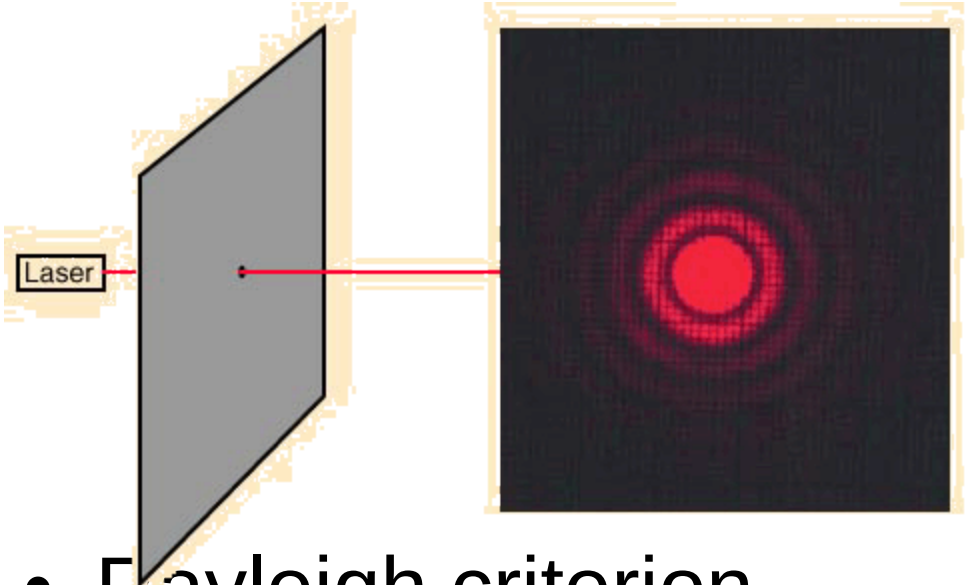
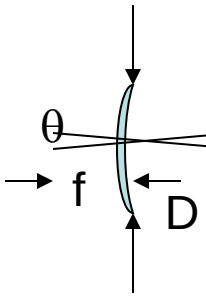
Microscopy Outline

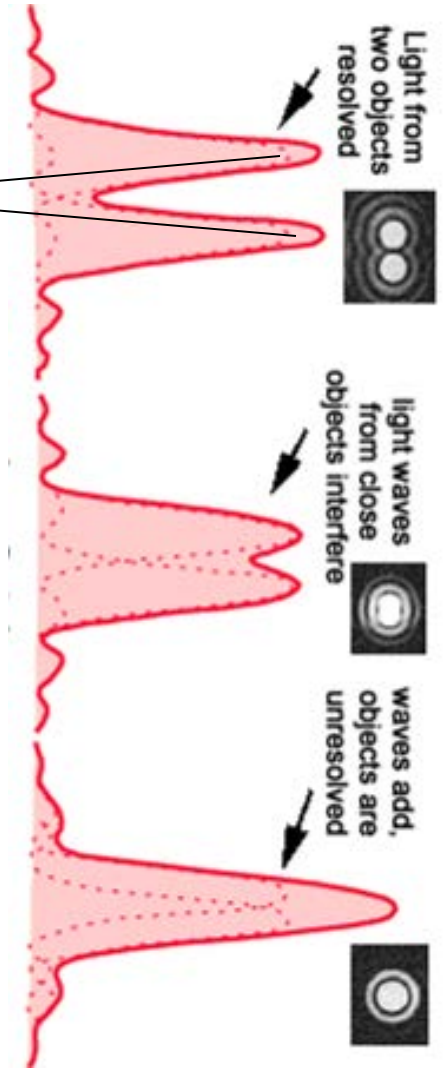
1. Resolution and Simple Optical Microscope
2. Contrast enhancement: Dark field, Fluorescence (Chelsea & Peter), Phase Contrast, DIC
3. Newer Methods: Scanning Tunneling microscopy (STM), Atomic Force Microscopy (AFM – Andrew R & Kyle); confocal, Laser Tweezers
4. Electron Microscopy(Chelsea & Peter): Transmission, Scanning (SEM), Scanning Transmission (STEM)

Resolution

- Diffraction from apertures limits resolution







- Rayleigh criterion

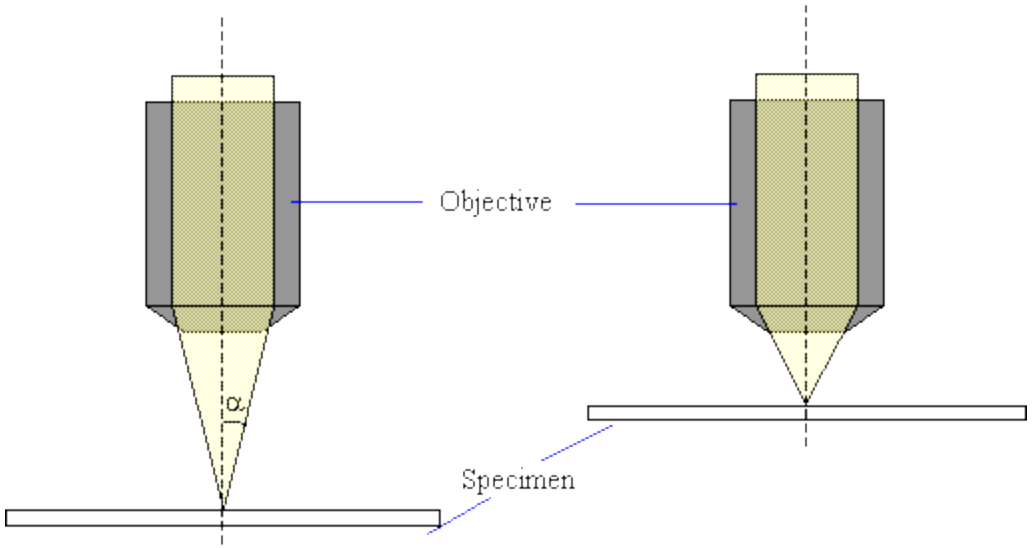
$\theta_{Rayleigh} = 1.22\ \lambda/D$

1 peak at 2nd minimum

Resolving power = minimum separation of object = $f\theta$ = RP= $f(1.22\lambda/D)$

Resolving power

- Can show that resolving power also equals RP = $1.22\lambda/(2n\sin\alpha)$ = 0.61λ/NA (NA = numerical aperture of lens)



Low numerical aperture
Low value for a
Low resolution

High numerical aperture
High value for a
High resolution

Eye + Improving Resolution

- What is resolution of eye?

  $\theta = 1.22\lambda/D = 1.22\ (550\ nm)/(0.1\ cm)$

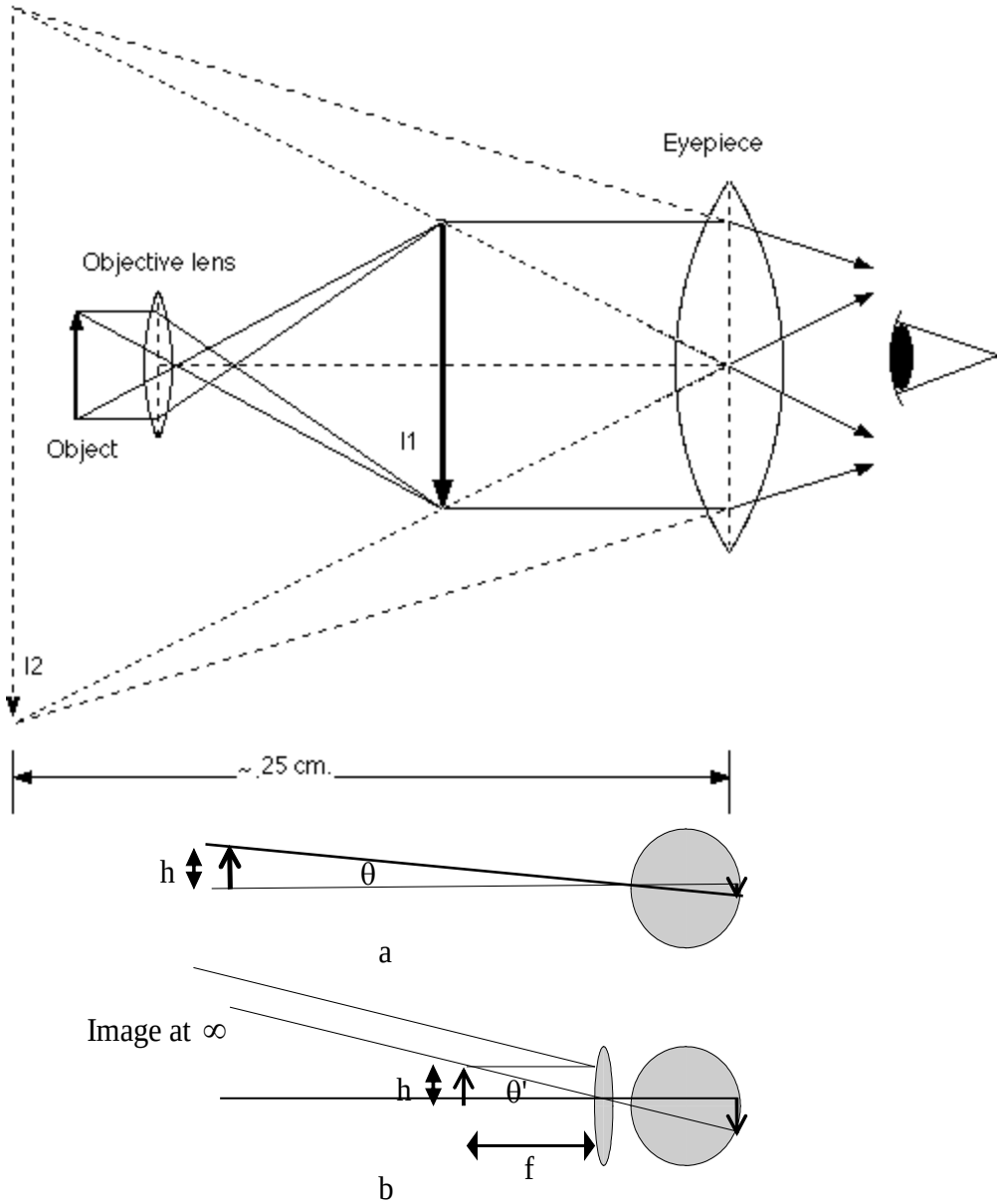
  $\sim 6 \times 10^{-4}$ rad or 1 cm at 20 m!

Highest sensitivity of eye

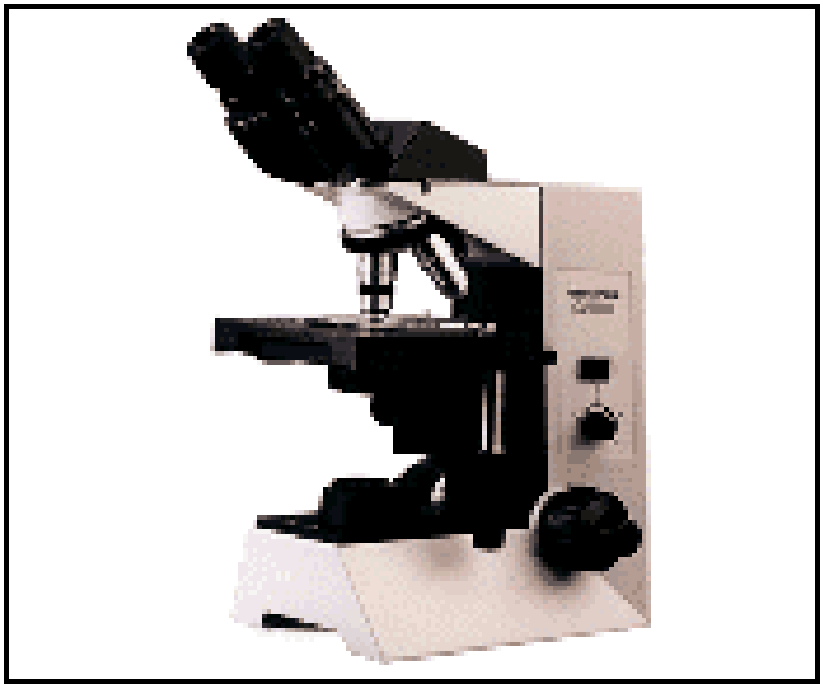
Pupil size

- On the retina (2 cm behind lens), separation of images corresponds to $s = f\theta$ = 12 μm – roughly single cone cell size
- Resolving power at near point of eye = $N\theta$ ~ 0.1 mm so max. magnification of microscope is from 0.1 mm to $\lambda/3$ ~ 200 nm or about 2000 X

Compound Microscope Optics



- $M_{obj}=d_i/d_o \sim (L-f_e)/f_o$
- $M_{eye}=\theta'/\theta = (h/d_e)/(h/N)=N/d_e \sim N/f_e$
- $M_{overall} \sim NL/(f_e f_o)$



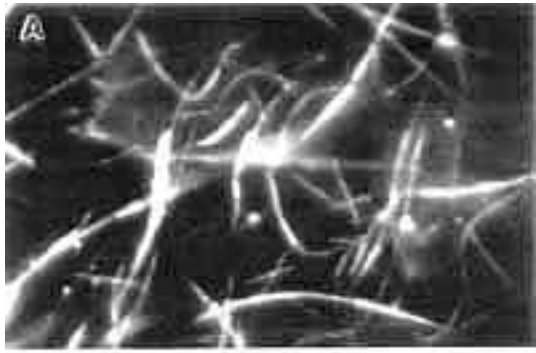
Contrast Problem

Hard to Read

Easy to Read

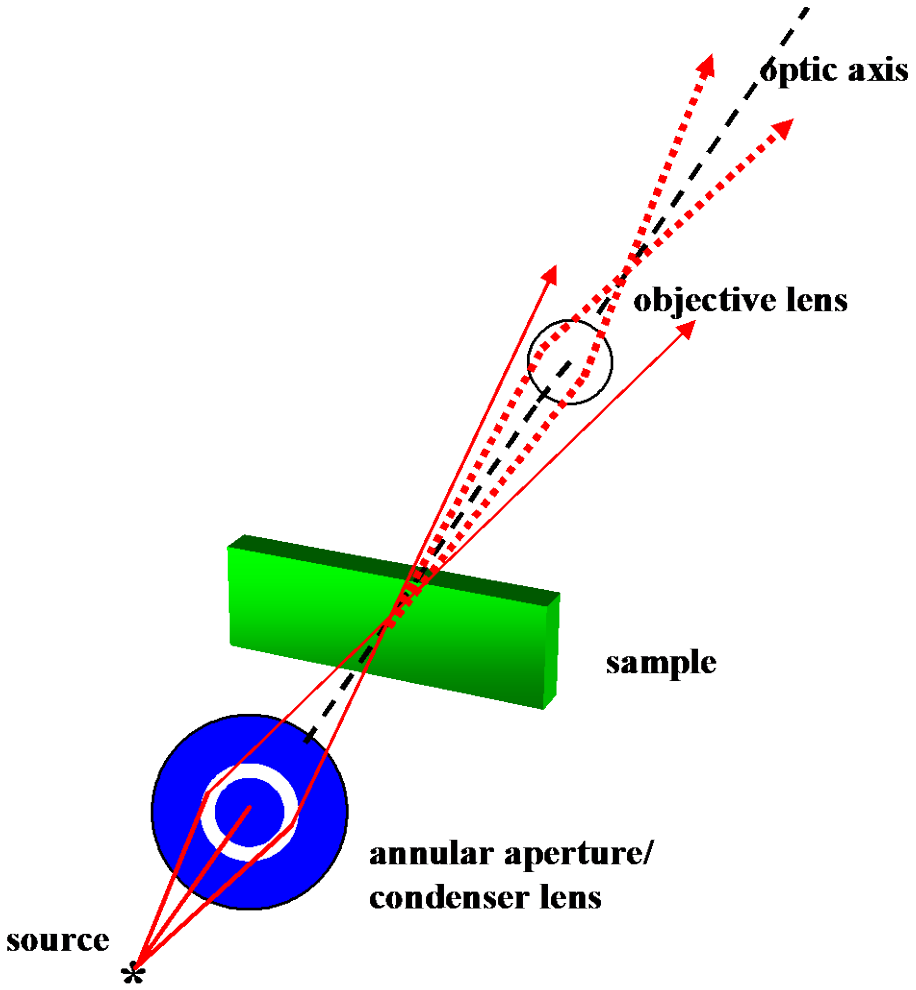
- Contrast in microscopy is given in % contrast:

  % contrast = $\{(I_{bkgd} - I_{sample})/I_{bkgd}\}$ X 100
- Low contrast since biological materials are fairly transparent to visible light
- Solve contrast problem by:
  - Staining
  - Dark field
  - Other

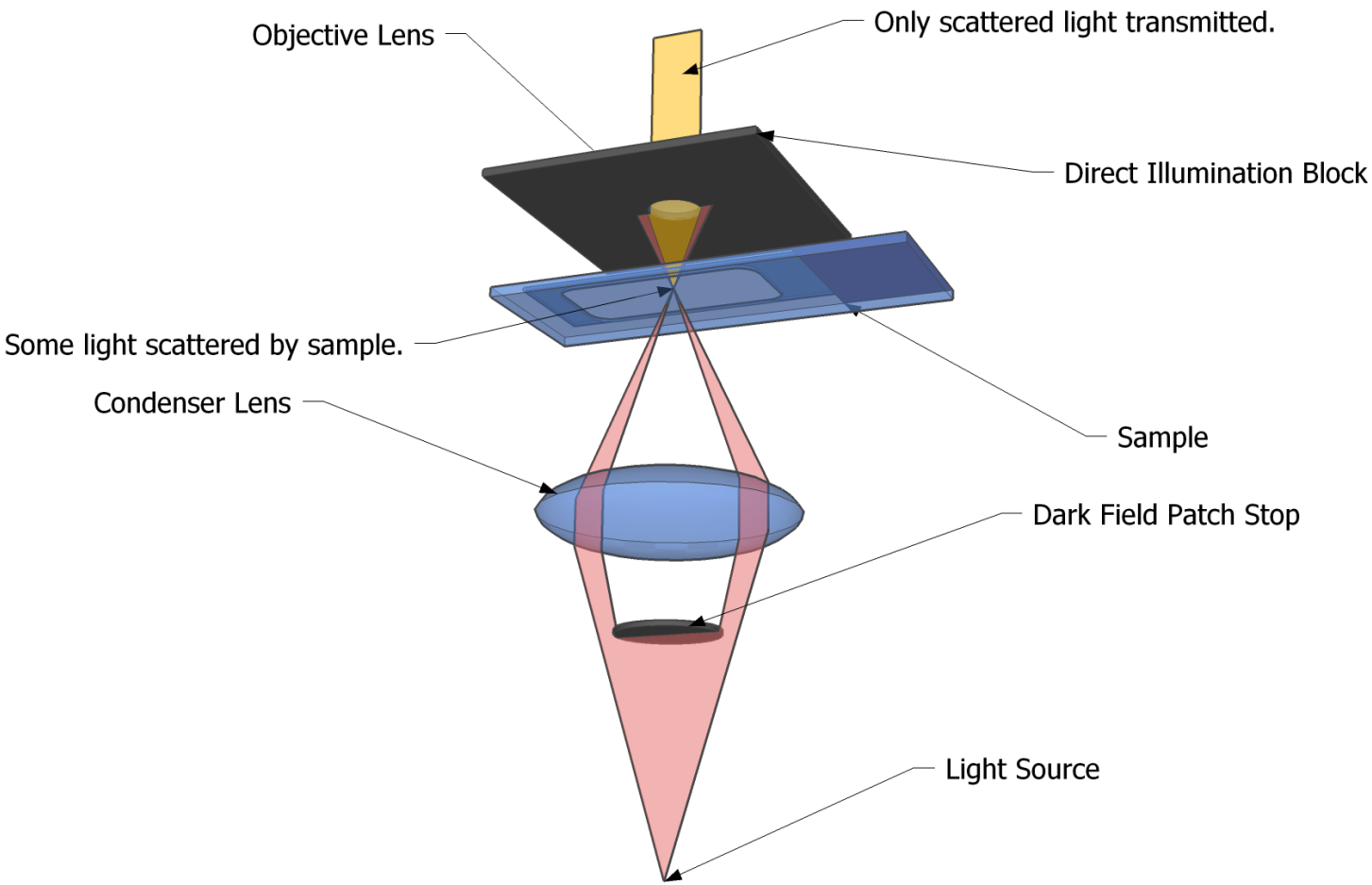


Dark Field

- Special aperture used to define incident light so that it is not collected unless scattered by sample- hence dark background

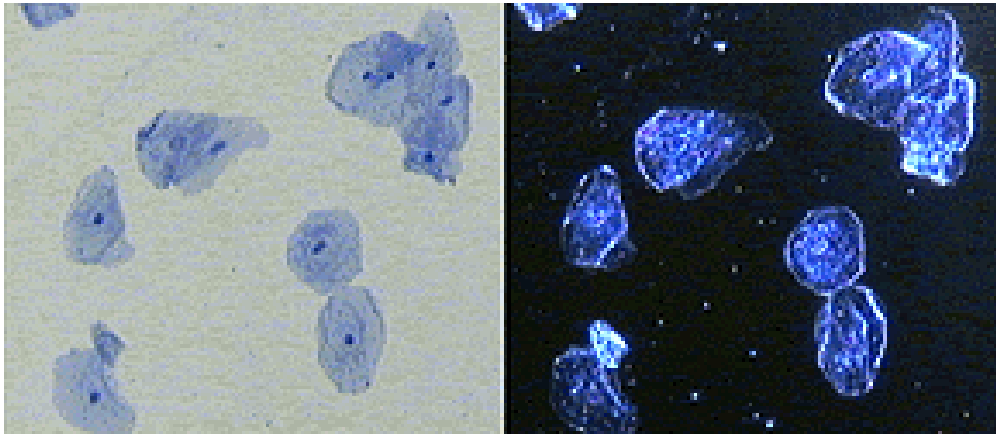


Bright-field vs Dark-field



A Dark-Field Microscope

Bright-field vs Dark-field

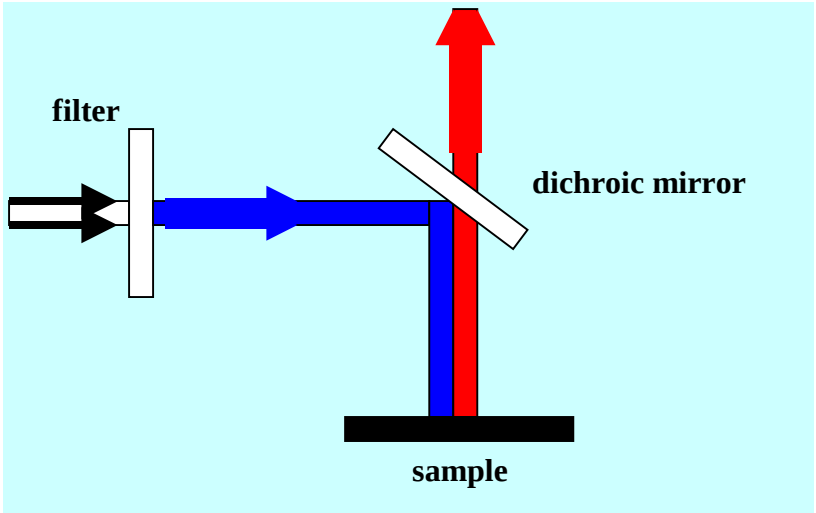


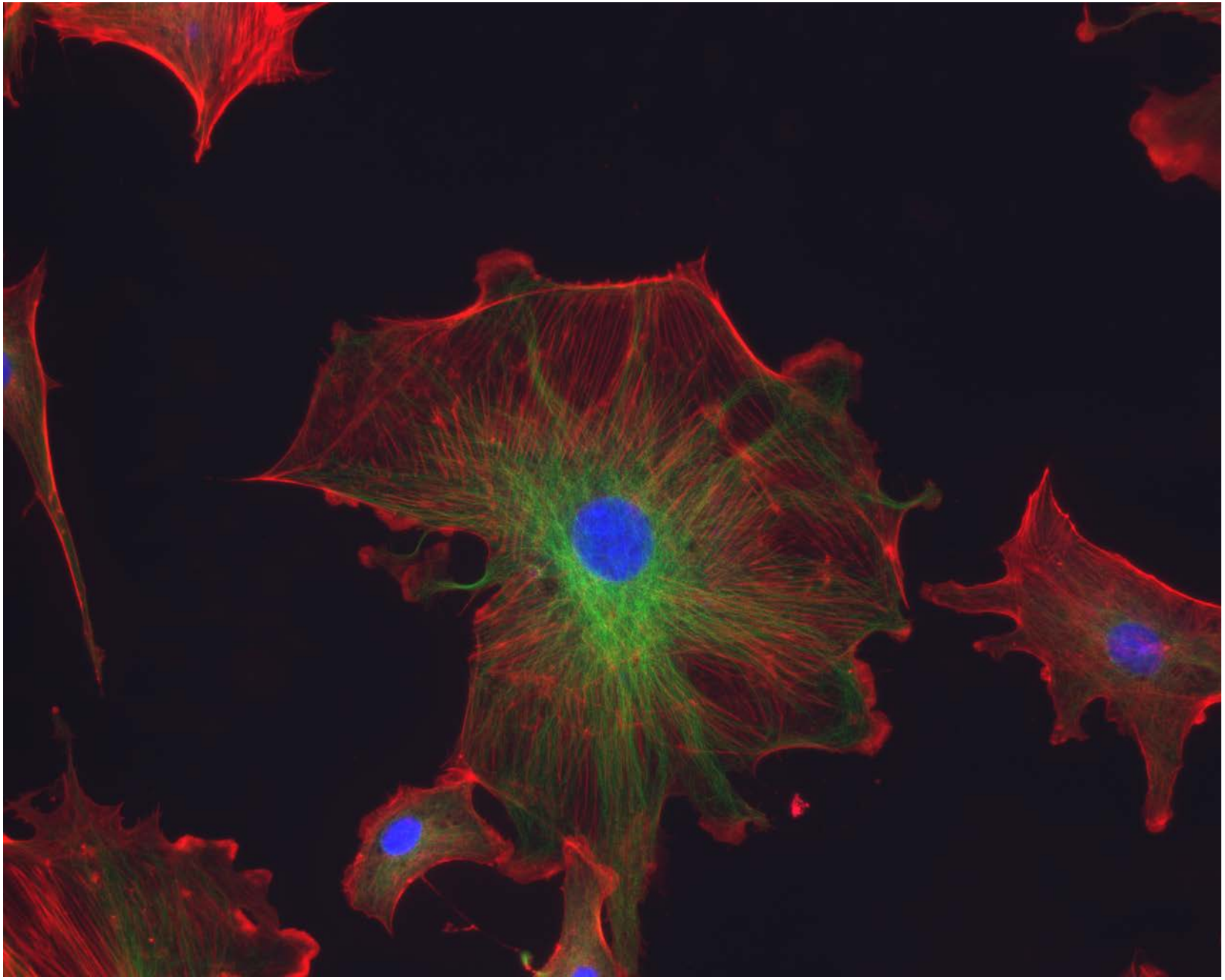
Bright-Field Dark-Field

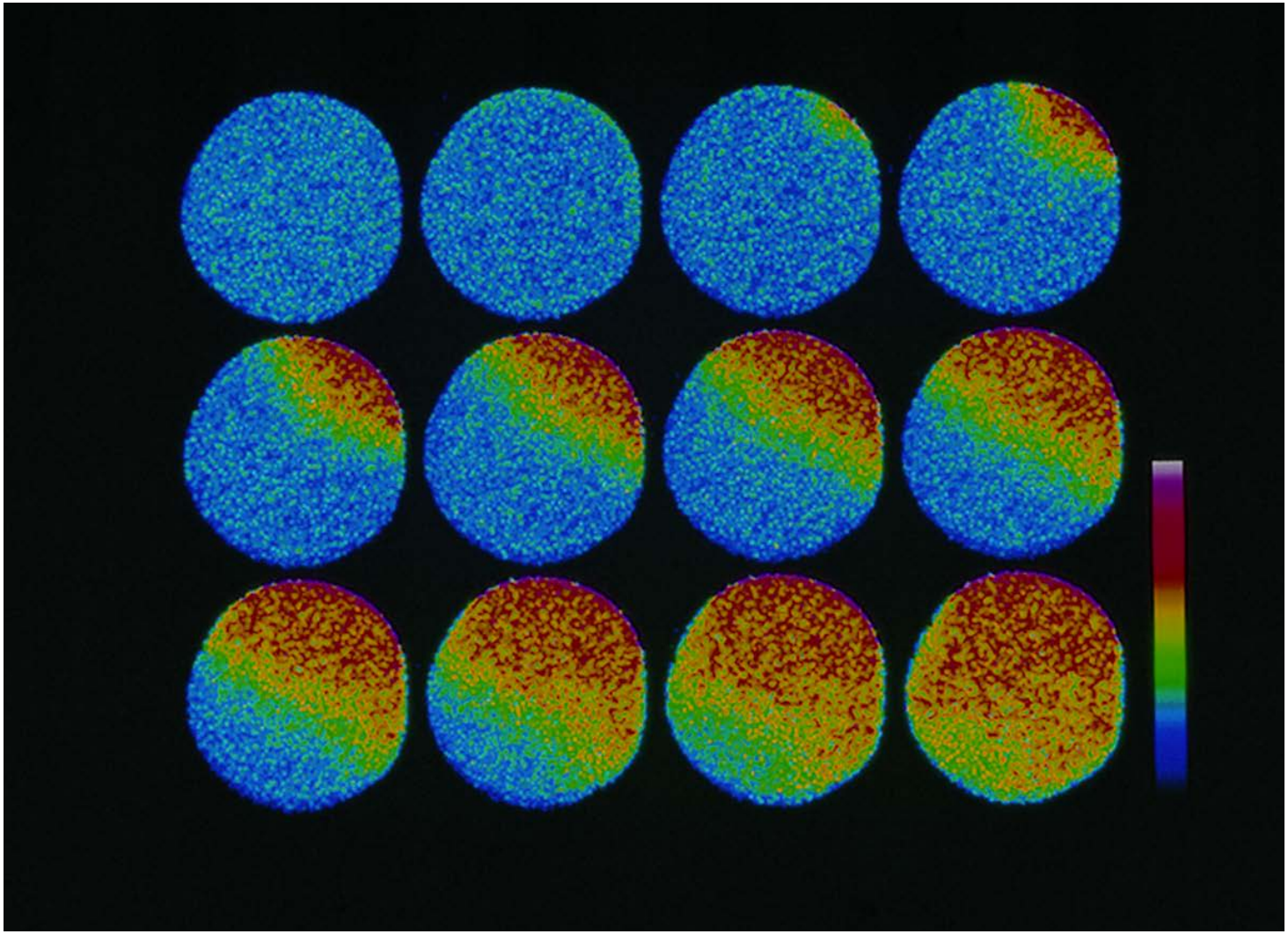
As shown above, the bright-field microscope shows far more opaque and indistinguishable elements than the dark-field. In addition, specks of material are shown on the dark-field image that are not even seen in the light-field.

Fluoresence Microscopy

- We studied fluorescence spectroscopy a bit already – recall that fluorescence is a process where light is absorbed at one energy and re-emitted, after losing some to non-radiative processes, at a lower energy.
- This implies that the light is red-shifted, meaning shifted toward the red end of the spectrum, the lower energy end-
- Special optics in microscope:

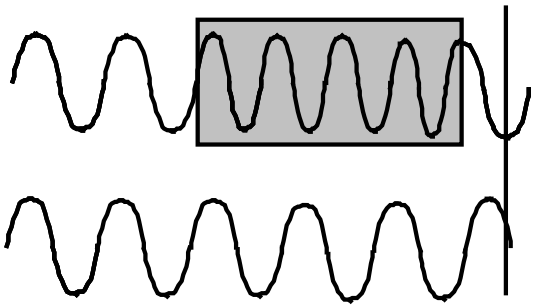






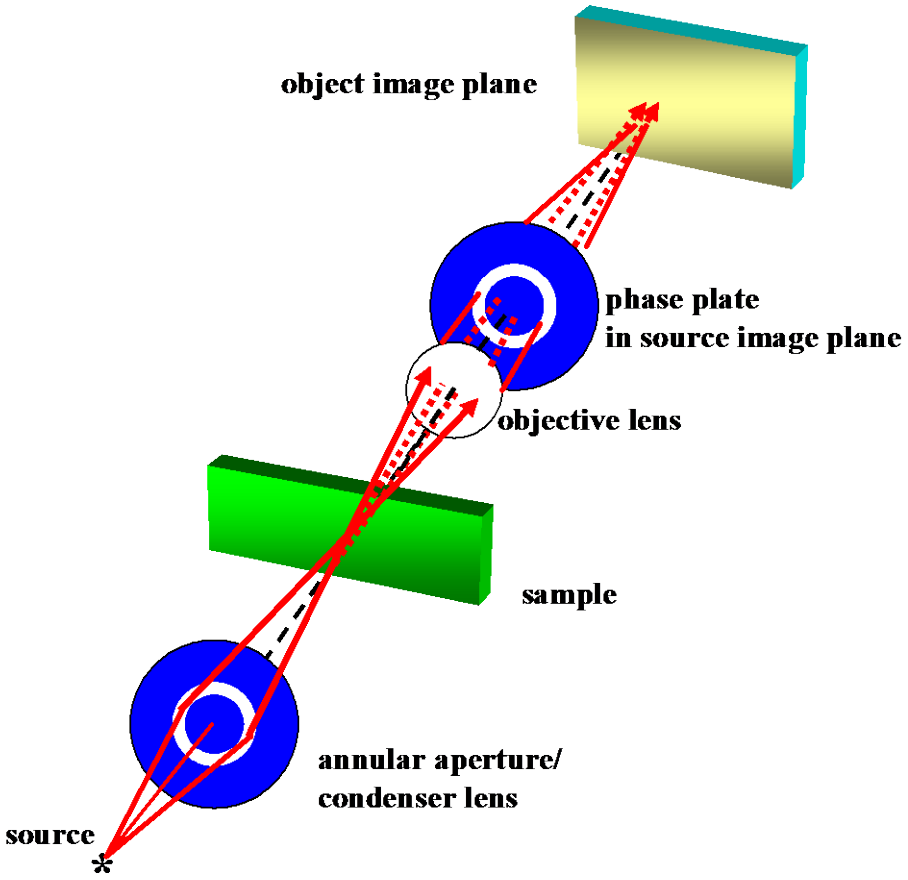
Phase Contrast

- While biological samples have little amplitude contrast (little absorbance in visible), they do have phase contrast due to refractive index differences from the solvent
- Optical path difference = n (physical path difference)

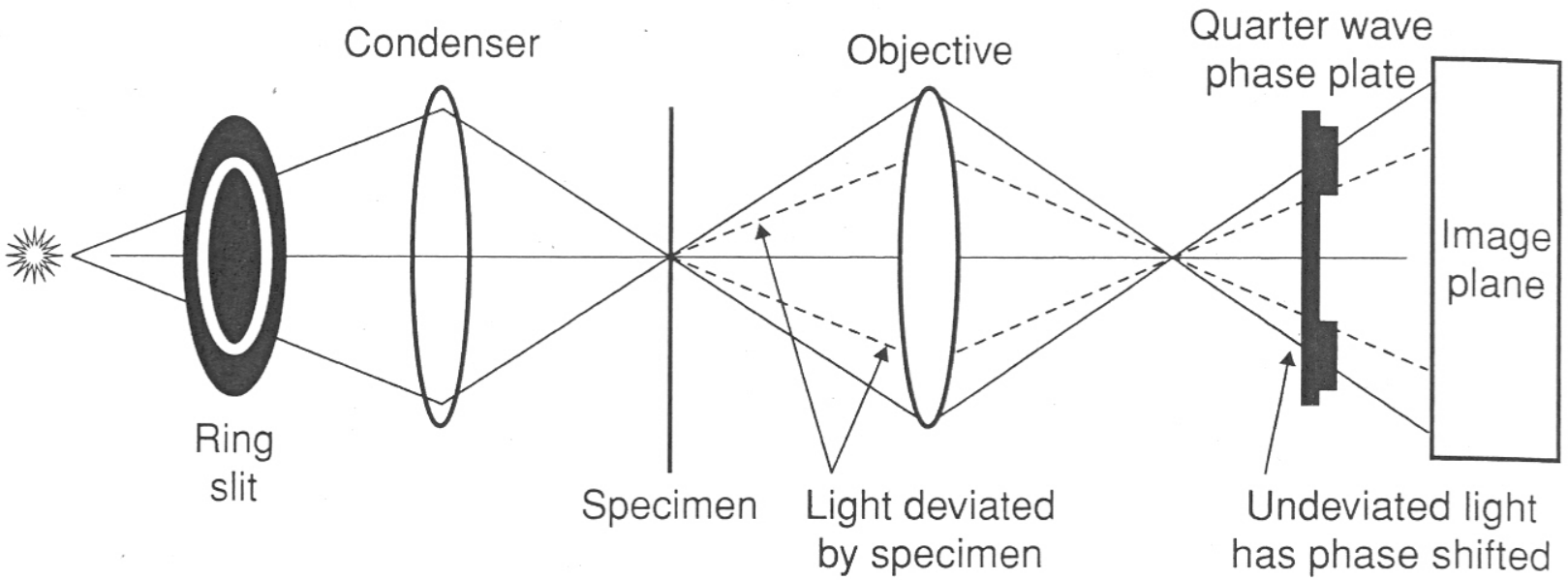


Phase Contrast Microscopy

- Similar to dark field with annular aperture
- Collect incident light and image undeviated light on “phase plate” – built into objective lens
- Phase shifted undeviated light then interferes with scattered light to produce images of phase objects

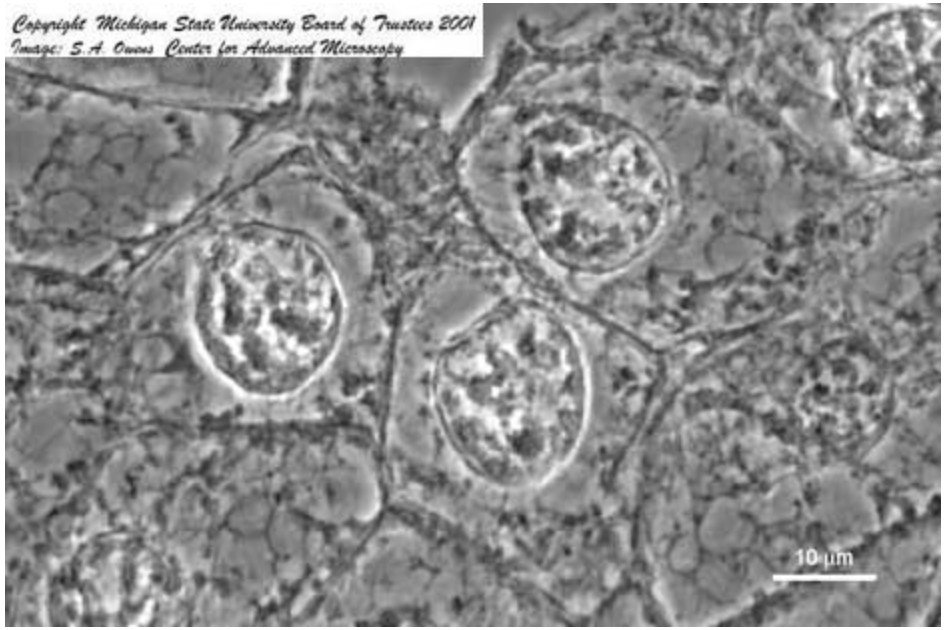


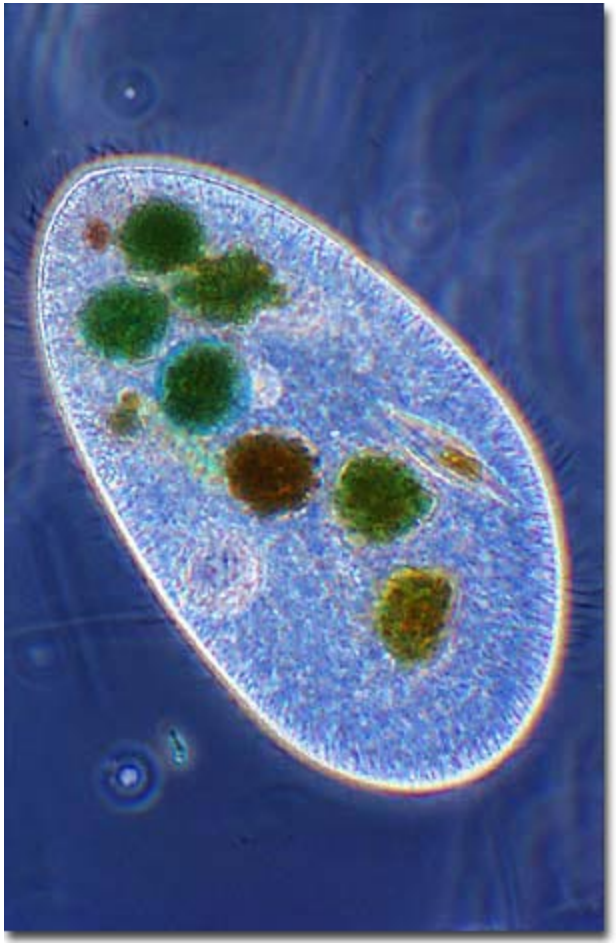
Phase-Contrast Microscopy



This phase plate shifts the phase of the light passing through it based upon where the light hits, because the phase plate has different areas that shift phase different amounts.

Phase contrast images

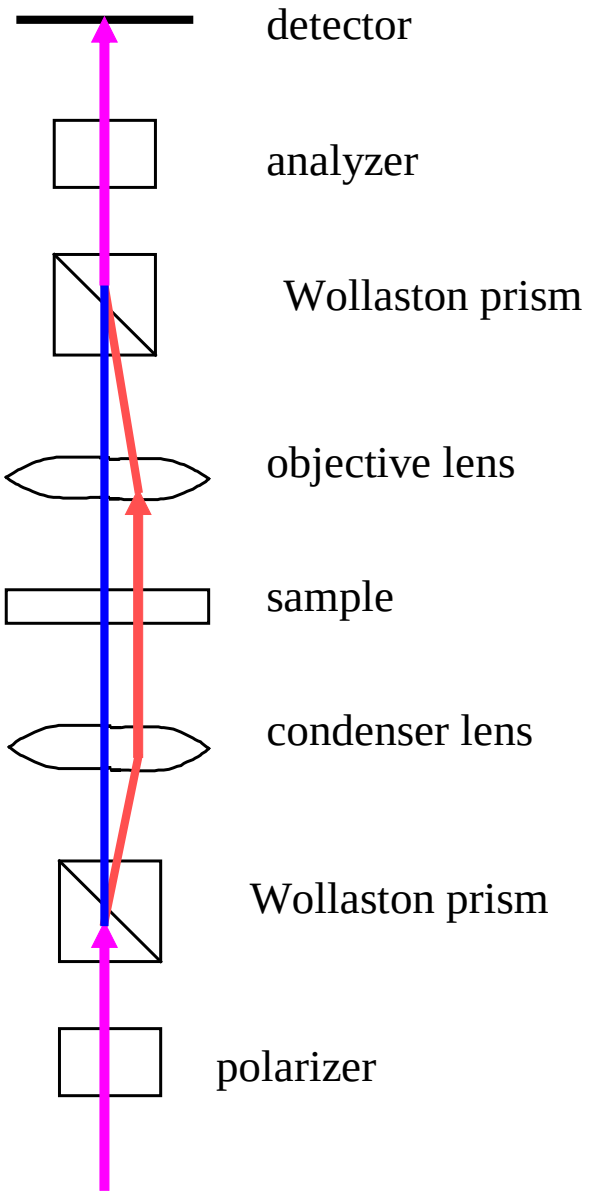




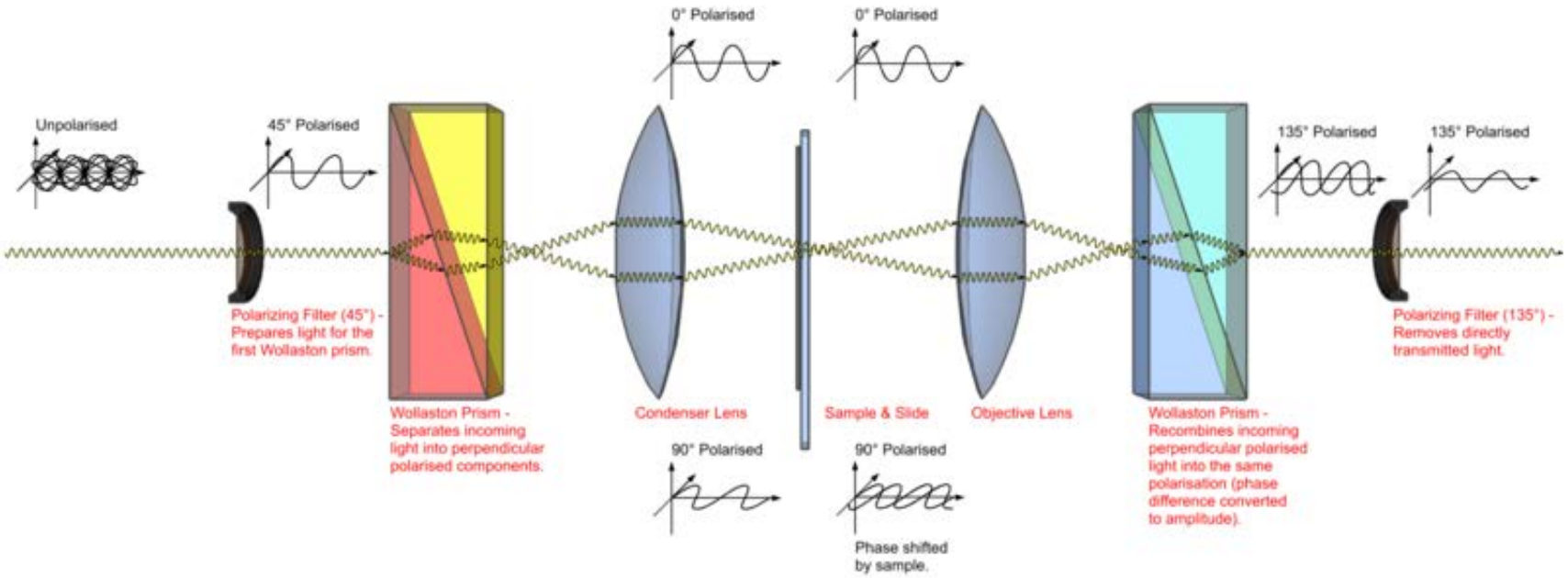
Differential Interference Contrast DIC

- Two closely spaced parallel beams are generated and made to interfere after passing through an unstained sample. The background is made dark and the interference pattern is particularly sharp at boundaries where n changes rapidly – hence the name -

- The two beams are generated using Wollaston prisms – which generate beams of different polarization. The polarization is not important in the technique – the beams are recombined and analyzed to produce an interference pattern



DIC Microscope



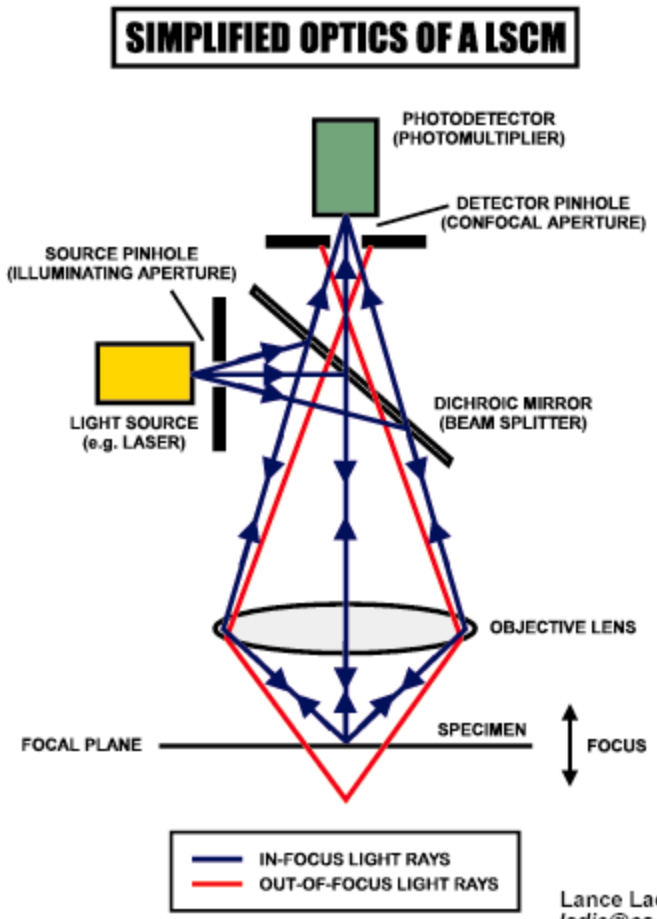
Fly muscle

Deer tick

Newer Microscopies: confocal

- Laser beam is focused to very tight spot and scanned over the sample (fluorescent)

The incident light is reflected toward the sample by the dichroic mirror and spread out so that the focus is very tight. The spot is scanned over the surface and fluorescent and reflected light is collected by the same lens. The dichroic mirror blocks the reflected light and transmits the fluorescent light. The pinhole in front of the detector images only light from the focal plane and blocks out of focus fluorescent light for a sharp image, especially in thick samples.



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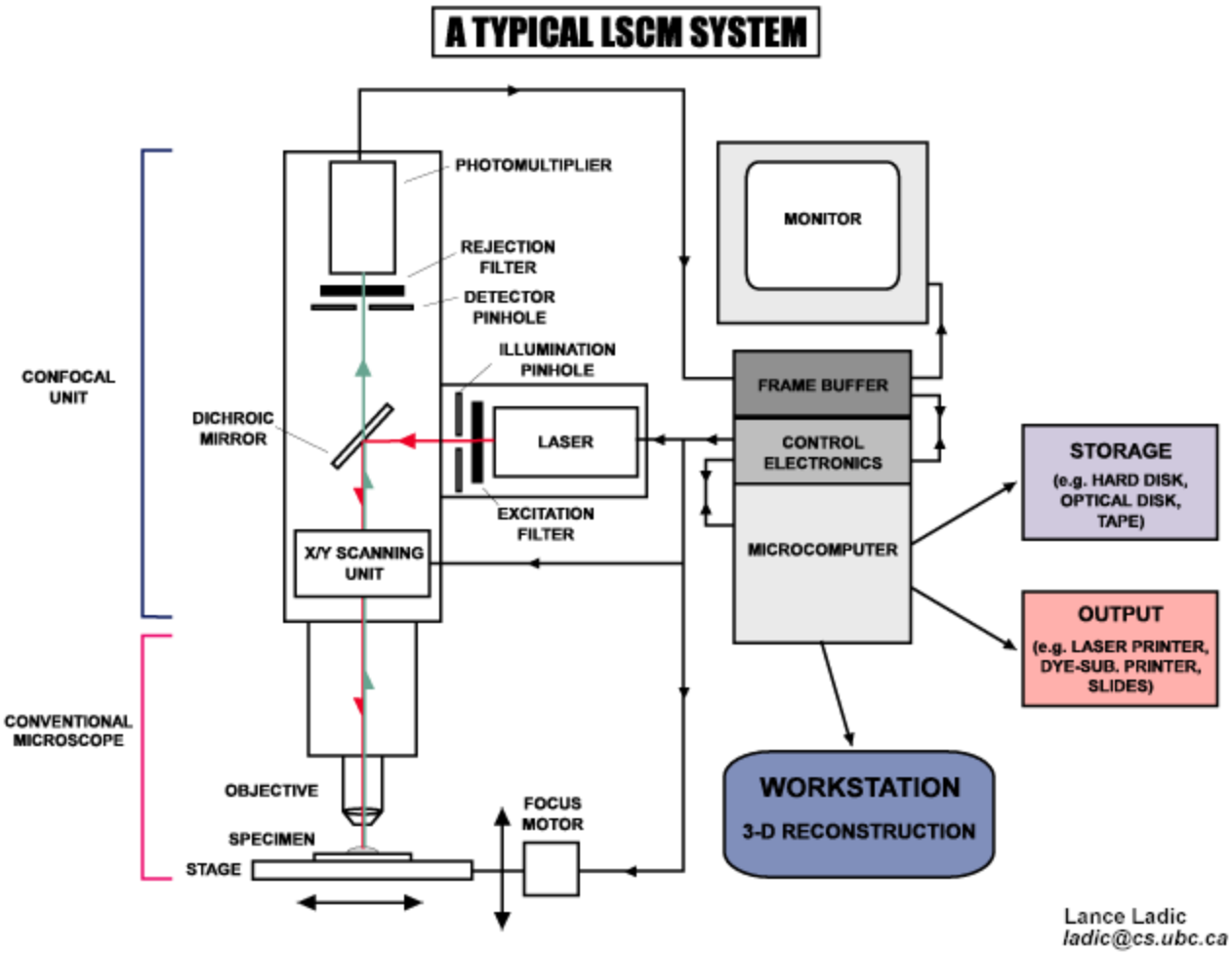
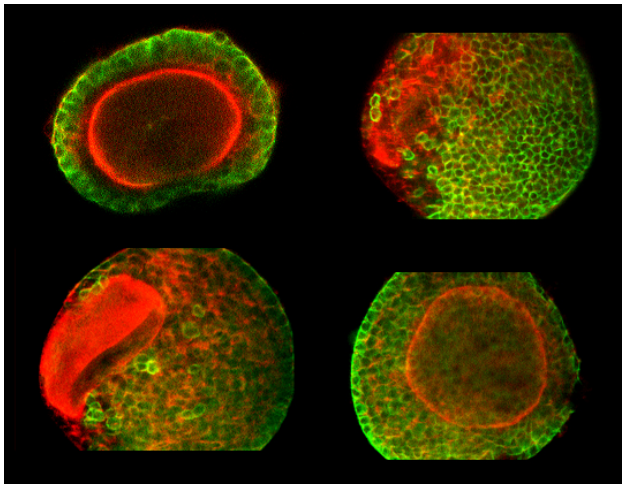
Confocal

- System is complex since need to scan and process image- can scan depth and make a movie of going through the cross-section

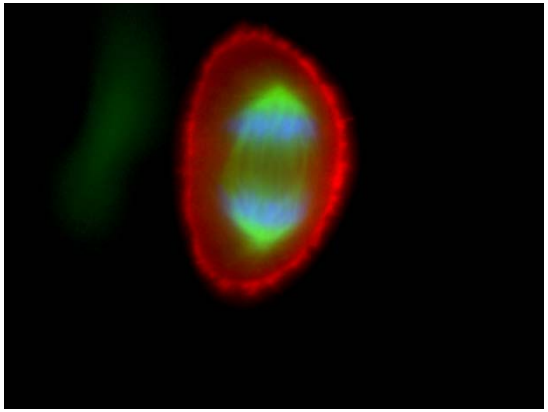


Confocal II

- Imaging done as scanned to get 2 – dim images or even 3 – dim images if scanned through different focal planes
- False color is added



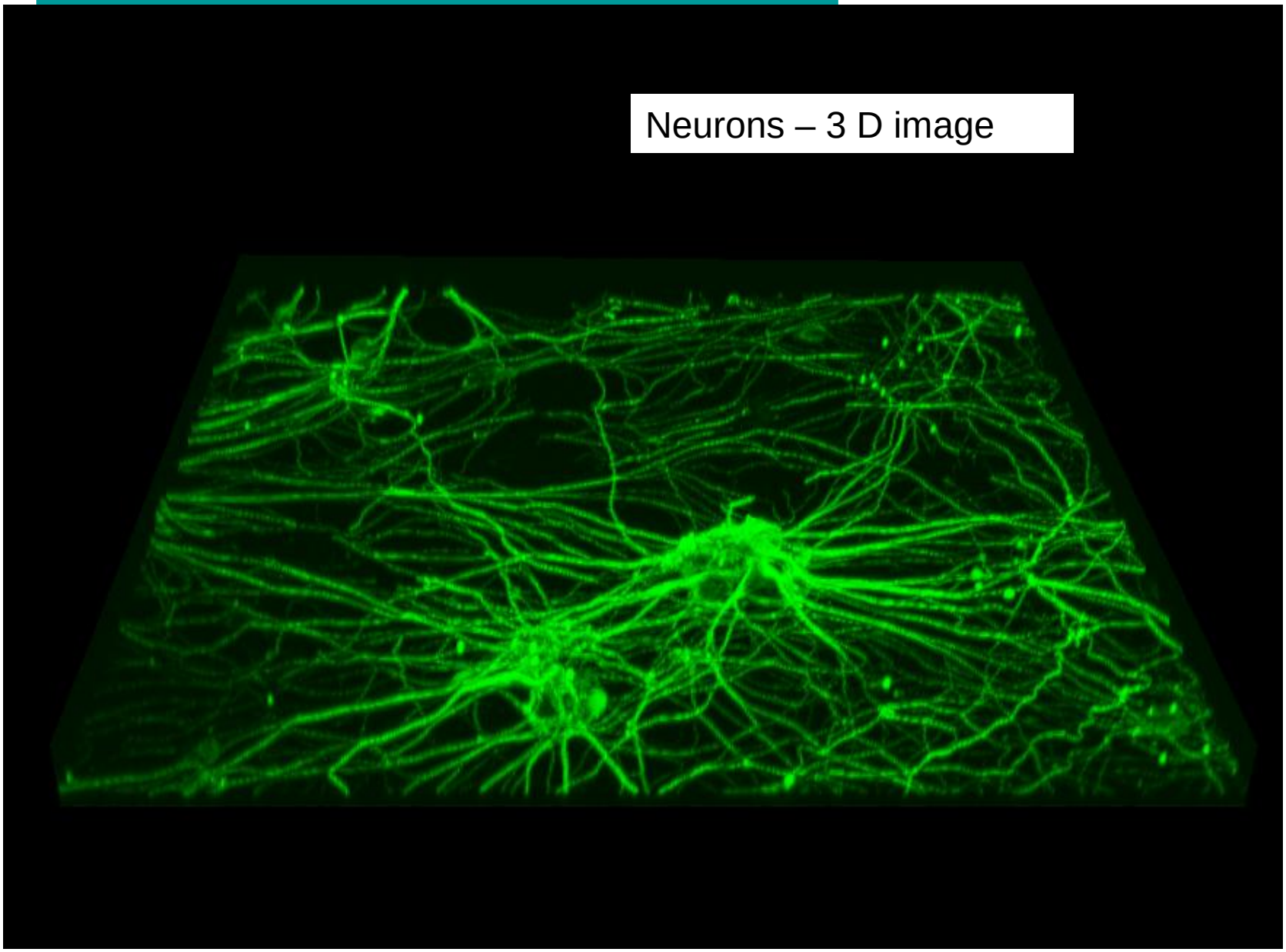
- mouse oocytes showing microtubules in red and actin filaments in green



anaphase in a cultured epithelial cell showing chromosomes (blue), spindle apparatus

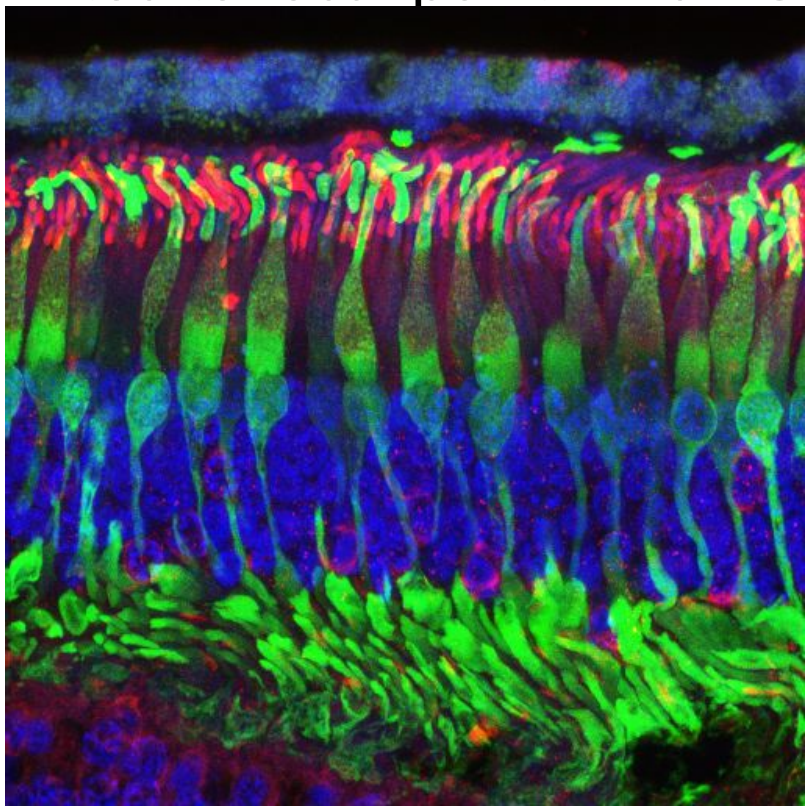
(green) and actin (red).

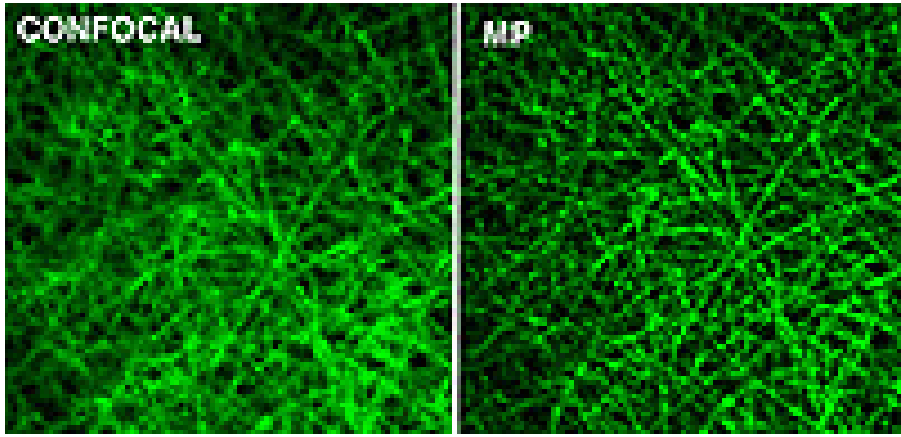
- Nikon Confocal movie page



Multiphoton microscopy

- Variation on confocal microscopy – uses high flux, low energy photon laser beam – at focal point, intensity is so high that there is high probability to absorb 2 or more photons to excite fluorescence. Out of focus there is no absorption and so photodamage, photobleaching is limited to focal point which is scanned.



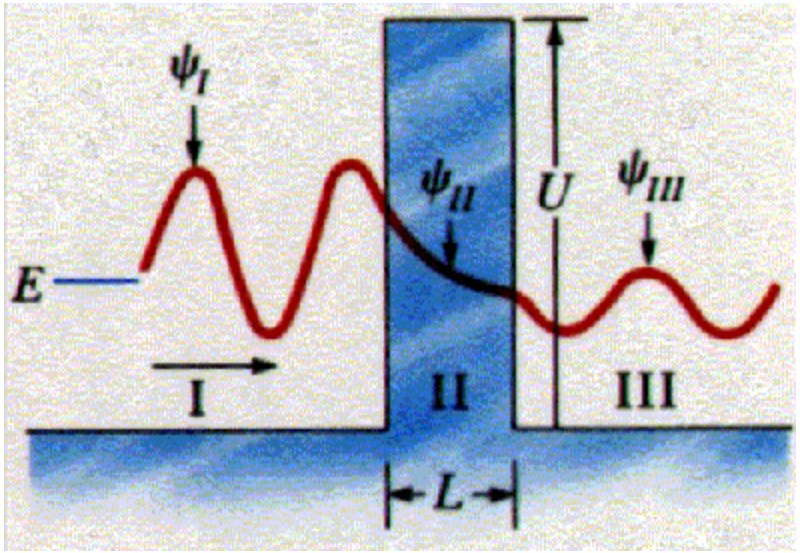


Human retina

Egg membrane proteins

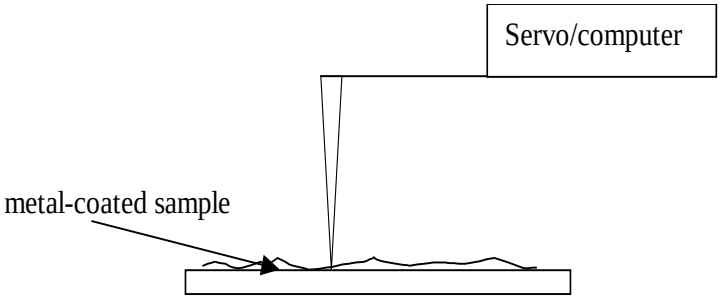
STM: Scanning Tunneling Microscopy

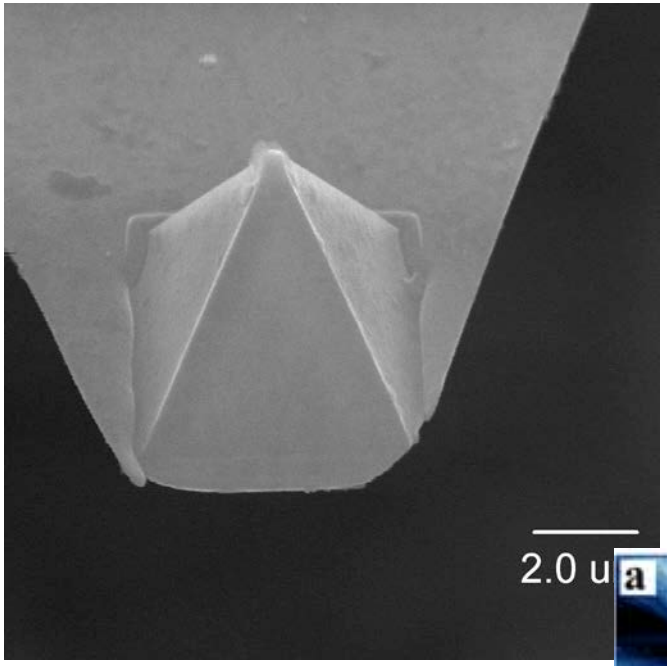
- Based on quantum mechanical phenomenon = tunneling; illustrate with electron in 1 dimensional box with walls:



Probability of tunneling depends on barrier height and thickness as well as energy of particle

Particle can get through barrier because of uncertainty principle: $\Delta E\, \Delta t > \hbar$ - if Δt is short enough, ΔE can be large enough for the particle to get over the barrier


Servo/computer
metal-coated sample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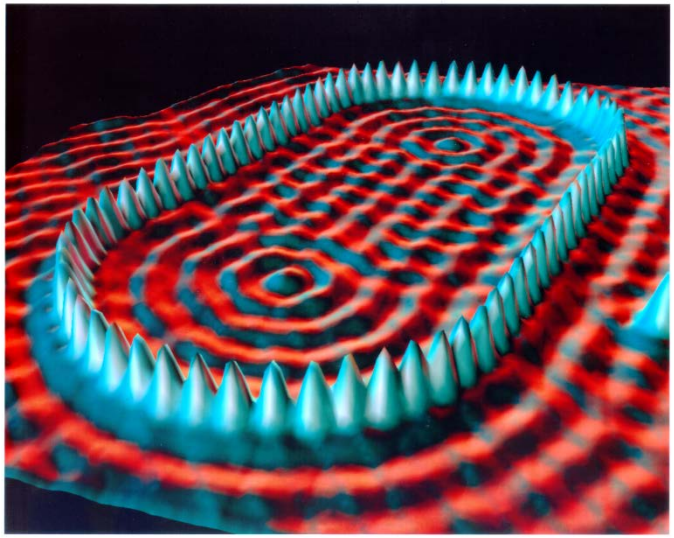

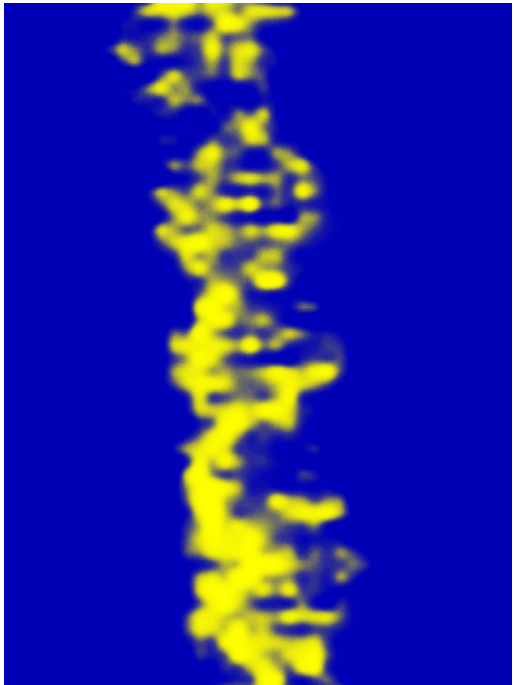

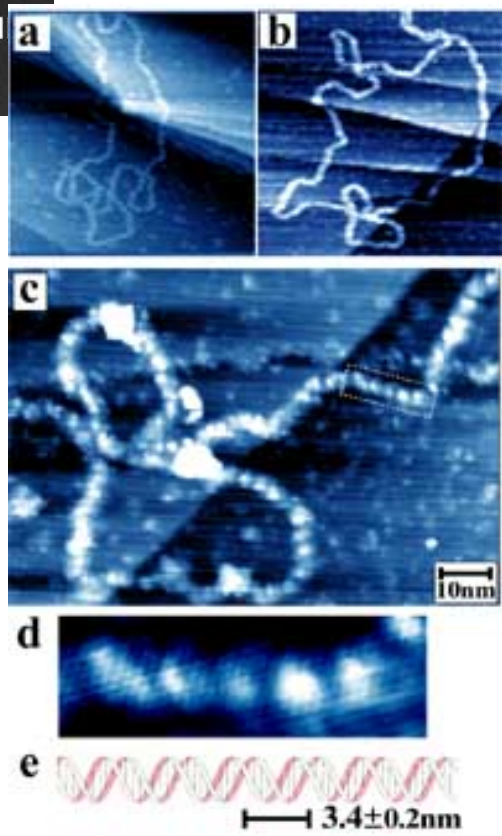
a
b
c
10nm
d
e
3.4±0.2nm