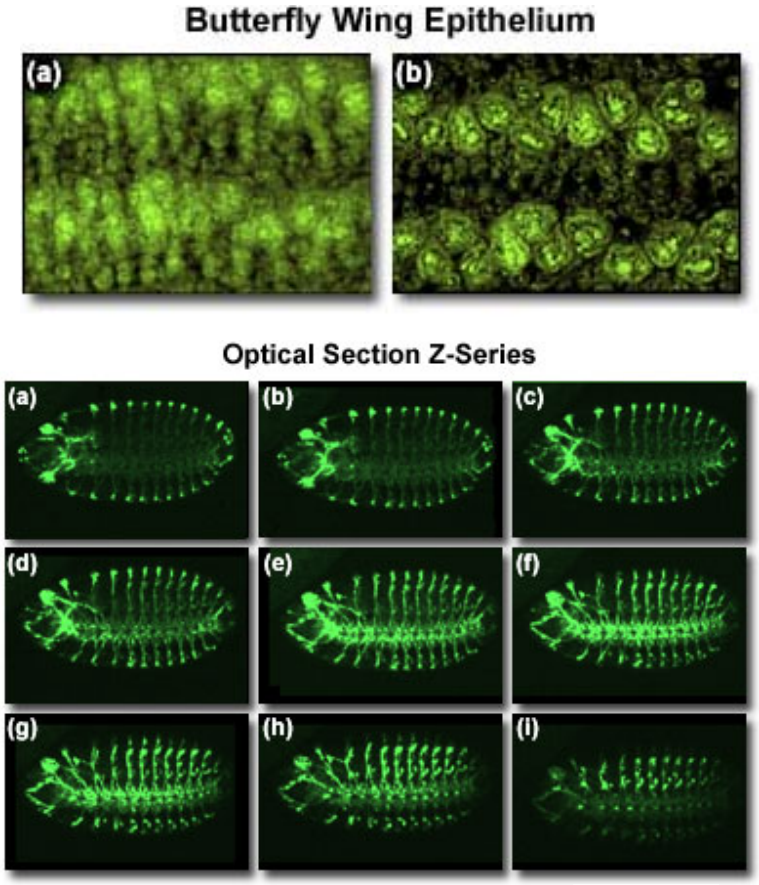
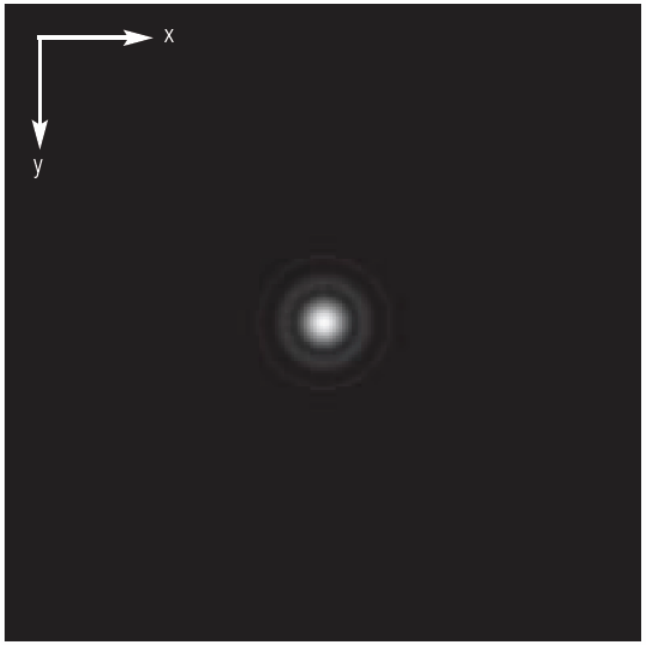


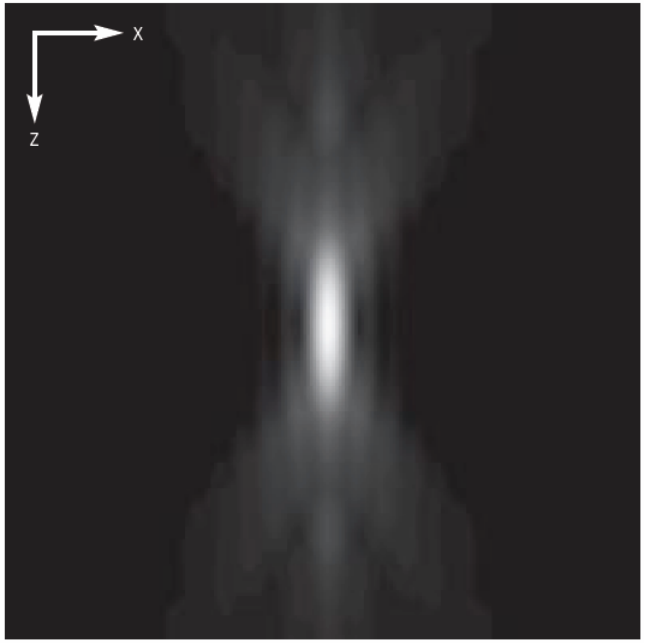
Figure 1



Images are from http://micro.magnet.fsu.edu/

Three-dimensional point-spread function





Lateral:

$$FWHM_{ill,lateral} = 0.51 \frac{\lambda_{exc}}{NA}$$

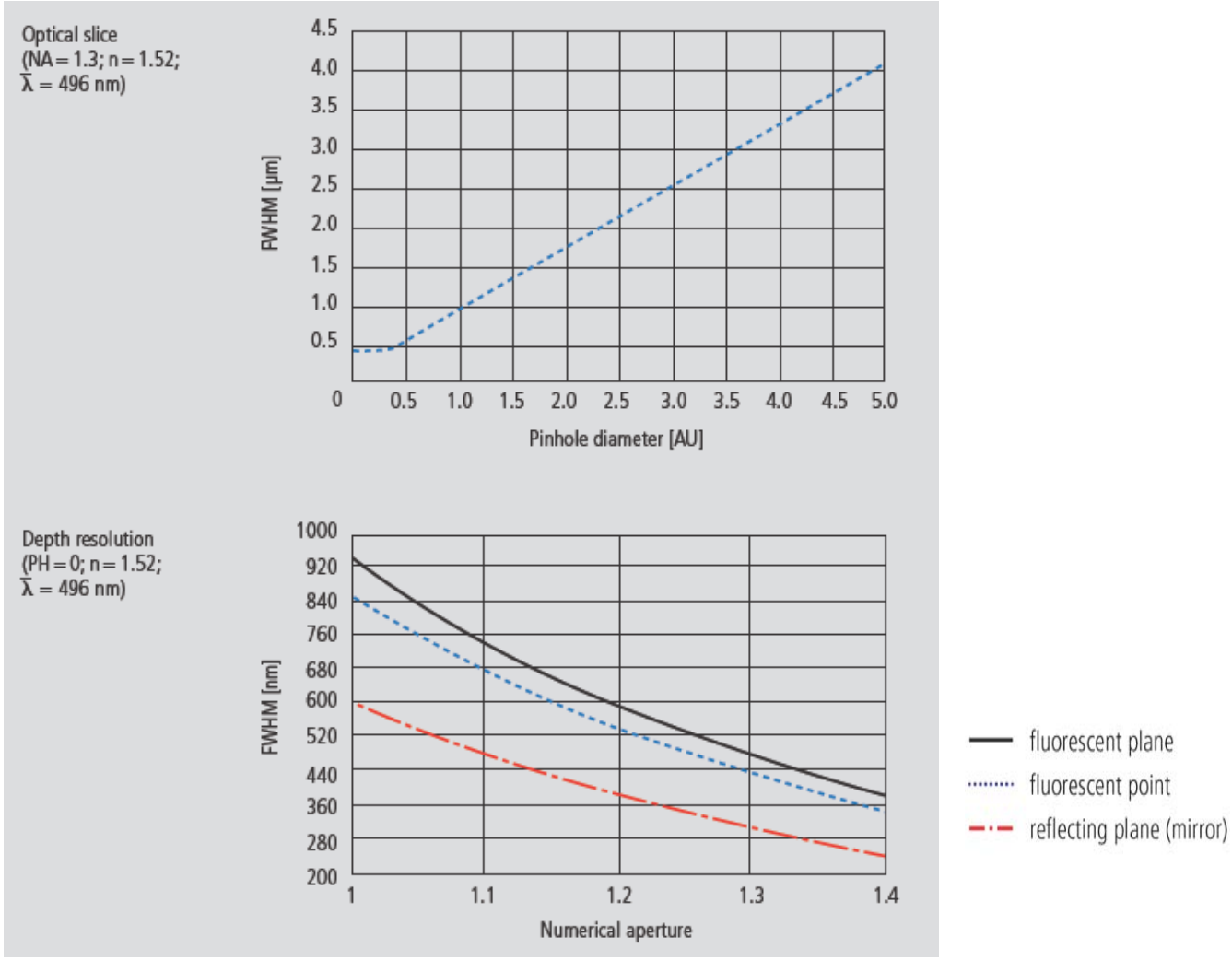
Axial:

$$FWHM_{ill,axial} = \frac{0.88 \cdot \lambda_{exc}}{(n - \sqrt{n^2 - NA^2})}$$

n = *refractive index of immersion liquid,*
NA = *numerical aperture of the microscope objective,*
λ_{exc} = *wavelength of the excitation light*

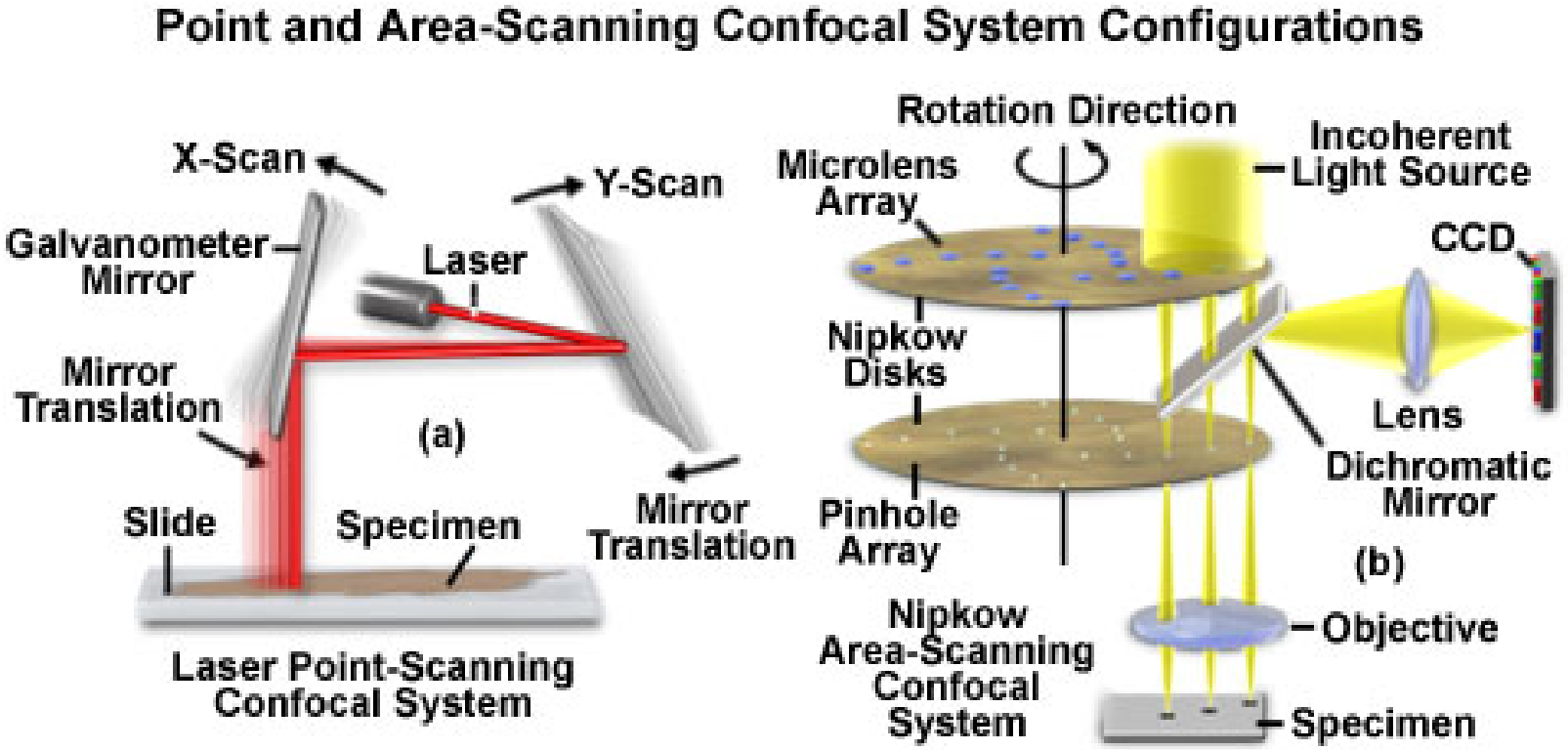
Ref: Carl Zeiss, *Confocal Laser Scanning Microscopy*

Effect of the pinhole diameter and NA



Ref: Carl Zeiss, *Confocal Laser Scanning Microscopy*

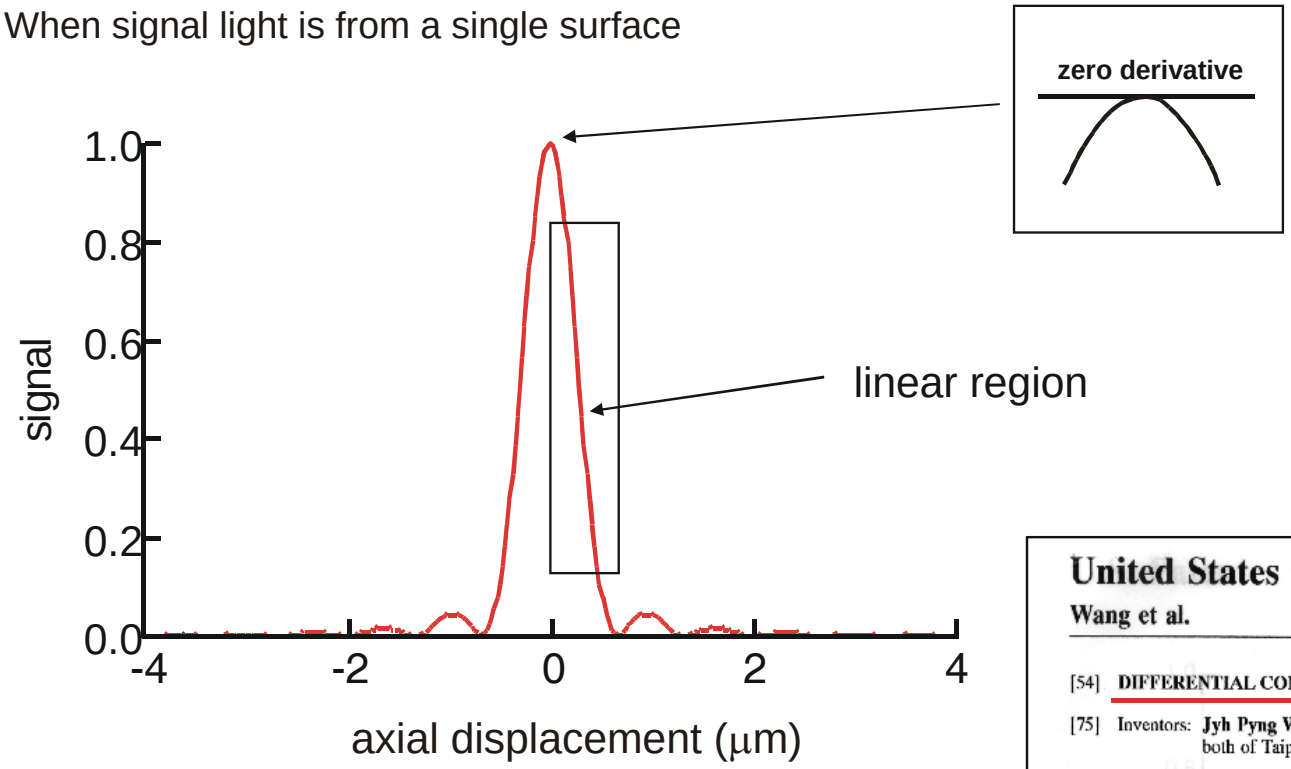
Scanning system



Images are from http://micro.magnet.fsu.edu/

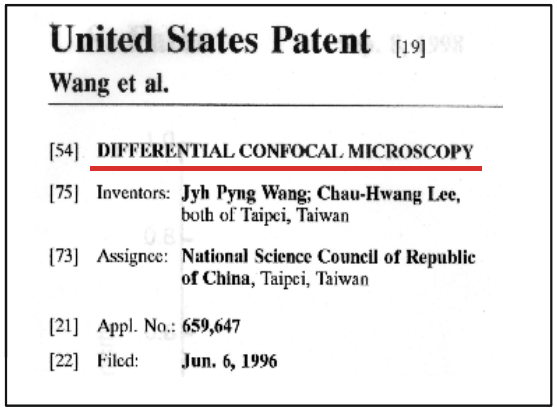
Nanometer depth resolution: differential confocal microscopy

When signal light is from a single surface



Typical slope in the linear region = 1/μm

→ 10 nm displacement = 1% signal variation



United States Patent [19]

Wang et al.

[54] DIFFERENTIAL CONFOCAL MICROSCOPY

[75] Inventors: Jyh Pyng Wang; Chau-Hwang Lee, both of Taipei, Taiwan

[73] Assignee: National Science Council of Republic of China, Taipei, Taiwan

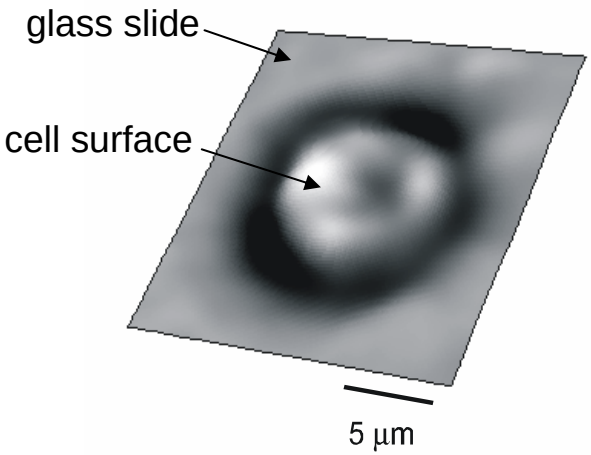
[21] Appl. No.: 659,647

[22] Filed: Jun. 6, 1996

Ref: C.-H. Lee and J. Wang, *Opt. Commun.* **135,** 233 (1997).

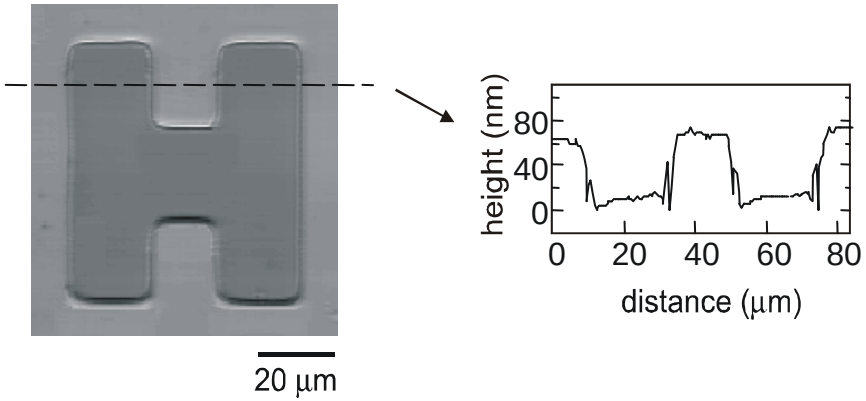
Sample images of DCM

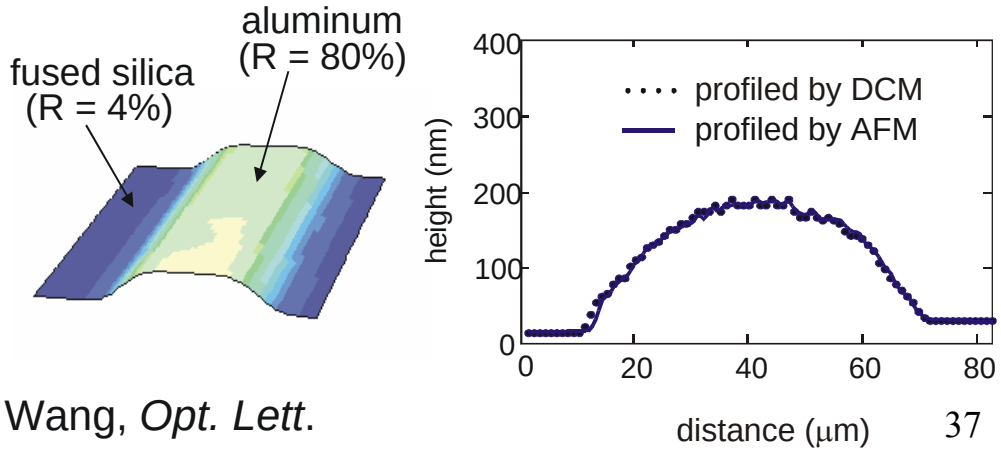
human red blood cell



The center recess is 570 nm.

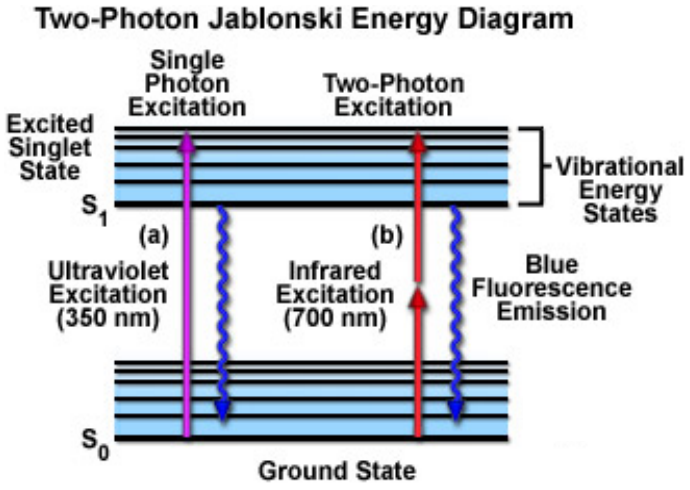
70-nm deep H-trench on InGaAs

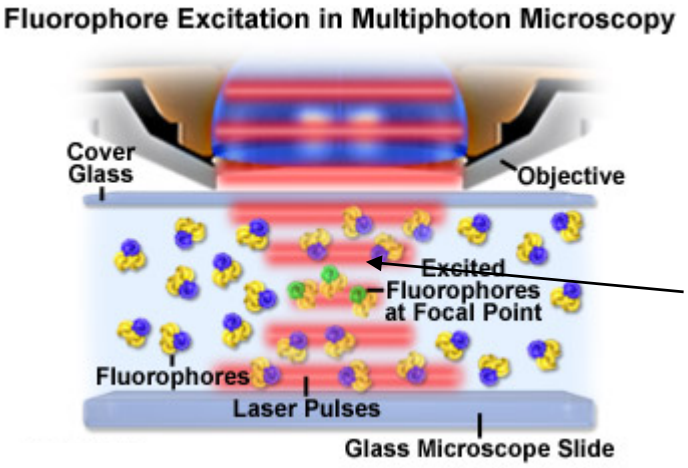


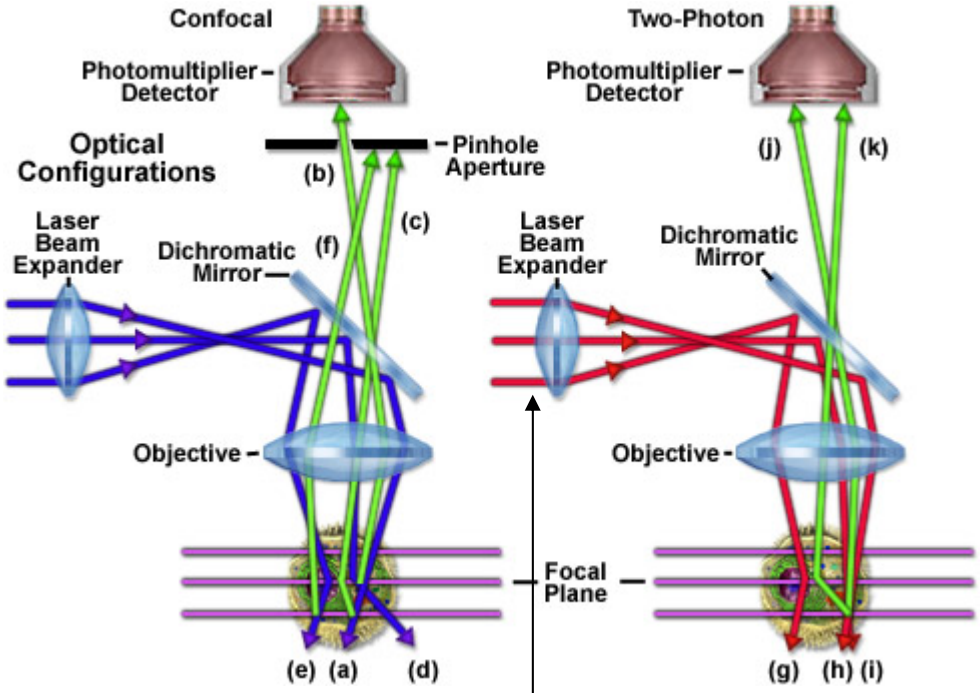


Ref: C.-W. Tsai, C.-H. Lee, and J. Wang, *Opt. Lett*. **24,** 1732 (1999).

Multiphoton Microscopy







IR light can penetrate deeper into the tissues.

Femtosecond laser pulses are required to perform two-photon excitation.

Images are from http://micro.magnet.fsu.edu/

Limits to Imaging Quality

Image formation

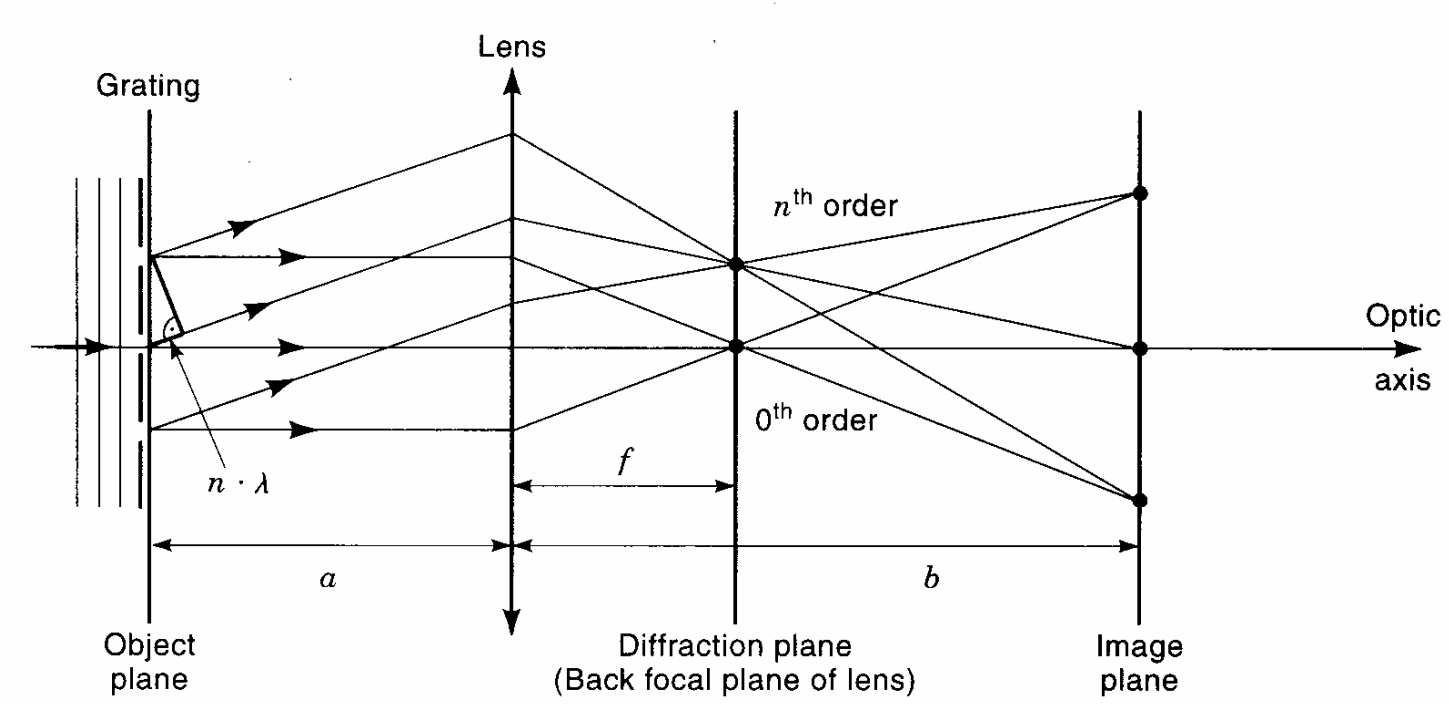
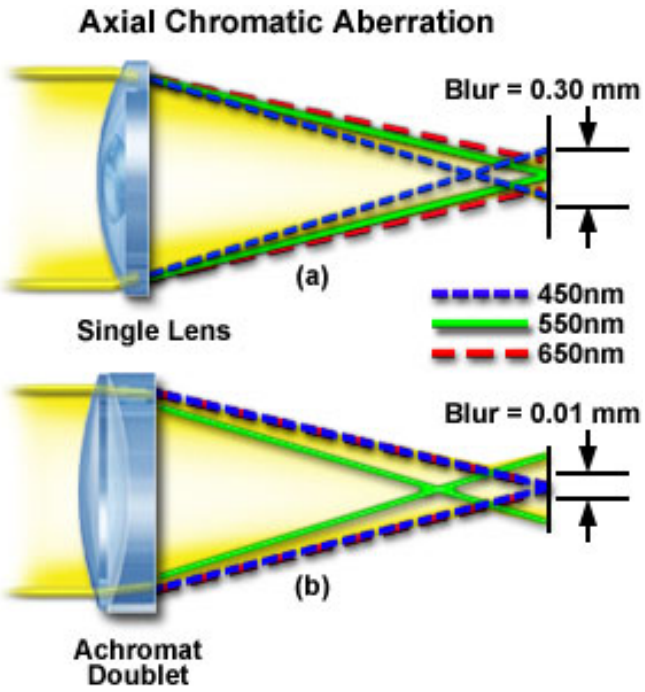


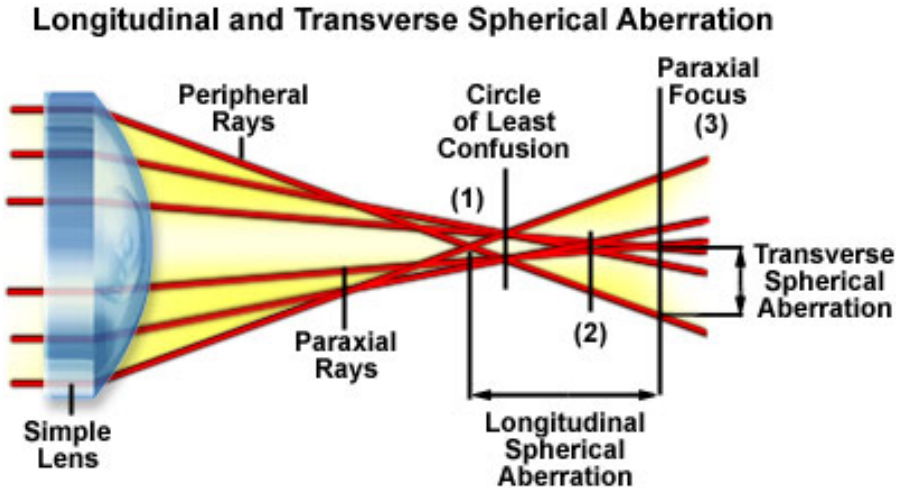
Figure 5-14

Abbe's theory for image formation in a light microscope. An objective lens focused on a grating ($2f > a > f$) in the object plane produces a magnified real image of the grating in the image plane. The diffraction plane is located at $1f$ in the back aperture of the lens. An incident planar wavefront is shown. Diffracted nth-order and nondiffracted 0th-order rays are separated in the diffraction plane, but are combined in the image plane.

Ref: D. B. Murphy, *Fundamentals of Light Microscopy and Electronic Imaging* (Wiley-Liss, New York, 2001).

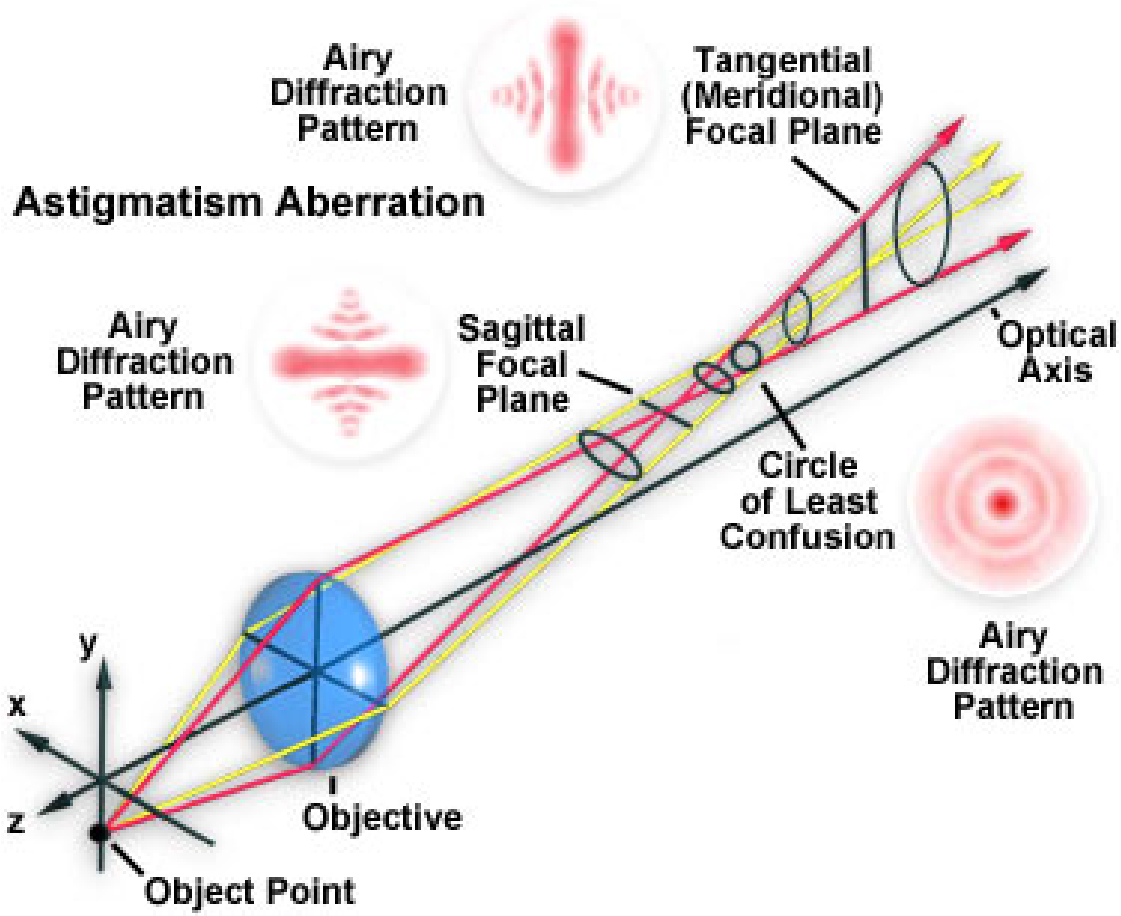
Aberrations





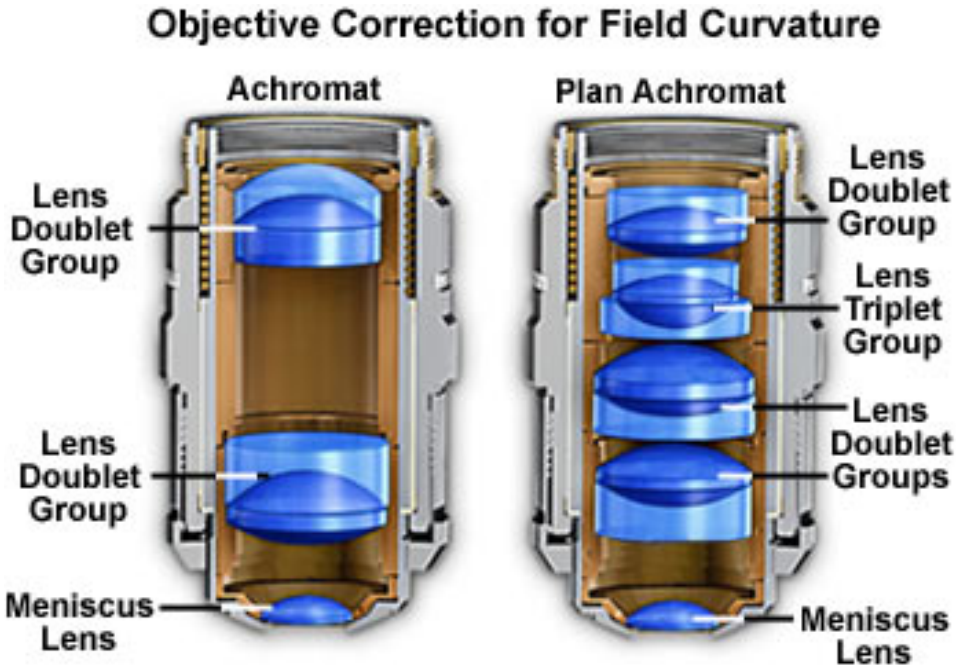
Images are from http://micro.magnet.fsu.edu/

Astigma



Images are from http://micro.magnet.fsu.edu/

Achromatic



Images are from http://micro.magnet.fsu.edu/

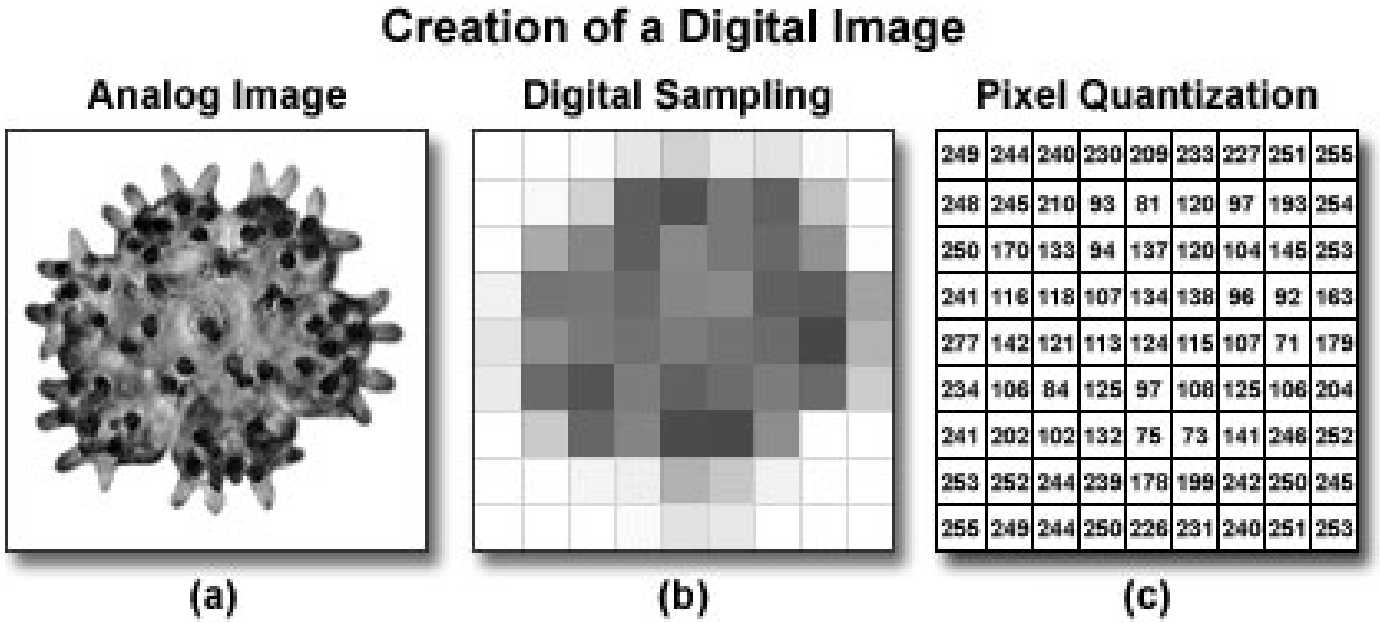
Types of objectives

Objective Type	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

Images are from http://micro.magnet.fsu.edu/

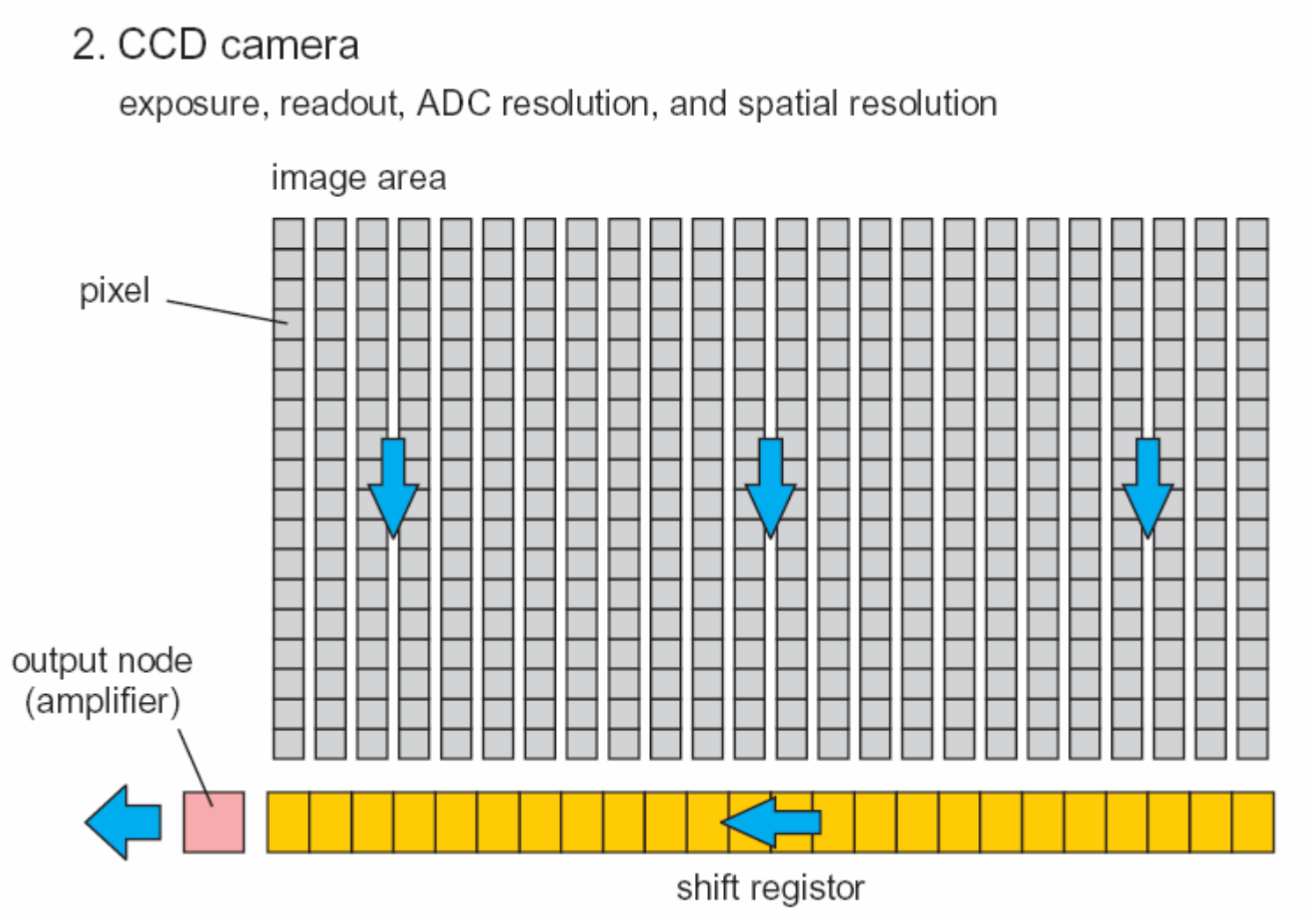
Digital Images

A digital image



Images are from http://micro.magnet.fsu.edu/

Charge-coupled device (CCD)



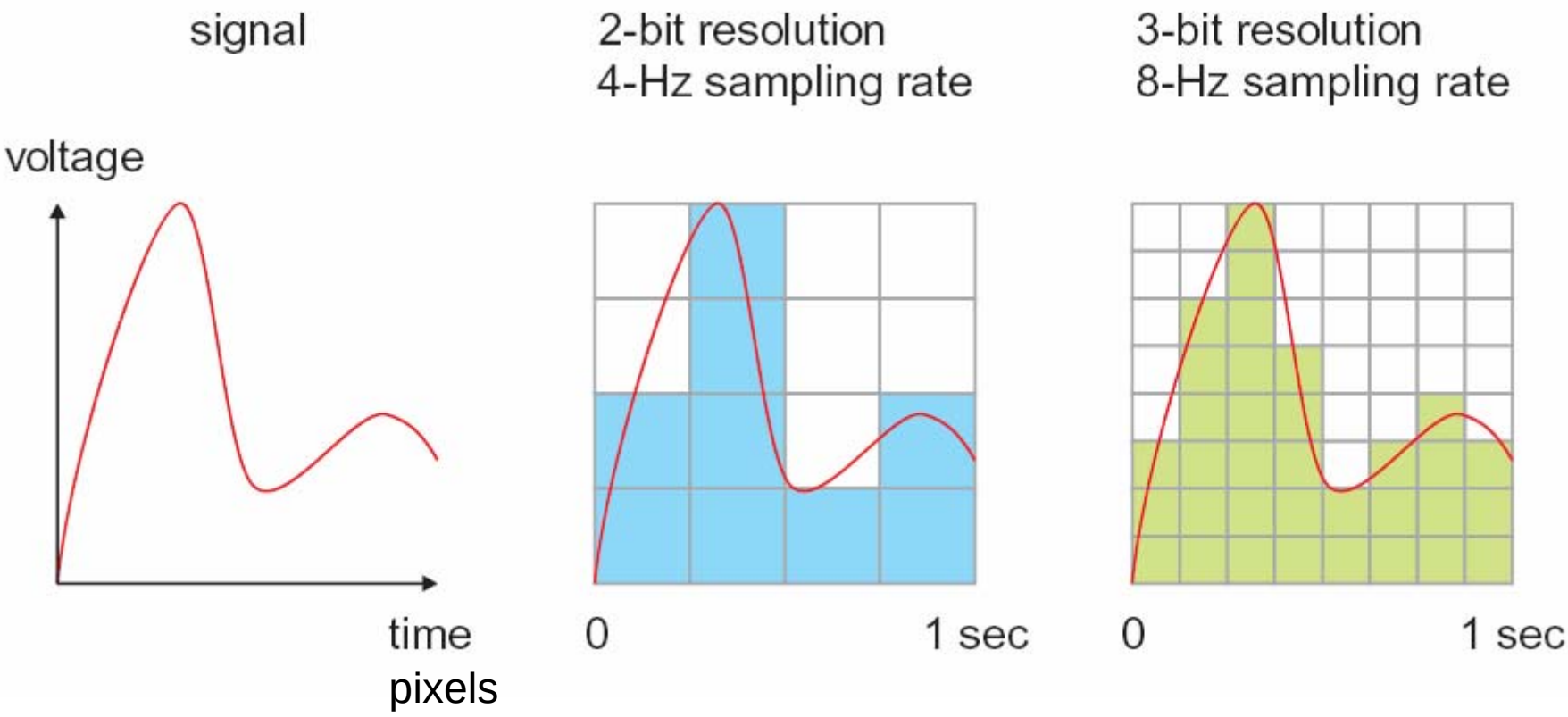
Specifications of CCD cameras

- pixel size (8 μm; 23 μm)
- pixel resolution (640 x 480; 1024 x 1024)
- spectral response (300 nm to 1000 nm)
- well depth (> 300,000 e^-)
- dark current (50 pA/cm^2 at 20 ^oC)
- dynamic range (> 85 dB)
- digital or analog
- bit depth (10 bit; 12 bit; 14 bit...)

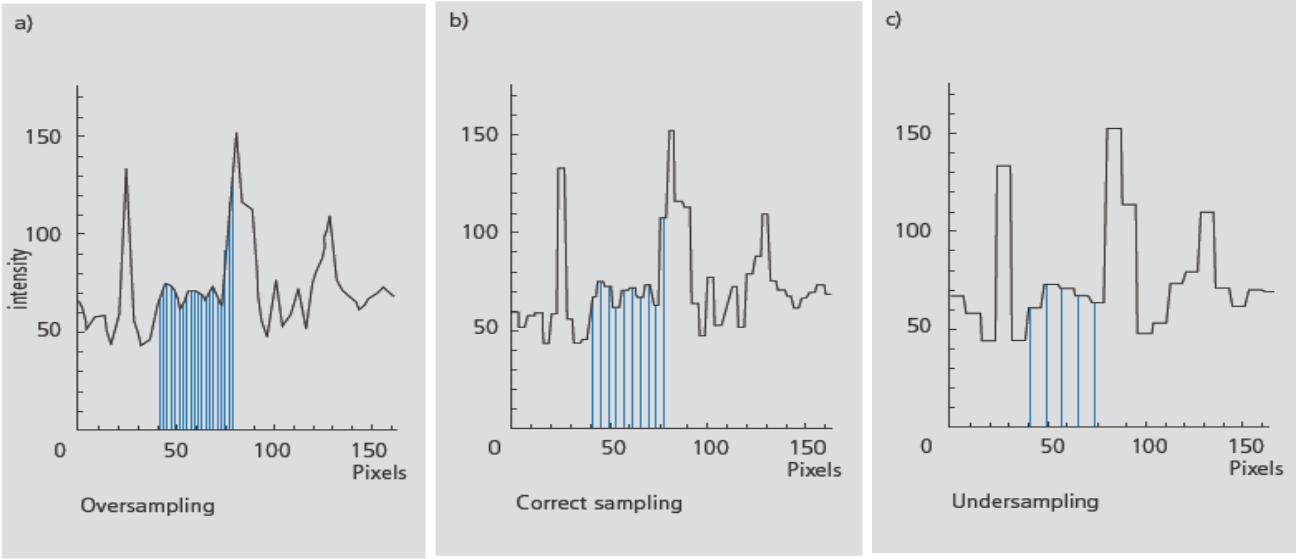
Bandwidth of digital camera interfaces

Interface	Bandwidth (Mbit/s)
IEEE-1394a	50
IEEE-1394b	100
USB 2.0	60
Camera Link	400
GigE	1250
PCI Bus	125
PCI-X Bus	4300

Signal digitization



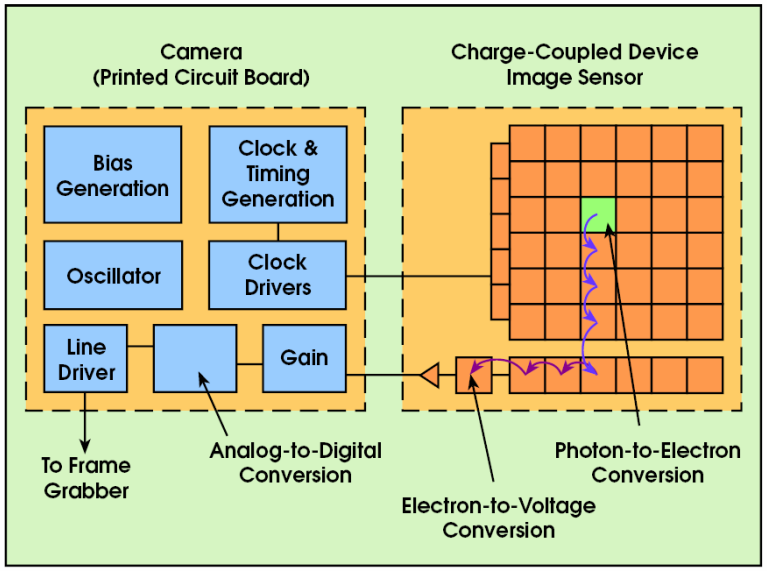
Sampling

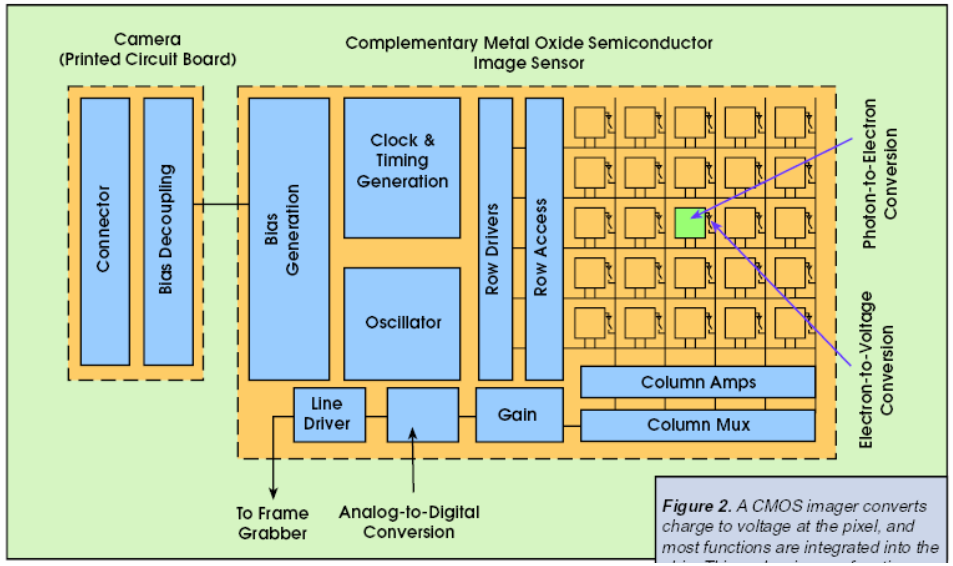


Sampling frequency $\geq$ 2 x signal bandwidth
For CCD cameras, the pixel size on the image should be smaller than half the optical resolution.

From: Carl Zeiss, *Confocal Laser Scanning Microscopy*

CCD vs. CMOS





CCD: larger dynamic range, smaller dark current.

CMOS: better responsivity, lower power consumption, compactness.

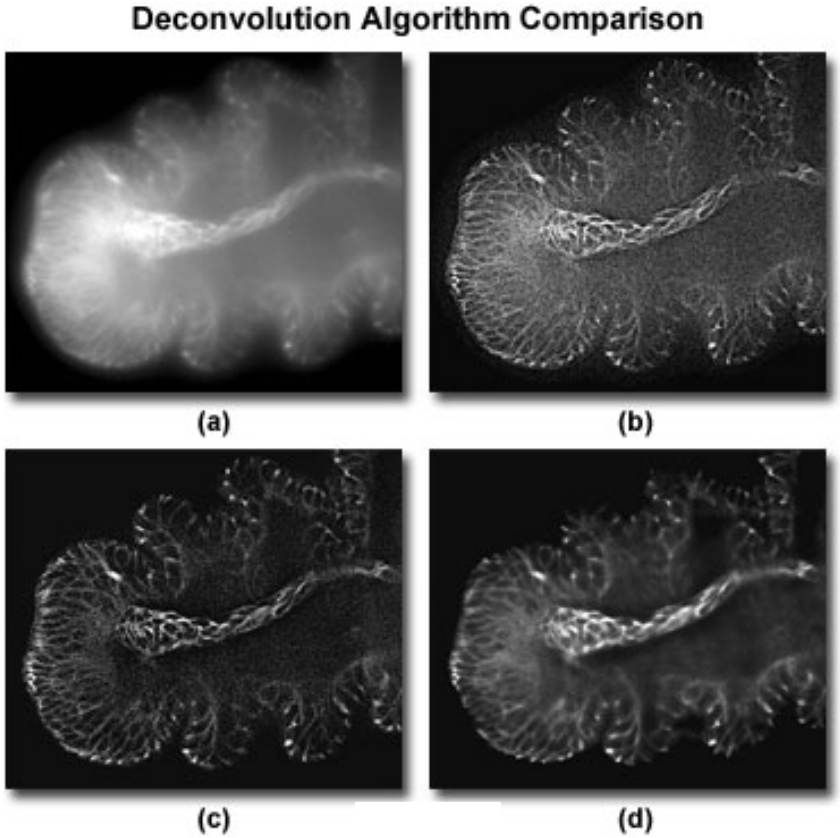
Ref: D. Litwiller, “CCD vs. CMOS: Facts and Fiction,” *Photonics Spectra*, **Jan. 2001.**

Noise in Optical Detection

Receiver	Noise	Also called	Physical source	Equation
Solid state	**Johnson**	Nyquist, white, thermal	Thermal motion of charges in circuit components	$V_{noise} = (4kTRB)^{1/2}$ k: Boltzmann's constant T: absolute temperature R: resistance B: bandwidth in Hz $4k = 5.53*10^{-23}$ $V^2/Hz/K-\Omega$
Solid state	**Shot**	Dark current, leakage current	Statistical fluctuation in carriers at p-n junction	$I_{noise} = (2qIB)^{1/2}$ q: electron charge I: average dc current B: bandwidth in Hz
Photo-emissive	**Quantum**	Photon	Statistical fluctuation in arrival of signal photons	Linear

Resolution Enhancement

Deconvolution in optical microscopy



(a) original (raw) image

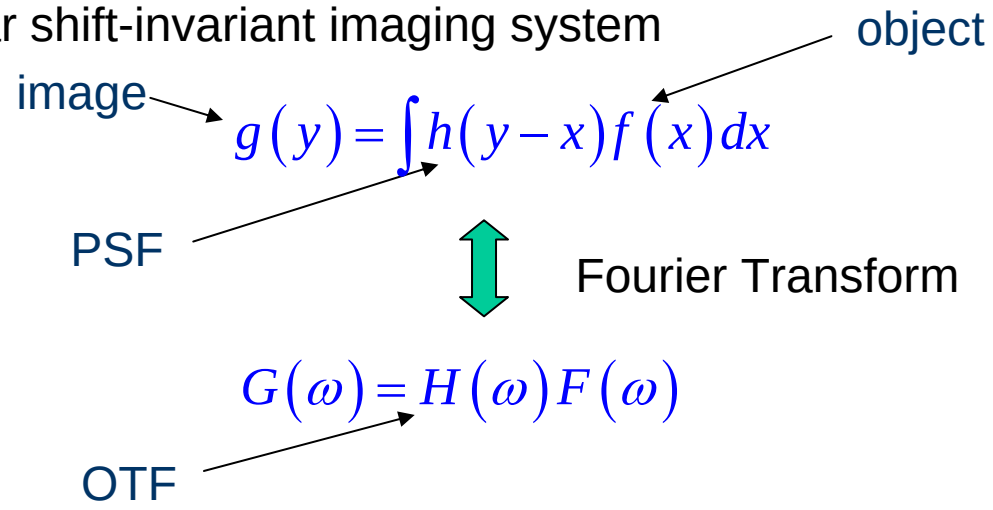
(b) deblurring by a nearest neighbor algorithm

(c) deconvolution by an inverse (Wiener) filter

(d) by iterative blind deconvolution incorporating an adaptive point spread function

Image-formation of an optical system

- Ideal linear shift-invariant imaging system



- Practical linear shift-invariant imaging system

$$g(y) = \int h(y-x) f(x) dx + n(y)$$

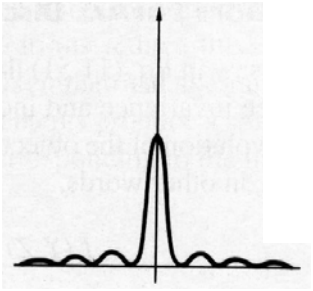
Fourier Transform

$$G(\omega) = H(\omega) F(\omega) + N(\omega)$$

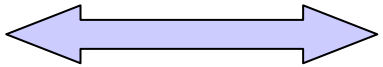
Why direct deconvolution fails

$$F(\omega) = \frac{G(\omega) - N(\omega)}{H(\omega)}$$

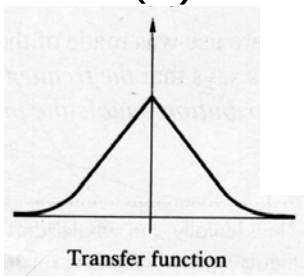
h(y-x)



Fourier Transform



H(ω)

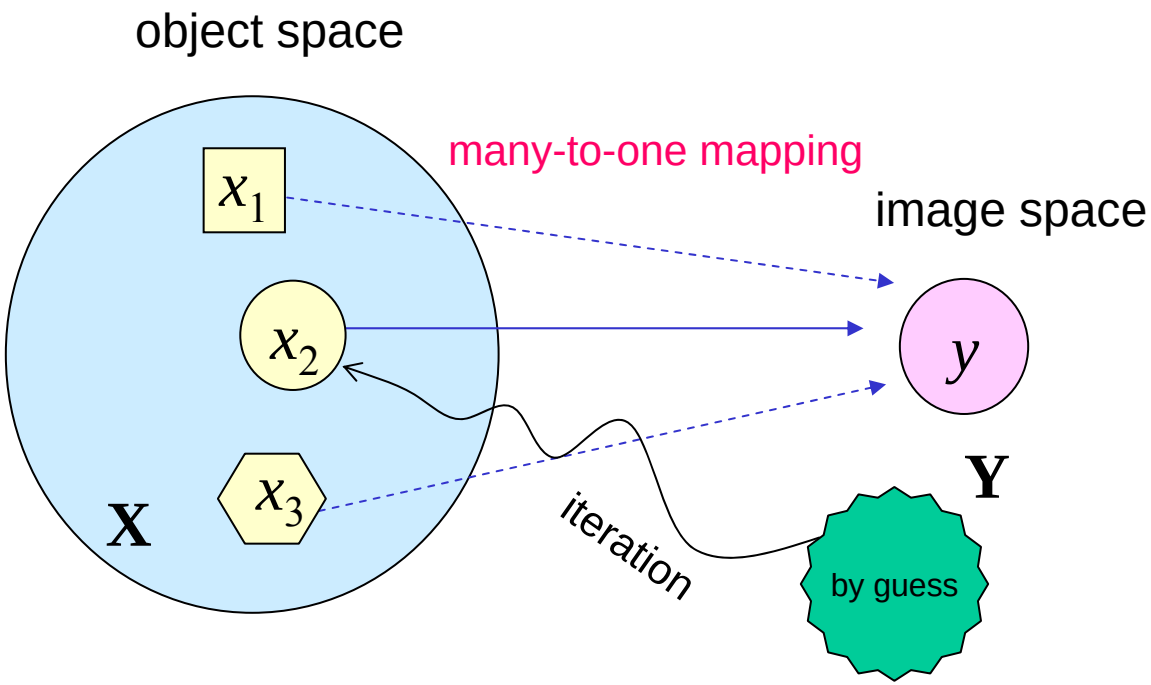


- Band-limited character of the optical transfer function (OTF) $H(\omega)$

  ➔ results in divide-by-zero for some spatial frequencies;

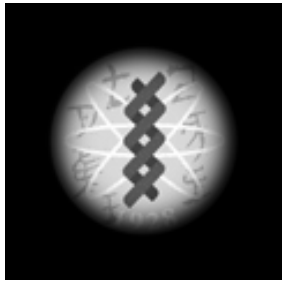
  ➔ enhances high-frequency noise.

Maximum-likelihood estimation

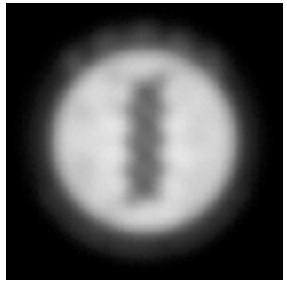


Maximum-likelihood estimation can be used to find a solution for such a “many-to-one” mapping problem.

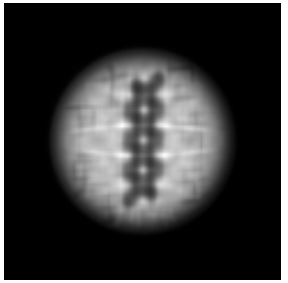
Deconvolution improves the resolution



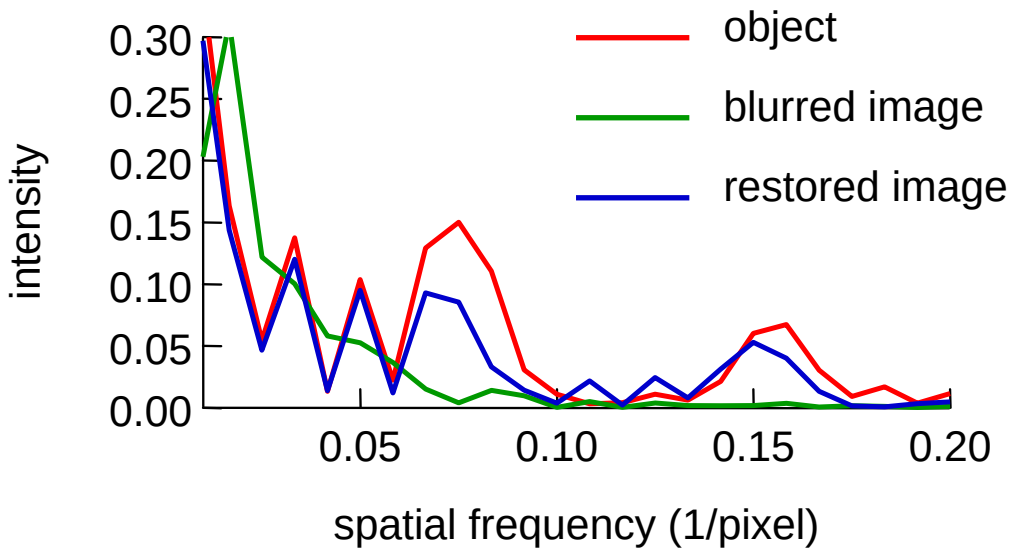
object



blurred image



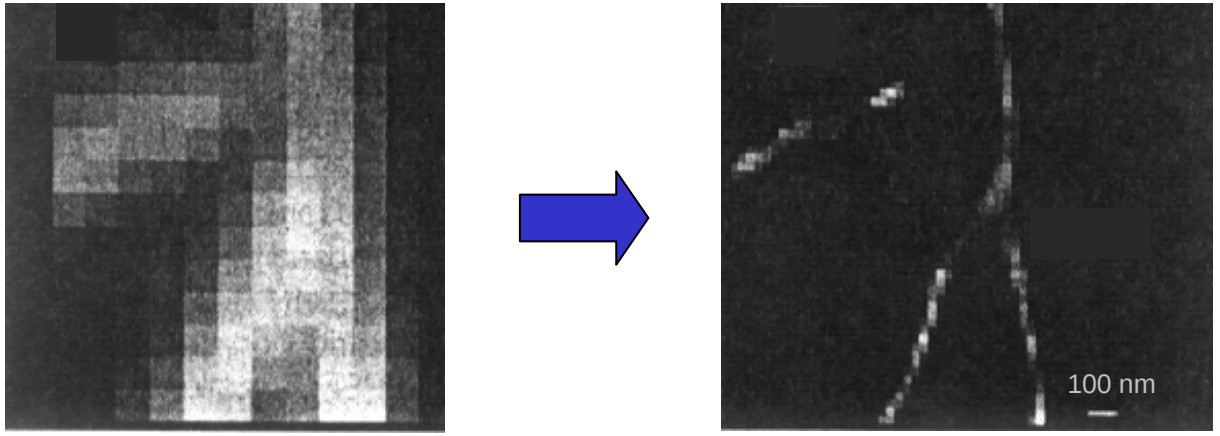
restored image
(1000 iterations)



Super resolution by restoring high-contrast image

To approach super resolution we need high contrast, such as that provided by fluorescent or scattering labelling.

Restored by an iterative algorithm



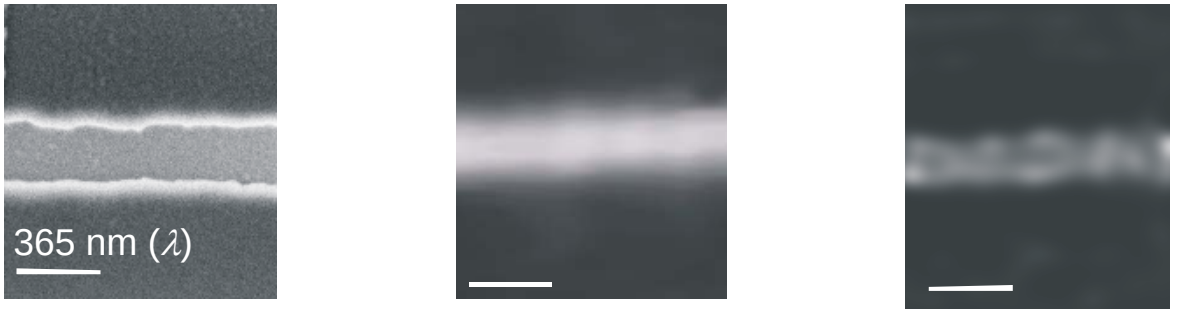
Sample: microtubules in a rat kidney cell

After 2000 iterations, ~ 50-nm lateral resolution is achieved.

Ref: W. A. Carrington *et al*., *Science* **268,** 1483 (1995).

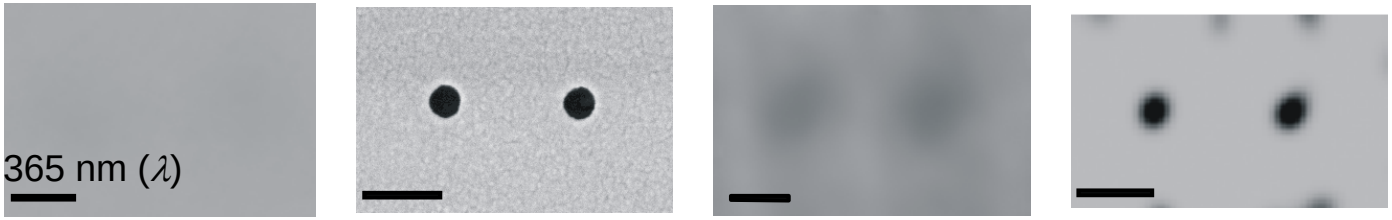
Resolution enhancement based on nano-topography

200-nm Cr line



SEM image | Topographic image | Restored topographic image

100-nm holes

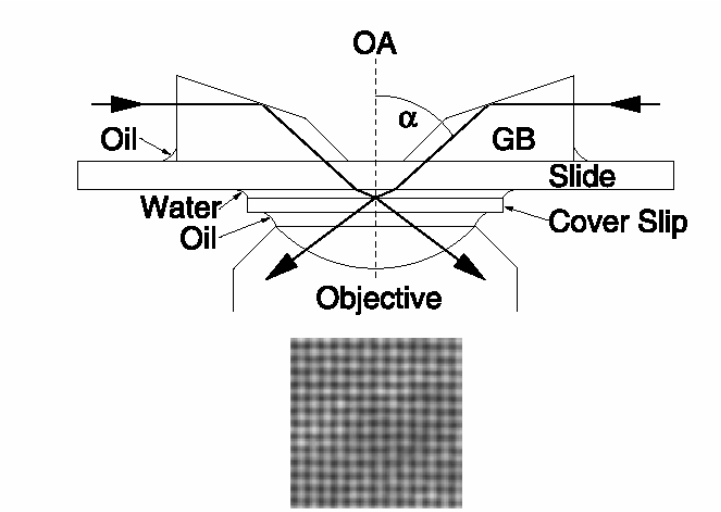


Optical image | SEM image | Topographic image | Restored topographic image

Ref: S.-W. Huang, H.-Y. Mong, and C.-H. Lee, *Microsc. Res. Tech.* **65,** 180 (2004).

Super-resolution by structured illumination

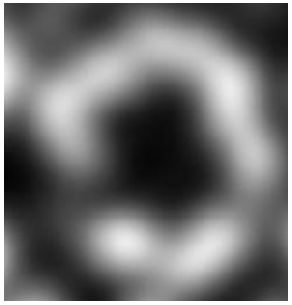
Harmonic excitation light microscopy (HELM)



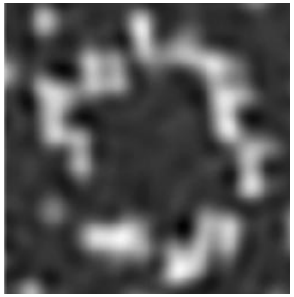
$$\tilde{\theta} = T \times (4A + e^{i\Delta_x}B^+ + e^{-i\Delta_x}B^- + e^{i\Delta_y}C^+ + e^{-i\Delta_y}C^-).$$

Every image is restored from 5 patterned images, without doing iterations.

100-nm fluorescence beads



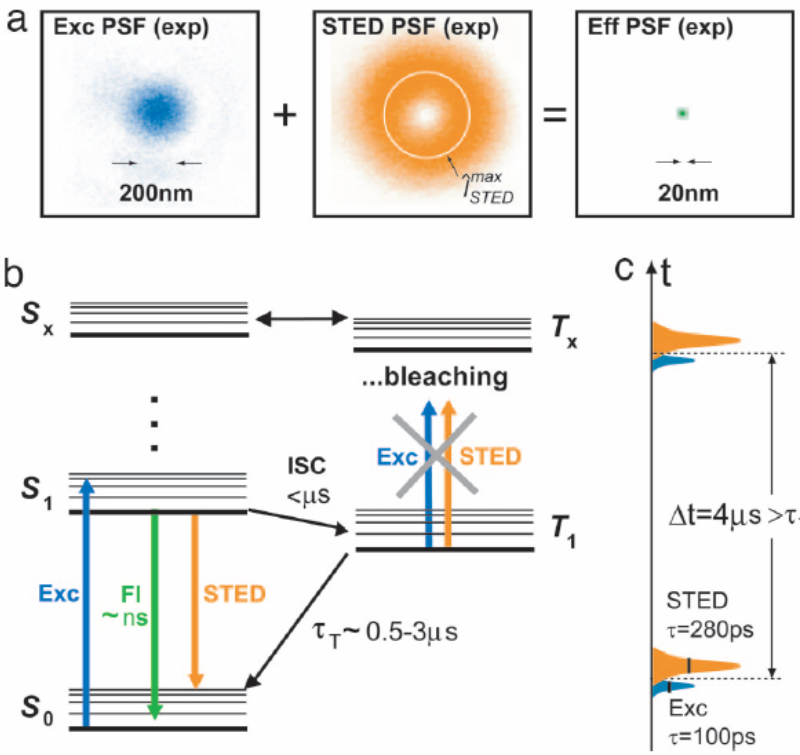
conventional wide-field image



HELM image

Ref: J. T. Frohn, H. F. Knapp, and A. Stemmer, *Proc. Natl. Acad. Sci. USA* **97,** 7232 (2000).

Stimulated emission depletion (STED) microscopy



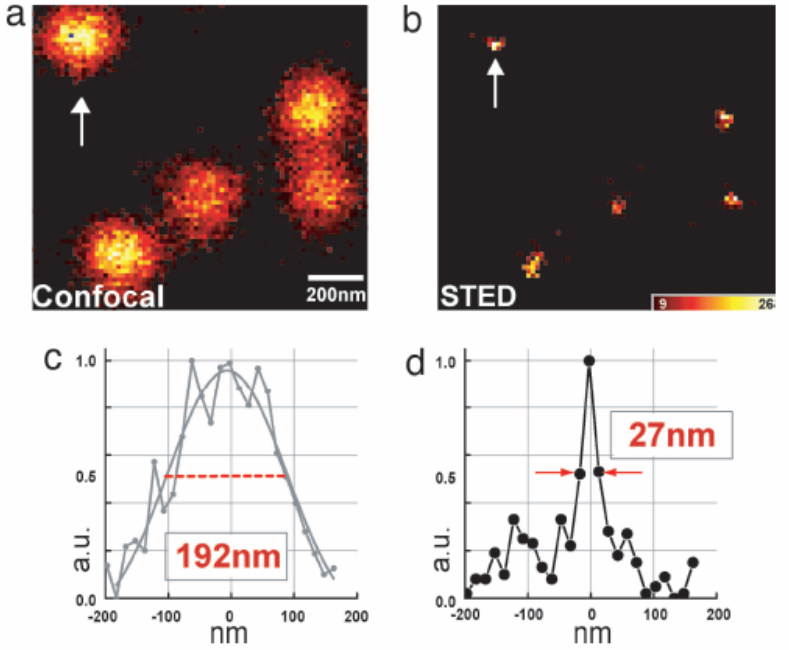
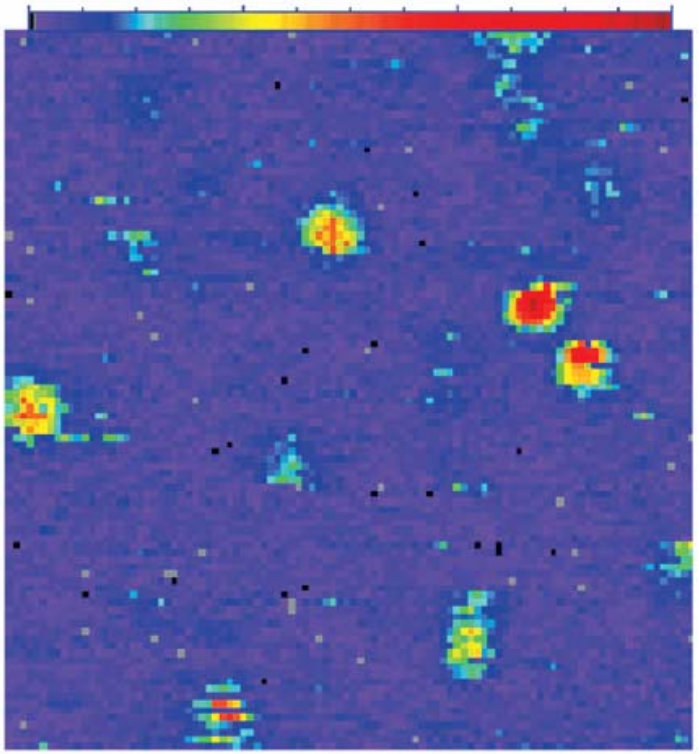


Fig. 3. Synaptotagmin I molecules form distinct spots on endosomes. (*a* and *b*) Whereas confocal microscopy exhibits a 190- to 200-nm diffraction-limited spot per endosome (*a*), STED microscopy recognizes sharp dots of 25–40 nm (*b*), both indicating its resolution as well as the punctated spatial arrangement of synaptotagmin I on the endosome. (*c* and *d*) Corresponding intensity profiles.

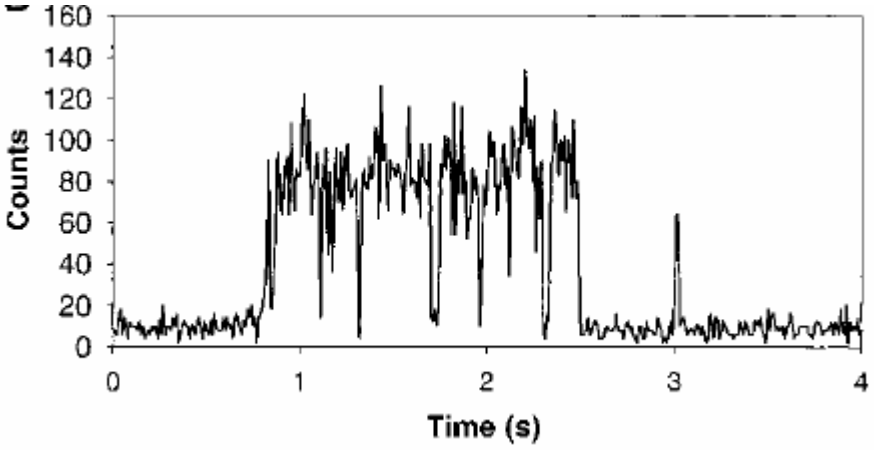
Ref: G. Donnert et al., *Proc. Natl. Acad. Sci. USA* **103,** 11440 (2006).

Molecular Imaging

Confocal microscopy

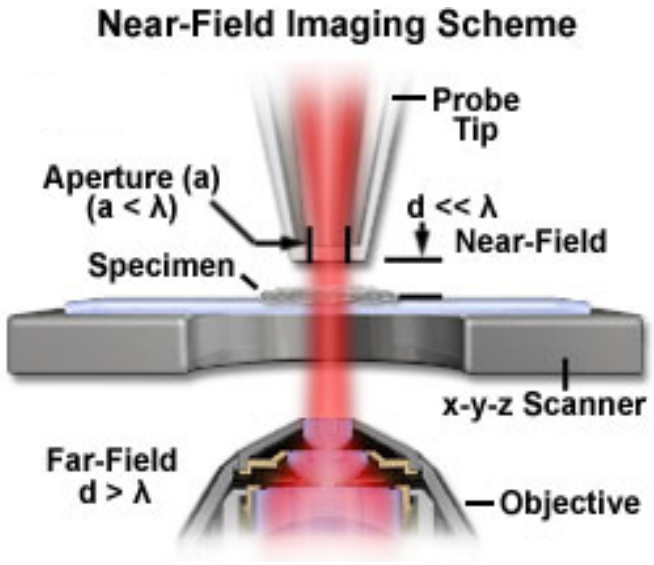


Blinking



Ref: W. E. Moerner and M. Orrit, *Science* **283,** 1670 (1999).

Scanning near-field optical microscopy (SNOM)



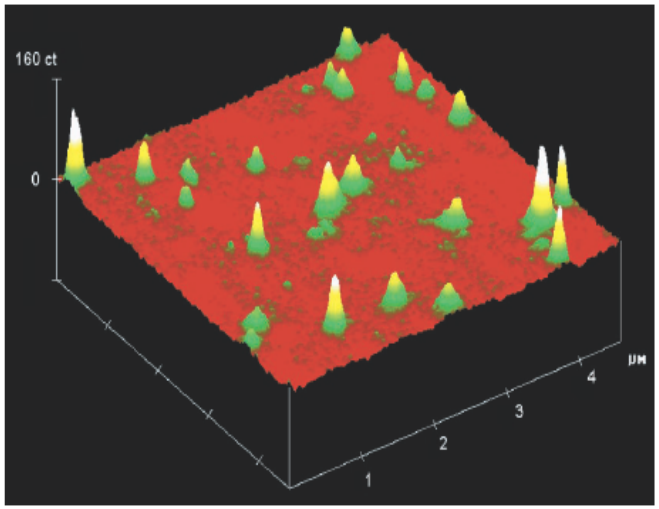
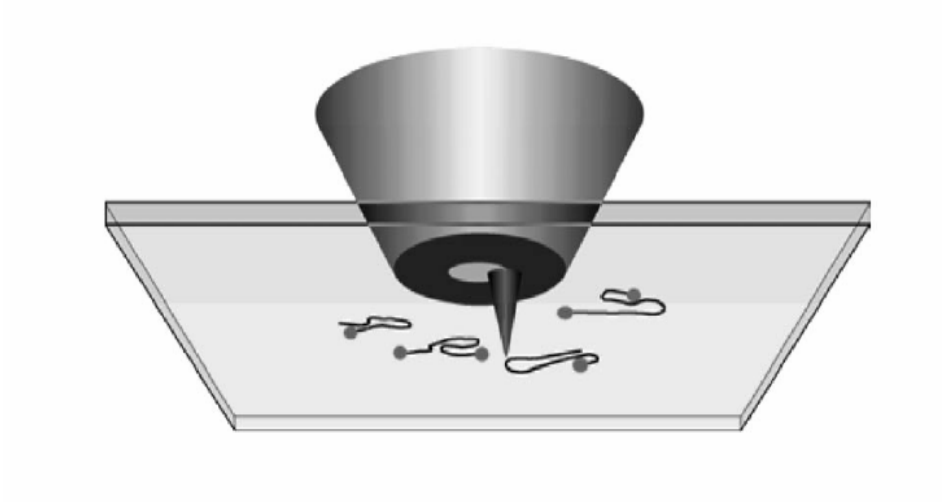


Fig. 3. Near-field fluorescence image (4.5 μm by 4.5 μm) of single oxazine 720 molecules dispersed on the surface of a poly(methylmethacrylate) film. Each subdiffraction peak (full width at half maximum, 100 nm) comes from a single molecule. Reproduced with permission from (*47*).

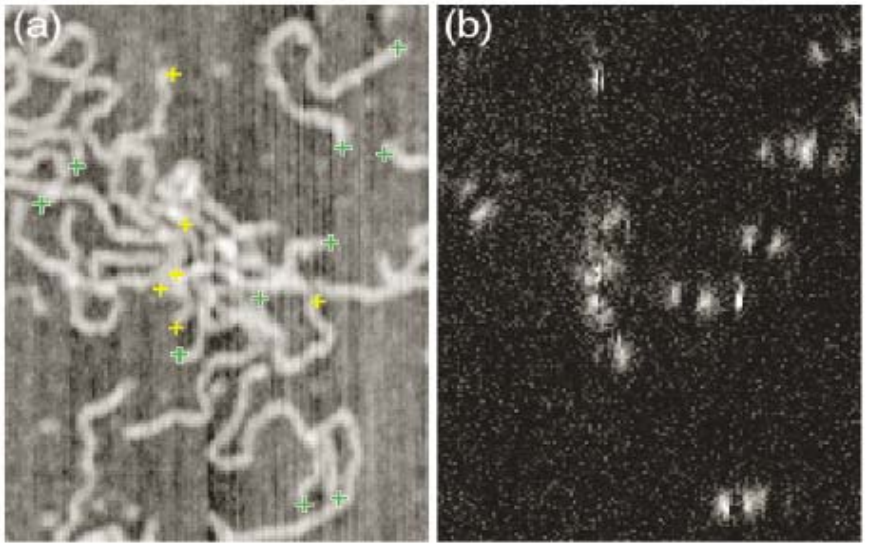
Ref: W. E. Moerner and M. Orrit, *Science* **283,** 1670 (1999).

http://micro.magnet.fsu.edu/

Metal tip near an aperture



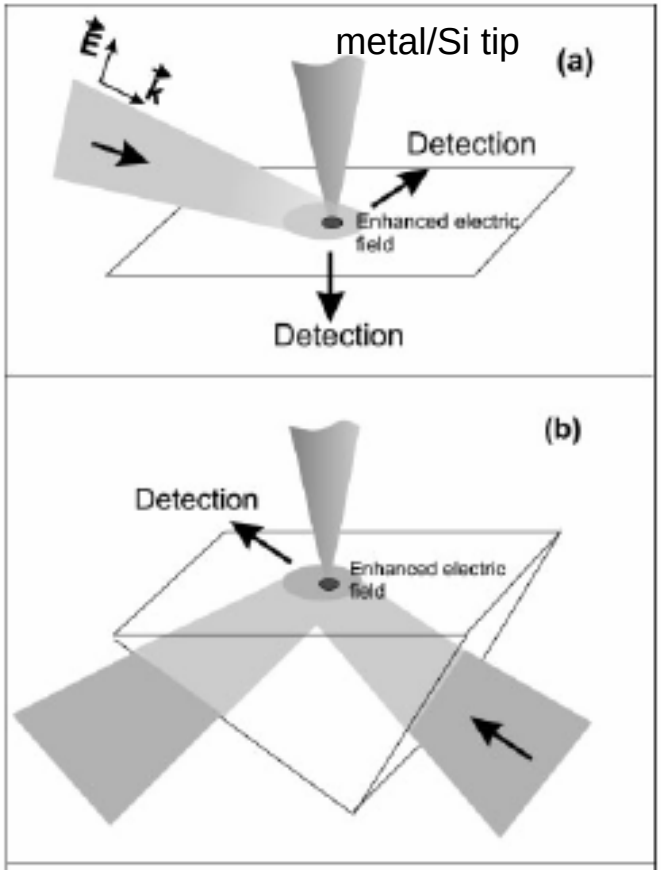
DNA labeled with Cy-3



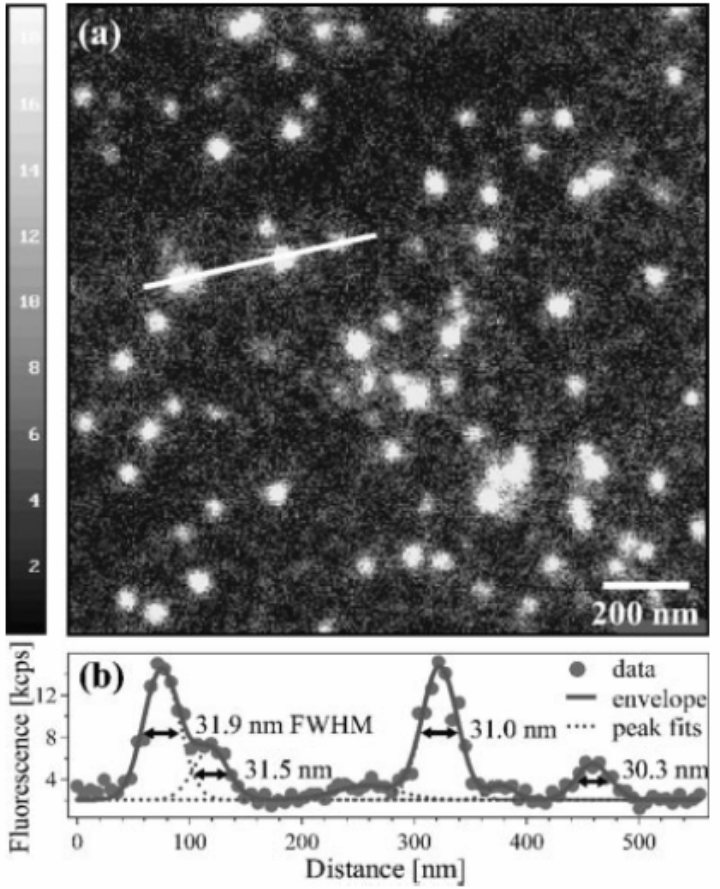
topography fluorescence

Ref: H. G. Frey, S. Witt, K. Felderer, and R. Guckenberger, *Phys. Rev. Lett.* **93,** 200801 (2004).

Apertureless SNOM

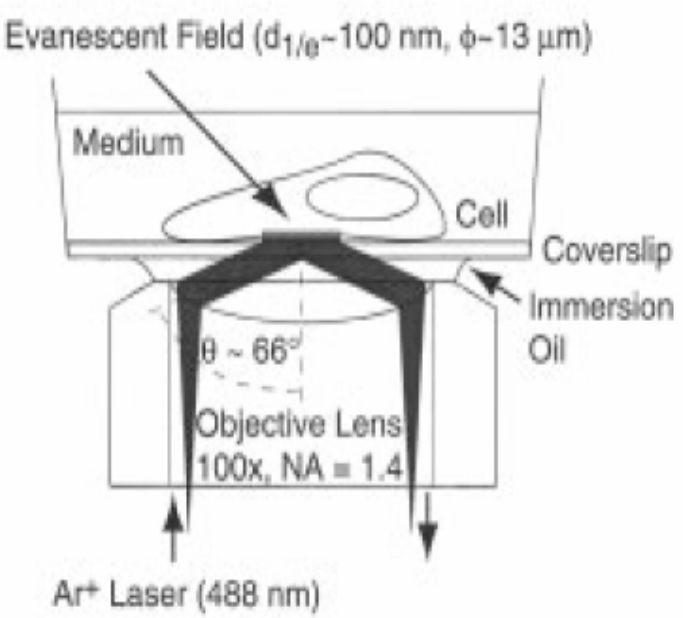


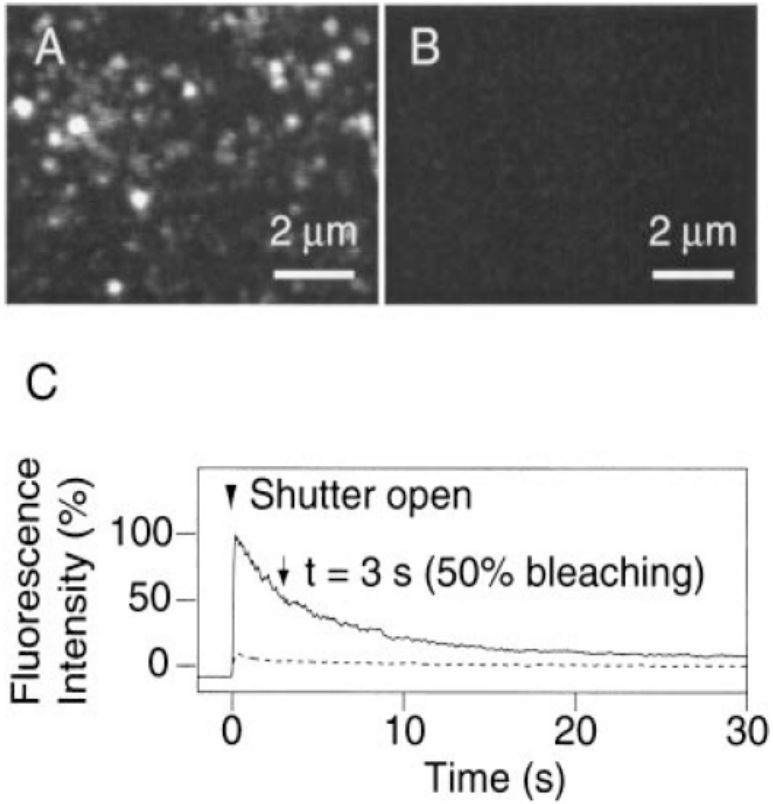
Single-molecule image of AlexaFluor 488



Ref: A. Bouhelier, *Microsc. Res. Tech.* **69,** 563 (2006).

Total-internal reflection fluorescence microscopy (TIRFM)

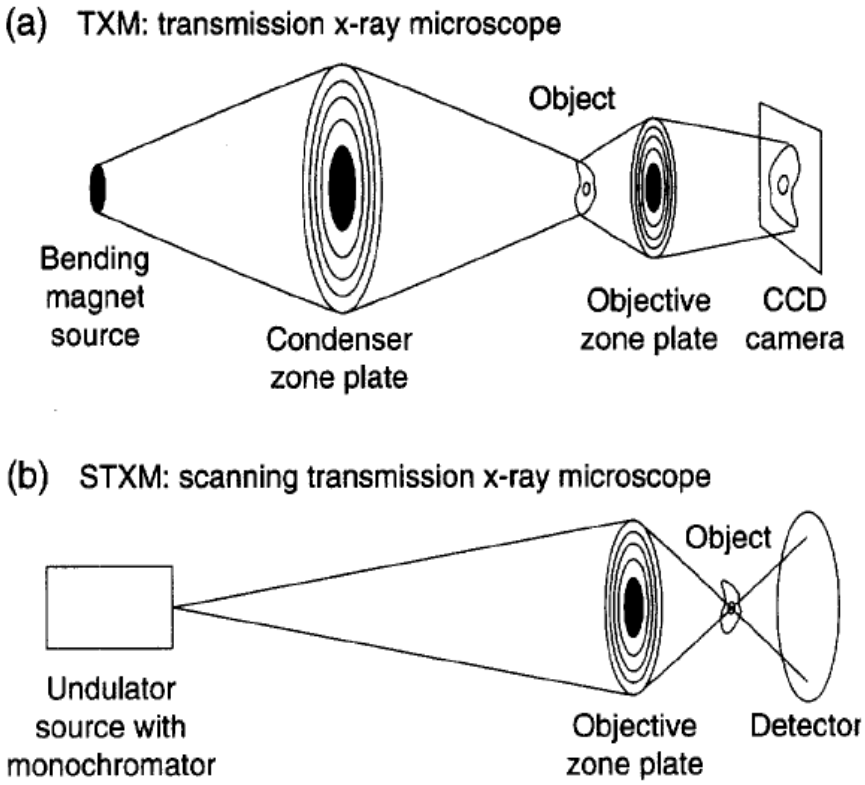




Ref: R. Iino and A. Kusumi, *J. Fluoresc*. **11,** 187 (2001).

X-ray Microscopy

Two types of x-ray microscopy



Ref: C. Jacobsen, *Trends Cell Biol*. **9,** 44 (1999).

The resolution of a zone plate is almost equal to the smallest (outermost) zone width. With current e-beam lithography, the smallest zone width can be ~15 nm.

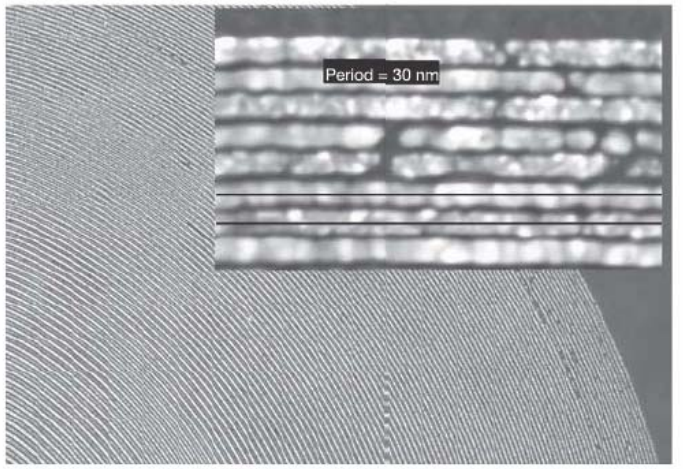
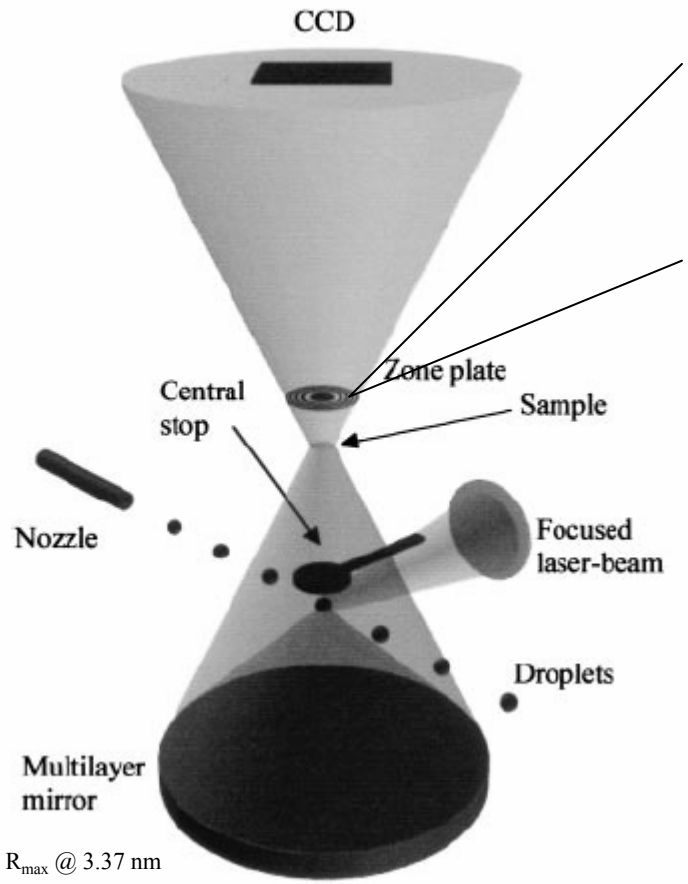
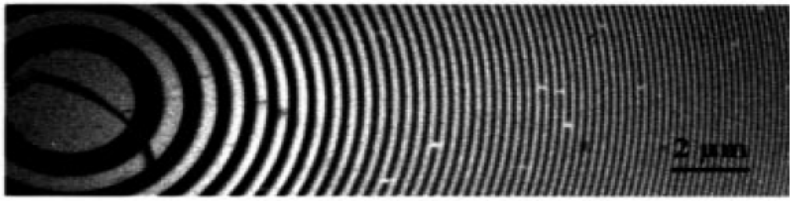


Figure 3 | Scanning electron micrograph of a zone plate with 15 nm outermost zone. Shown in the inset is a more detailed view of the outermost zones. The zonal period, as indicated by the two black lines, is measured to be 30 nm. The zone placement accuracy is measured to be 1.7 nm.

Ref: W. Chao et al, *Nature* **435,** 1210 (2005).

Compact soft x-ray microscope





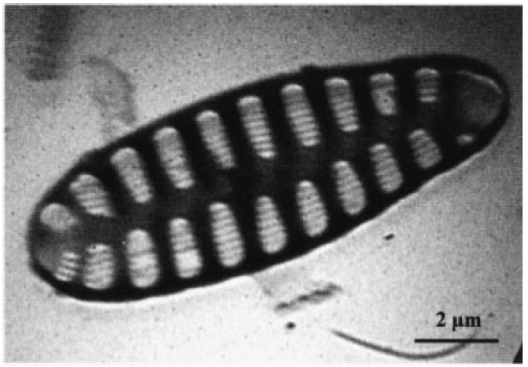
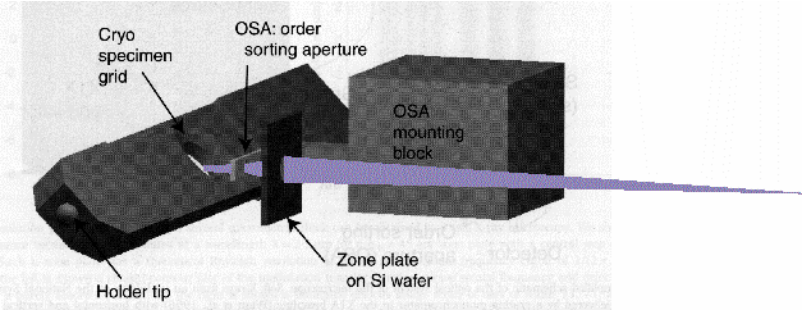


Image of diatom

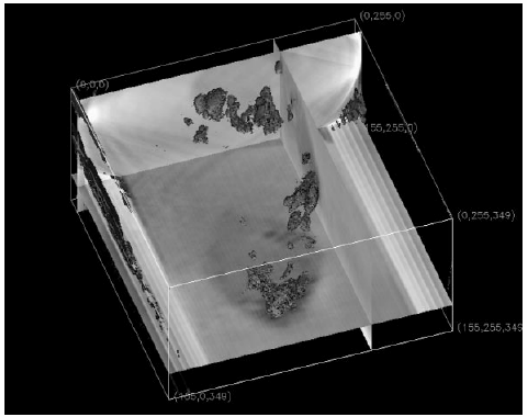
Resolution ~ 100 nm

Ref: M. Berglund et al., *J. Microsc*. **197,** 268 (2000).

X-ray microtomography



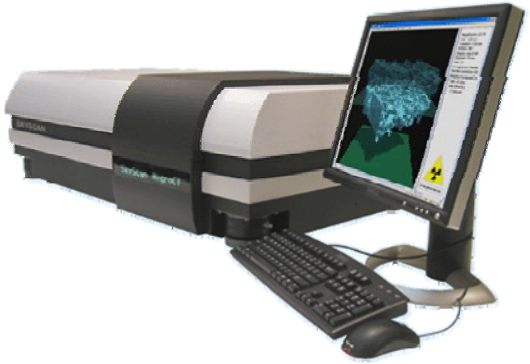
Vesicles inside a cell



Resolution ~ 250 nm

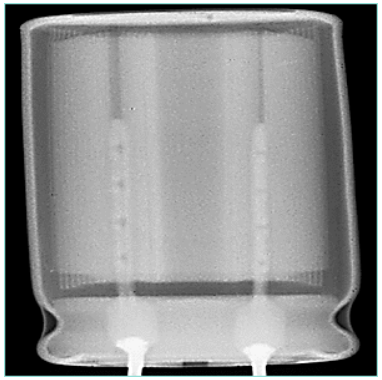
Ref: Y.Wang et al., *J. Microsc*. **197,** 80 (2000).

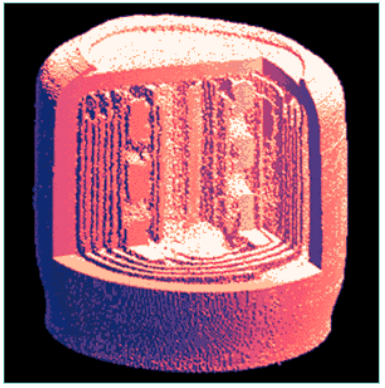
Commercial product available



http://www.microphotonics.com

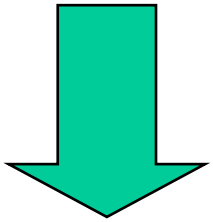
Capacitor





Resolution < 10 μm

Seeing is believing.



More than just watching.