Microscopy

Jan Kybic[1]

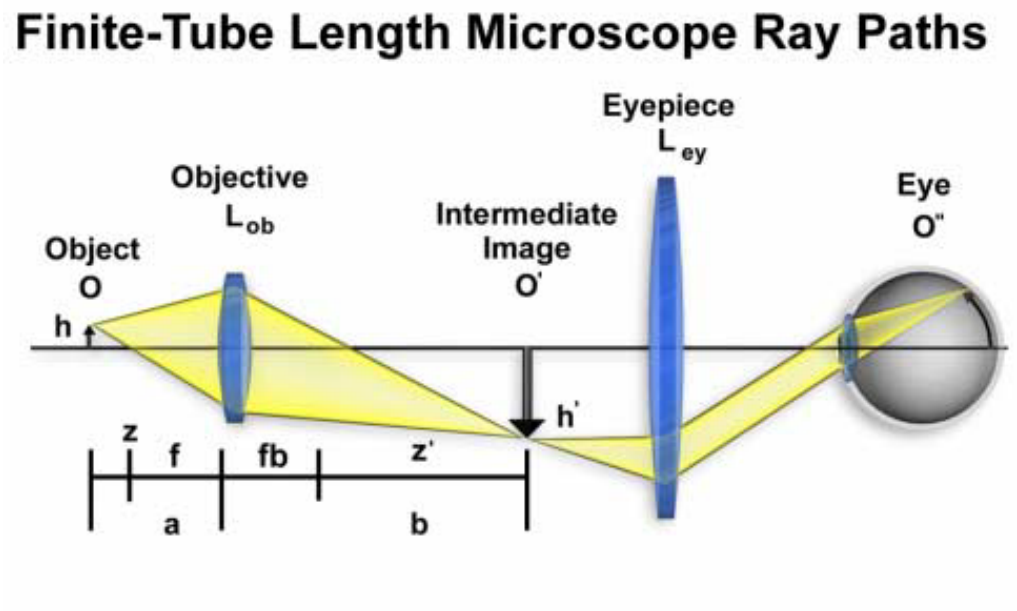
2011–2023

[1]Using material from Davidson and Abramowitz: Optical Microscopy

Microscopy

Optical microscopy – since 17th century; Jensen, van Leeuwenhoek, Galilei, ...

Finite-Tube Length Microscope

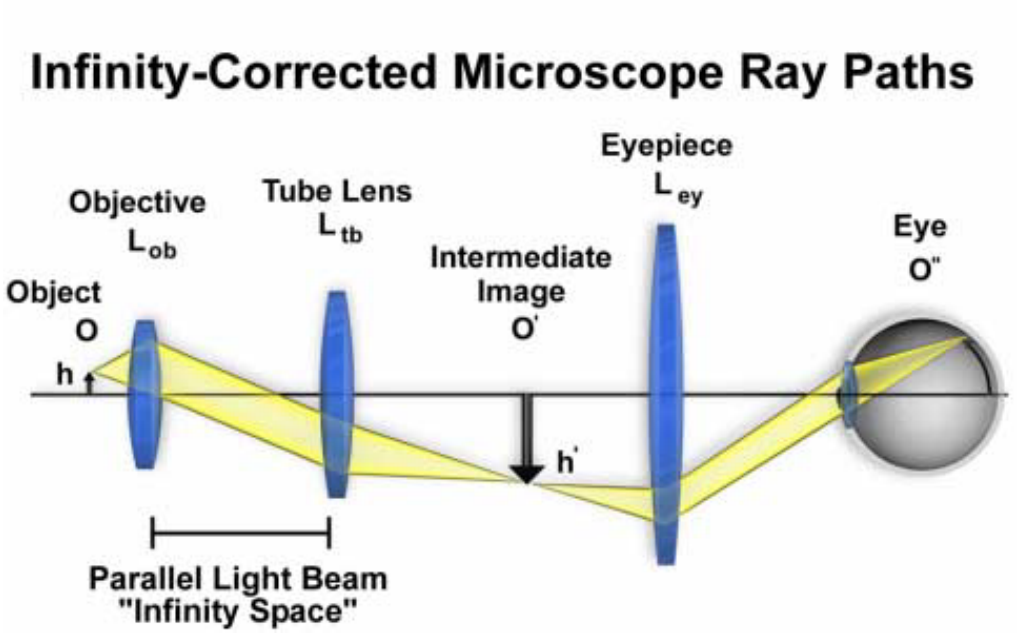


- magnification of the objective $\frac{b}{a}$
- magnification of the eyepiece $\frac{25\,\mathrm{cm}}{f_{\mathrm{eyepiece}}}$
- thin-lens equation

$$\frac{1}{a} + \frac{1}{b} = \frac{1}{f}$$

- narrow range of image distances
- specifically corrected optical systems with matching eyepieces

Infinite-Tube Length Microscope

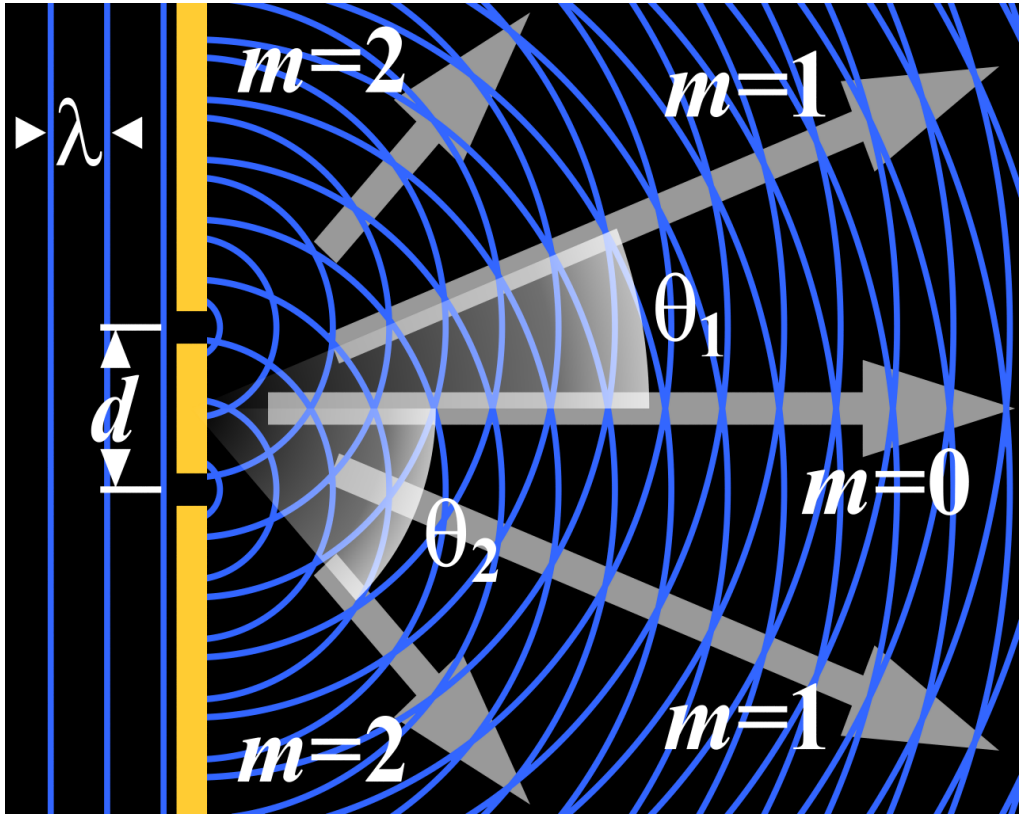


- Modern design (since 1980s)
- Objective magnification determined by $\frac{f_{\text{tb}}}{f_{\text{ob}}}$
- Infinity space to add polarizers, prisms, retardation plates...
- Independently changeable objective and eyepiece

Image Formation

- Direct/undeviated light
- Deviated/diffracted light, out of phase
- Constructive/destructive interference

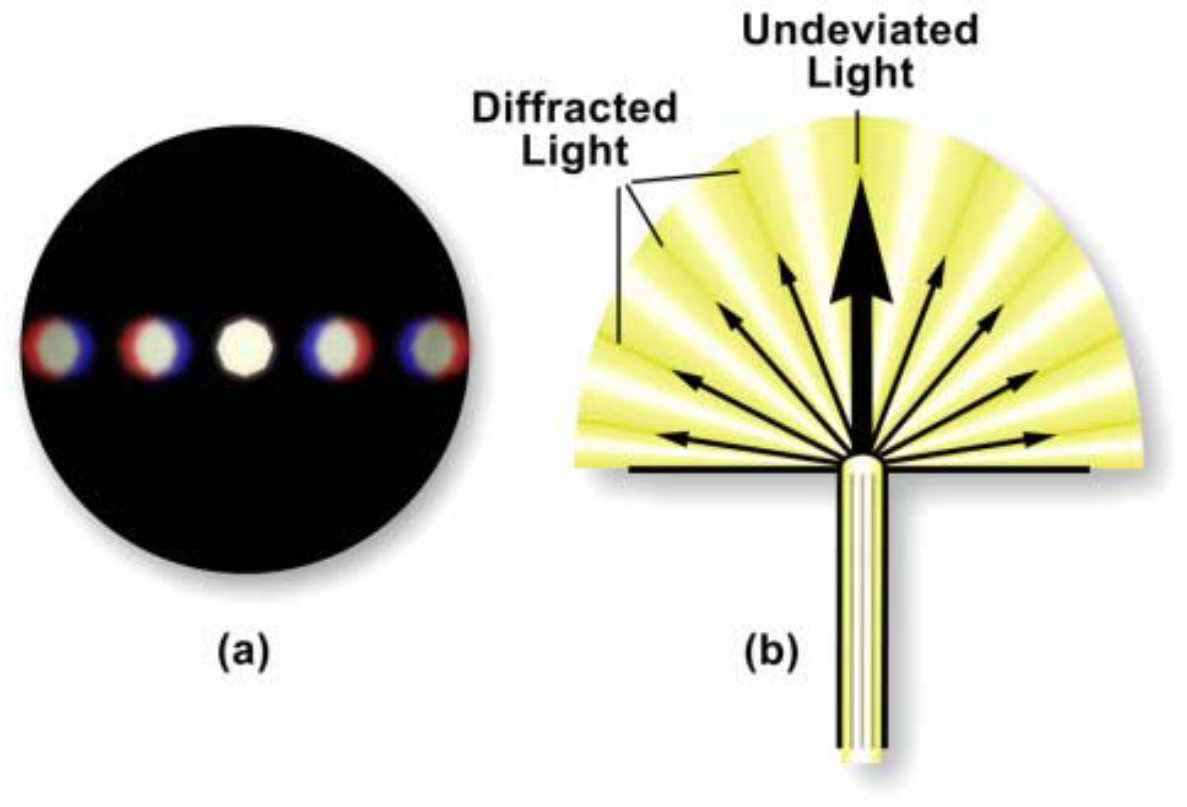
Diffraction



Position of maxima:

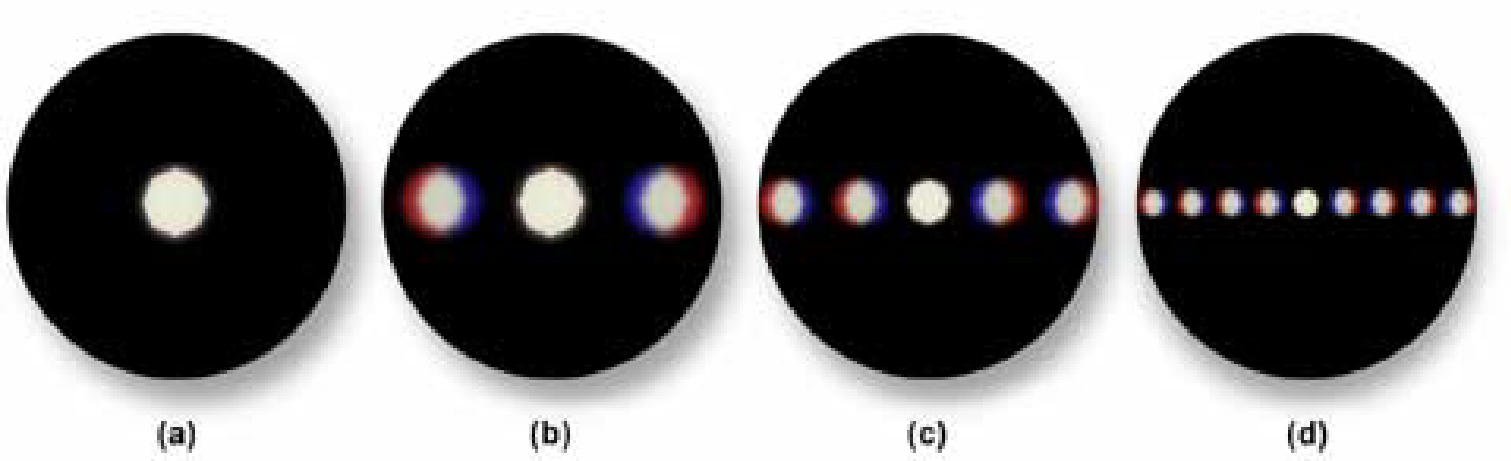
$$d \sin\theta = m\lambda, \quad m \in \mathrm{Z}$$

Diffraction



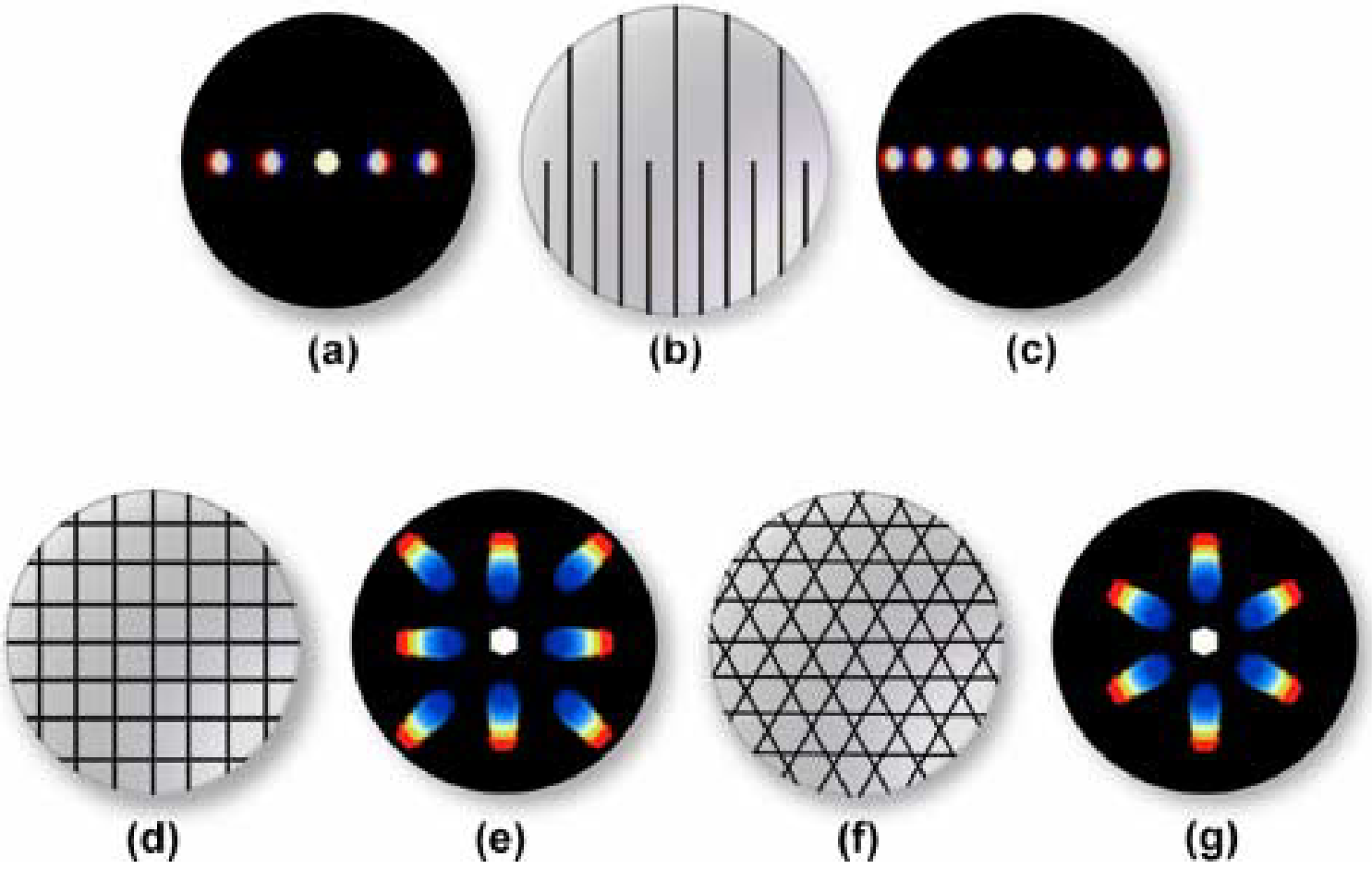
- constructive/desctructive interference
- specimen = superposition of complex gratings *(Ernst Abbe)*
- to resolve image, at least 0^{th} order and 1^{st} order images must be captured
- more orders captured $\rightarrow$ better accuracy

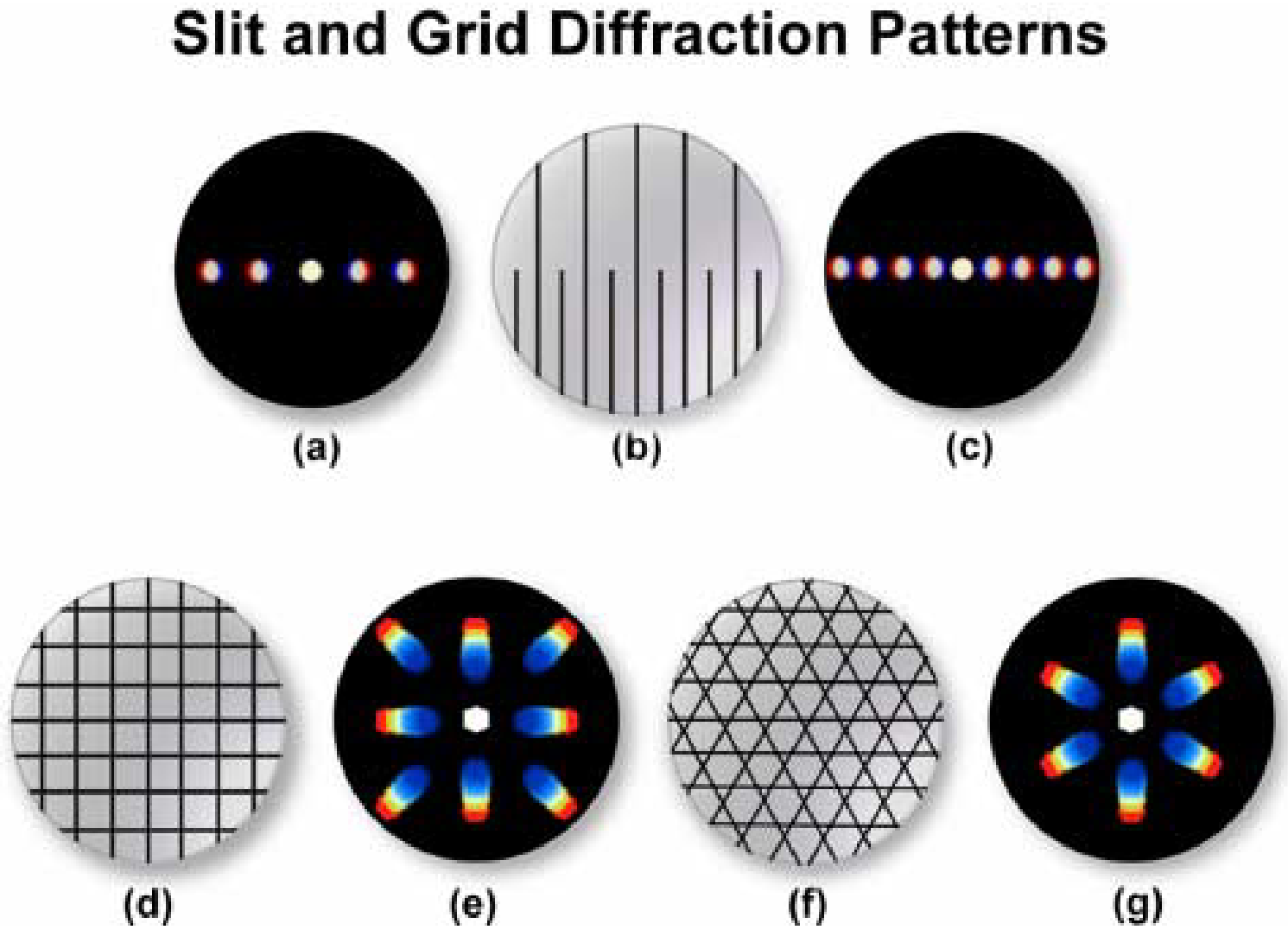
Line Grating Diffraction Patterns



- line phantom
- close diaphragm
- telescope, observe the rear focal plane of the objective
- (a) no phantom, (b) 10×, (b) 40× (higher NA), (c) 60× (highest NA)
- 0^{th} order, 1^{st} order image

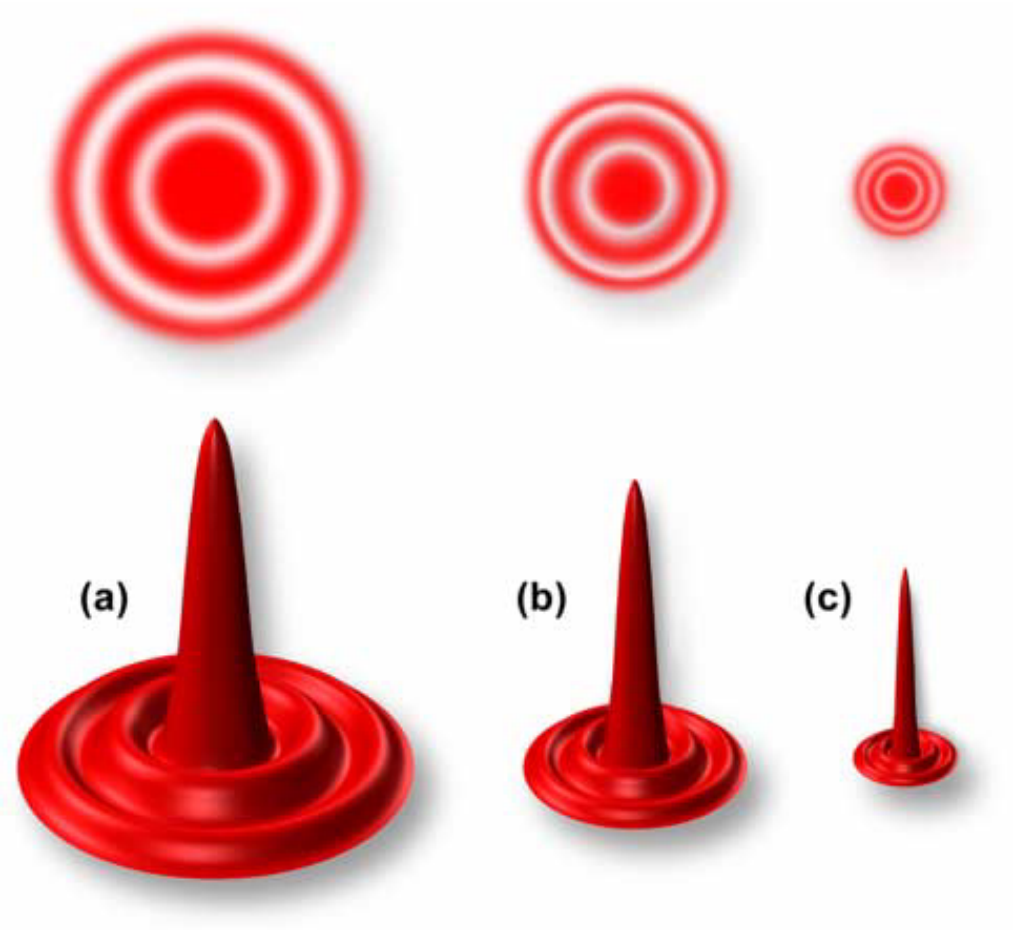
Slit and Grid Diffraction Patterns





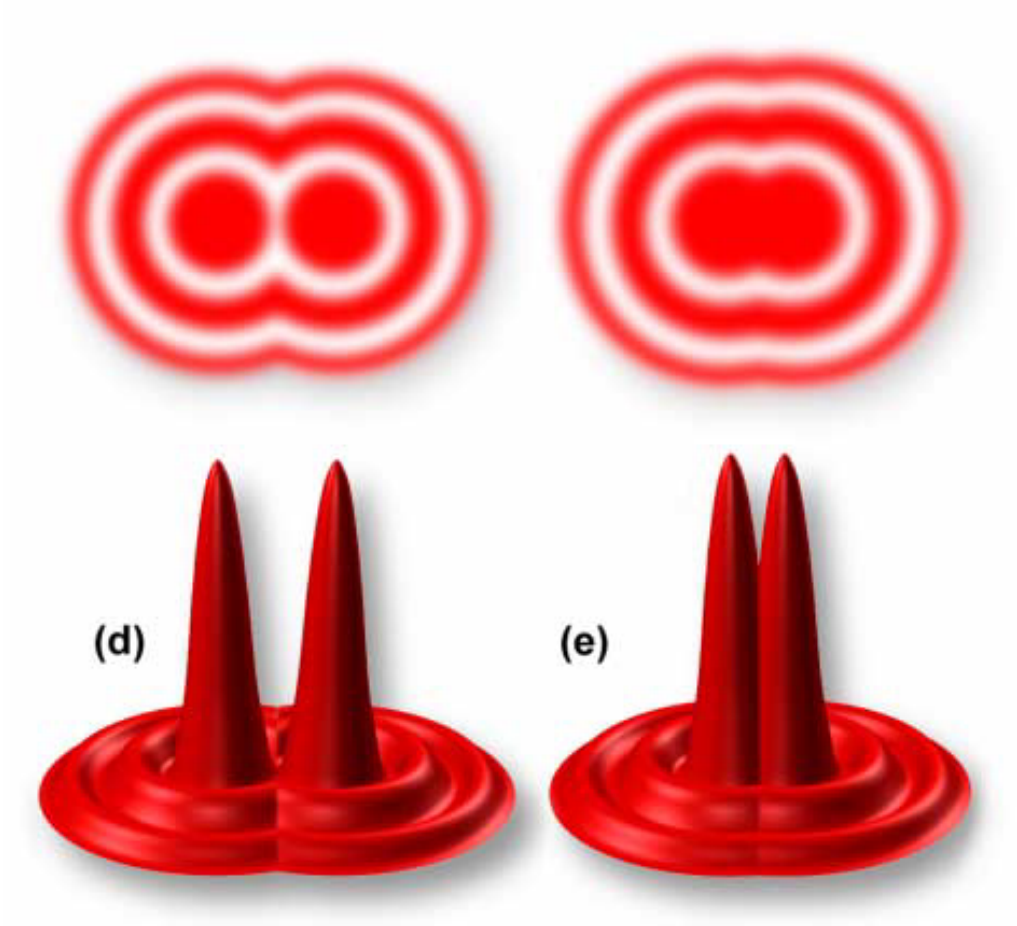
- Diffraction patterns behave like Fourier transforms of the sample
- Fourier optics

Airy disks



- NA increases left to right.
- Impulse response (PSF)

Airy disks (2)



Resolution limit.

Resolution limit

Rayleigh equation:

$$d \approx 1.22 \frac{\lambda}{2\,\mathrm{NA}}$$

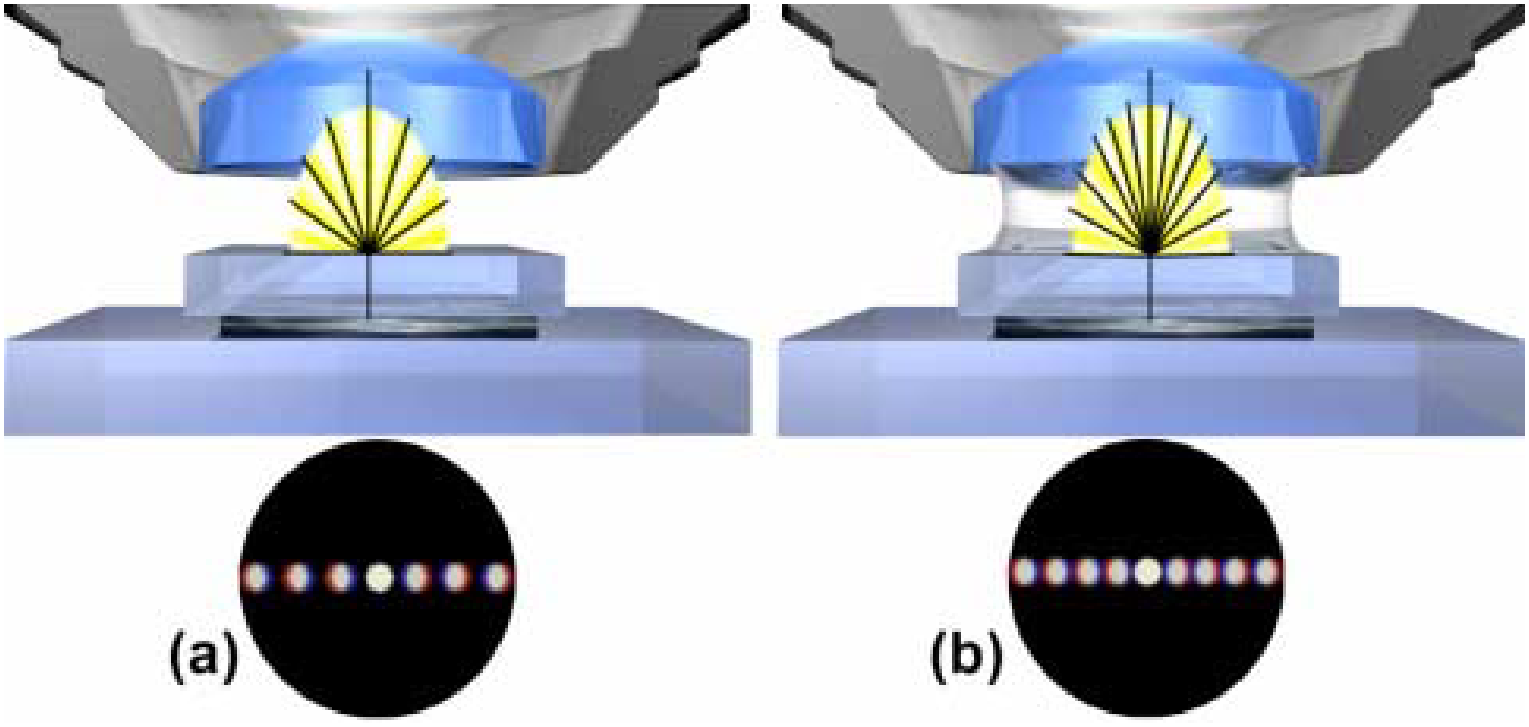
To improve resolution, use:

- Big lenses (big NA)
- Short wavelength (blue)

Numerical aperture:

- $NA = n \sin\theta$, with *half-cone angle* θ
- f-number $N = f/D \approx 1/(2NA)$, written as f/N

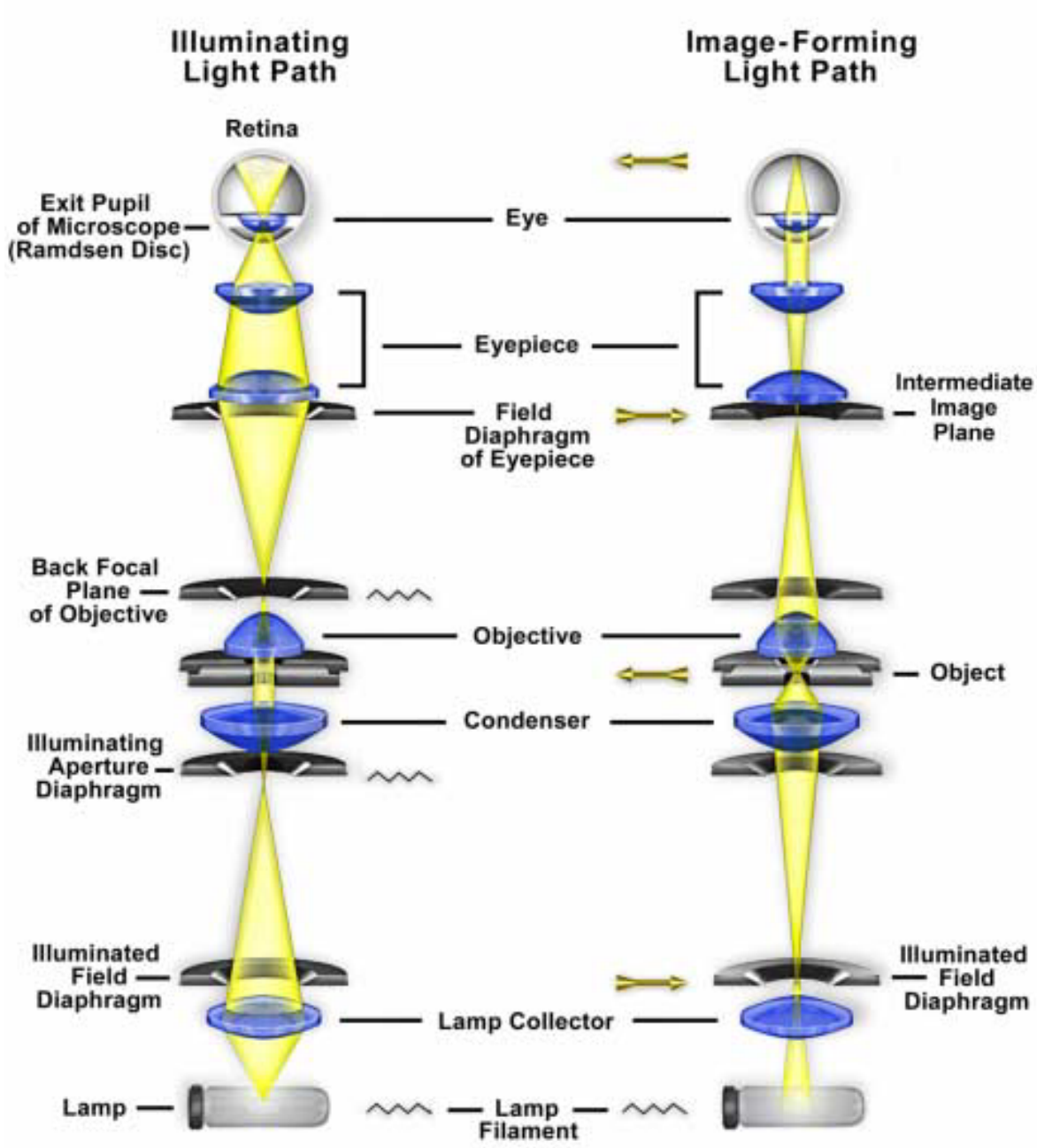
Immersion optics



- High refractive-index media (immersion oil) reduce diffraction angle
- $\rightarrow$ More orders are captured
- $\rightarrow$ Better image

Köhler illumination

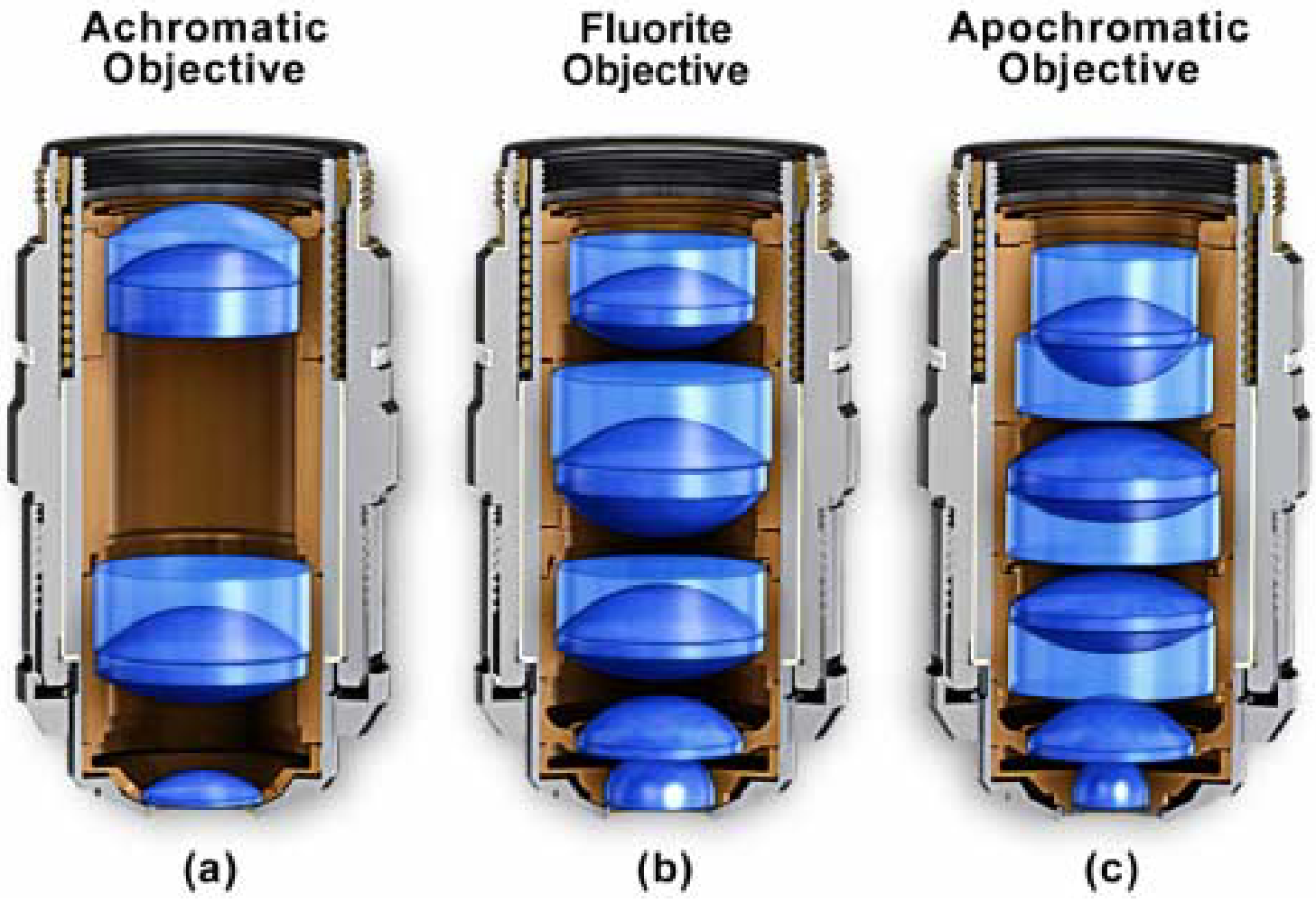
- Focused lamp image is projected to the diaphraghm of a condenser.
- Field diaphraghm controls width of the light bundle.
- Apperture diaphraghm controls the light intensity. Trade-off between diffraction artifacts and glare.
- Light is not focused on the specimen, illumination is homogeneous.
- The focal point of image-forming rays is at the level of the specimen.


Illuminating Light Path
Image-Forming Light Path
Retina
Exit Pupil of Microscope (Ramdsen Disc)
Eye
Eyepiece
Field Diaphragm of Eyepiece
Intermediate Image Plane
Back Focal Plane of Objective
Objective
Object
Condenser
Illuminating Aperture Diaphragm
Illuminated Field Diaphragm
Lamp Collector
Illuminated Field Diaphragm
Lamp
Lamp Filament

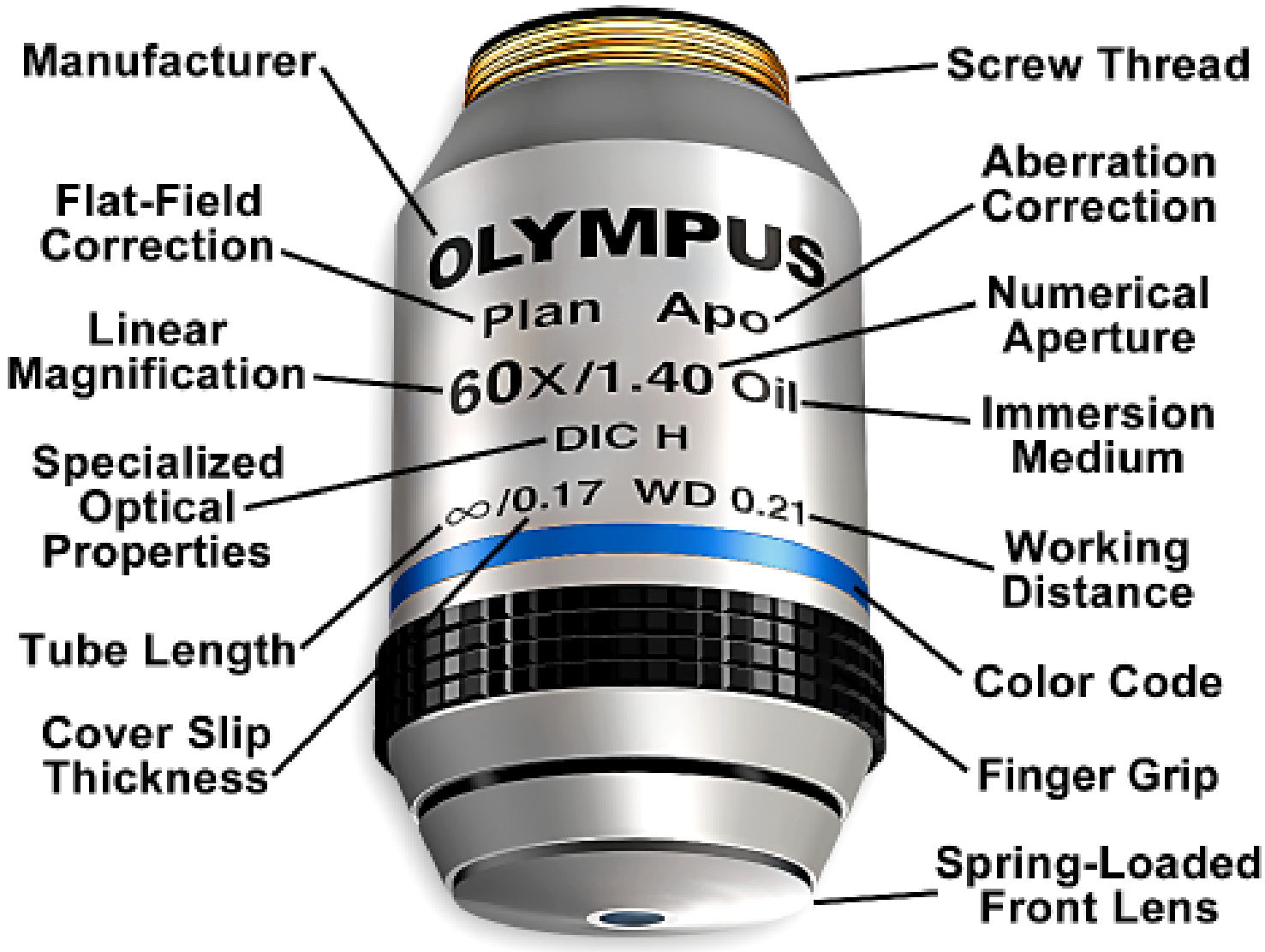
Optical Aberrations

- Geometric aberrations
  - Spherical — rays on axis and far from the axis do not converge to the same point. Blurred images.
  - Flat-field — because lenses are curved, the image is curved. Center and off-center not simultaneously in focuss.
- Chromatic aberrations — rays of different color do not converge to the same point

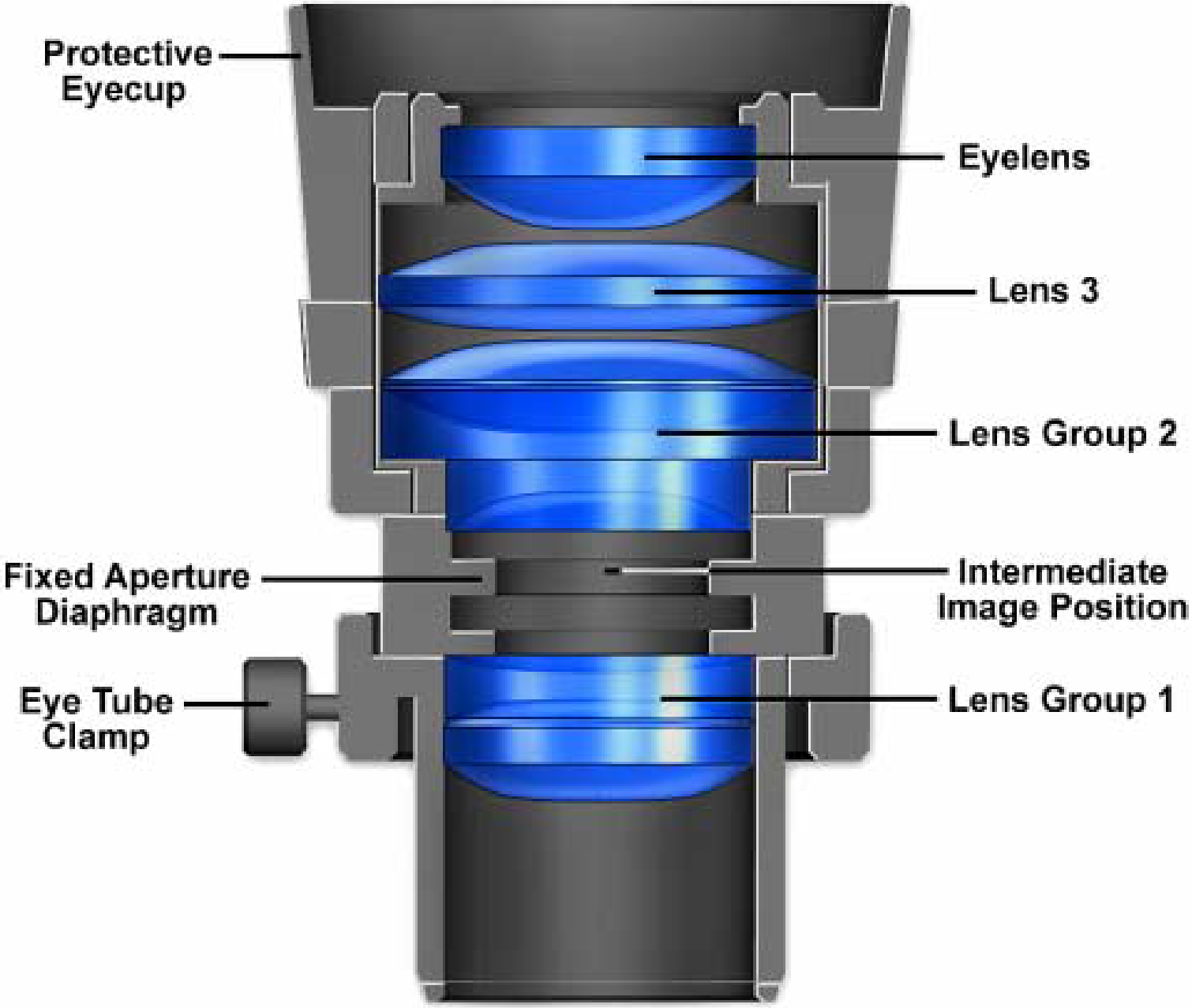
Optical Correction in Objectives



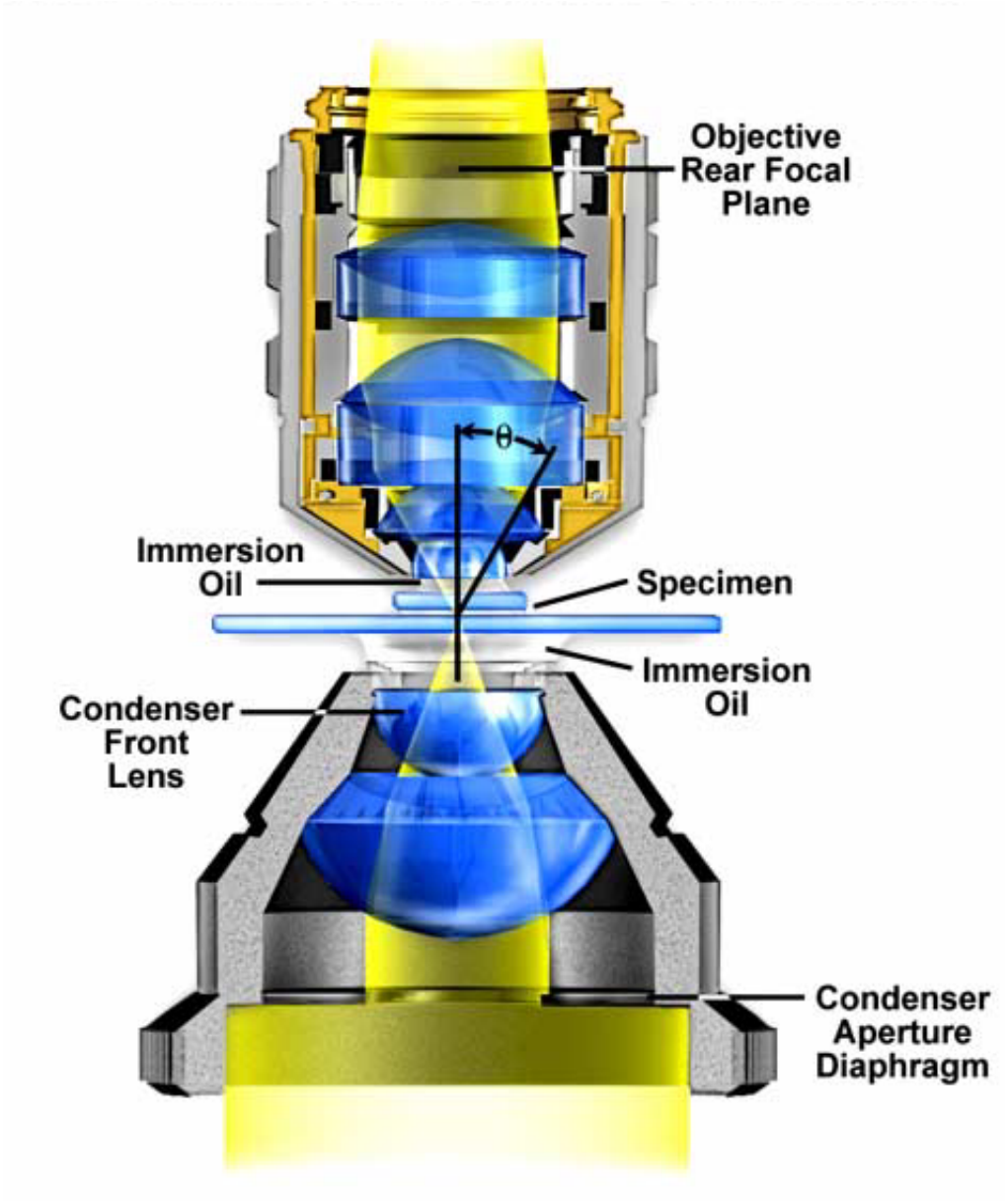
Objective Specifications



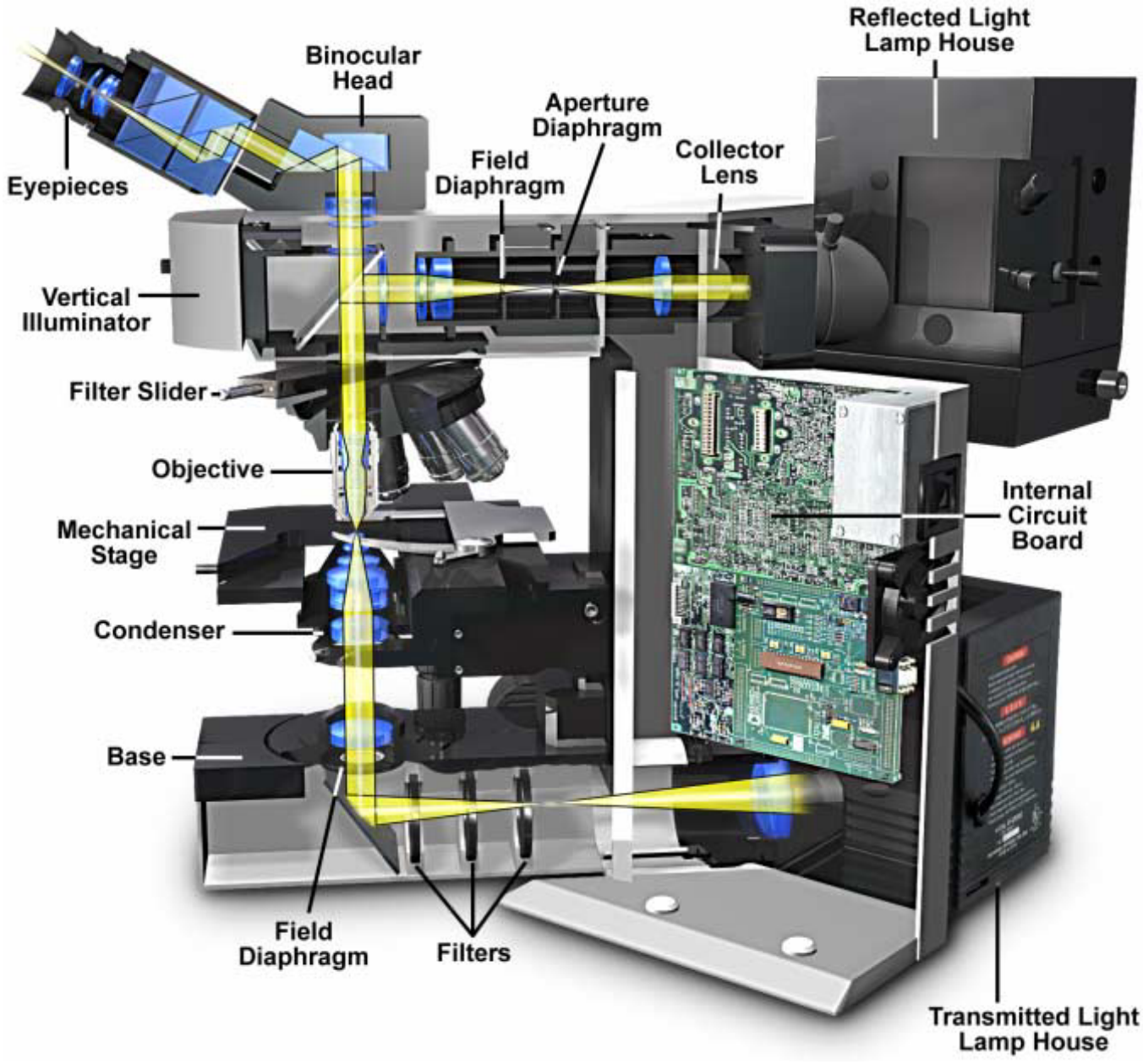
Eyepiece Cutaway Diagram



Condenser

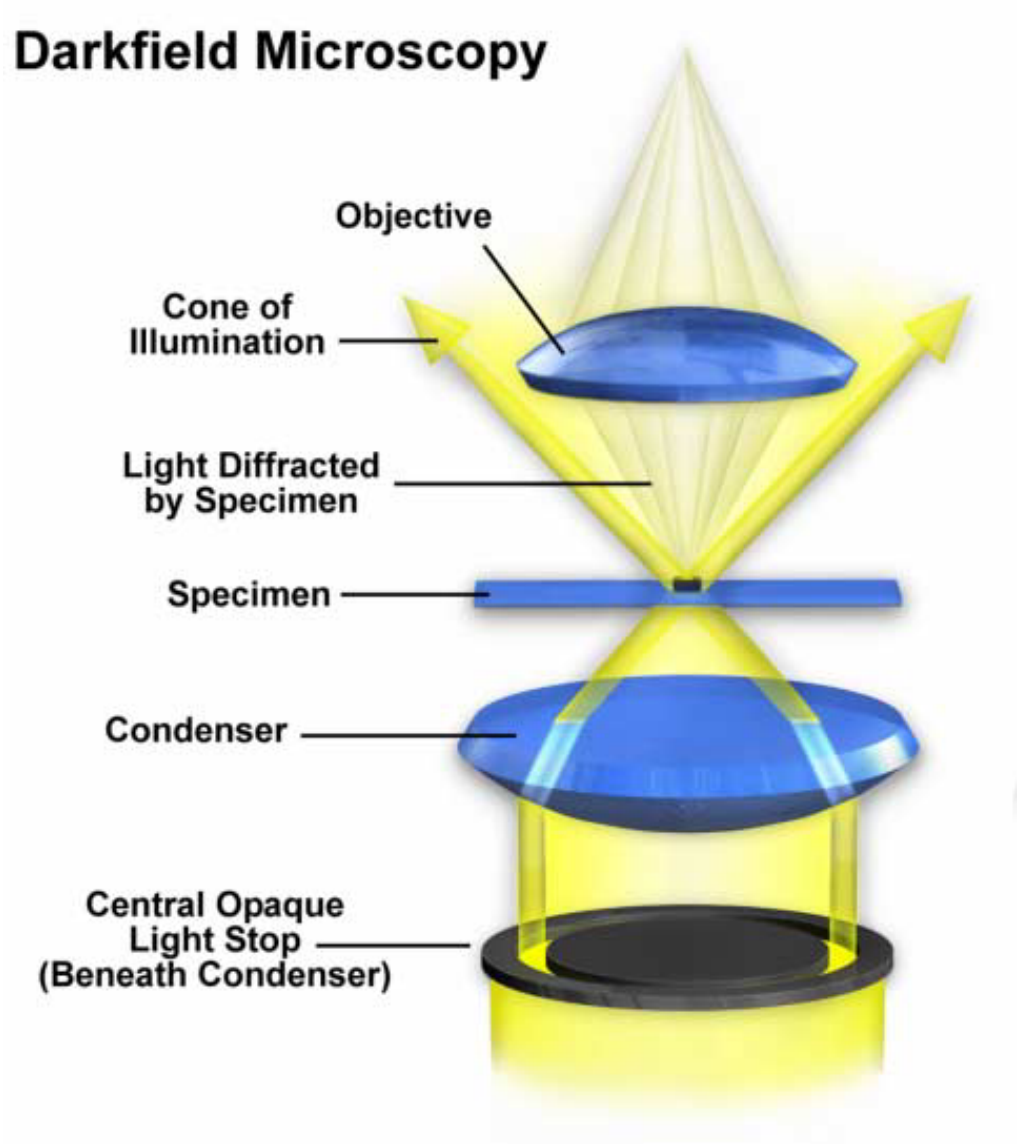

Objective
Rear Focal
Plane
θ
Immersion
Oil
Specimen
Immersion
Oil
Condenser
Front
Lens
Condenser
Aperture
Diaphragm

Transmitted/Reflected light microscope



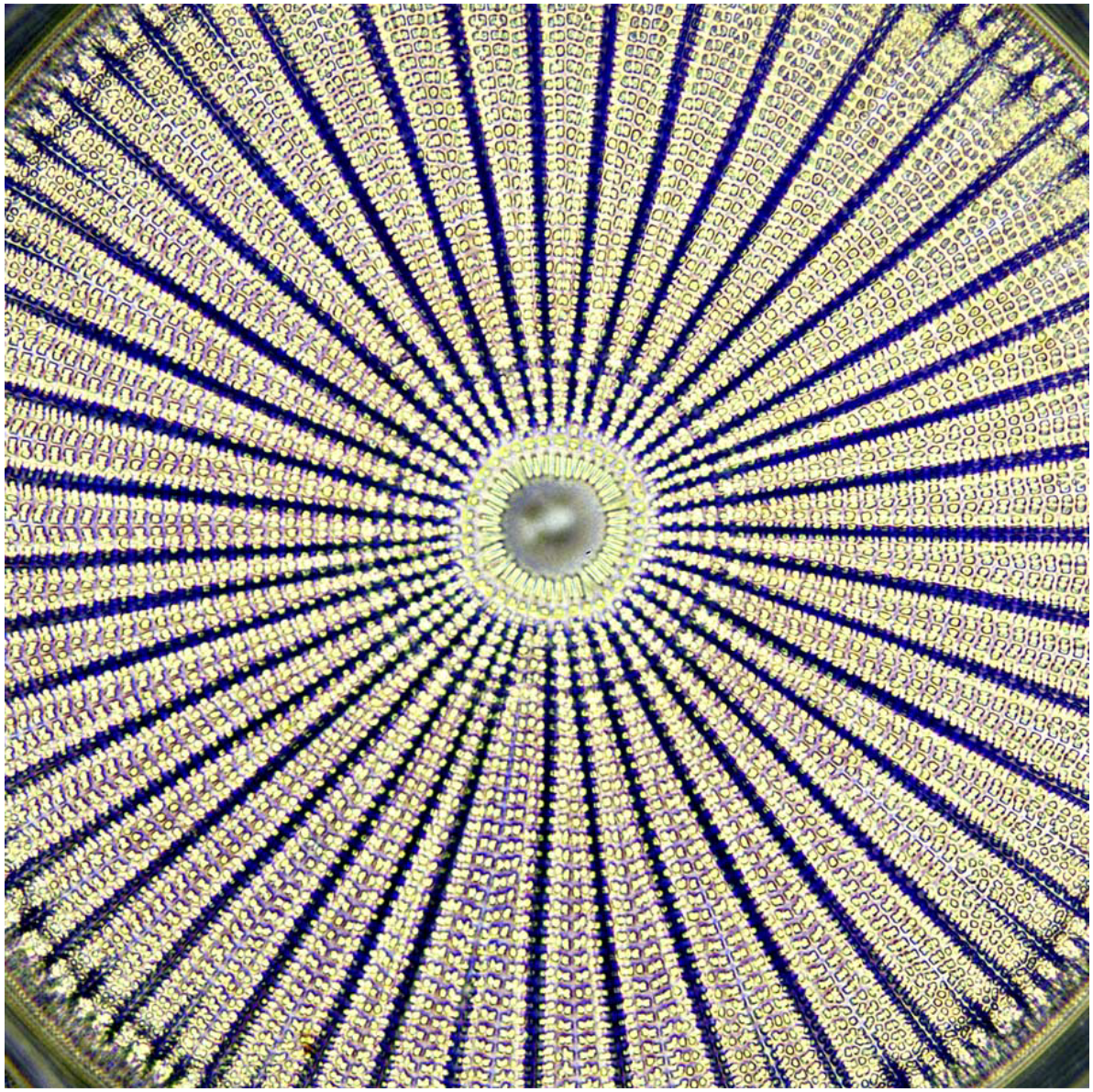
Contrast enhancing techniques

- Dark field microscopy
- Rheinberg illumination
- Phase contrast microscopy
- Polarized light
- Hoffman modulation
- Differential interfence contrast



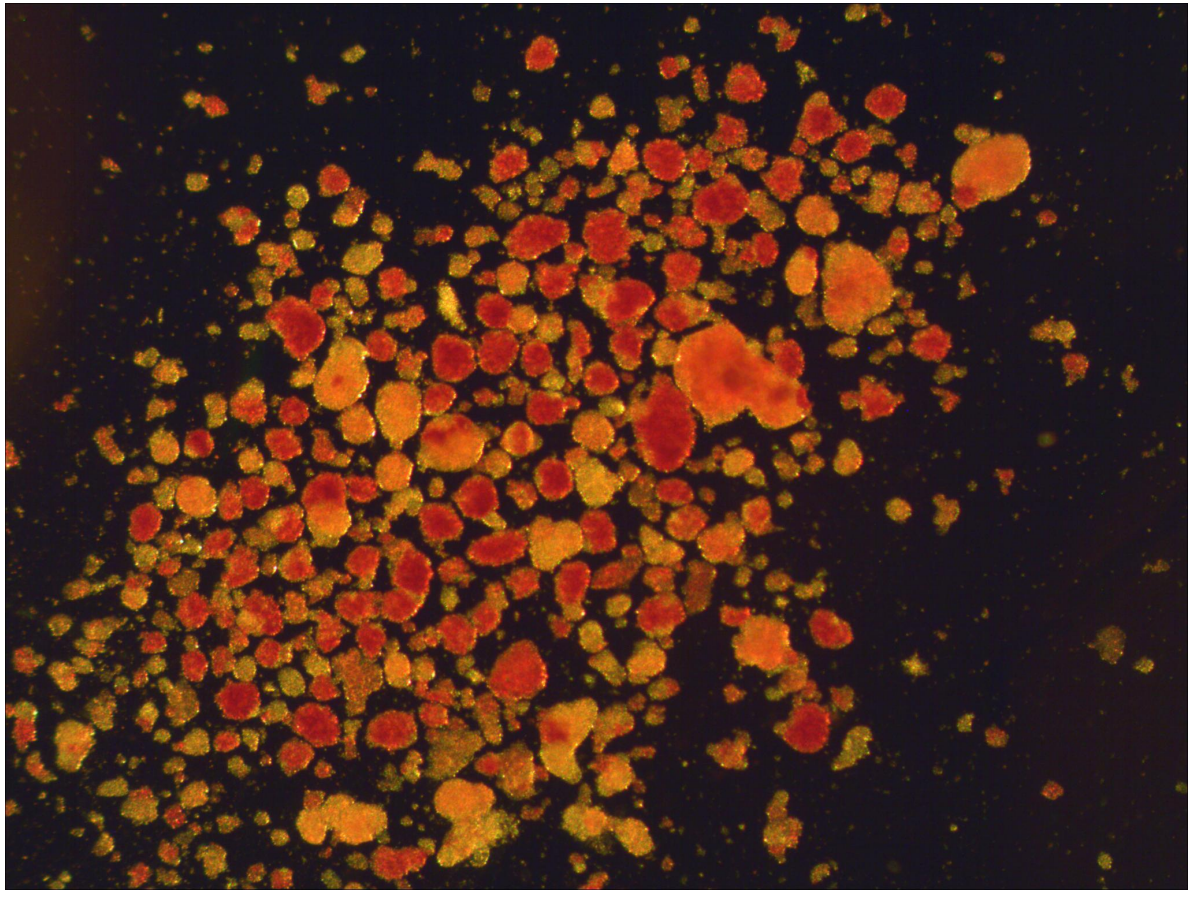
For unstained objects. Appear bright on dark background.

Darkfield microscopy (2)



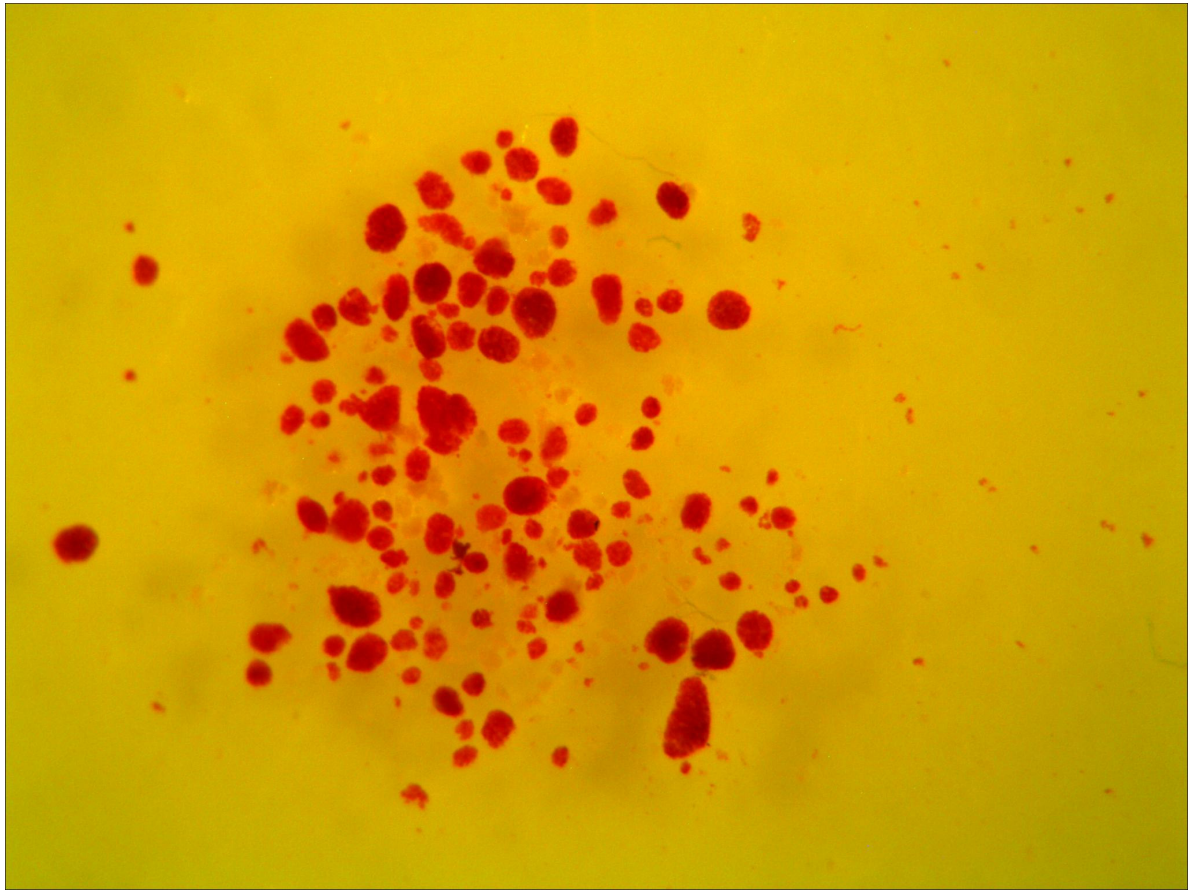
Arachnoidiscus ehrenbergi

Darkfield microscopy (3)

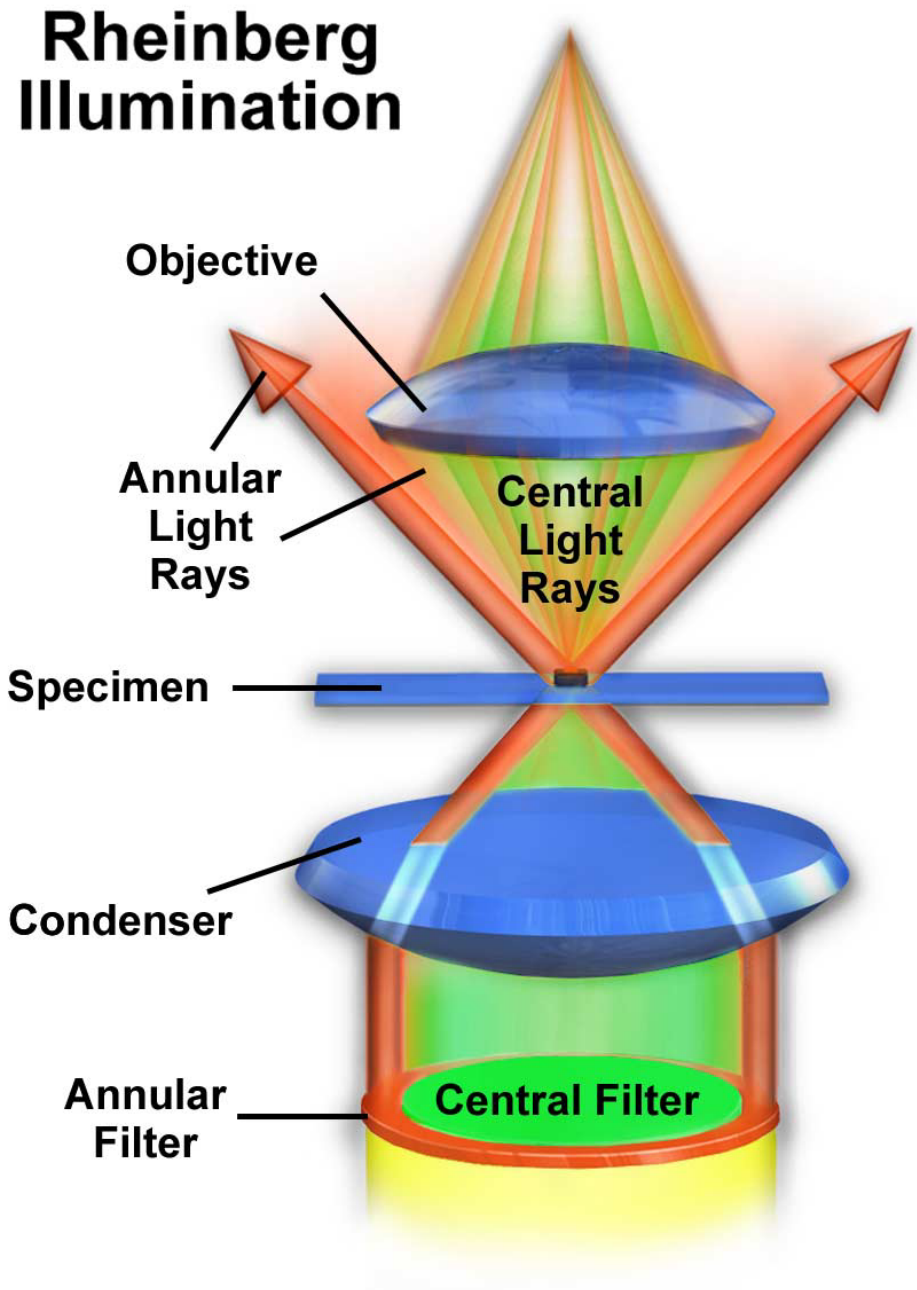


Langerhans islets

Brightfield microscopy

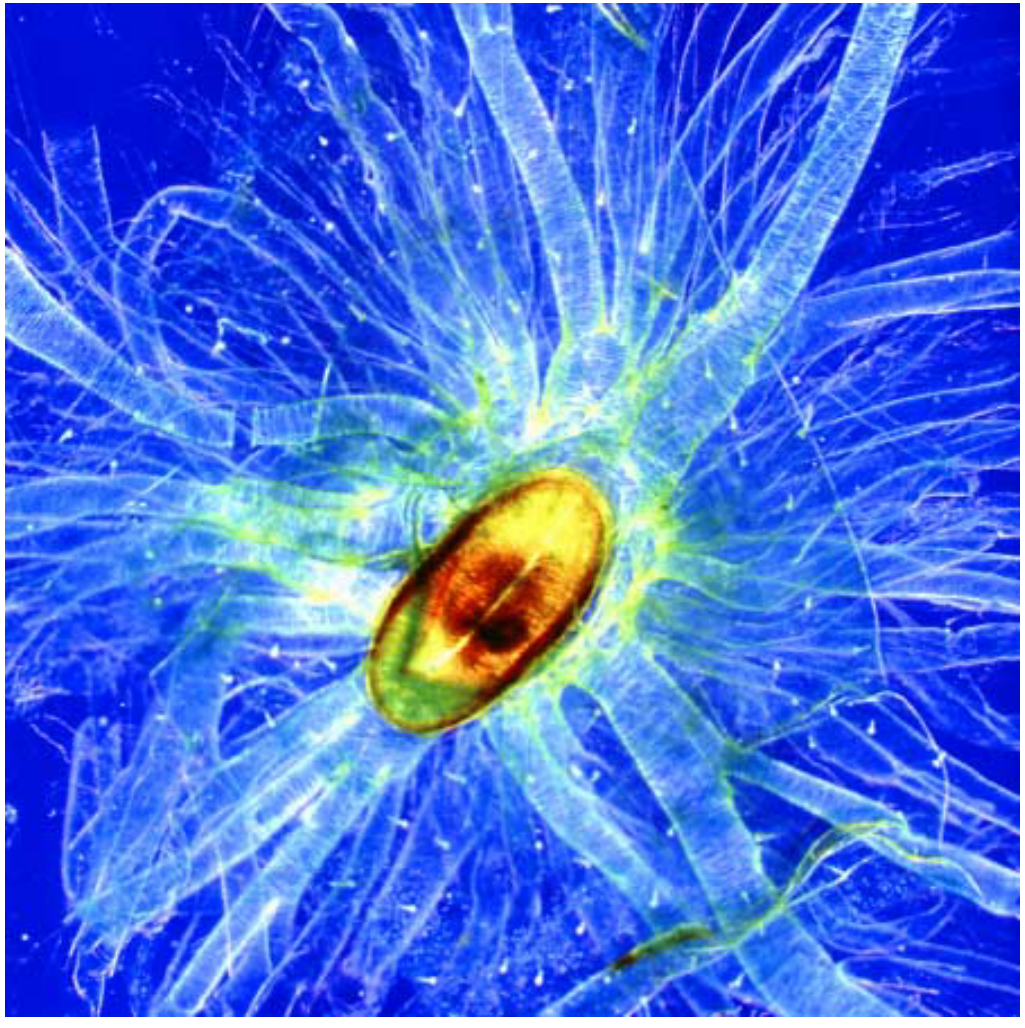


Langerhans islets

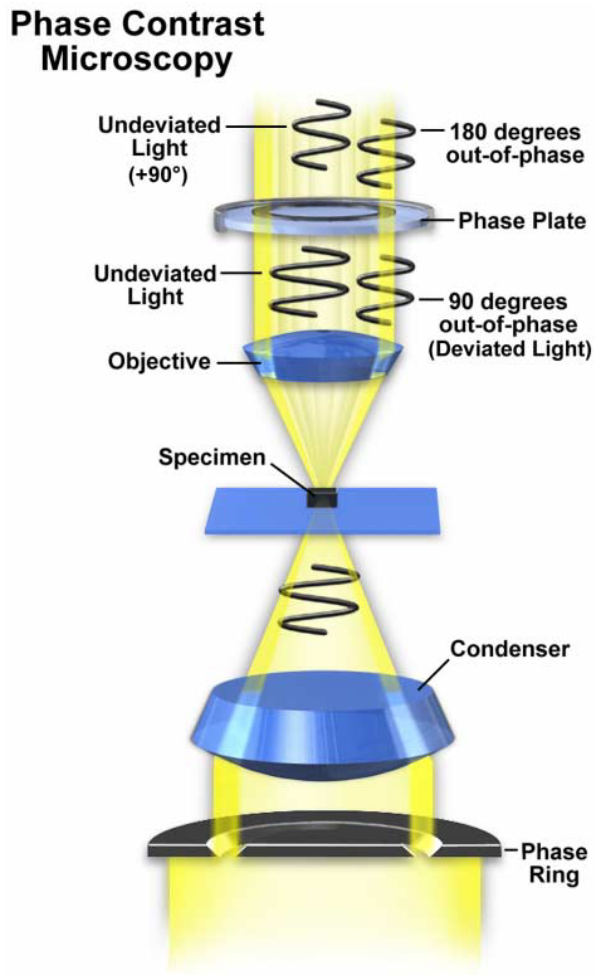


Color annular filters instead of the darkfield stop.

Rheinberg illumination (2)



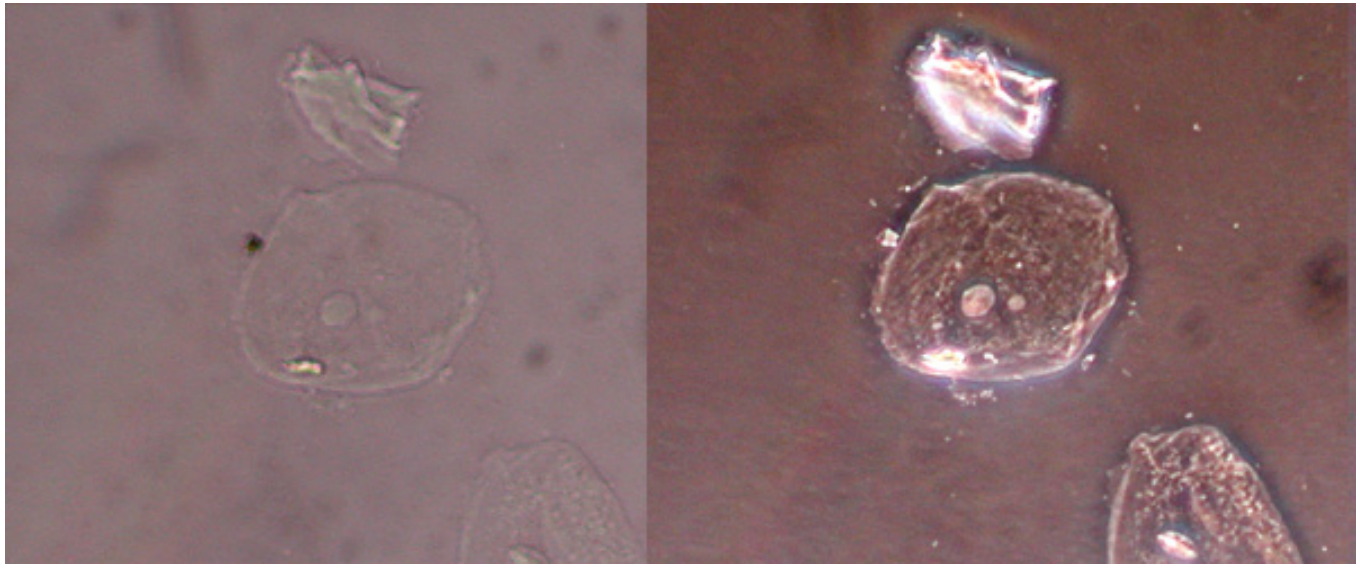
silkworm larva



Frits Zernike (1930s, Nobel price 1953). Show differences in phase/refractive index.

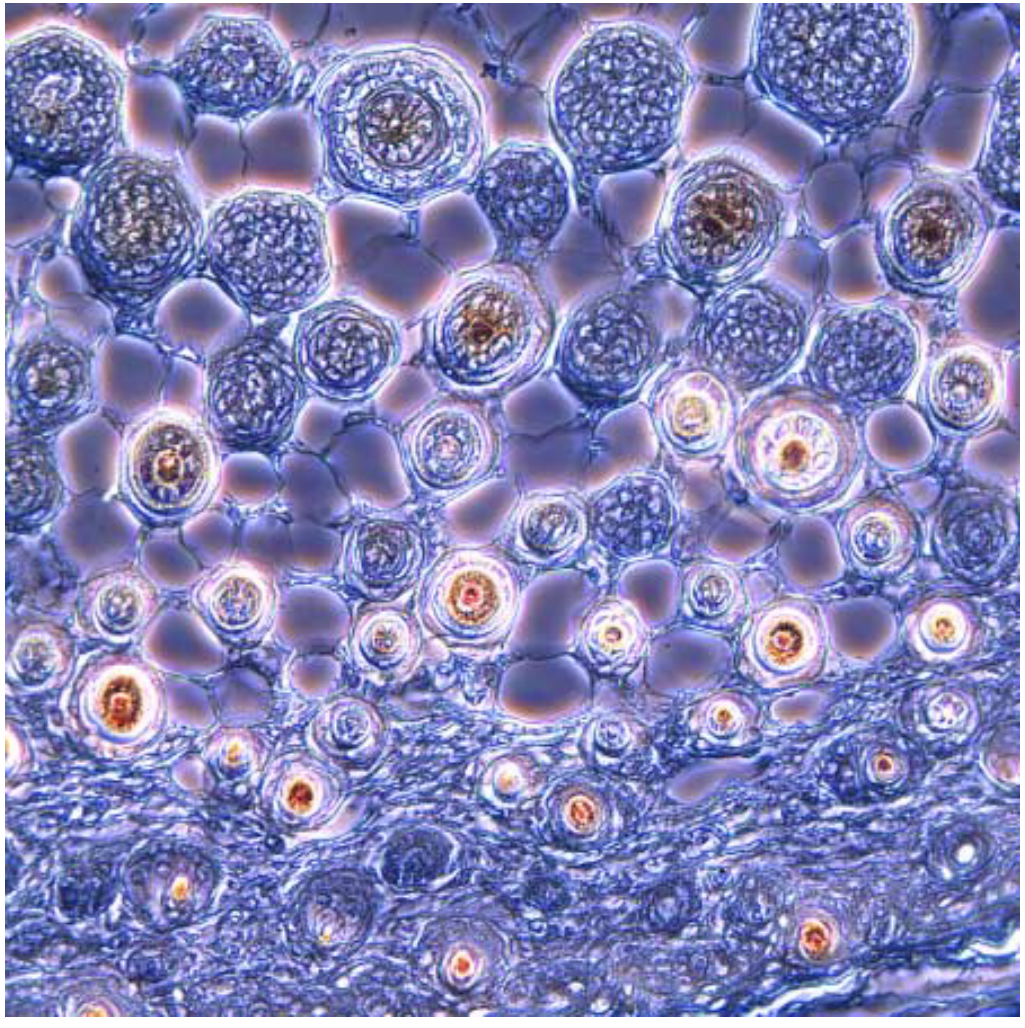
Interference. Slow down/Speed up. direct light $\rightarrow$ bright/dark contrast

Phase contrast microscopy (2)



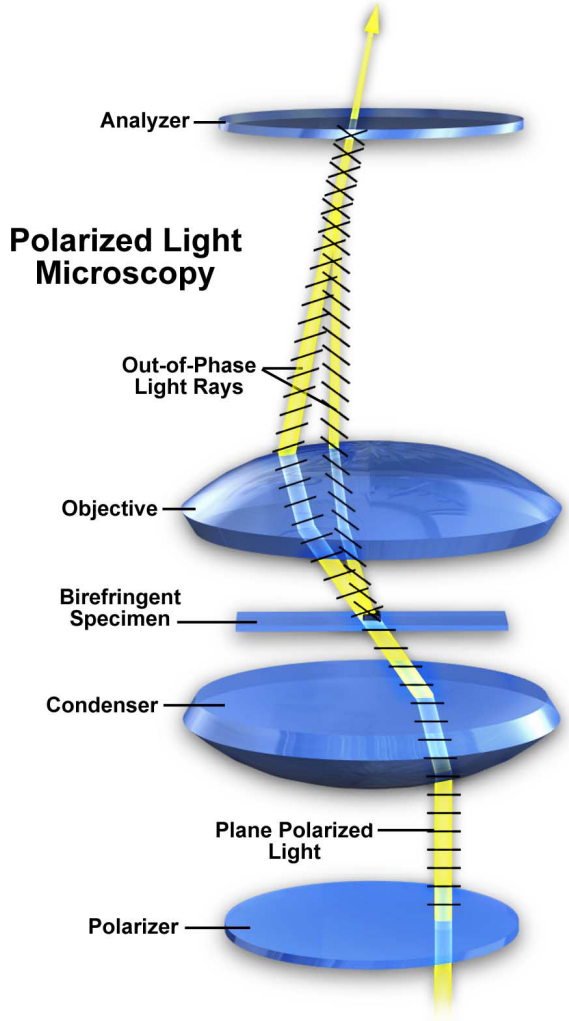
cells

Phase contrast microscopy (2)



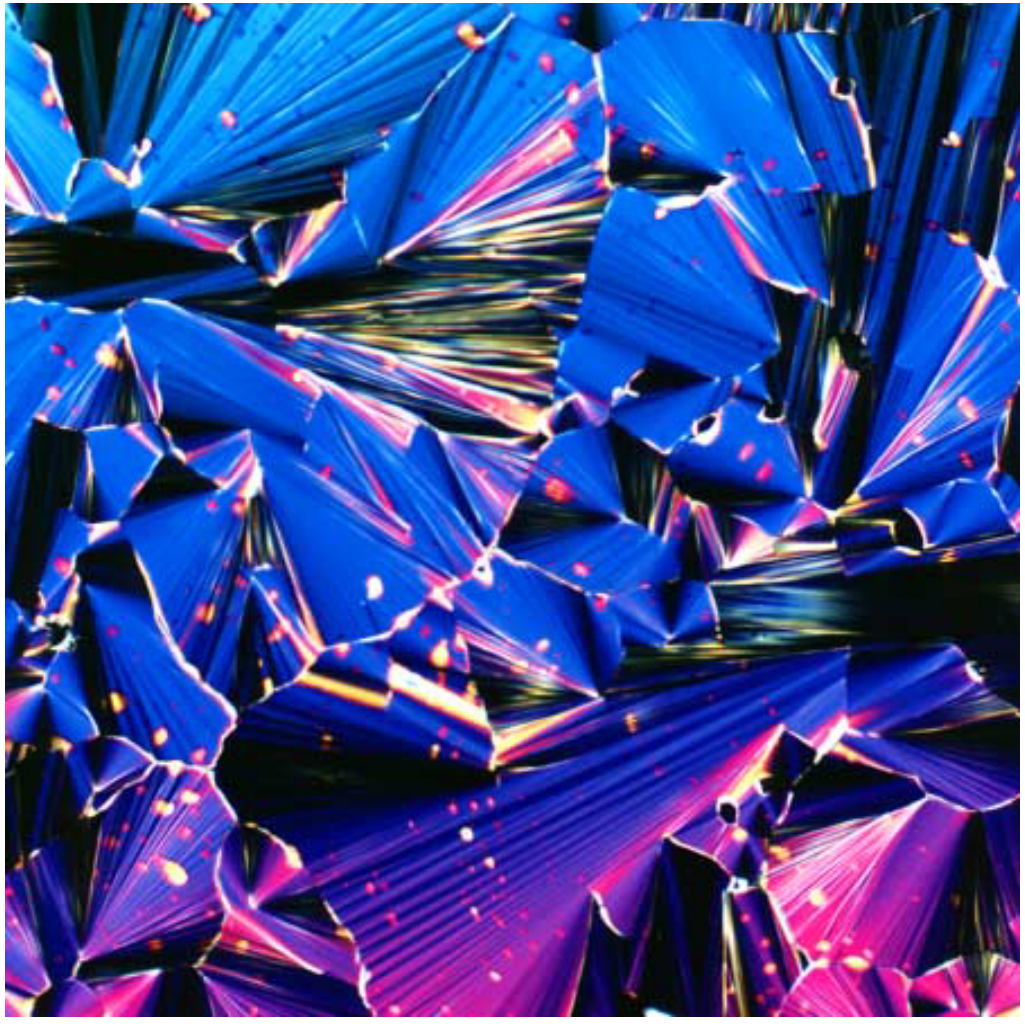
mouse hair cross-section

Polarized light microscopy

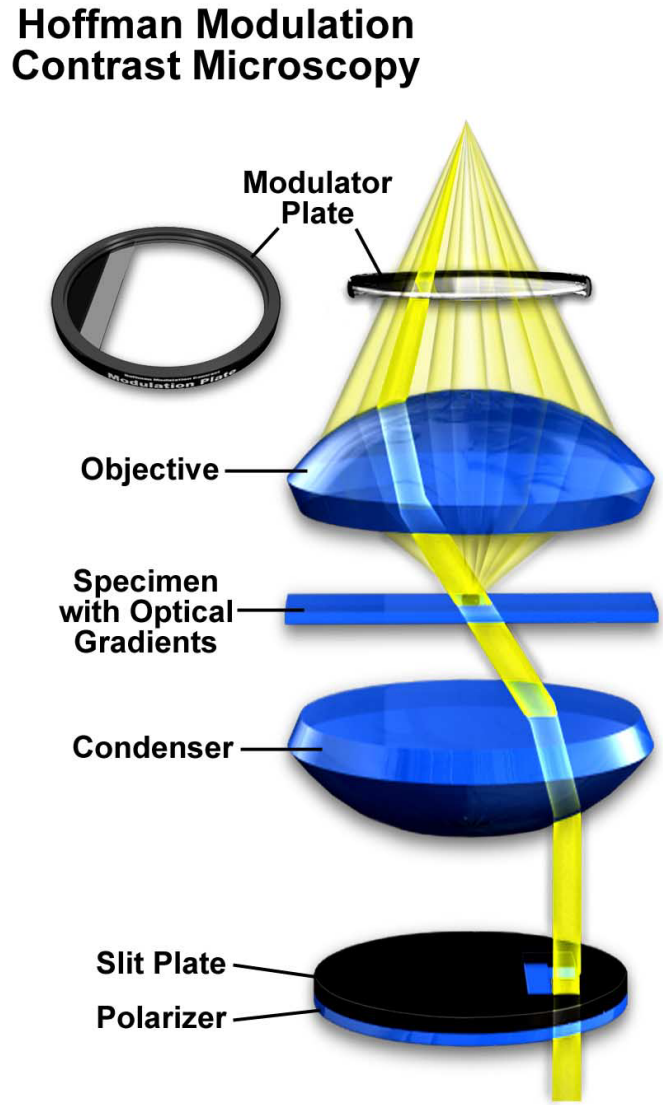


- different refractive indices for different polarizations
- interference subtracts some wavelength $\rightarrow$ colors

Polarized light microscopy (2)

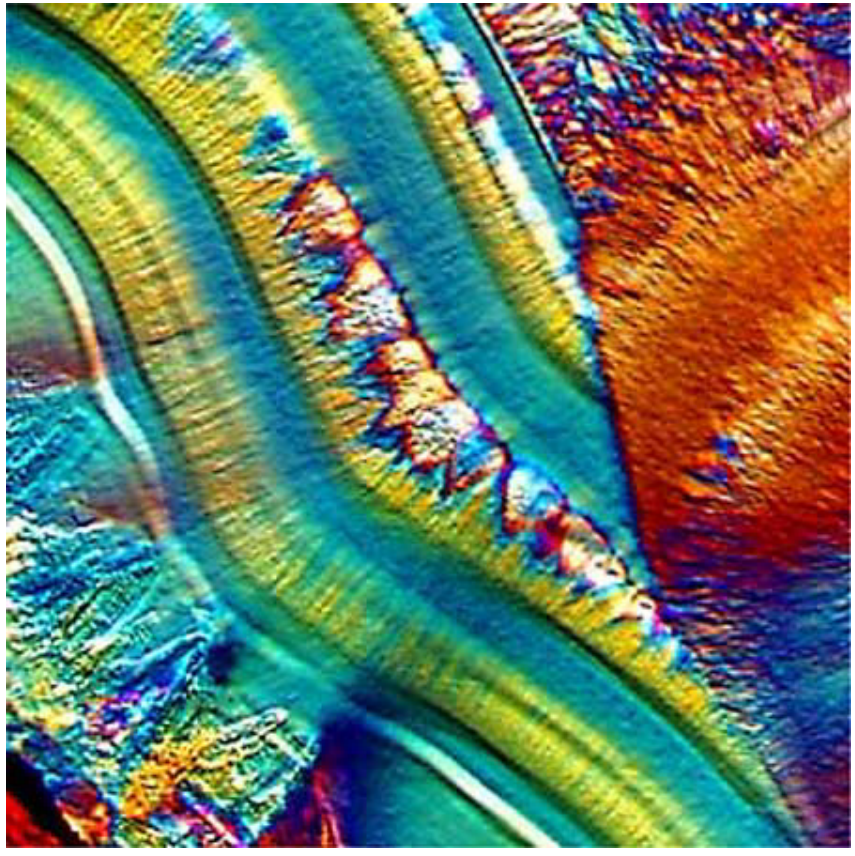


DNA

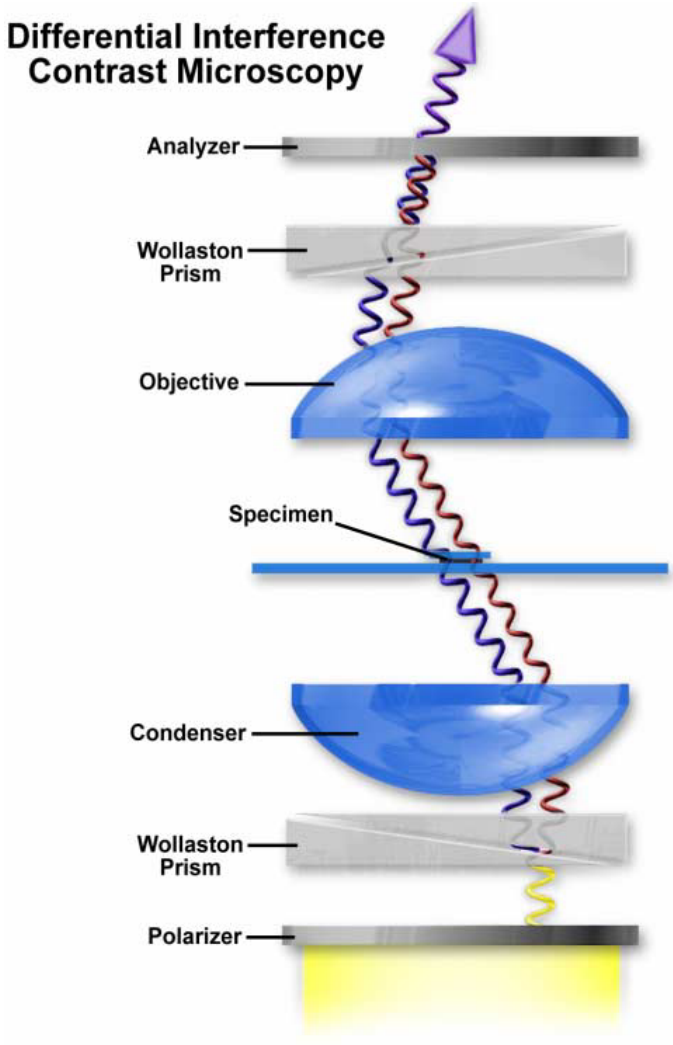


Robert Hoffman (1975). For living and unstained specimens. Detects optical gradients. Image intensity proportional to the derivative of the optical intensity of the specimen.

Hoffman modulation contrast (2)

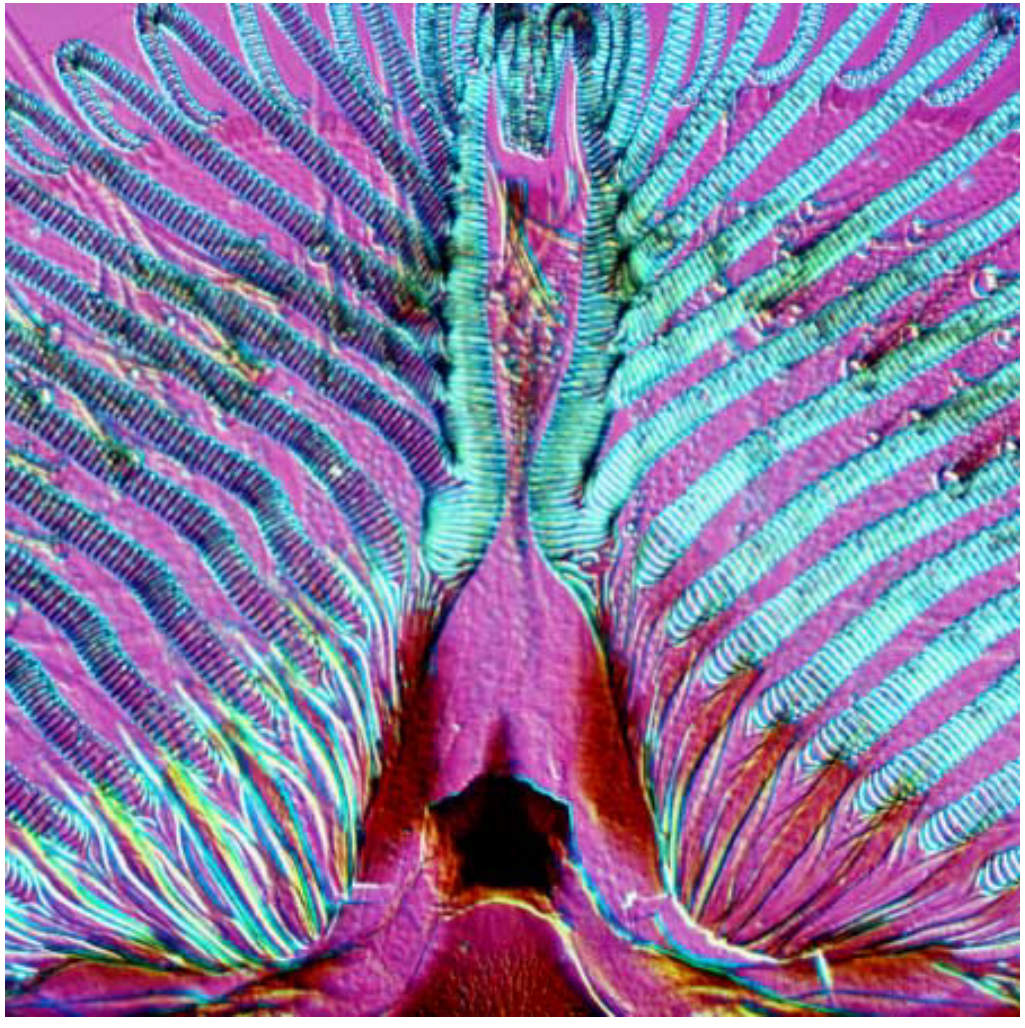


Dinosaur bone



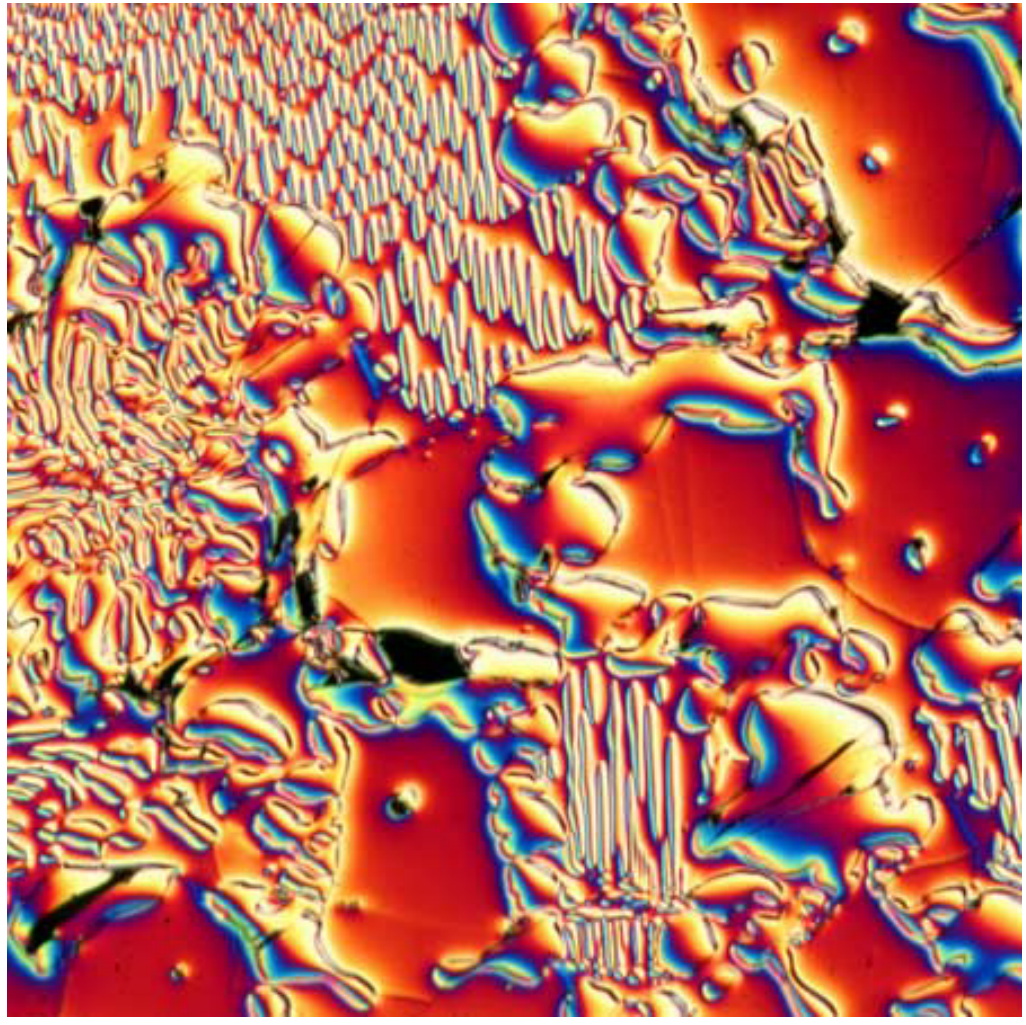
Detects differences in optical paths between two close slightly offset rays (shear). Wollaston prism $\rightarrow$ orthogonal polarizations.

Differential interference contrast microscopy (2)



Mouth part of a blowfly.

Differential interference contrast microscopy (3)

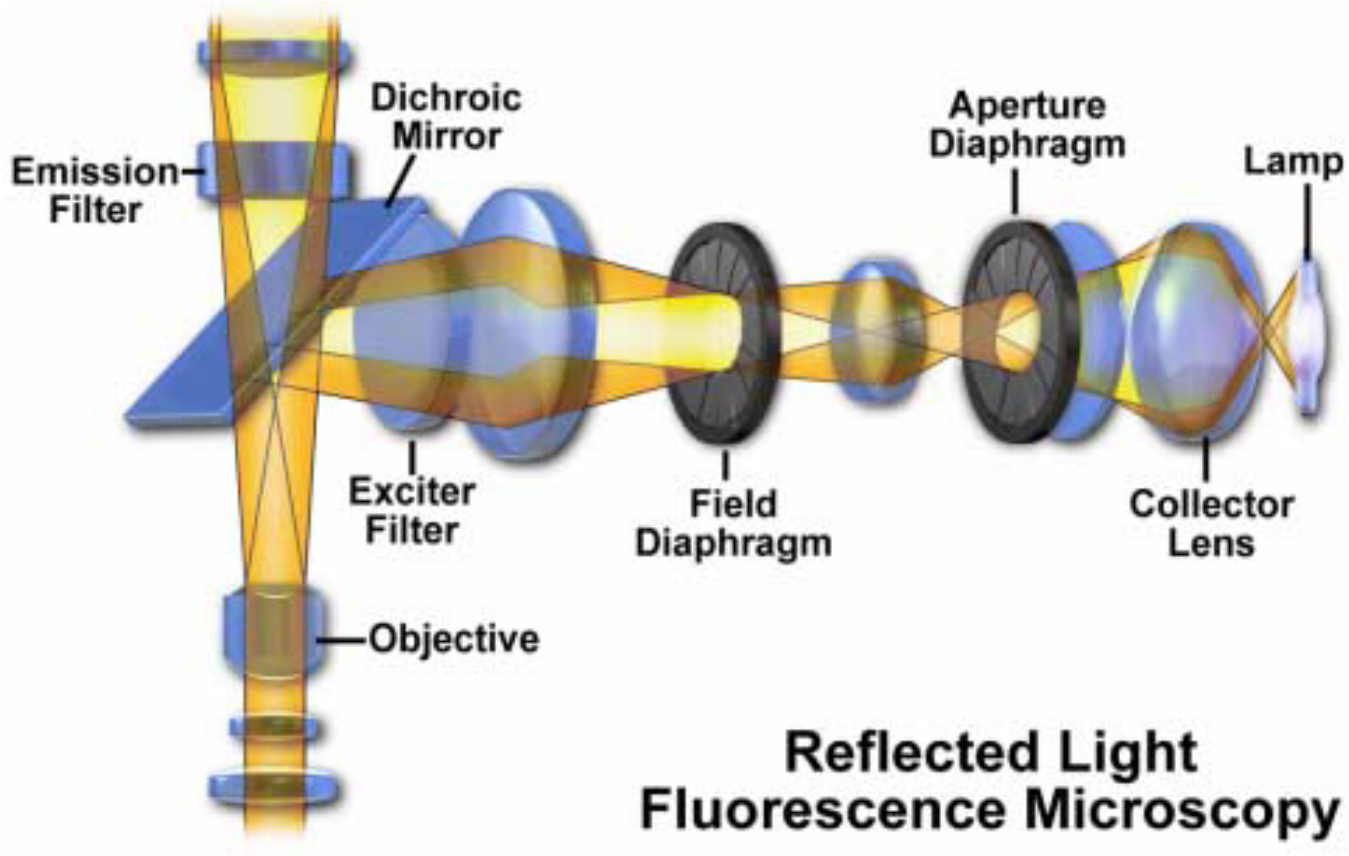


Defects in ferro-silicon alloy.

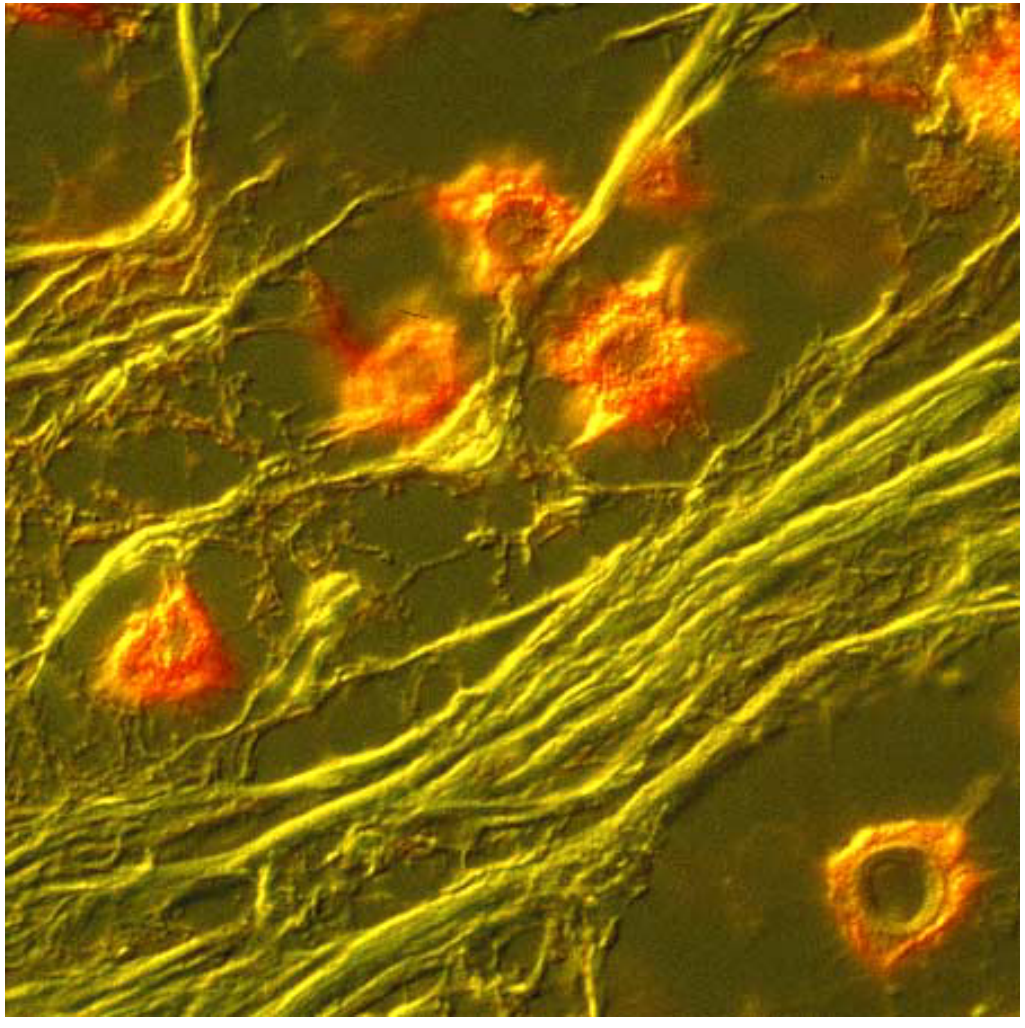
Fluorescence microscopy

- fluorescent dyes
- multiple sensing channels/filters
- multiple light sources – visible, UV

Fluorescence microscopy (2)

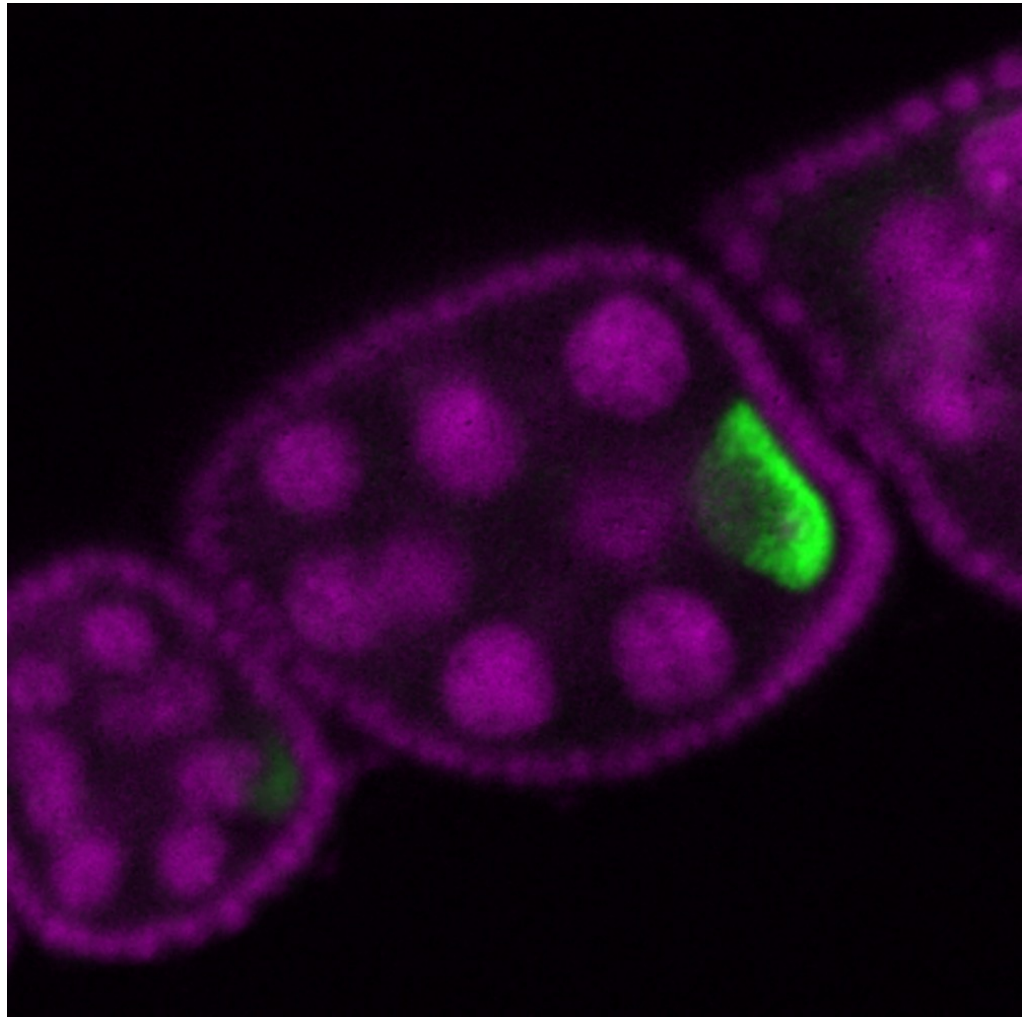


Fluorescence microscopy (3)



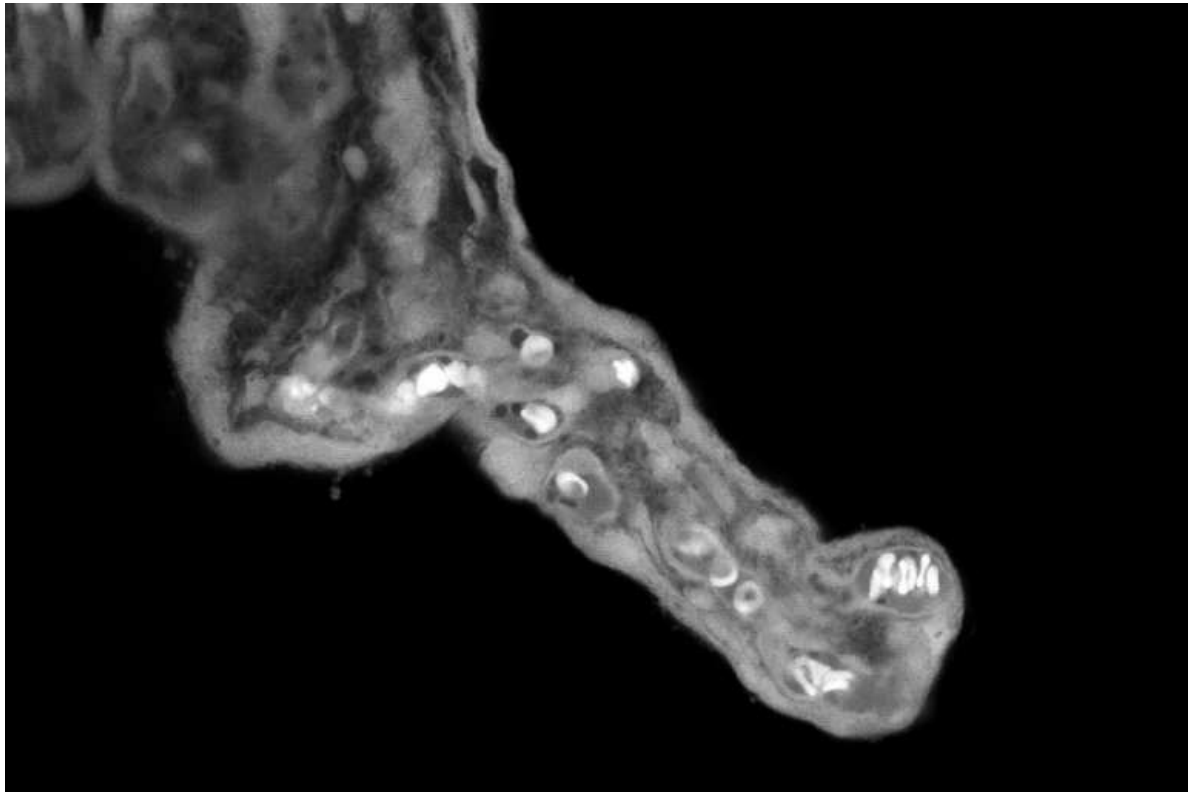
cat brain tissue infected with *Cryptococcus* (fungus)

Fluorescence microscopy (4)



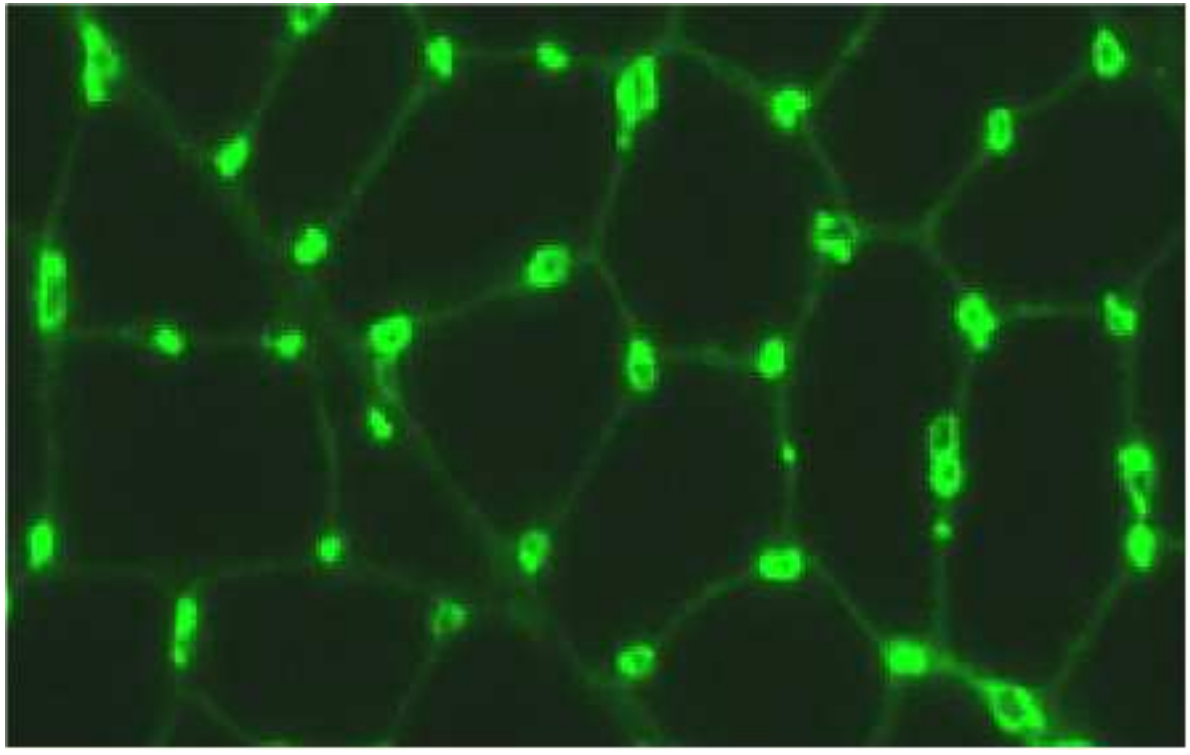
Drosophila eggs gene expression

Other examples images



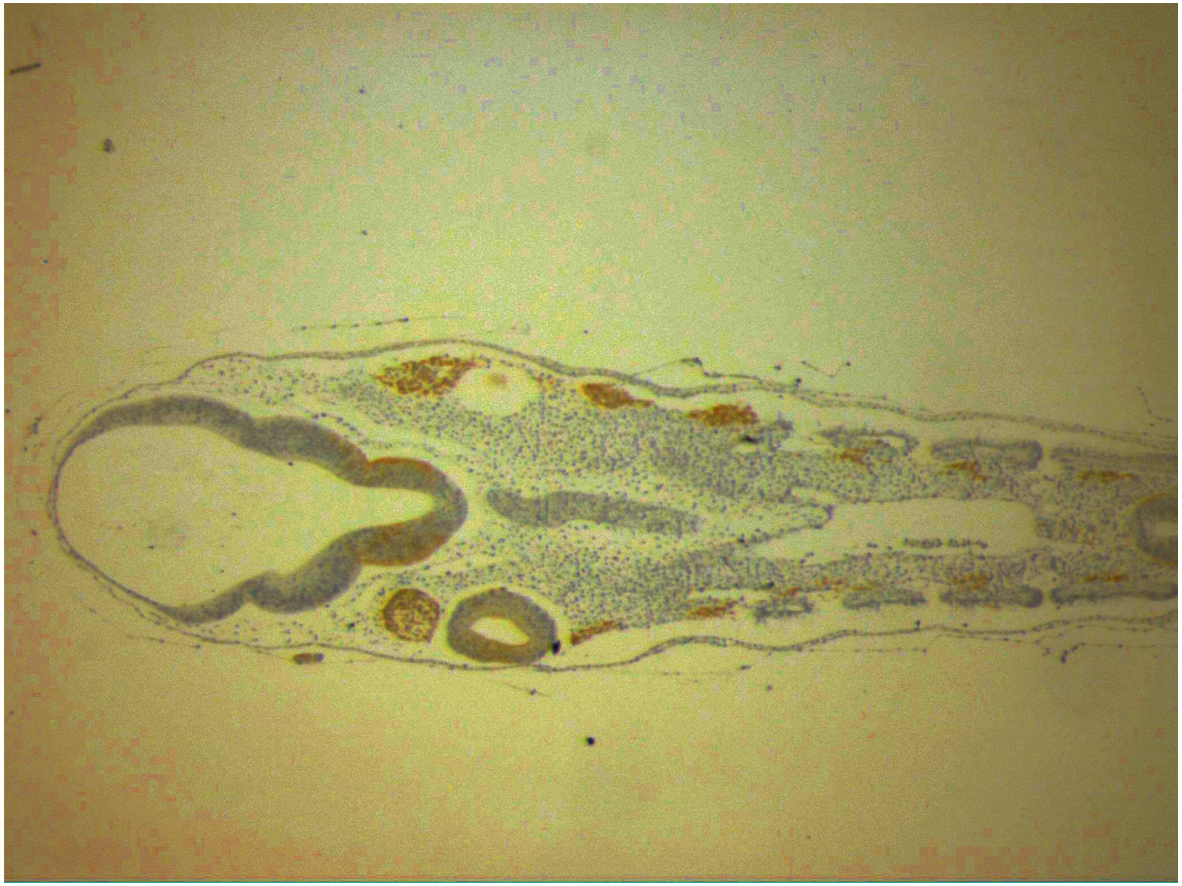
placenta cross-section

Other examples images



muscle capillaries

Other examples images

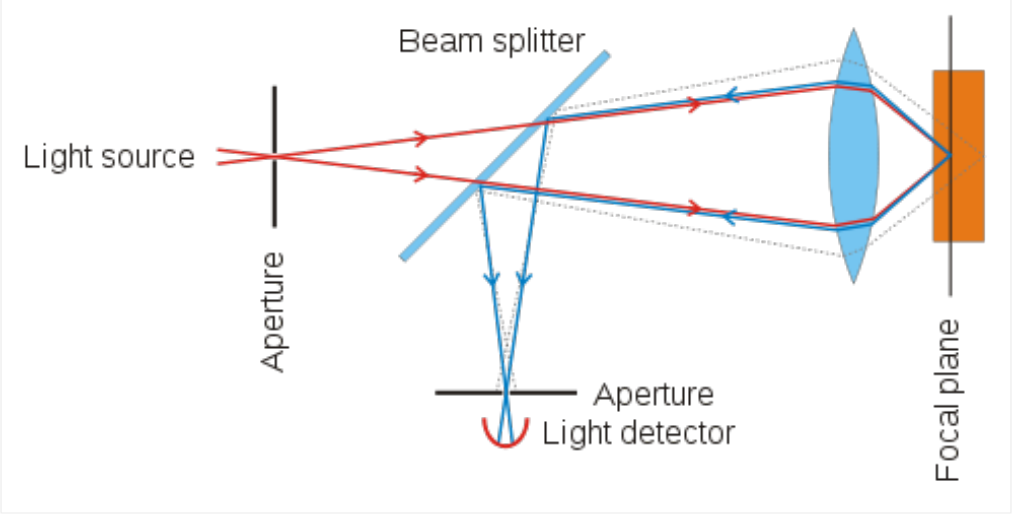


crocodile ear slice

Advanced microscopy techniques

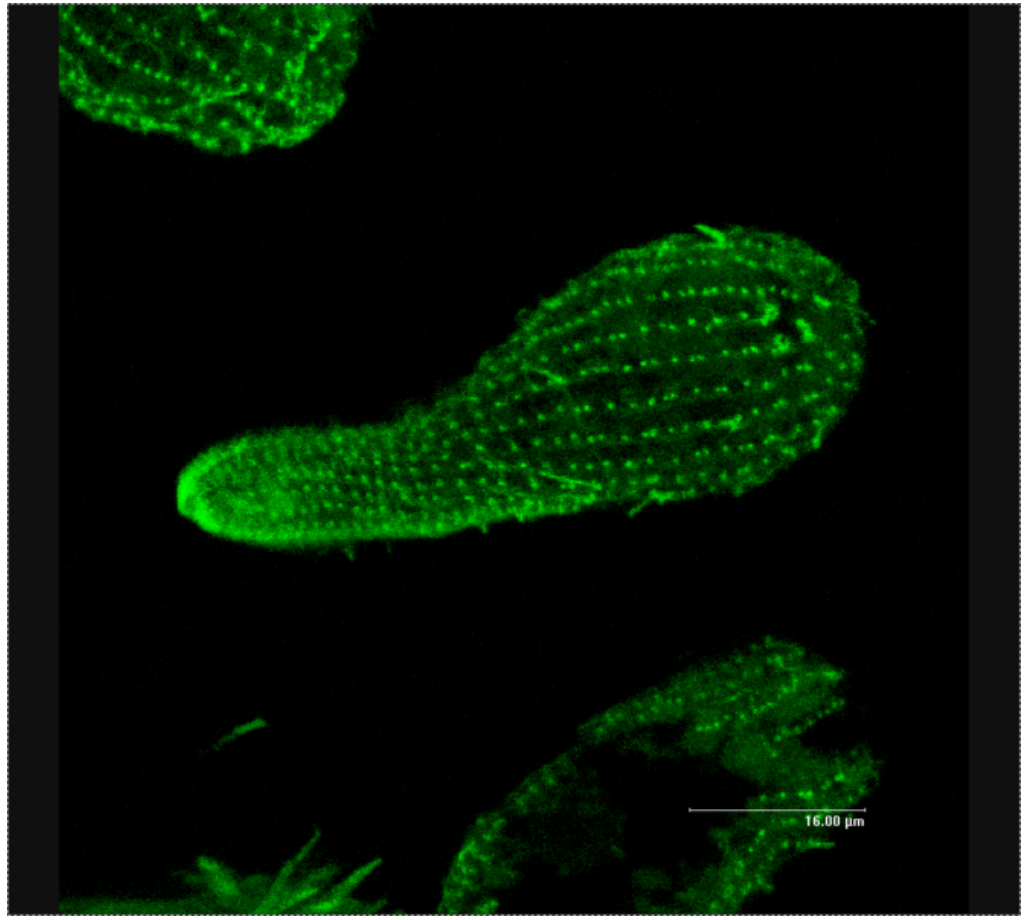
- **3D microscopy**
  - Confocal microscopy
  - Optical coherence tomography (OCT)
  - Multiphoton / two-photon microscopy
- **High resolution microscopy**
  - Stimulated emission depletion (STED)
  - Stochastic optical reconstruction microscopy (STORM)
  - Photo-activated localization microscopy (PALM)
- **Electron microscopy**
  - Scanning electron microscopy (SEM)
  - Serial section EM (3D)
  - Focused ion beam (FIB) (3D)
  - Transmission electron microscopy (TEM)

Confocal microscopy



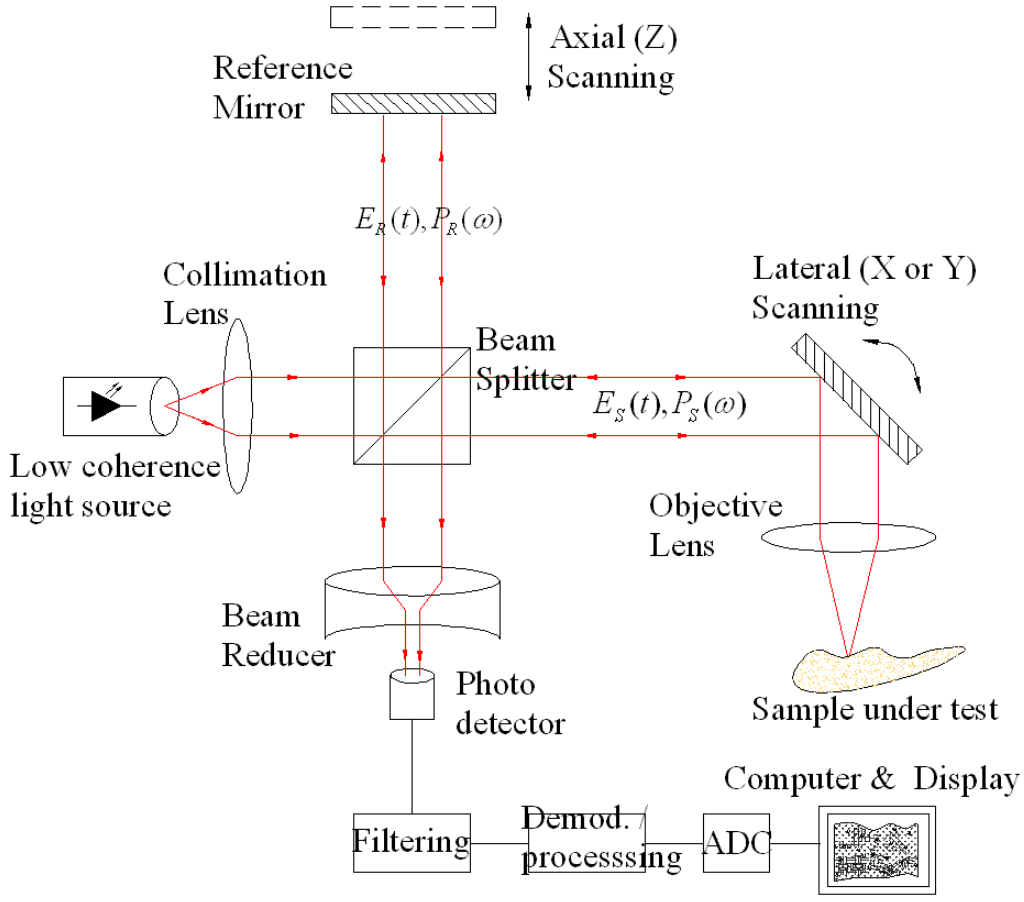
- Very good resolution
- Very thin focal plane — 3D imaging
- Confocal laser scanning
- Scanning — slow

Confocal microscopy example



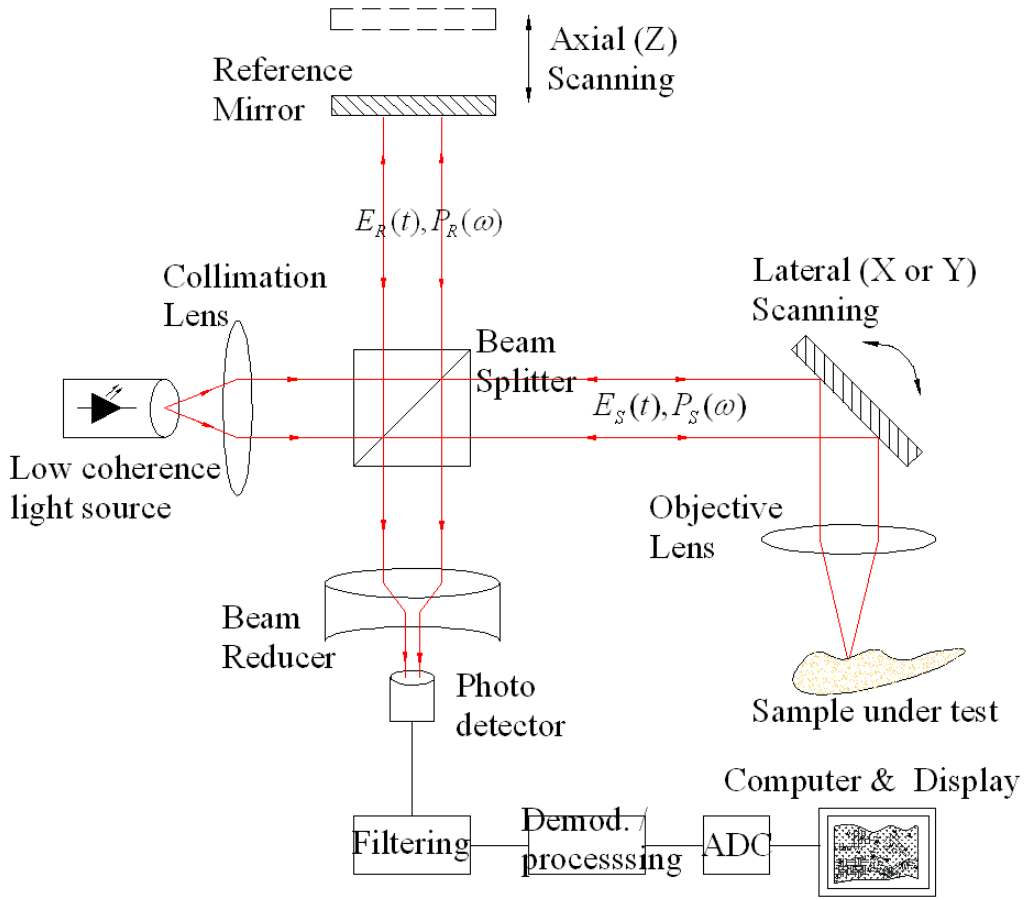
Tetrachimena

Optical coherence tomography (OCT)



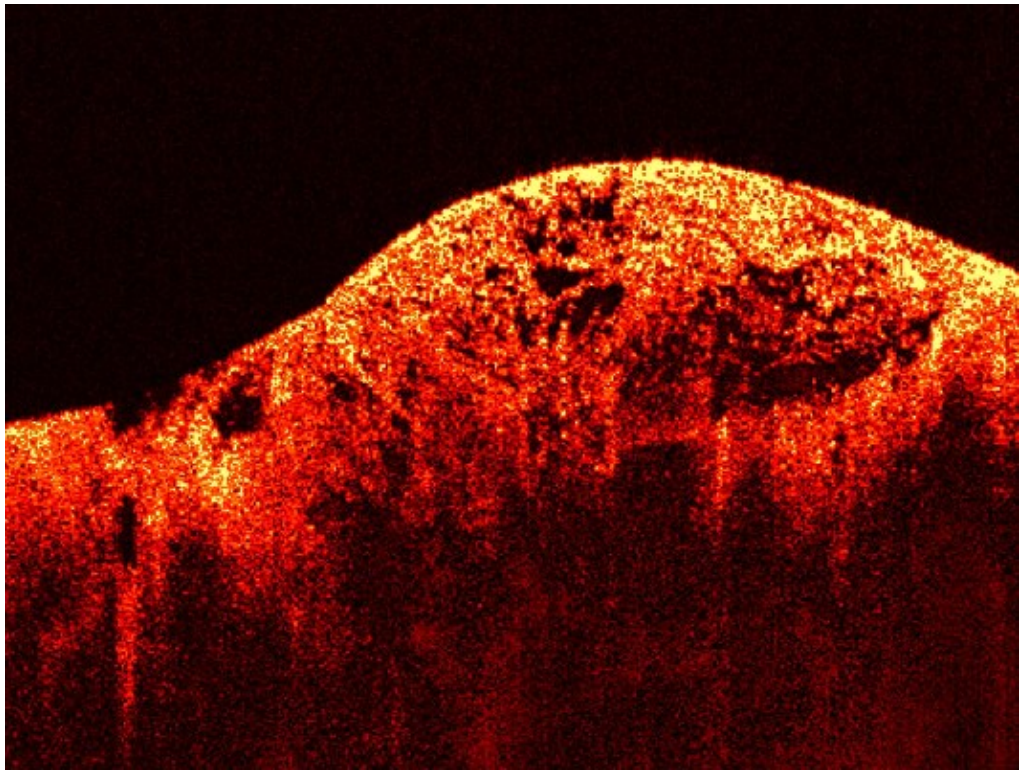
- 3D imaging
- Interferometry
- More penetration than confocal, especially near infrared

Optical coherence tomography (OCT)



- 3D imaging
- Interferometry
- More penetration than confocal, especially near infrared
- Fourier-domain OCT — one z column at a time

OCT example



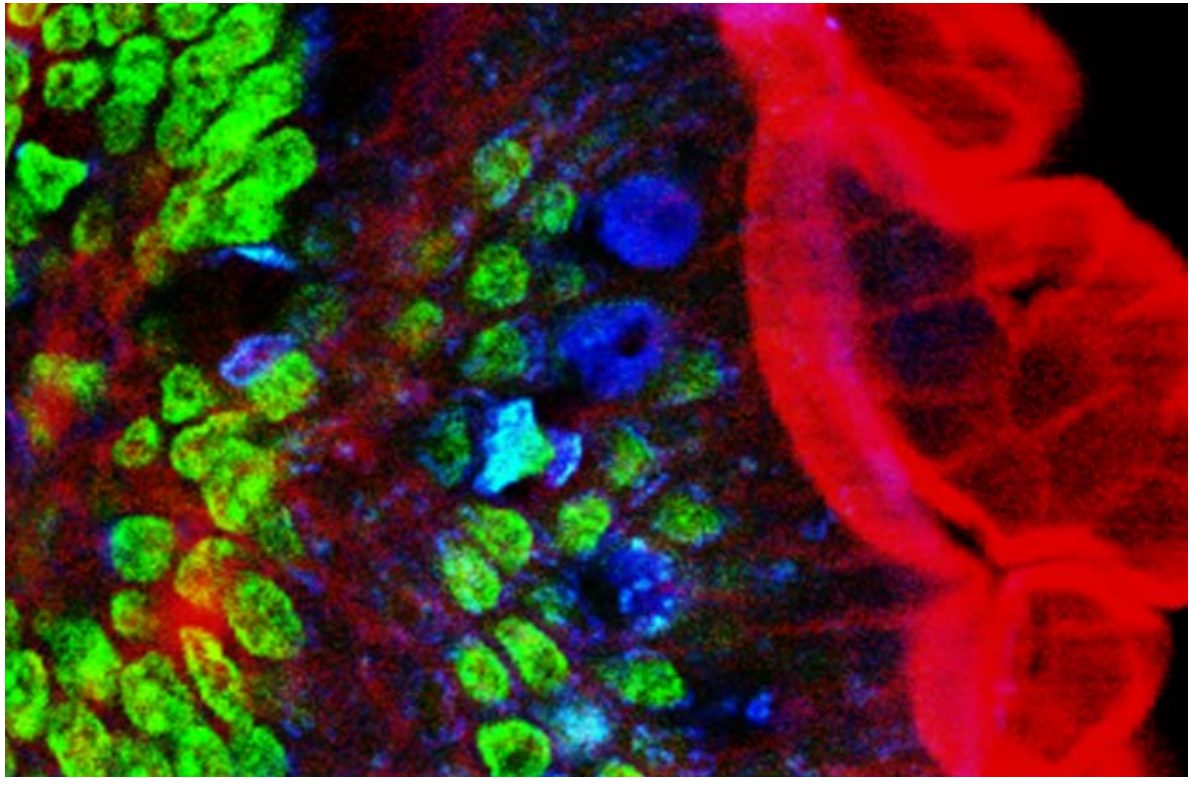
Sarcoma

Fluorescence — Two-photon microscopy

- two low-energy photons $\rightarrow$ fluorescence
- high-flux laser
- better penetration
- reduced phototoxicity
- better background suppression

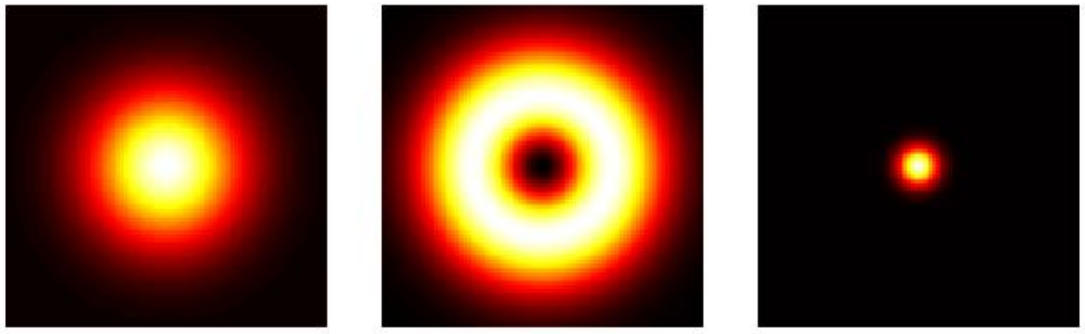
Maria Goeppert-Mayer (1931 publication, 1963 Nobel prize)

Two-photon microscopy (2)



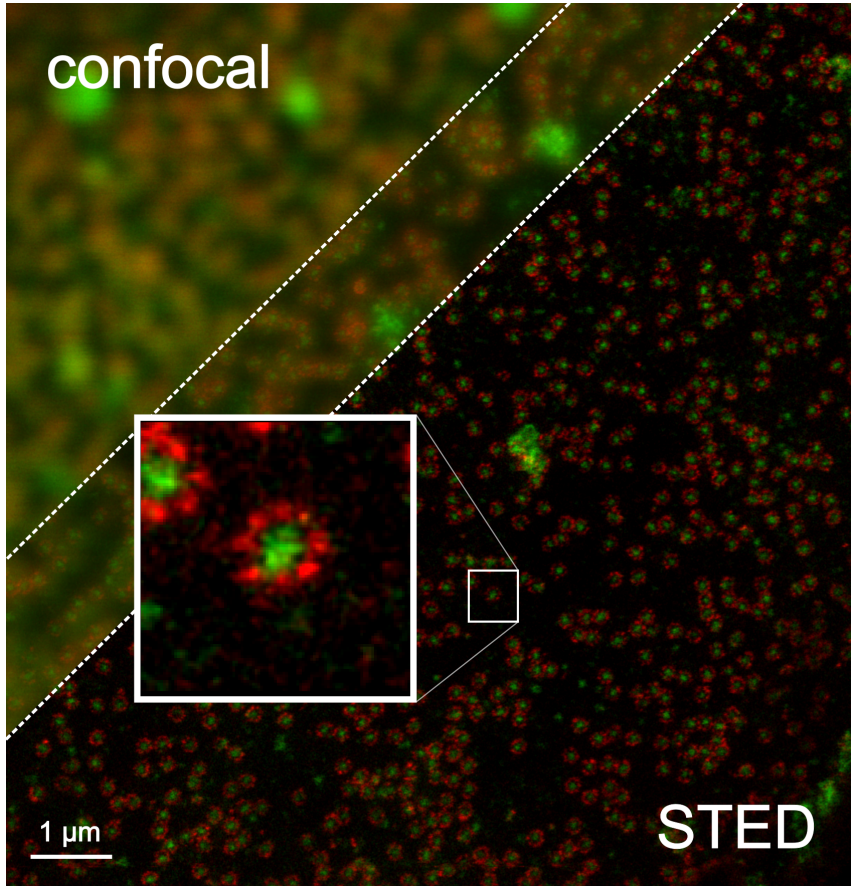
Two-photon excitation microscopy of mouse intestine. Red: actin. Green: cell nuclei. Blue: mucus of goblet cells. [Wikipedia]

Superresolution — Stimulated emission depletion (STED)



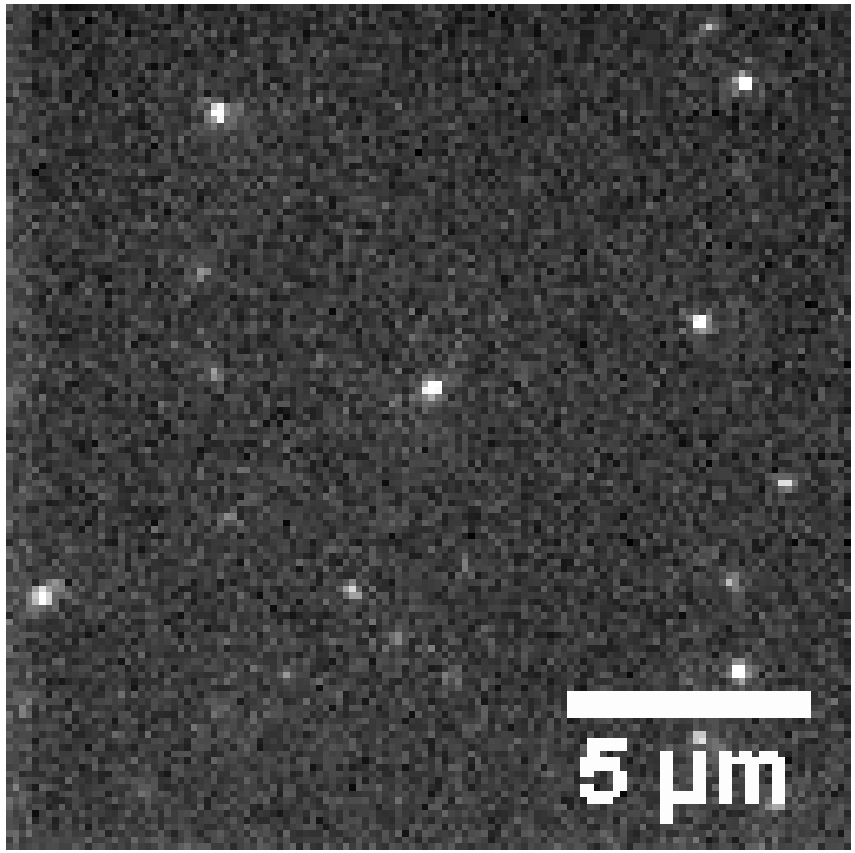
- excitation subpicosecond laser impulse
- depletion pulse around the focal spot, stimulating the emission
- fluorescence at the focal spot remains
- resolution $2 \sim 80\,\mathrm{nm}$
- Hell and Klar, 1999. Hell awarded the Nobel Prize in Chemistry in 2014

Stimulated emission depletion (STED) (2)



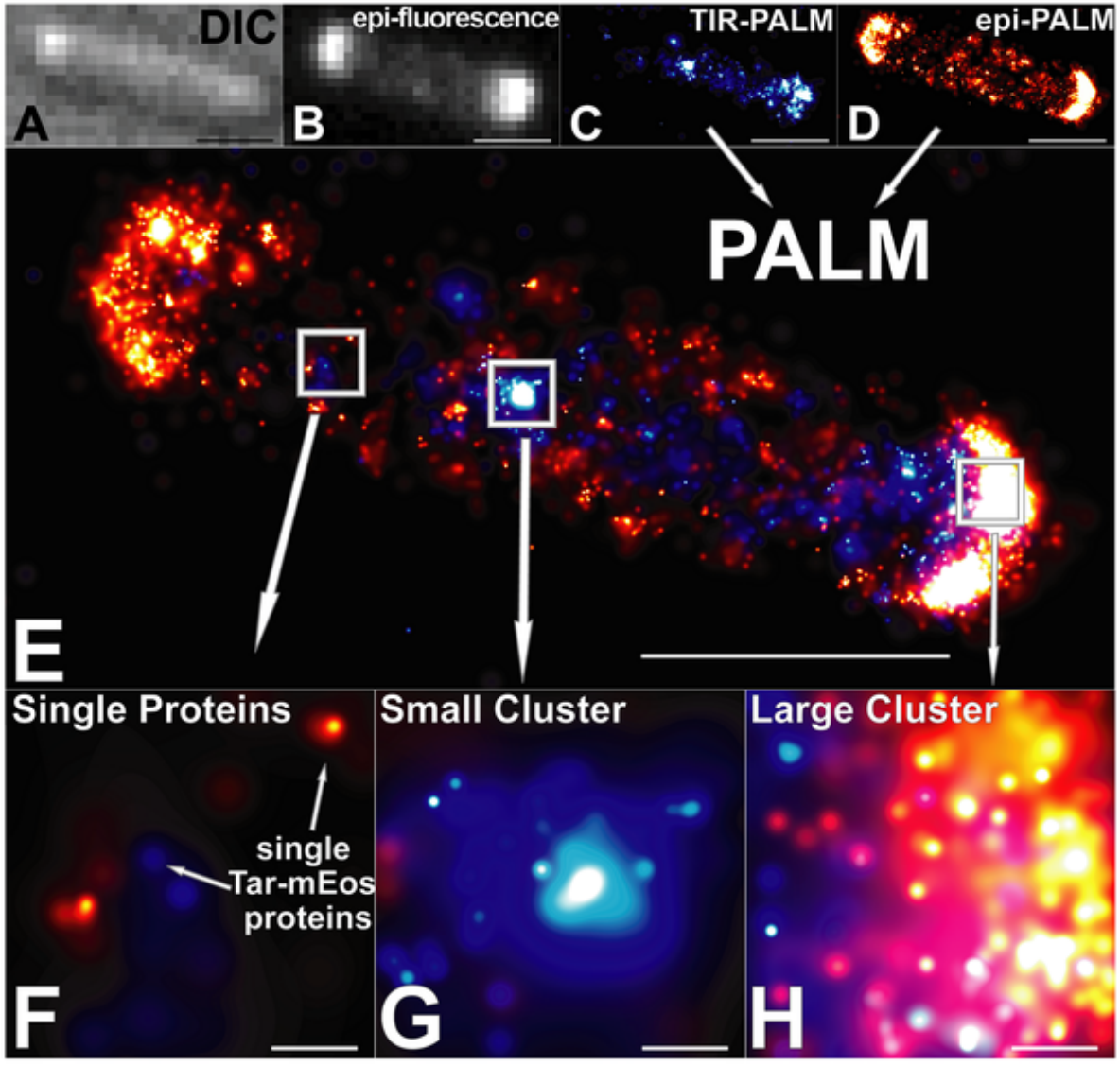
STED versus confocal

Stochastic optical reconstruction microscopy (STORM/PALM)

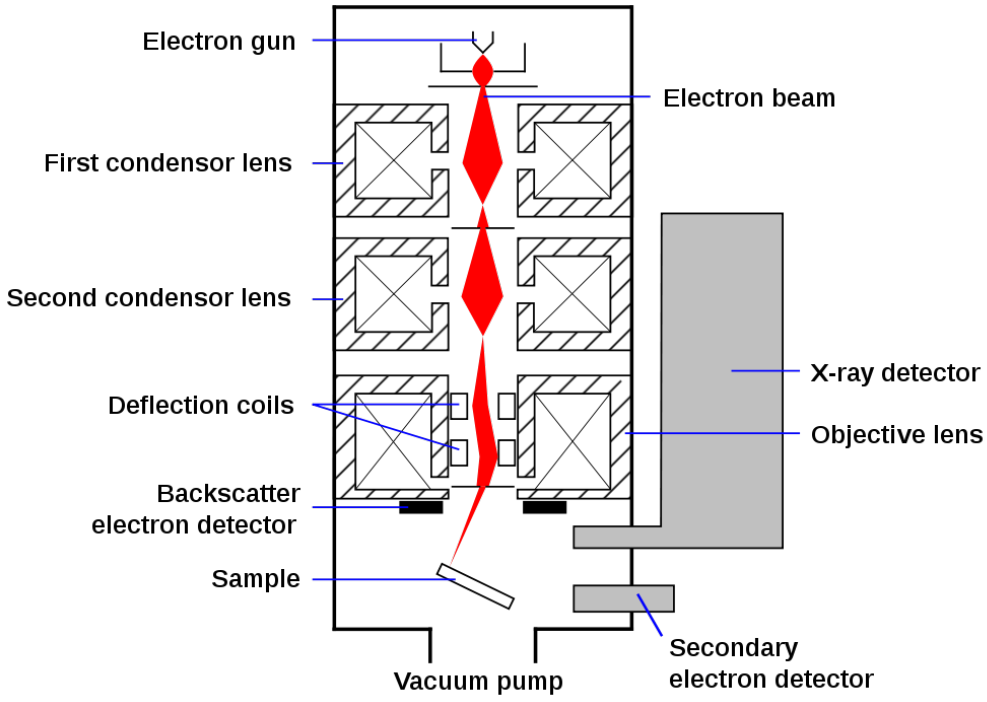


- sparse fluorophores localized by PSF fitting
- combine many images
  PALM photobleaching, STORM reversible switching

Stochastic optical reconstruction microscopy (STORM/PALM)

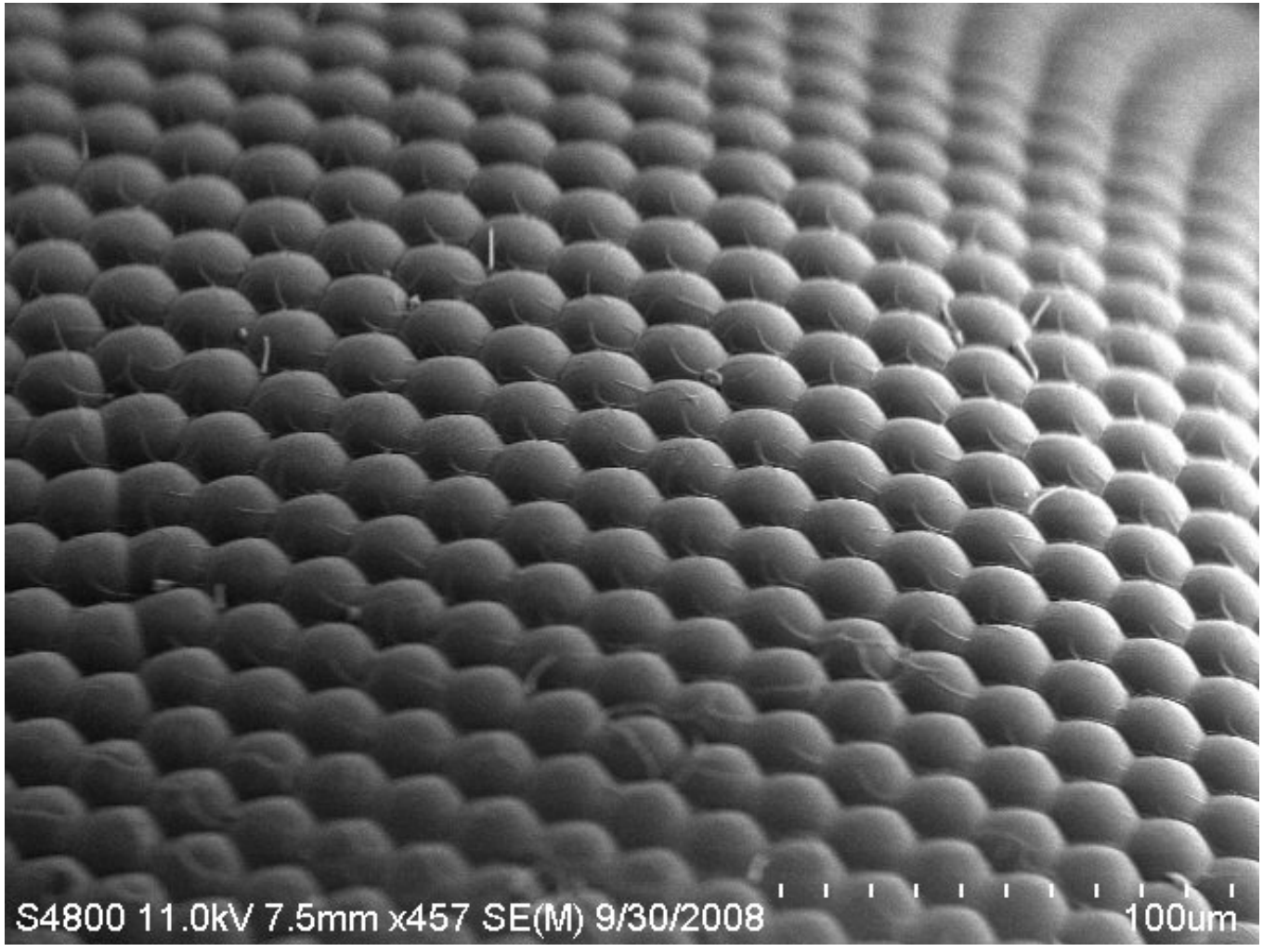


Scanning electron microscopy (SEM)



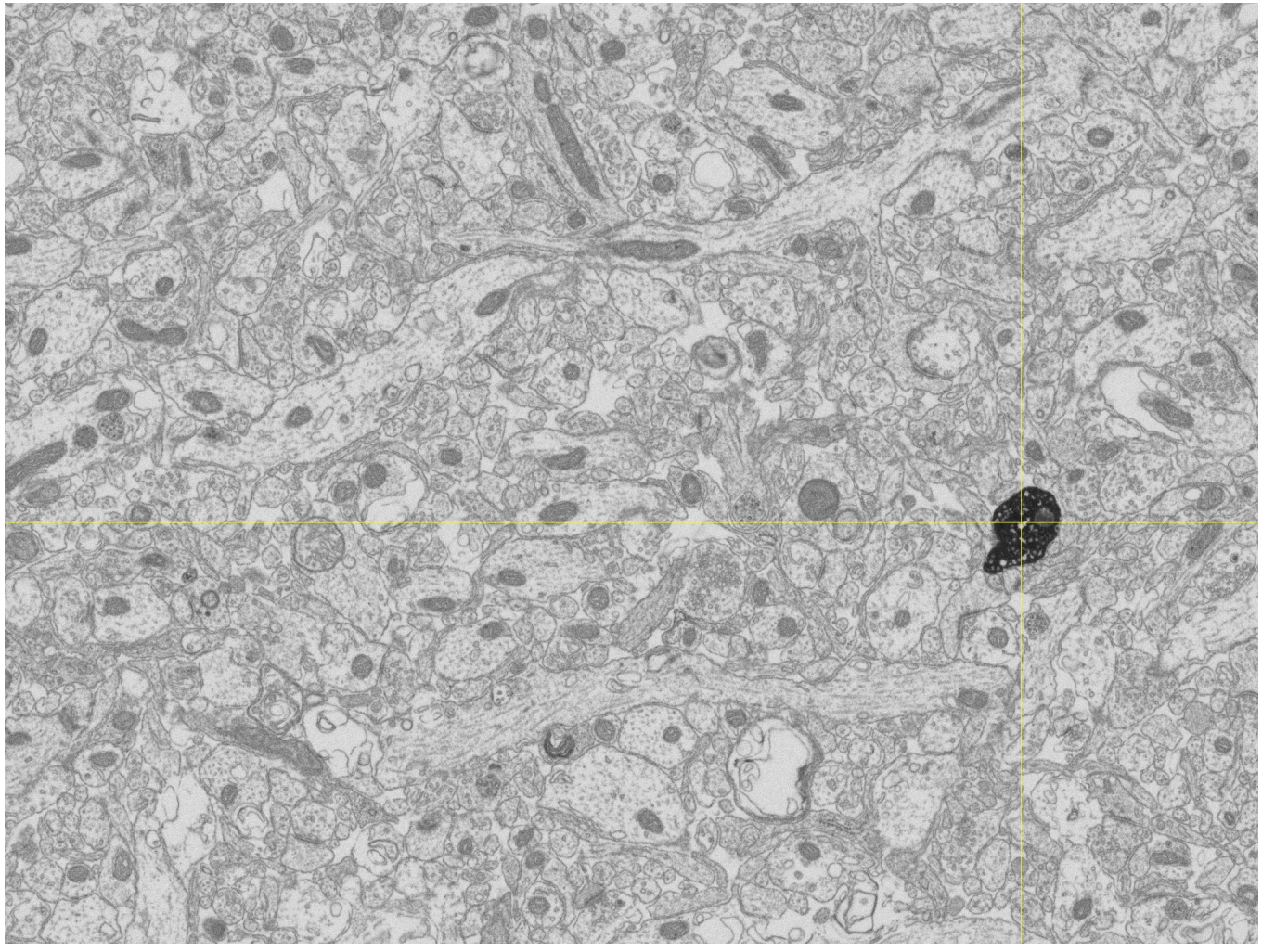
- Excellent resolution (a few nm)
- Needs vaccuum. Preparation — gold coating, osmium staining, cryofixation.

SEM example



Fly eye

FIB example



- Focused ion beam for slice cutting. True 3D

Microscopy — digitalization & automation

- CCD cameras
  - supercooled
  - superresolution
- Moveable specimen tray
  - Auto-focusing
  - Automated acquisition, mosaicking
- Automatic processing

Microscopy

- Advantages
  - High-spatial resolution
  - Colour and texture information
  - Affordable (optical microscopy)
  - Proven technique – large body of experts available
- Disadvantages
  - Difficulties of in-vivo observations
  - Mostly 2D
  - Missing large-scale perspective