Confocal Microscopy and Related Techniques

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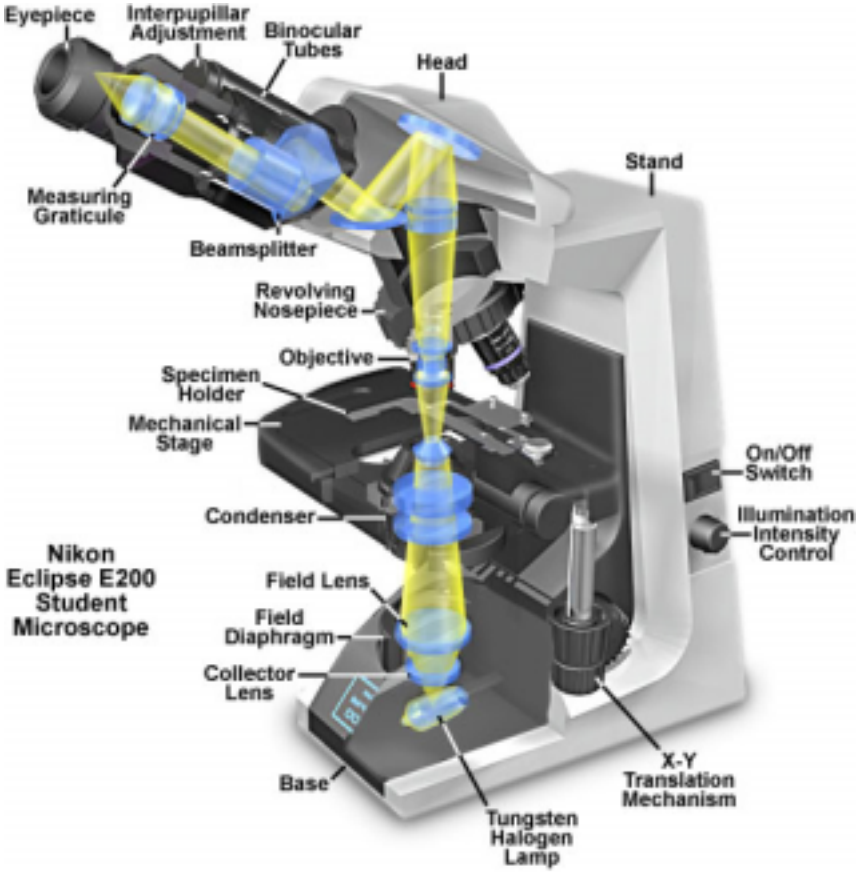
Imaging Microscopy

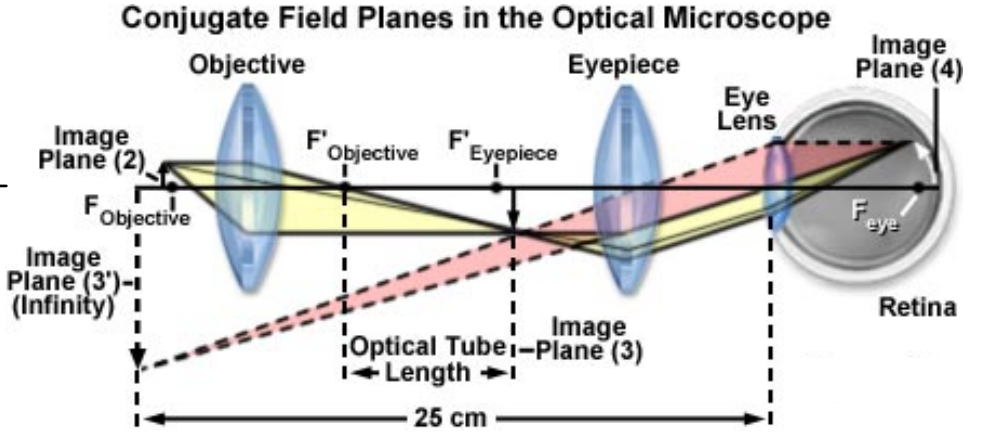
Light path in an optical imaging microscope

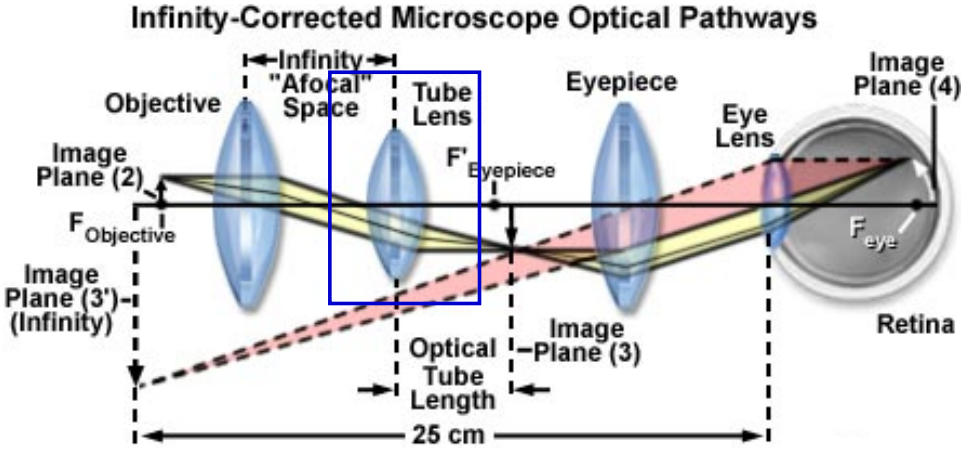


Images are from http://micro.magnet.fsu.edu/

Image formation

$$\frac{1}{d_o} + \frac{1}{d_i} = \frac{1}{f}$$





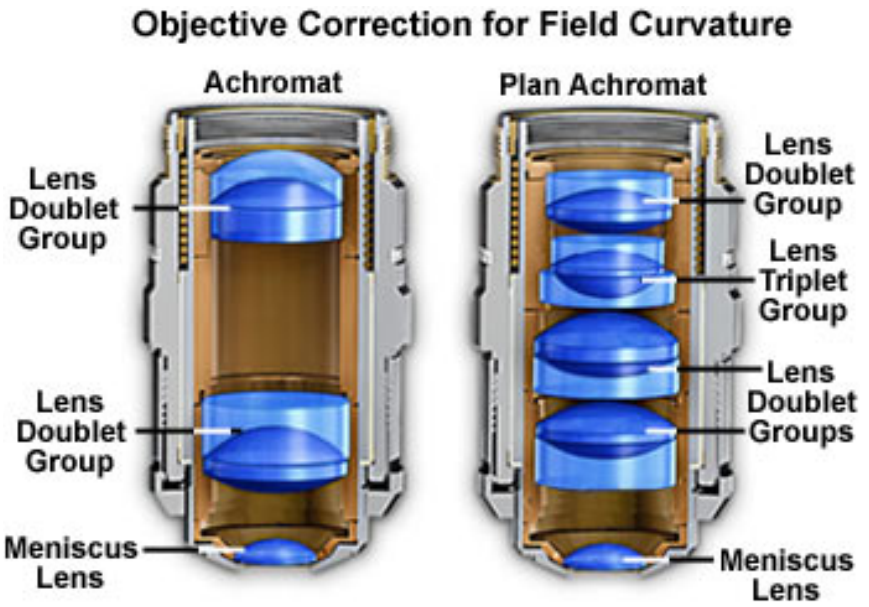
Images are from http://micro.magnet.fsu.edu/

Specifications of an objective

Figure 1

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

Achromatic



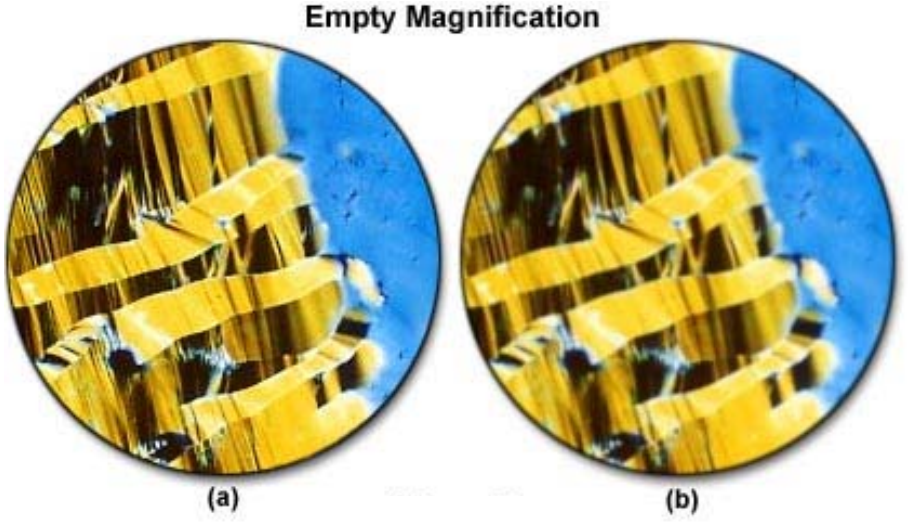
Images are from http://micro.magnet.fsu.edu/

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/

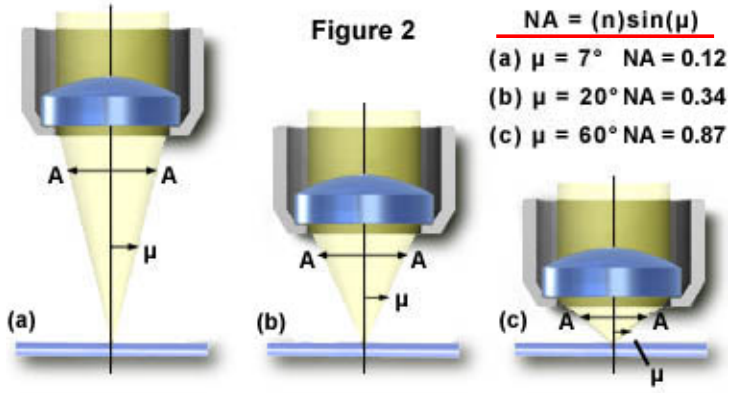
Resolution



Without resolution, magnified images cannot provide detailed information.

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

Numerical aperture



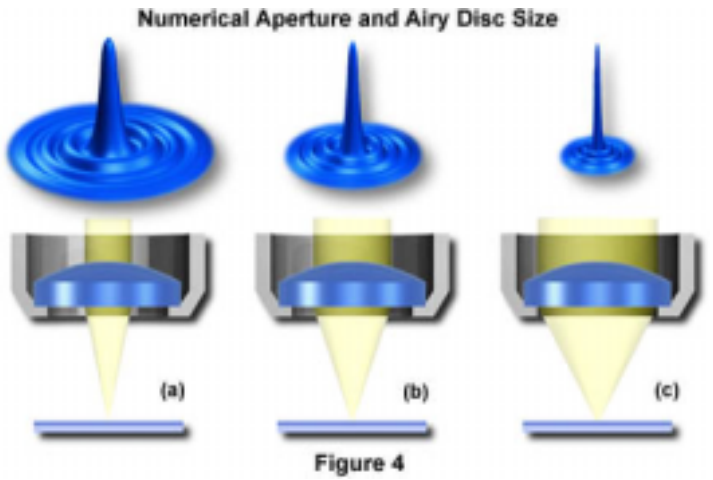


Figure 4

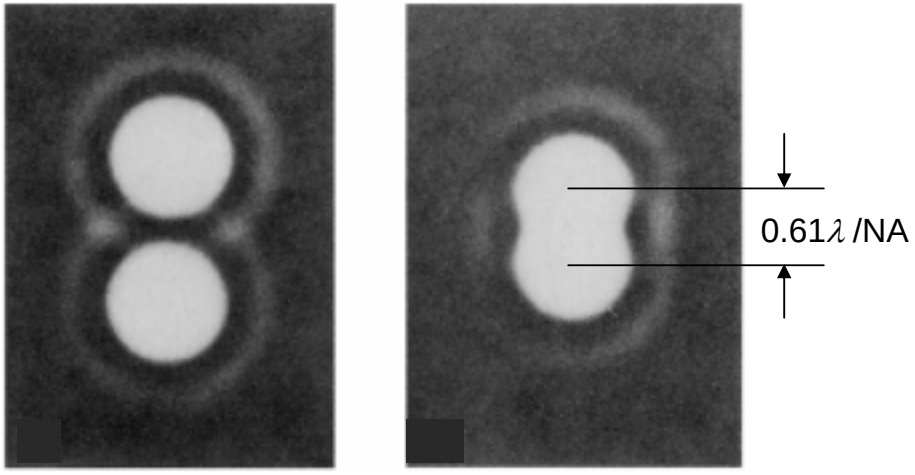
Images are from http://micro.magnet.fsu.edu/

Numerical aperture and resolution

Rayleigh criterion:

resolution ~ 0.61λ /NA

For dry samples, NA < 1.0

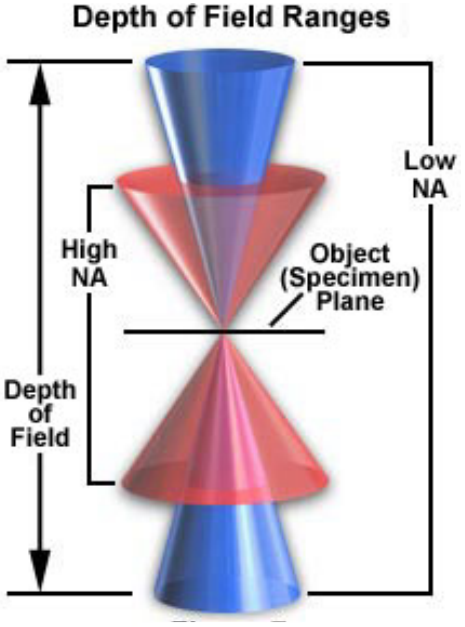


clearly resolved

resolution limit

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

Depth of field

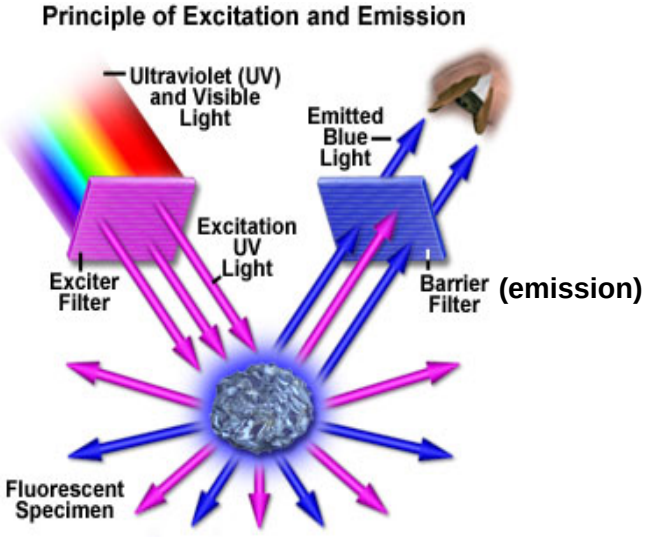


$$d = \lambda n/(\mathrm{NA})^2$$

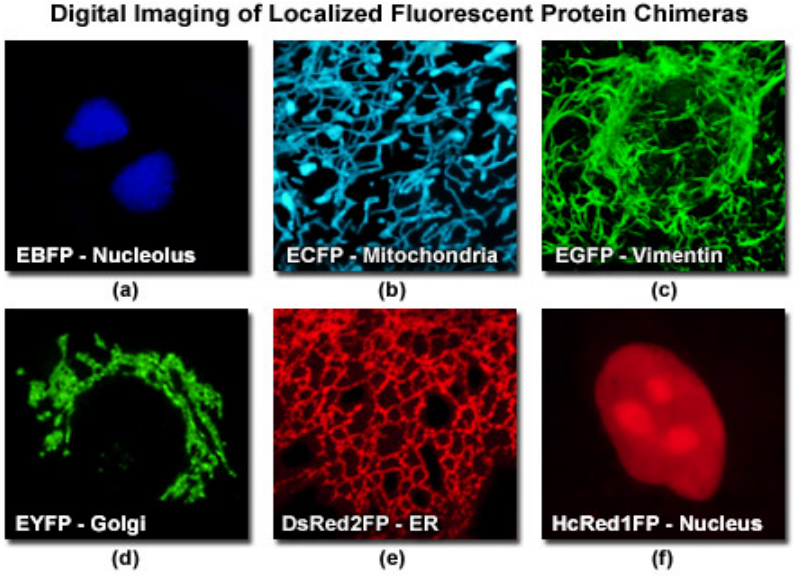
Images are from http://micro.magnet.fsu.edu/

Contrast

Fluorescence microscopy



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



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Fluorescence microscopy

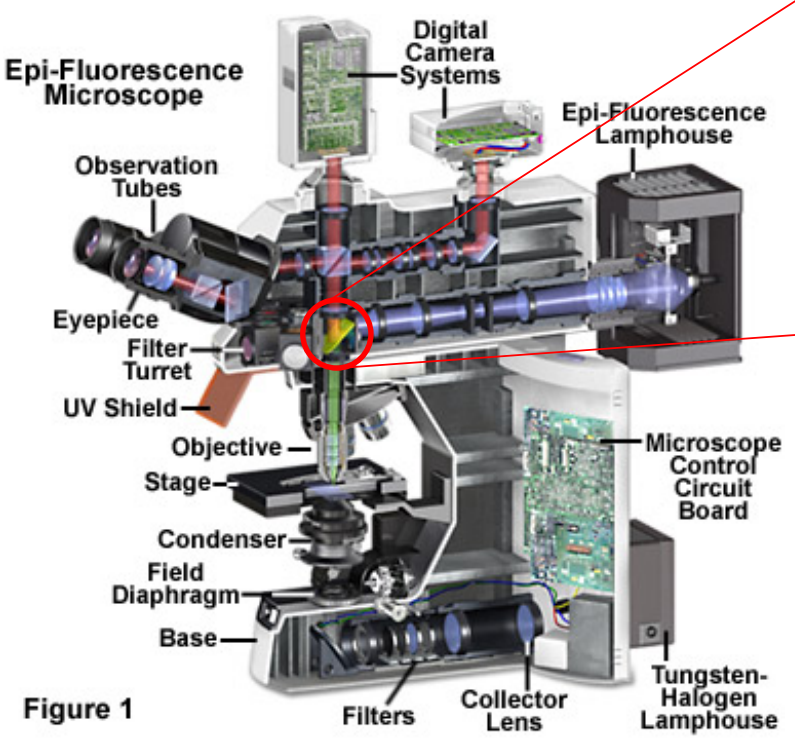


Figure 1

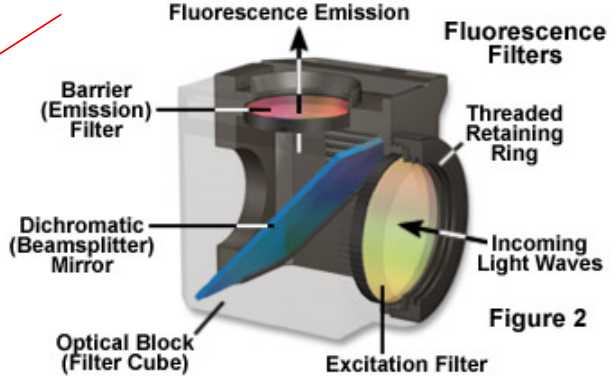


Figure 2

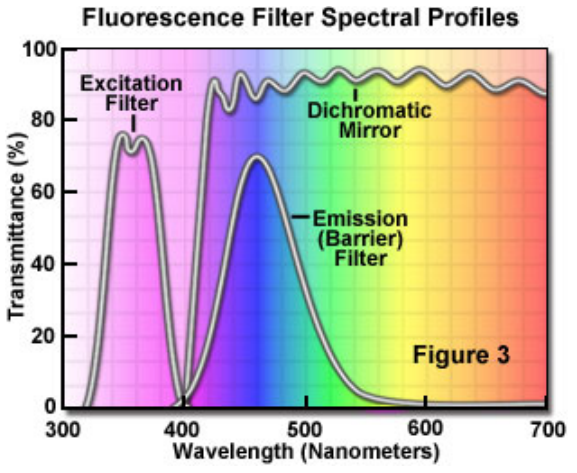
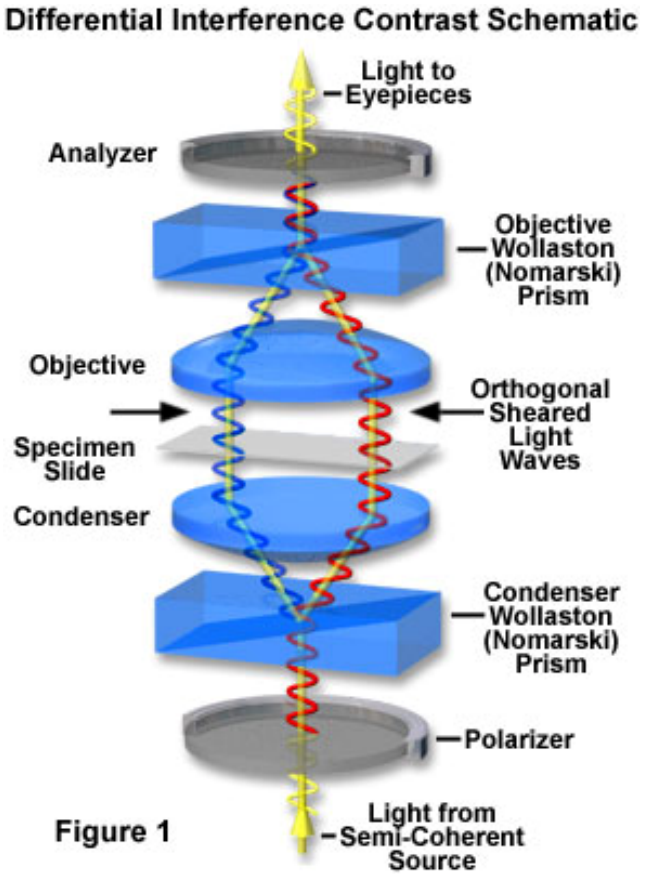


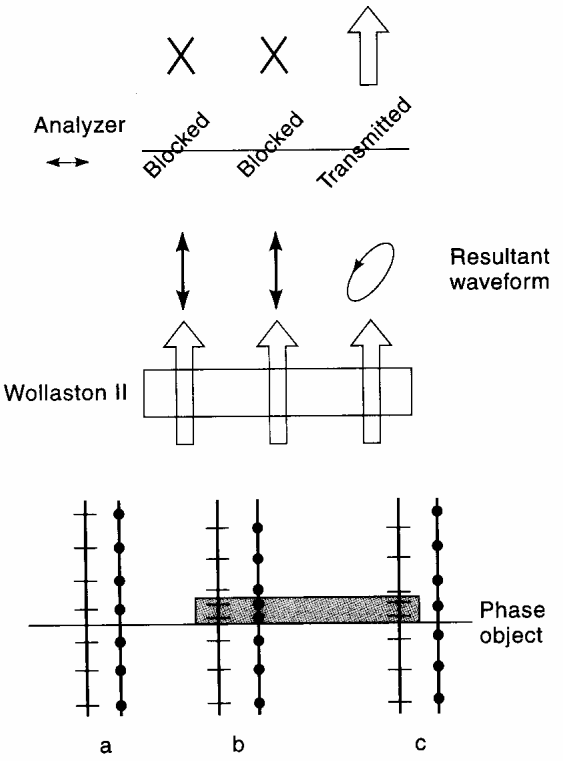
Figure 3

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

Differential interference contrast (DIC)



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



Orientation in DIC

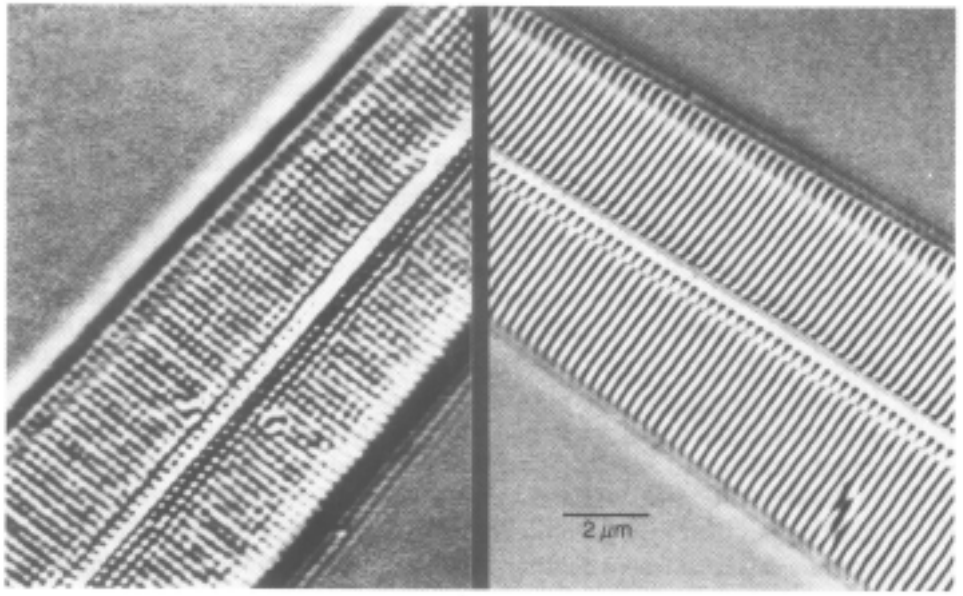
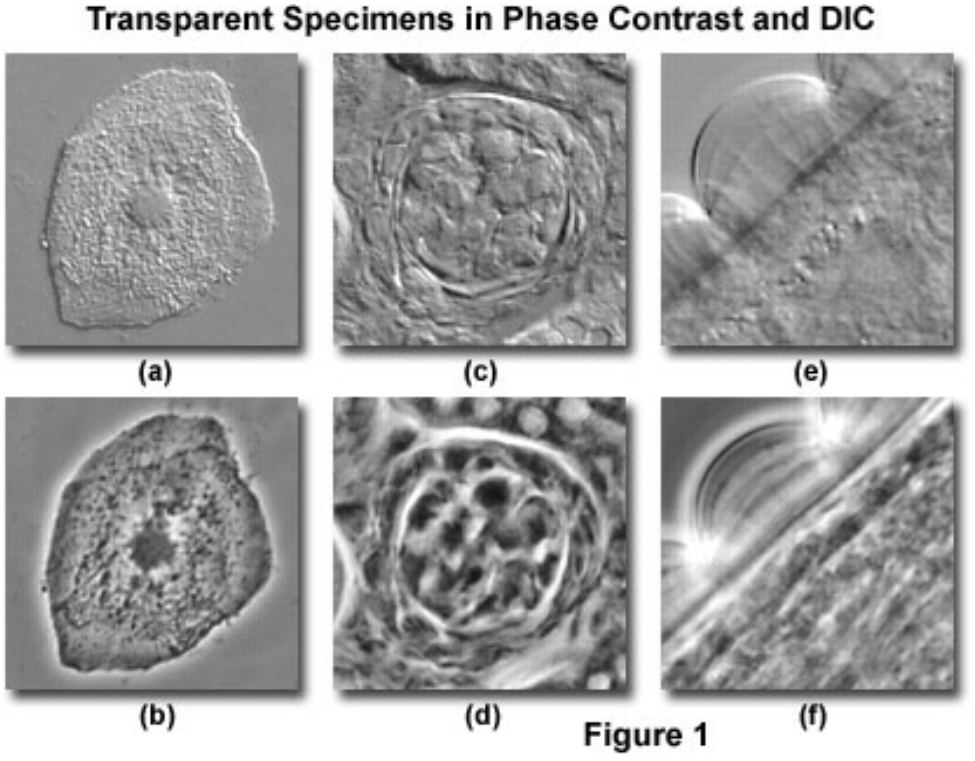


Figure 10-8

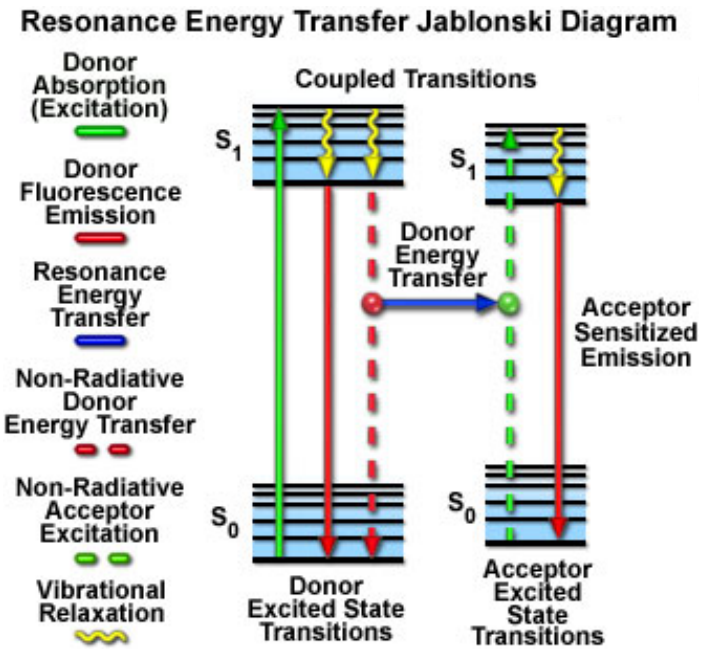
Effect of specimen orientation in DIC microscopy. Since the shear axis is fixed in DIC optics, the specimen itself must be rotated to highlight different features. Notice the differential emphasis of pores and striae in the shell of a diatom, *Amphipleura*, using video-enhanced DIC optics.

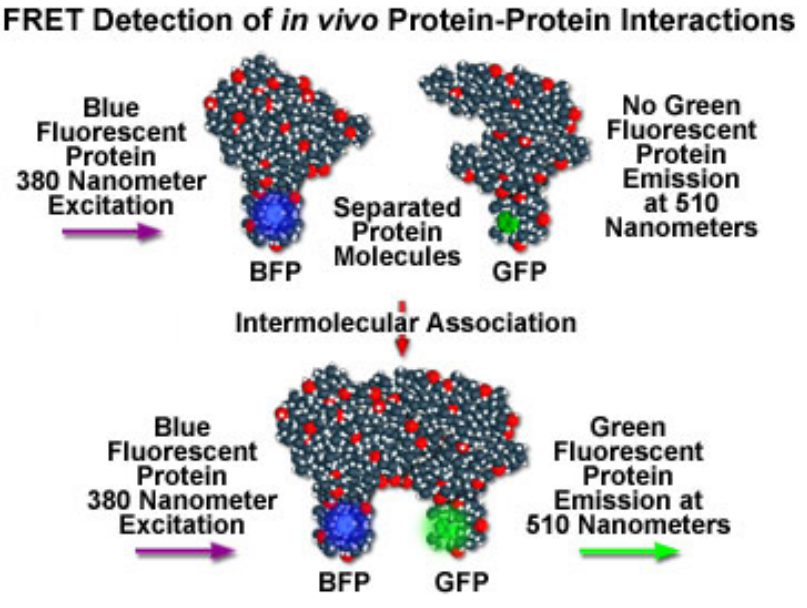
Ref: D. B. Murphy, *Fundamentals of Light Microscopy and Electronic Imaging* (Wiley-Liss, New York, 2001).

Comparison between phase contrast and DIC



Fluorescence resonance energy transfer (FRET) Microscopy

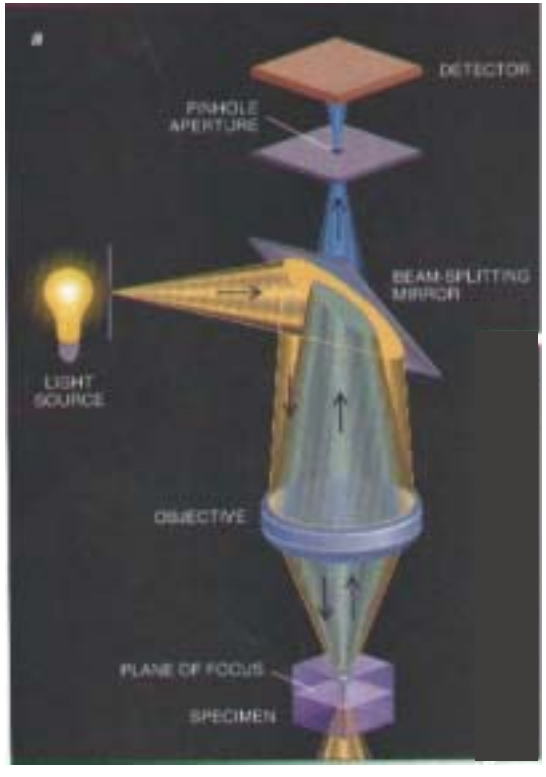




Images are from http://micro.magnet.fsu.edu/

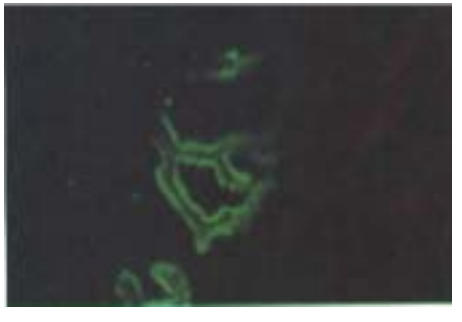
Confocal Microscopy

Confocal microscopy



Conventional fluorescence microscopy

Confocal microscopy



Images are from *Scientific American*, August 1994, p. 34.

Confocal images

Improved depth resolution

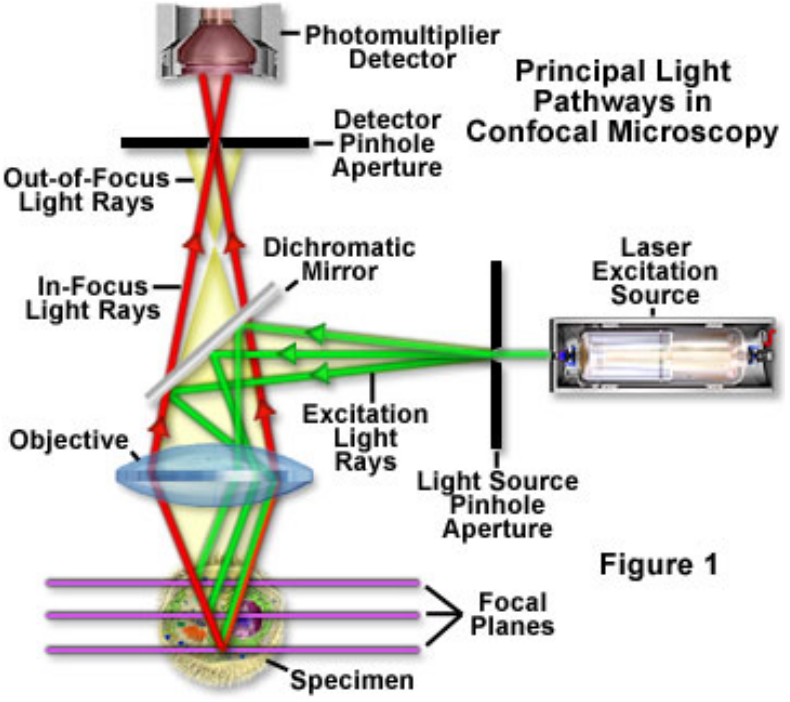
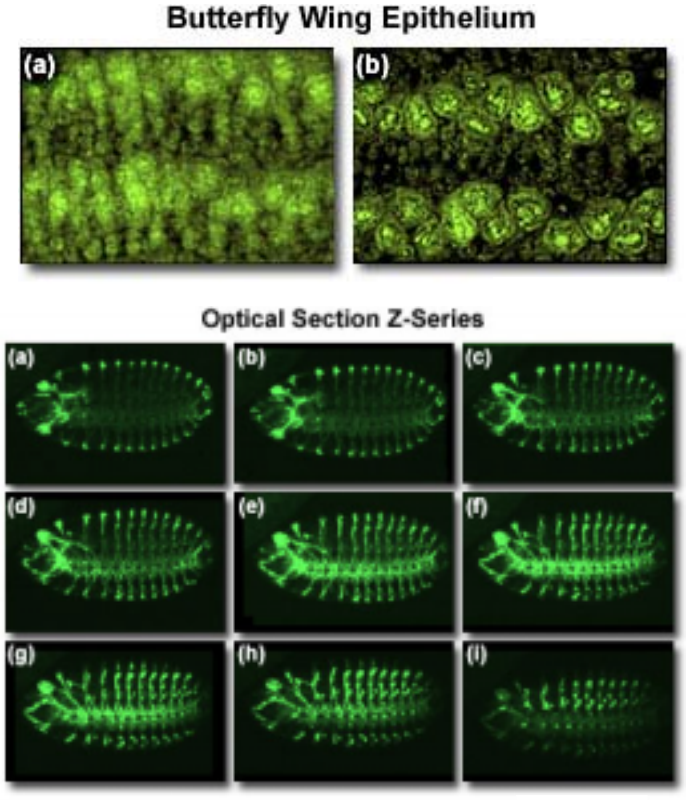
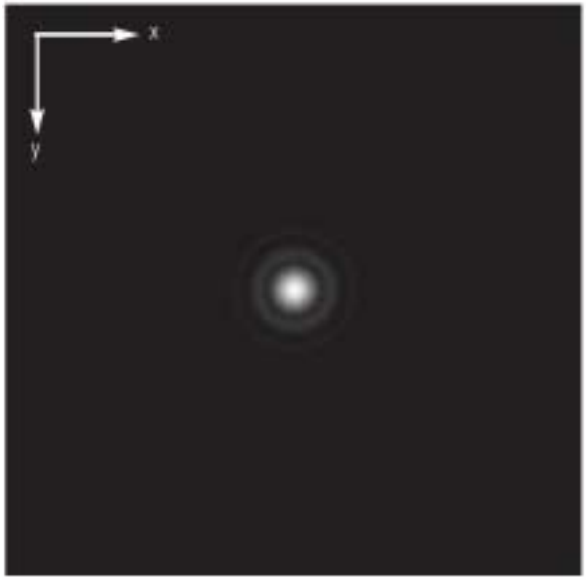


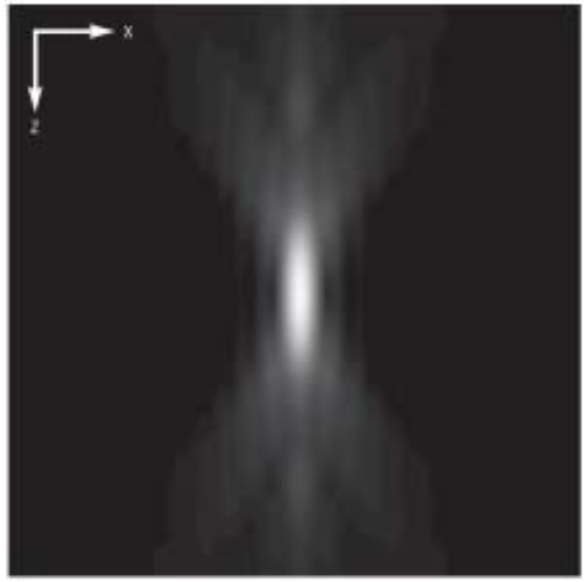
Figure 1



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

Three-dimensional point-spread function





Lateral:

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

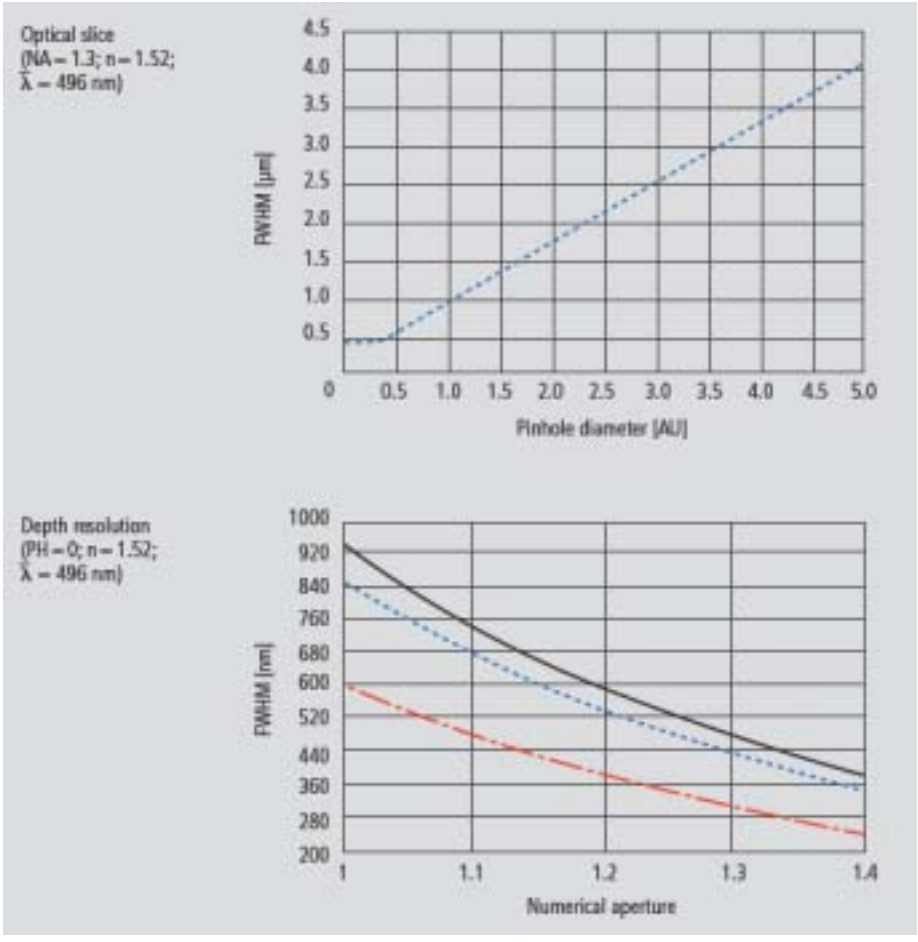
Axial:

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

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

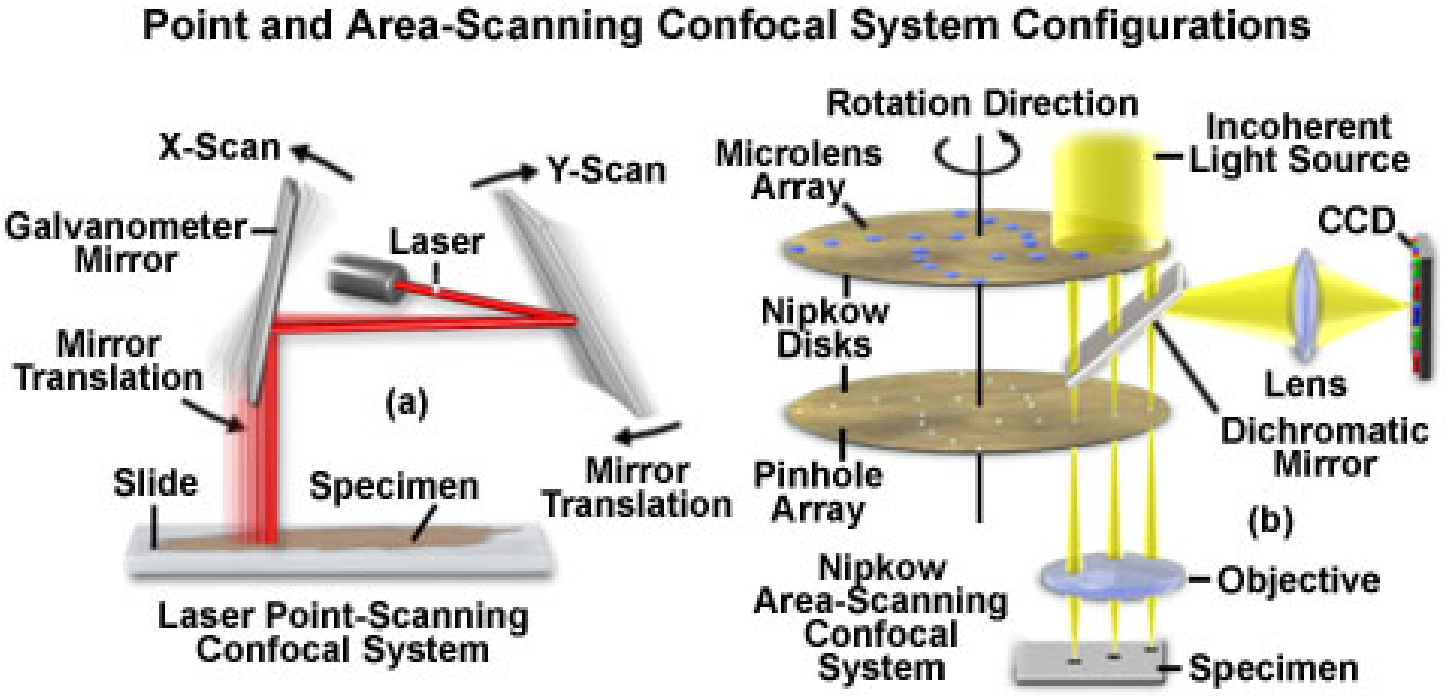
Ref: Carl Zeiss, *Confocal Laser Scanning Microscopy*

Effect of the pinhole diameter and NA



Ref: Carl Zeiss, *Confocal Laser Scanning Microscopy*

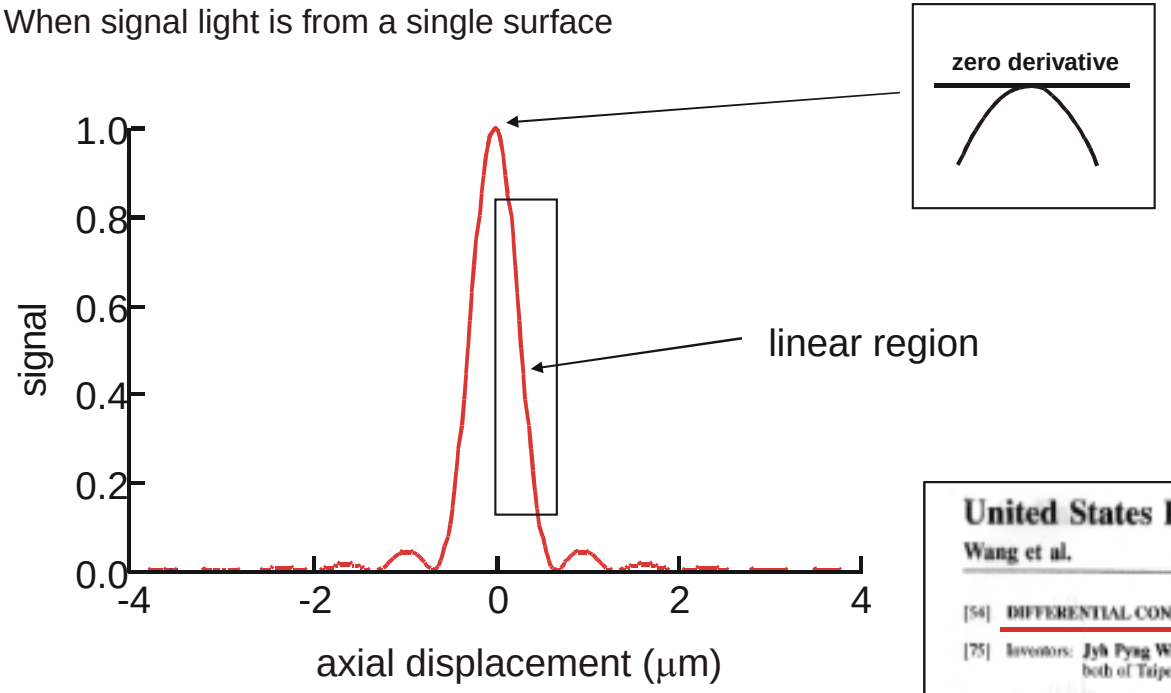
Scanning system



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

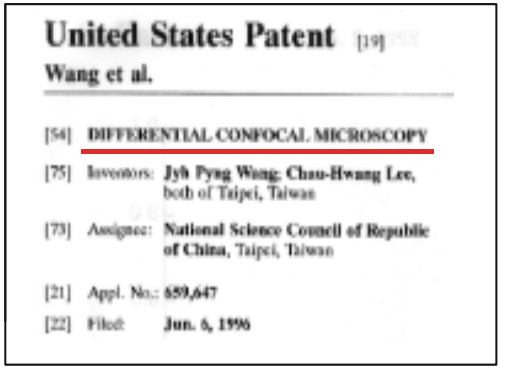
Nanometer depth resolution: differential confocal microscopy

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

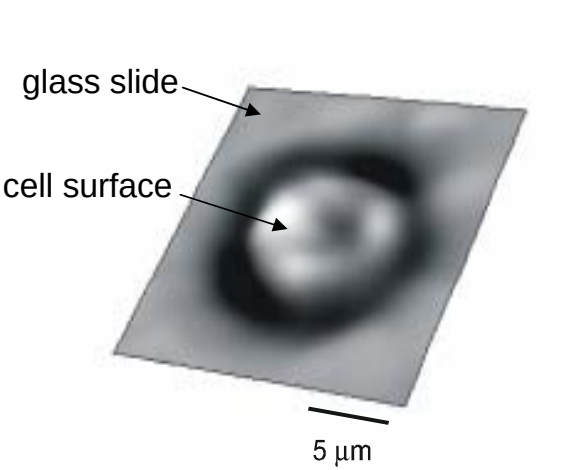
[21] Appl. No.: 659,647

[22] Filed: Jun. 6, 1996

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

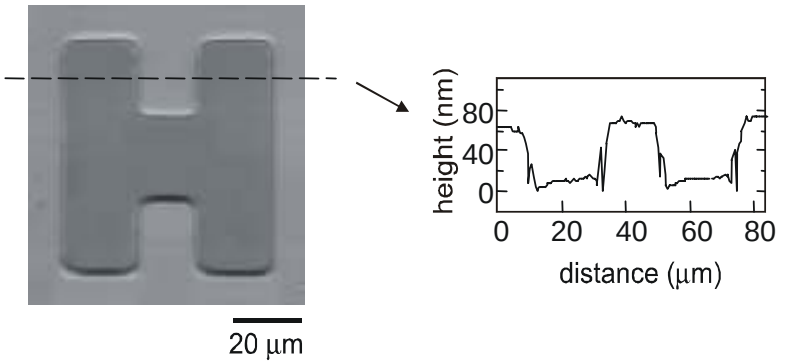
Sample images of DCM

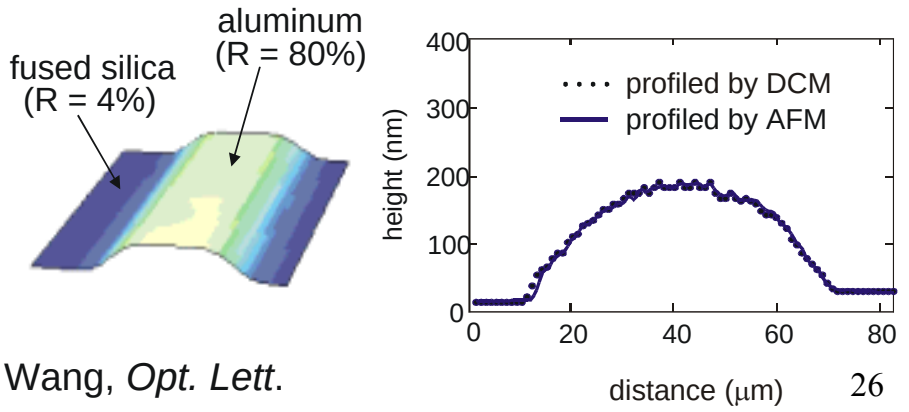
human red blood cell



The center recess is 570 nm.

70-nm deep H-trench on InGaAs

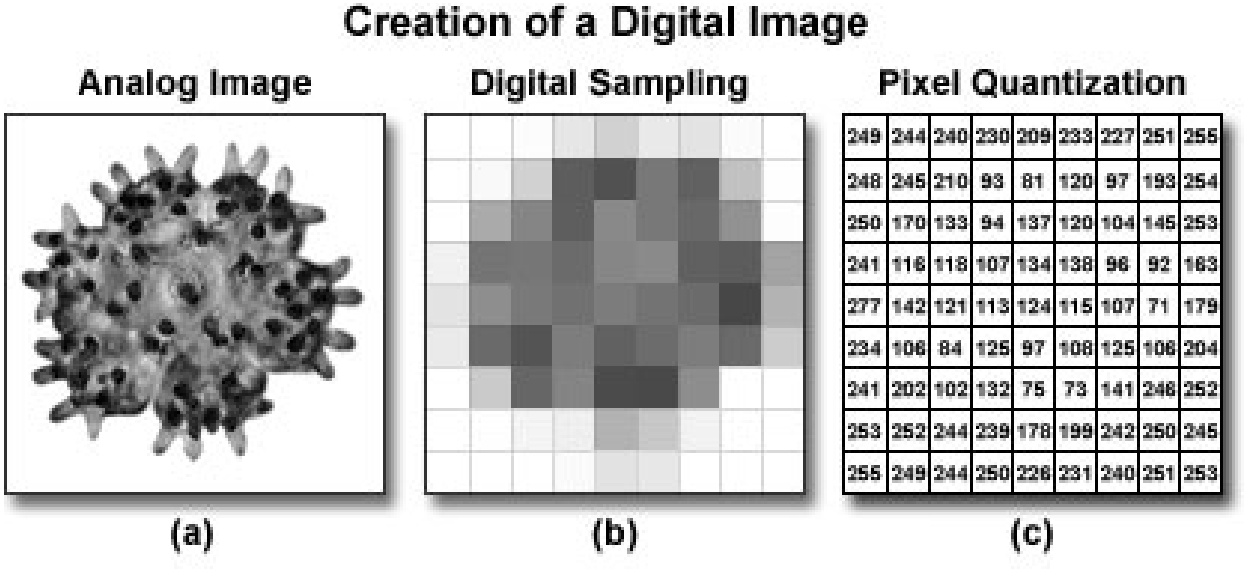




Ref: C.-W. Tsai, C.-H. Lee, and J. Wang, *Opt. Lett*. **24,** 1732 (1999).

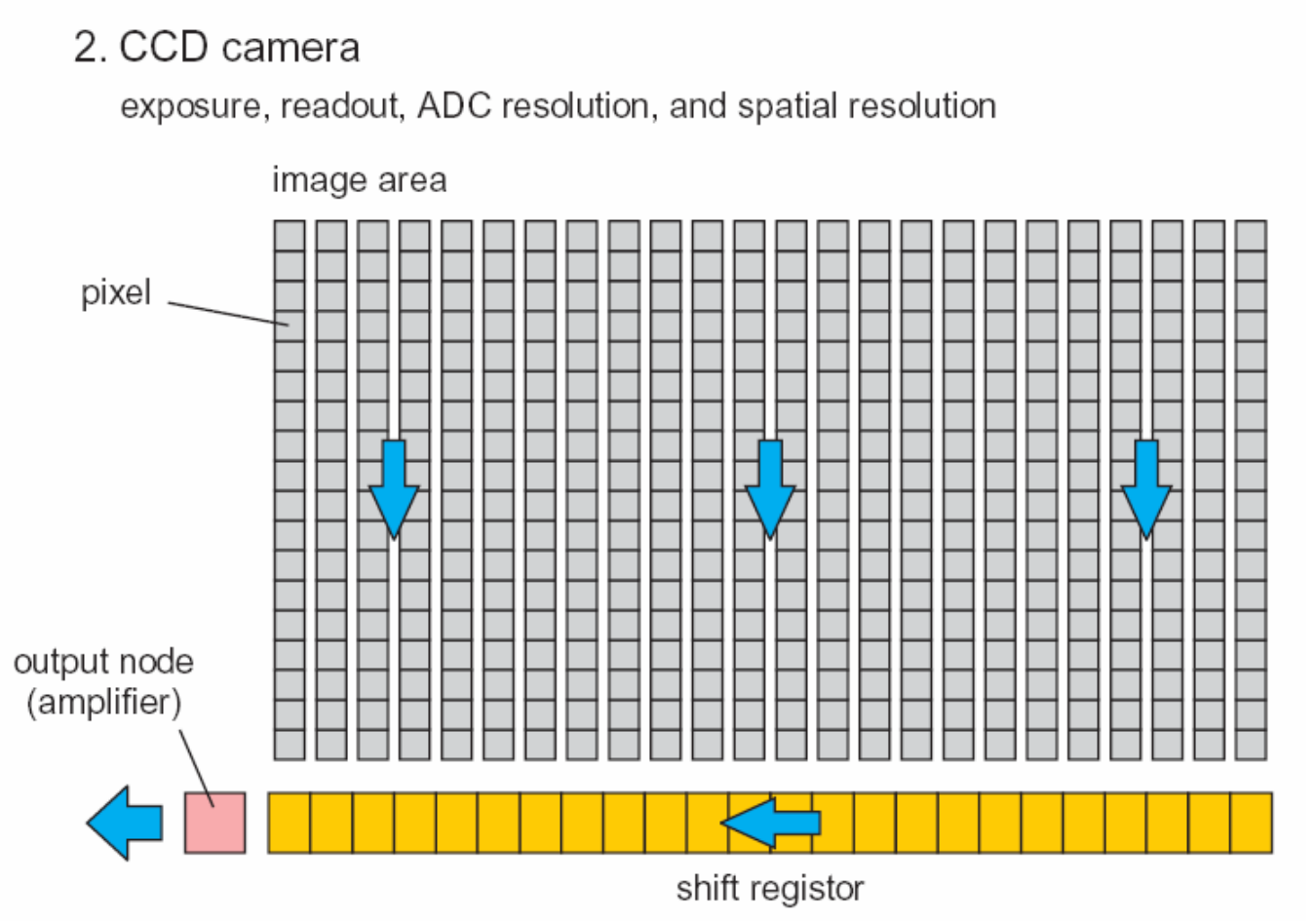
Digital Images

A digitized image



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

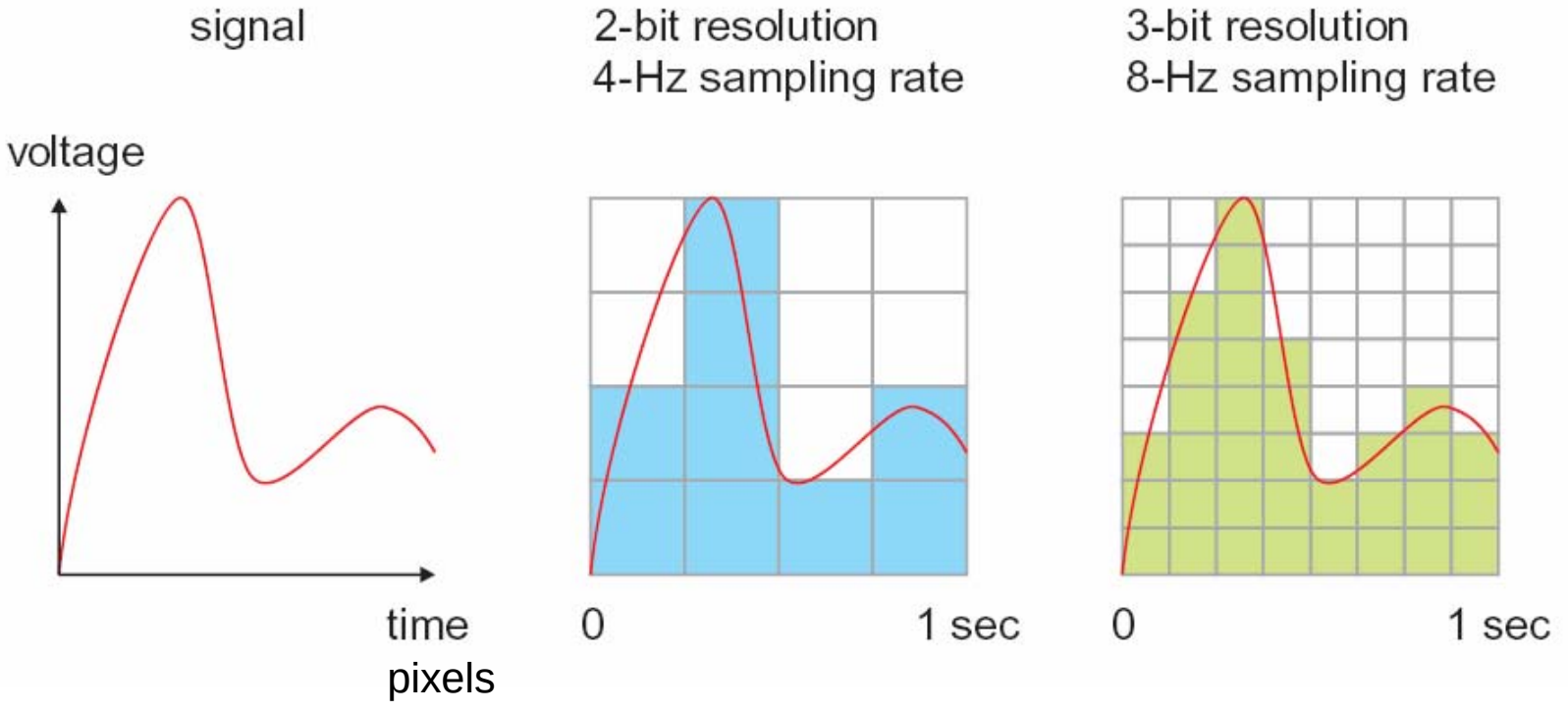
Charge-coupled device (CCD)



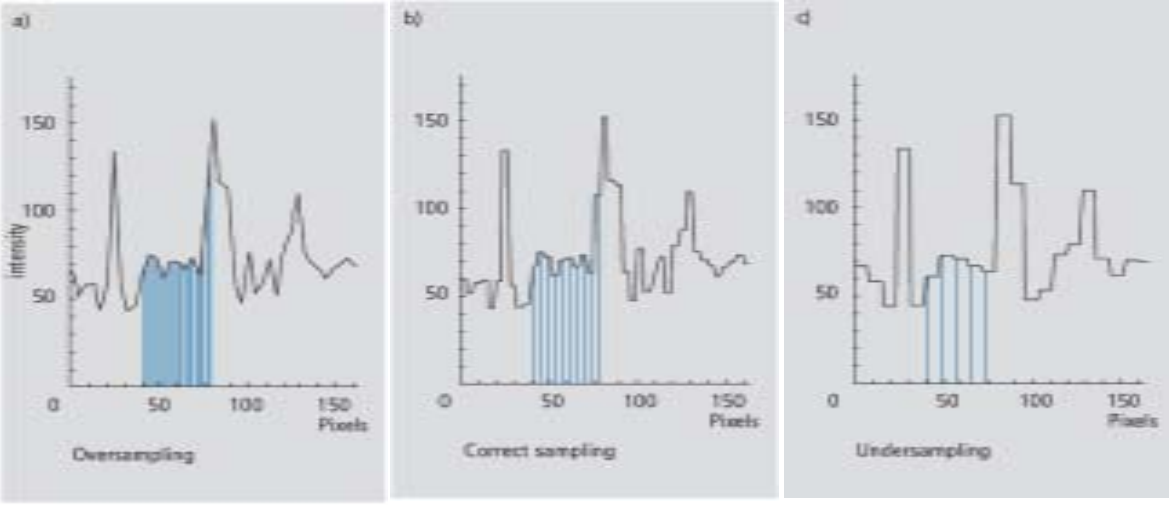
Specifications of CCD cameras

- 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...)

Signal digitization



Sufficient sampling

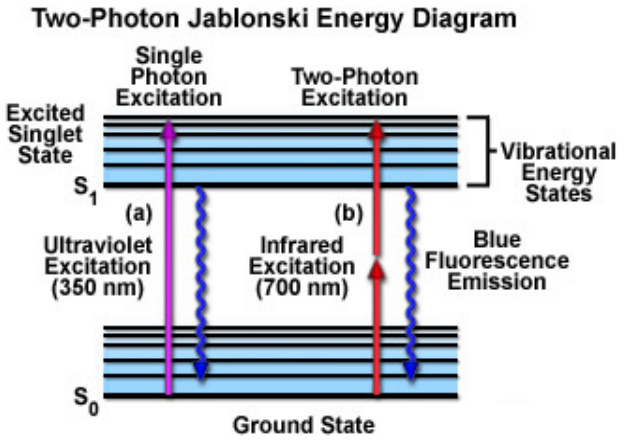


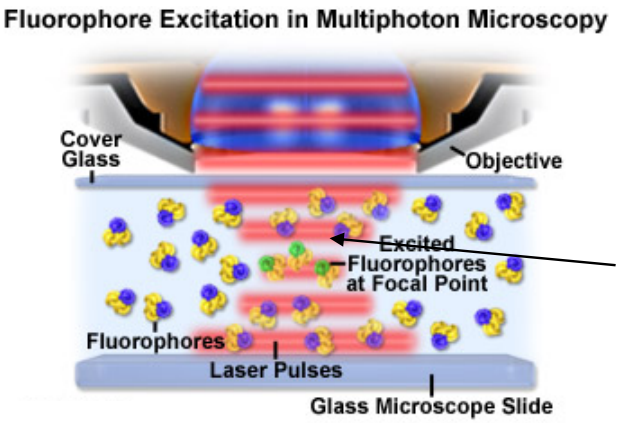
Sampling frequency $\geq$ 2 x signal bandwidth
For CCD cameras, the pixel size on the image should be smaller than half the optical resolution.

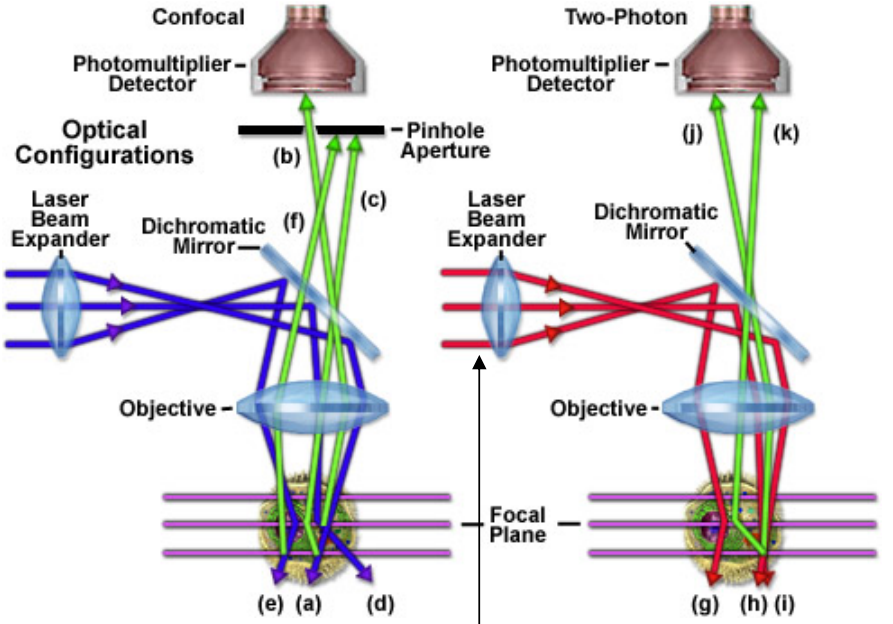
From Carl Zeiss, *Confocal Laser Scanning Microscopy*

Related Technologies

Multiphoton Microscopy





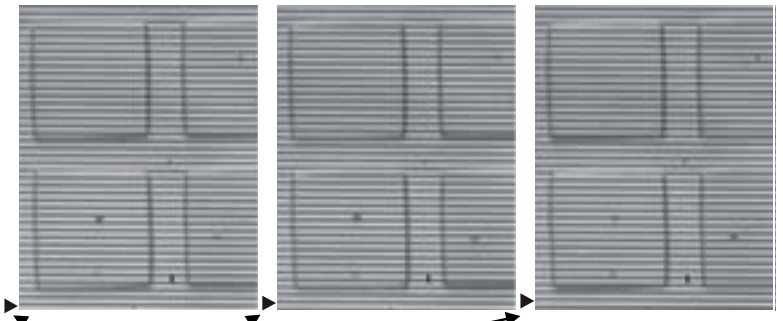


IR light can penetrate deeper into the tissues.

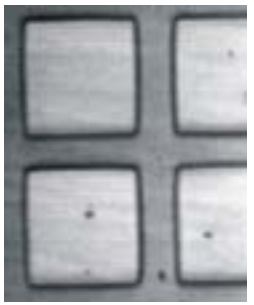
Femtosecond laser pulses are required to perform two-photon excitation.

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

Widefield optically sectioning microscopy



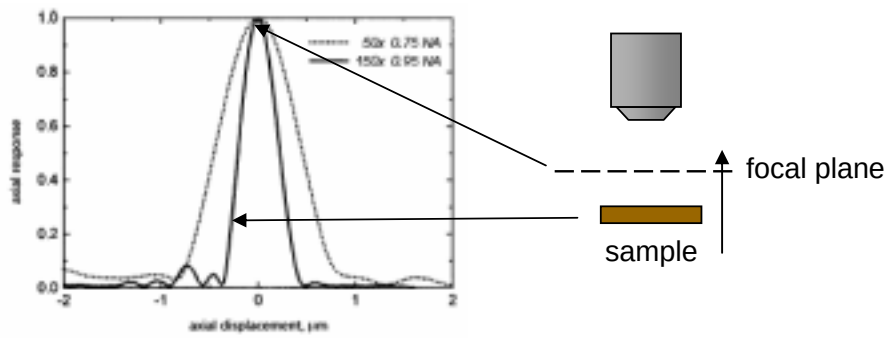
spatial phase shift: 2π/3



Homodyne detection principle

$$I_p = \sqrt{(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2}$$

Axial response curve:

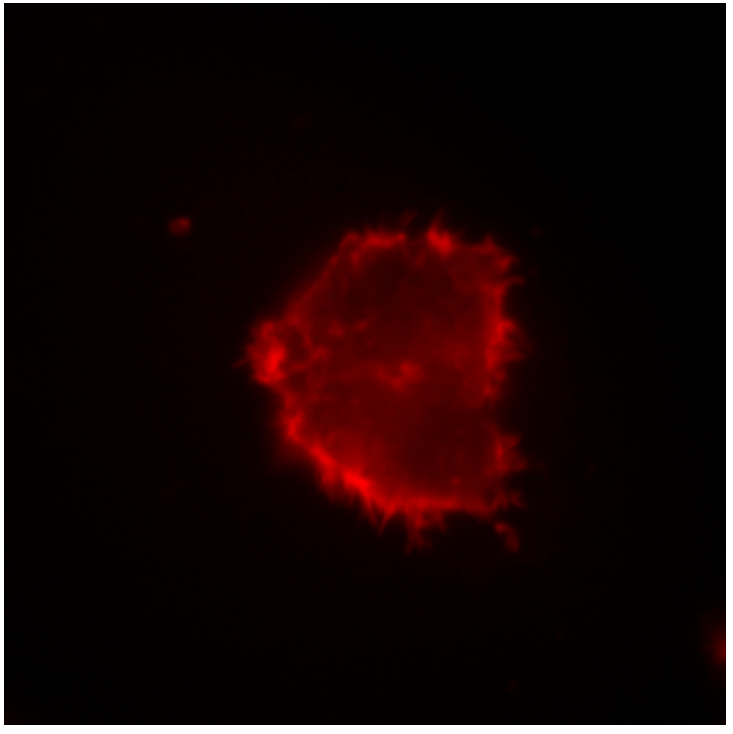


Carl Zeiss, ApoTome

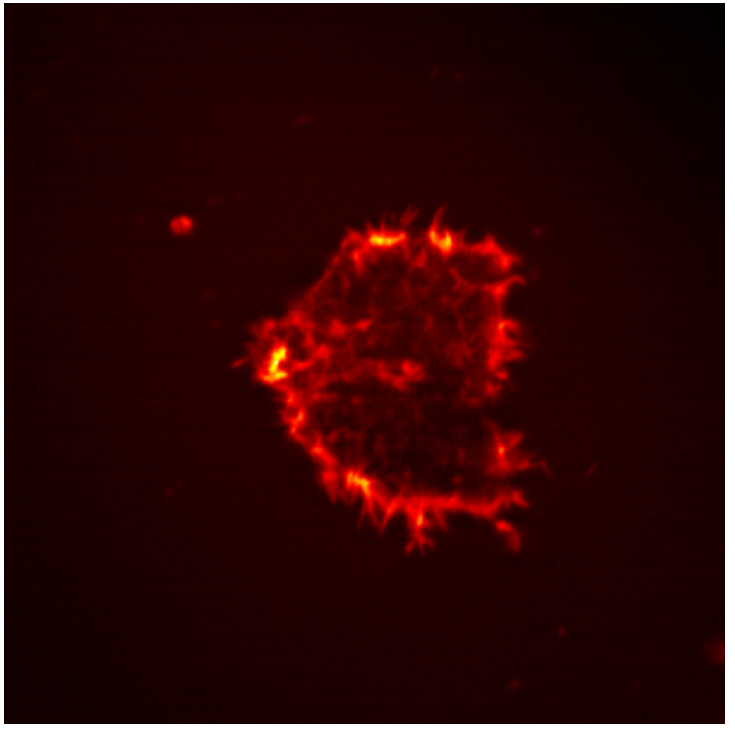
Ref: M. A. A. Neil, R. Juskaitis, and T. Wilson, *Optics Letters* **22,** 1905 (1997).

Sectioned fluorescence images without scanning

Fluorescence

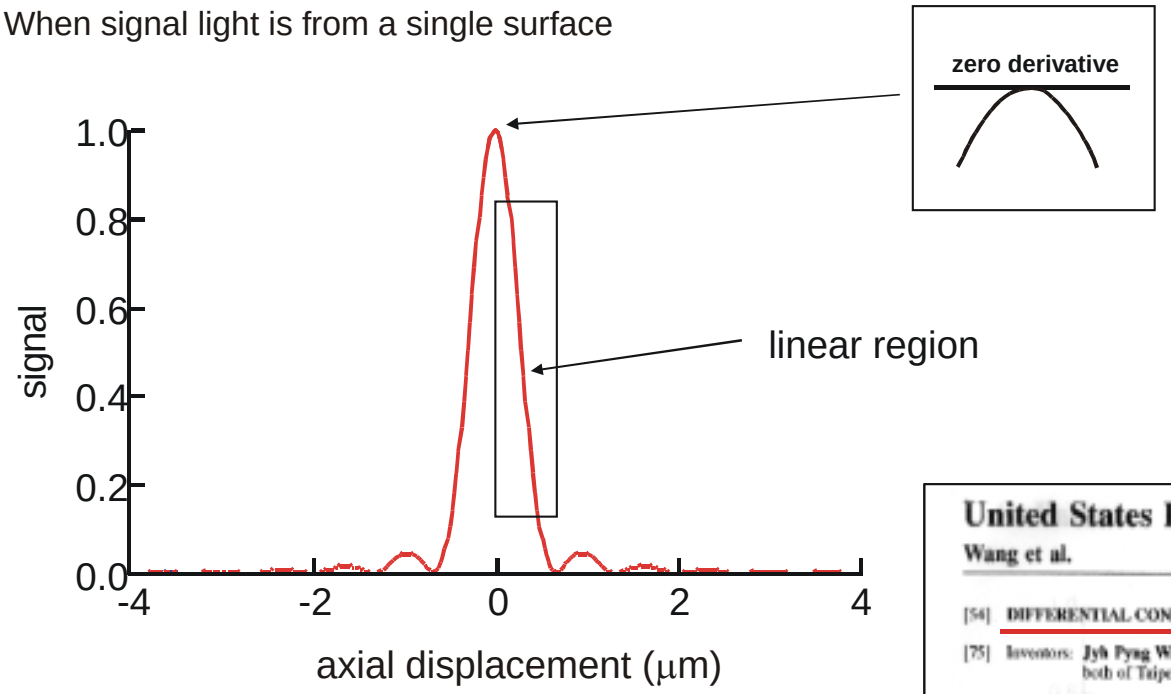


Optically sectioned



The concept of differential confocal microscopy

When signal light is from a single surface



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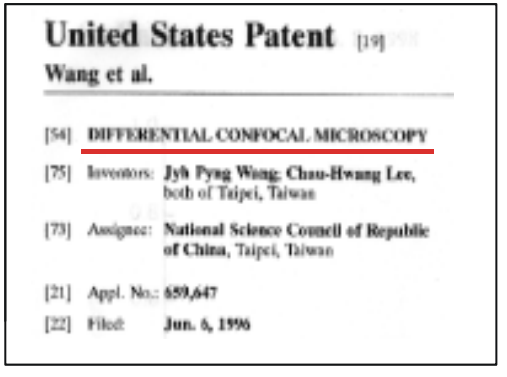
→ 10 nm displacement = 1% signal variation



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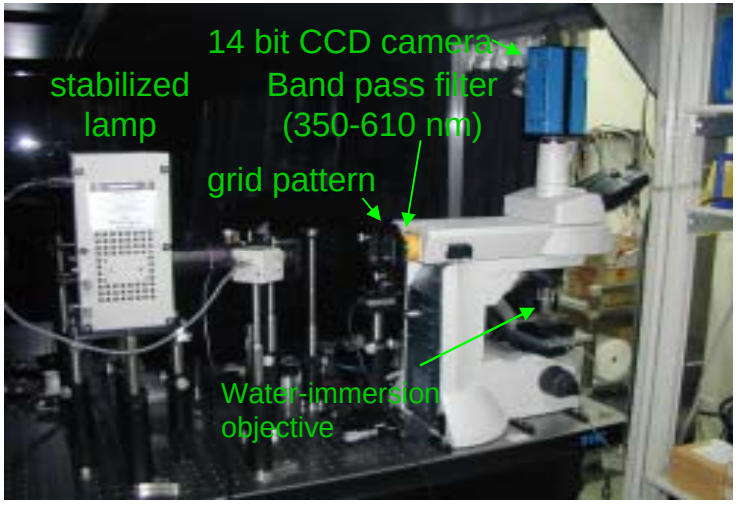
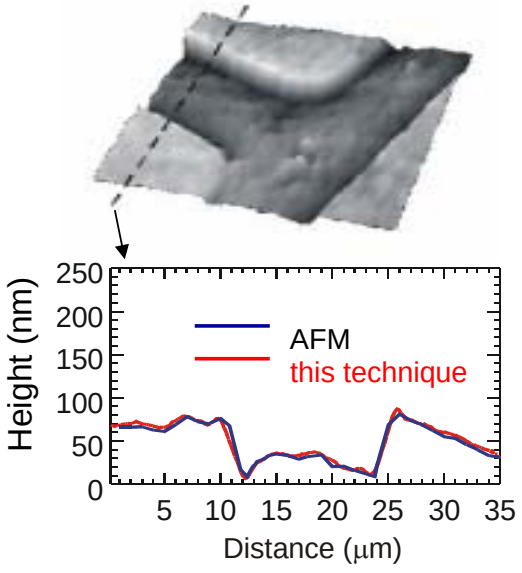
Ref: C.-H. Lee and J. Wang, *Opt. Commun.* **135,** 233 (1997).

The NIWOP technique



All the components are added outside a bench-top microscope.

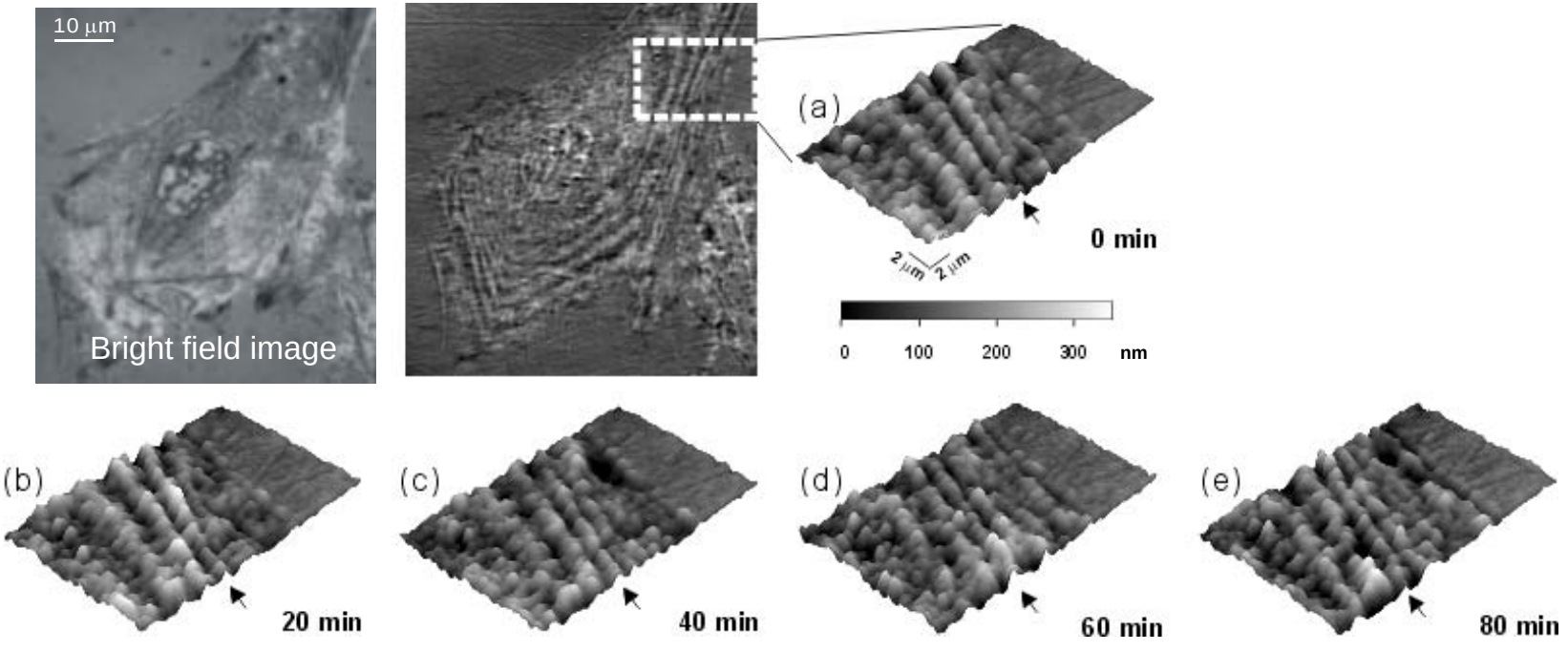
70 nm trench on InGaAs



This technique is called non-interferometric widefield optical profilometry (NIWOP).

C.-H. Lee, H.-Y. Mong, and W.-C. Lin, *Optics Letters* **27,** 1773 (2002).

Observation of membrane ripples of a living cell



The ripples are moving away from the cell edge with an average speed about 1.3 μm/h.

C.-C. Wang, J.-Y. Lin, and C.-H. Lee, *Optics Express* **13,** 10665 (2005).

Highlighted in *Virtual Journal for Biomedical Optics* (January 2006)

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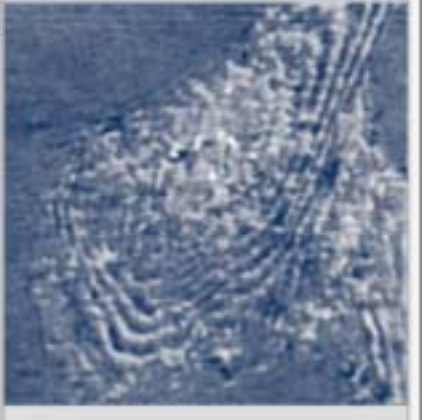

Membrane ripples on a living fibroblast cell measured using non-interferometric widefield optical profilometry. For details, see Opt. Express 13, 10665 (2005).

Editorial
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Microscopy and Optical Coherence Tomography
Optical Spectroscopies
Image Reconstruction and Data Processing
Biophotonics and Biosensors
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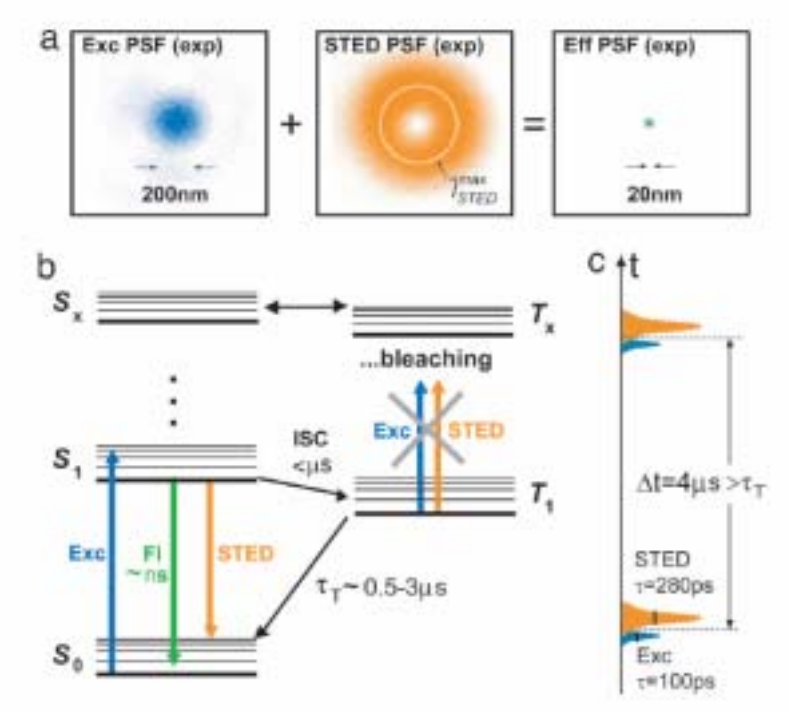
Stimulated emission depletion (STED) microscopy



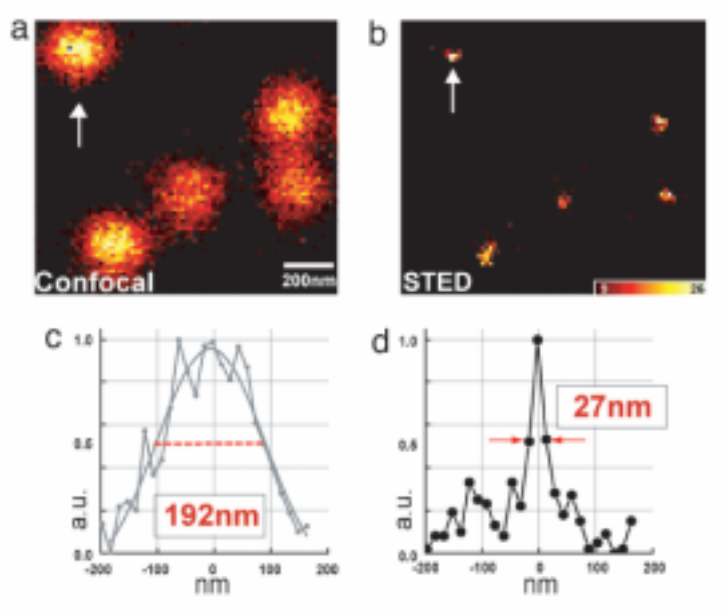
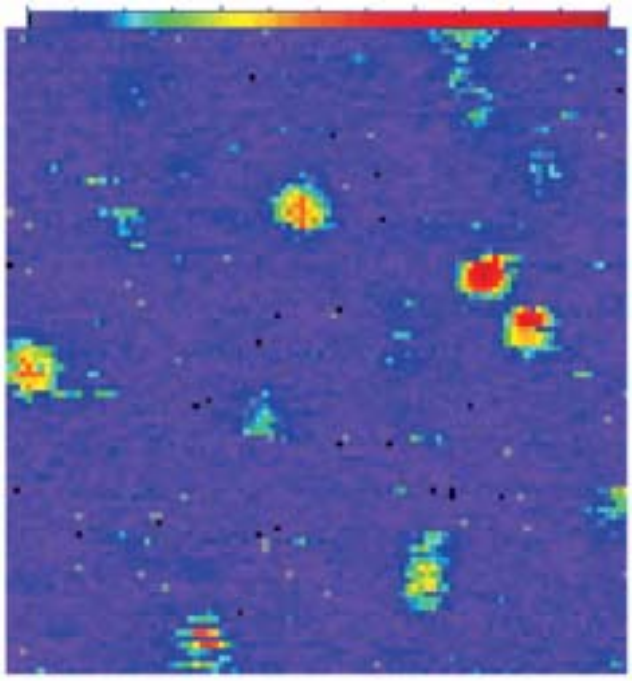


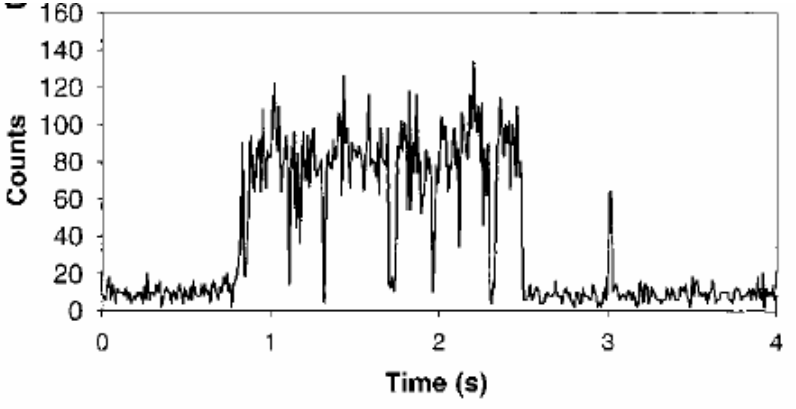
Fig. 3. Synaptotagmin I molecules form distinct spots on endosomes. (*a* and *b*) Whereas confocal microscopy exhibits a 190- to 200-nm diffraction-limited spot per endosome (*a*), STED microscopy recognizes sharp dots of 25–40 nm (*b*), both indicating its resolution as well as the punctated spatial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

Confocal microscopy for single molecules

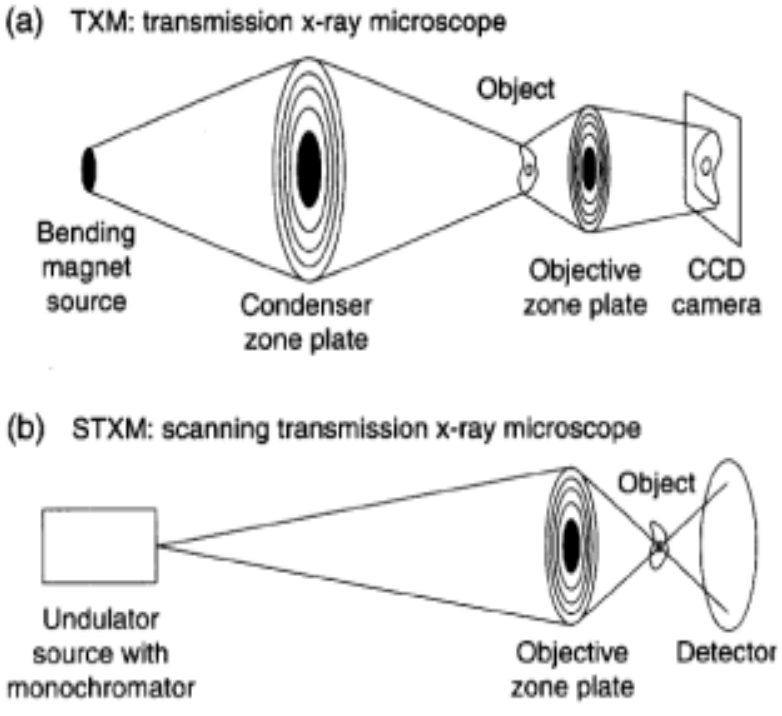


Blinking



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

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.

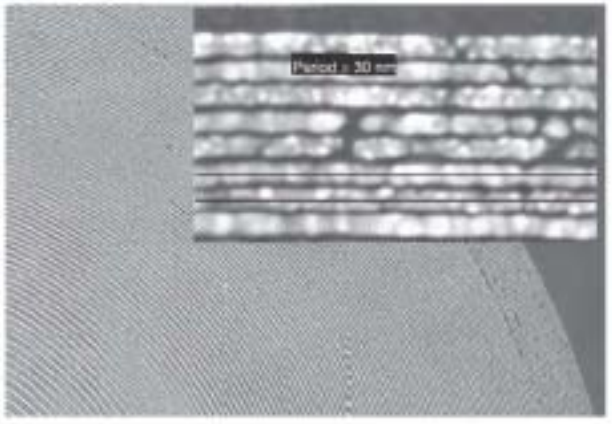
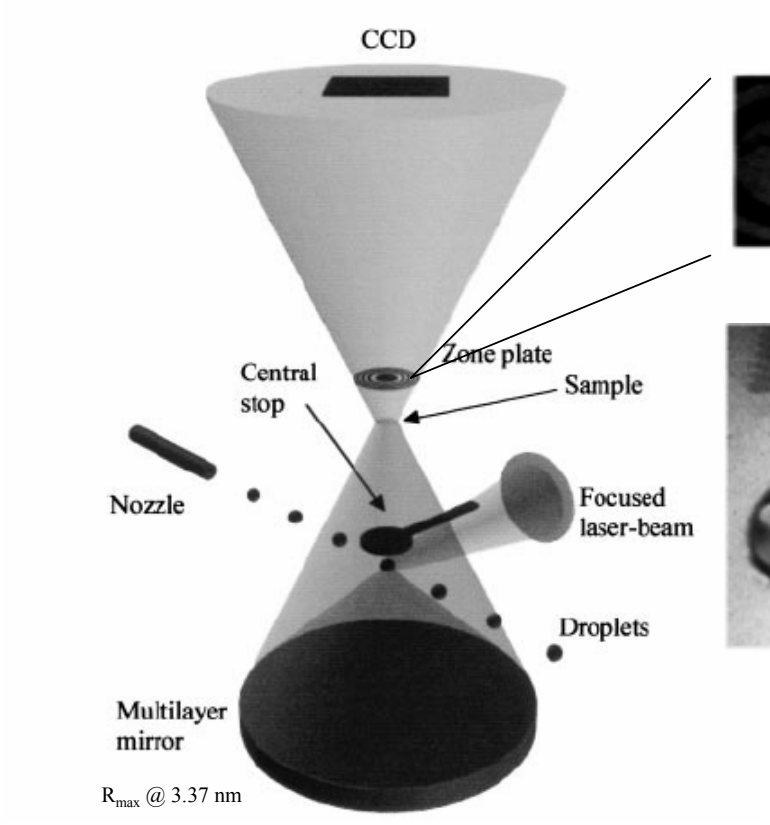
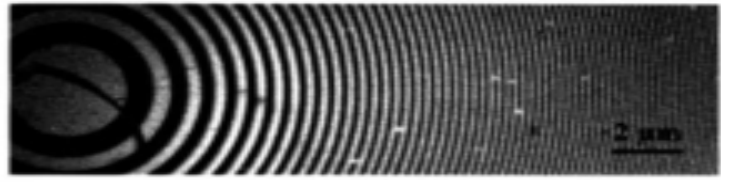


Figure 3 | Scanning electron micrograph of a zone plate with 15 nm outermost zone. Shown in the inset is a more detailed view of the outermost zones. The zonal period, as indicated by the two black lines, is measured to be 30 nm. The zone placement accuracy is measured to be 1.7 nm.

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

Compact soft x-ray microscope





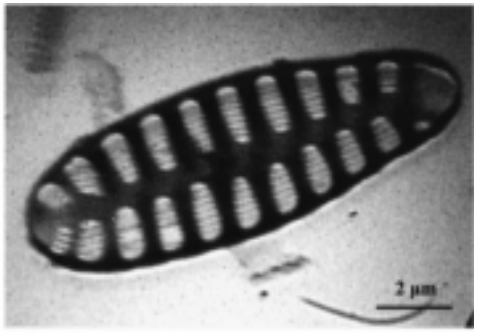
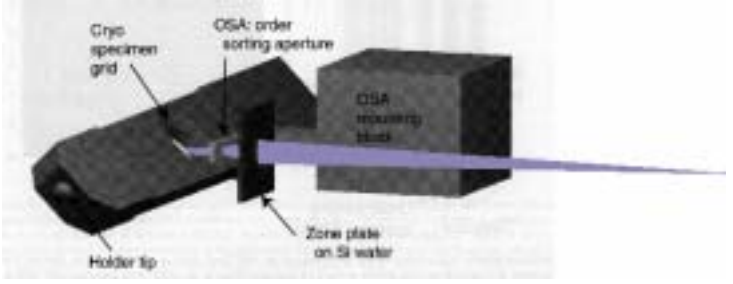


Image of diatom

Resolution ~ 100 nm

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

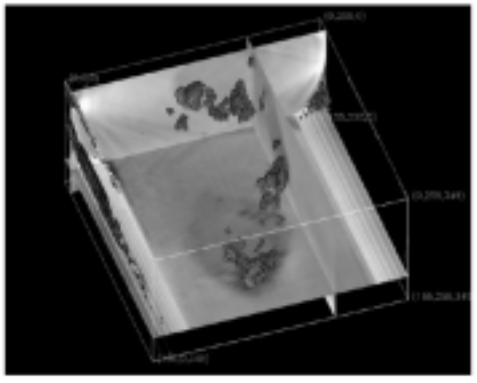
X-ray microtomography



Commercial product available

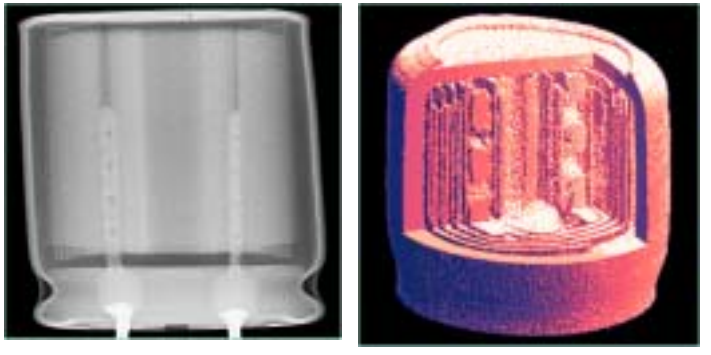
http://www.microphotonics.com

Vesicles inside a cell



Resolution ~ 250 nm

Capacitor



Resolution < 10 μm

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