Today's Announcements

1. Test given back next Wednesday
2. HW assigned next Wednesday.
3. Next Monday—1st discussion about Individual Projects.

Today's take-home lessons

(i.e. what you should be able to answer at end of lecture)

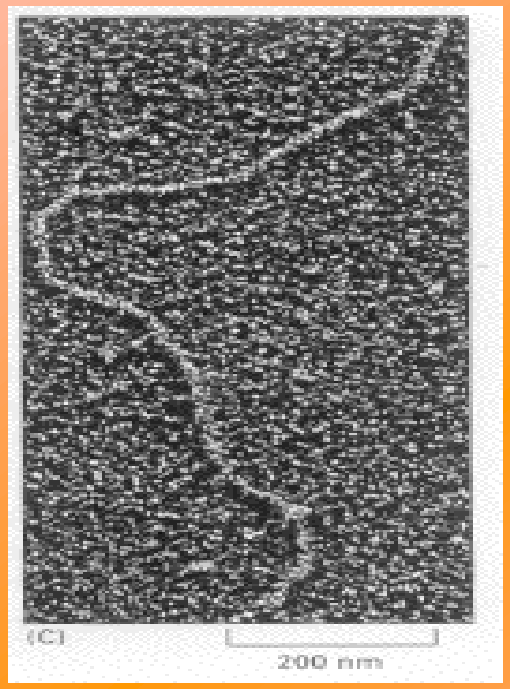
1. Seeing things with Light and Electron Microscopes
2. Accuracy and Resolution—how fine can you see.
3. Fluorescence. What is it (amplitude, time-scale)?

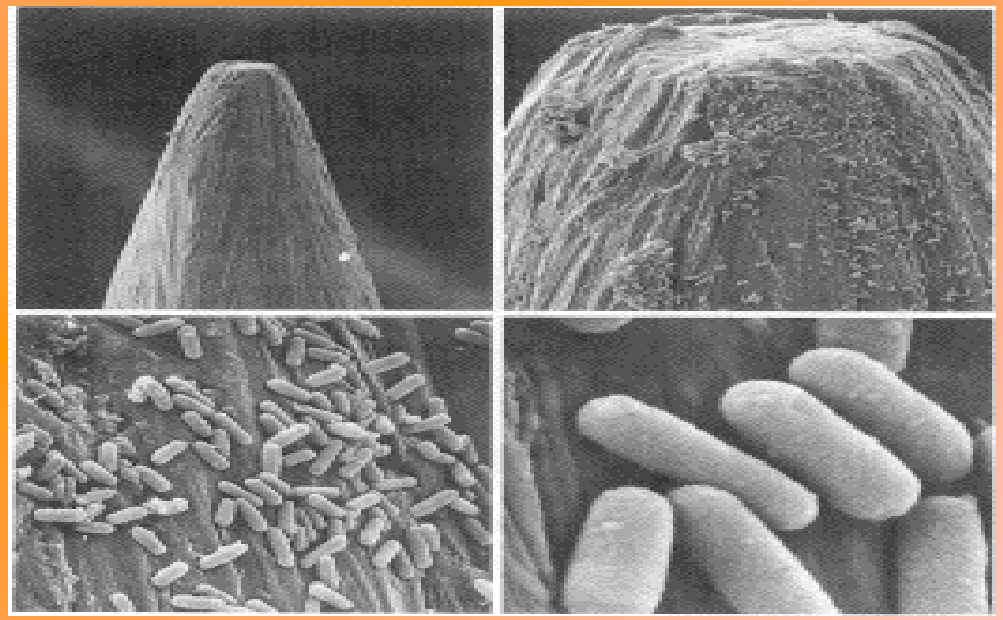
Today

Techniques for measuring distances

(where physicists have made a big impact on bio.)

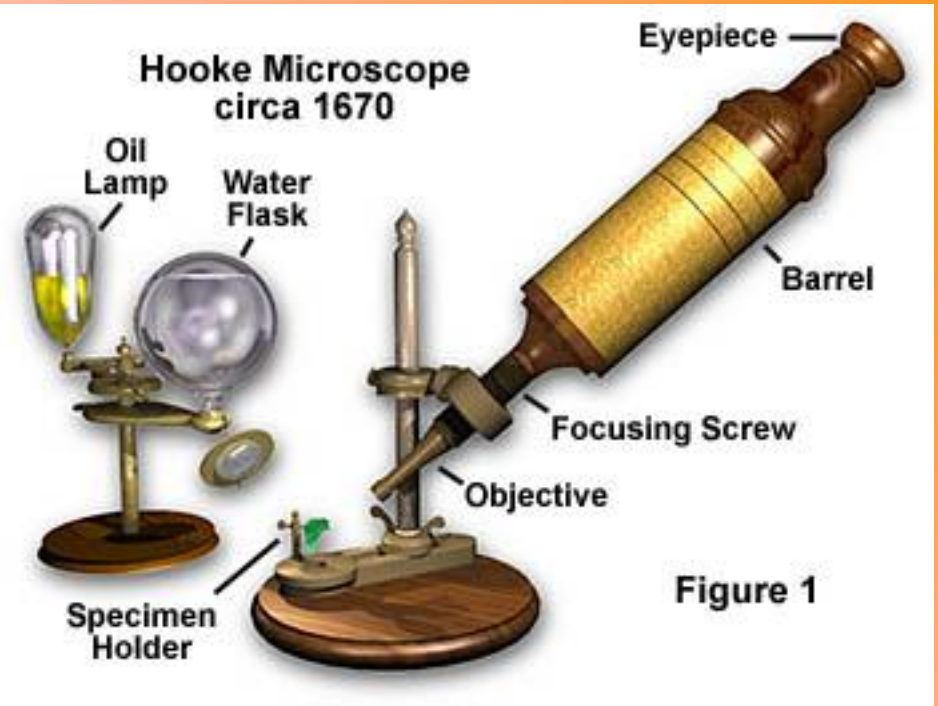
X-ray *diffraction* (atomic resolution)
Electron *(Imaging)* Microscopy (nm-scale)
Visible *(Imaging)* Microscopy (nm - μm)





Bacteria on head of a pin
at different magnifications

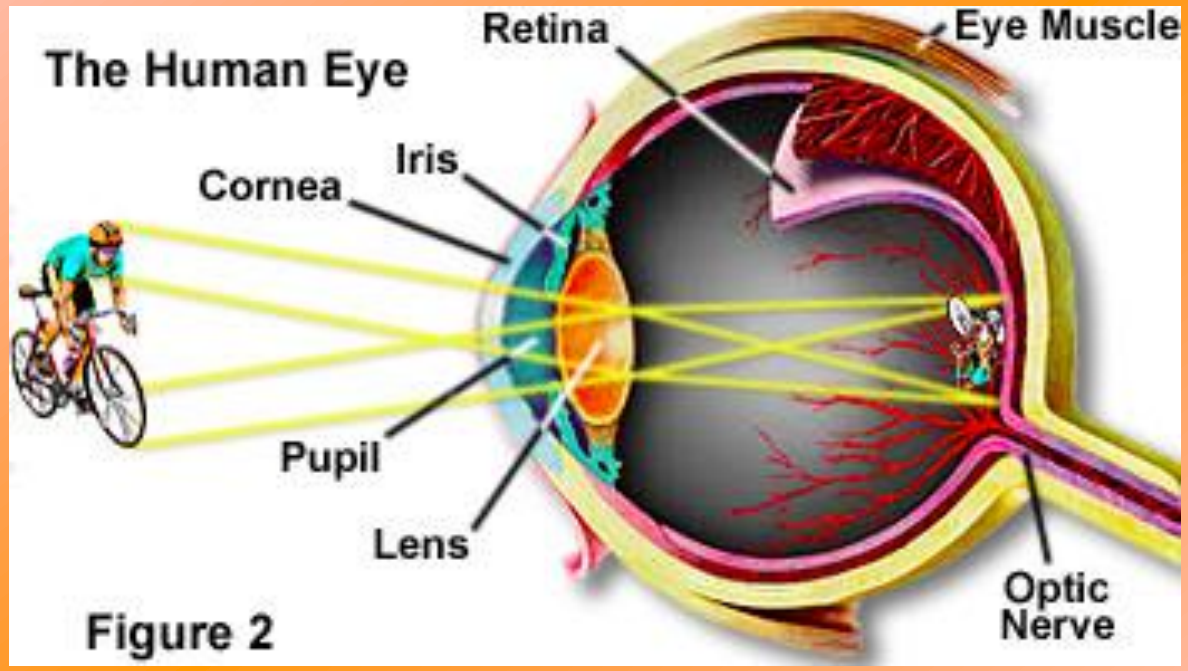
Beginning of microscopes



Microscope must produce a magnified image of the specimen, separate the details in the image, and render the details visible to the human eye or camera.

www.olympusmicro.com

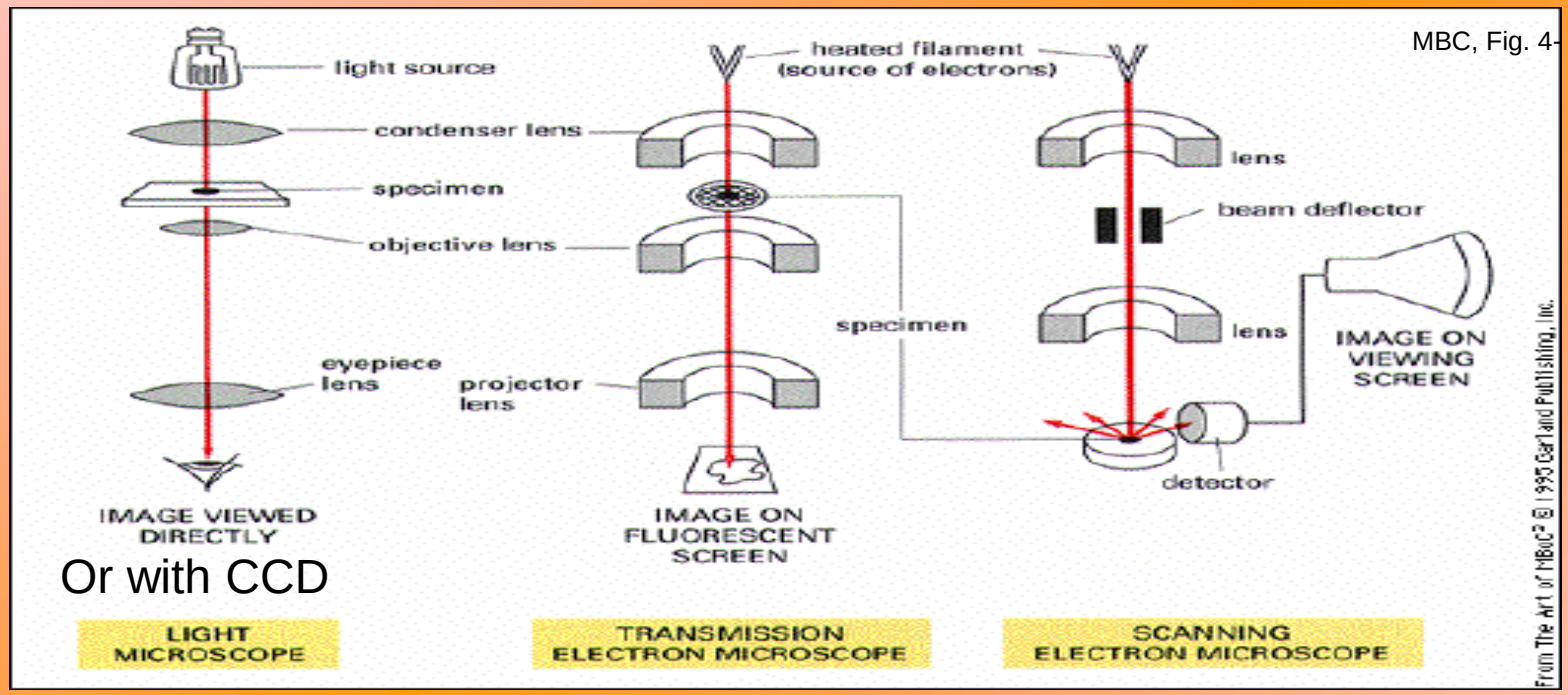
Your eye is a Microscope!



www.olympusmicro.com

Microscopes

Cells discovered with invention of microscope.



Or with CCD

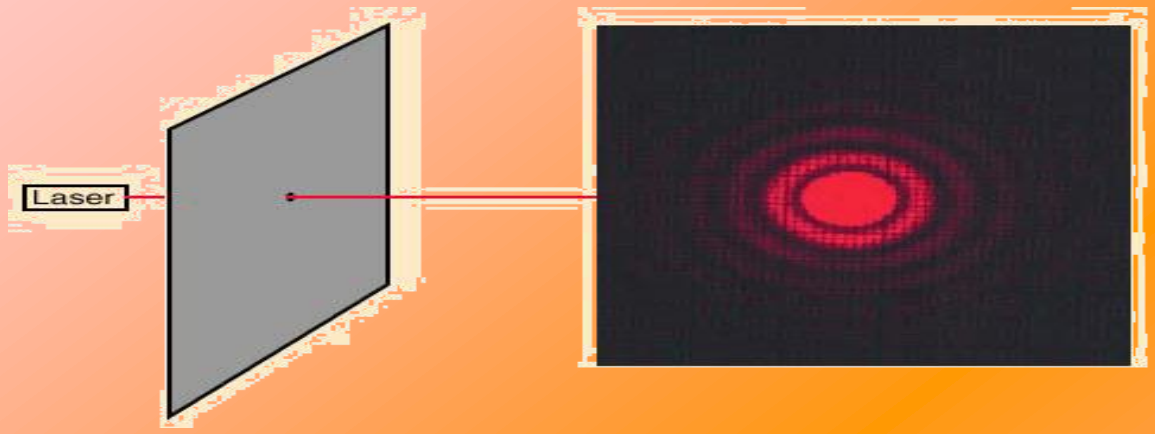
1000x, 0.2um

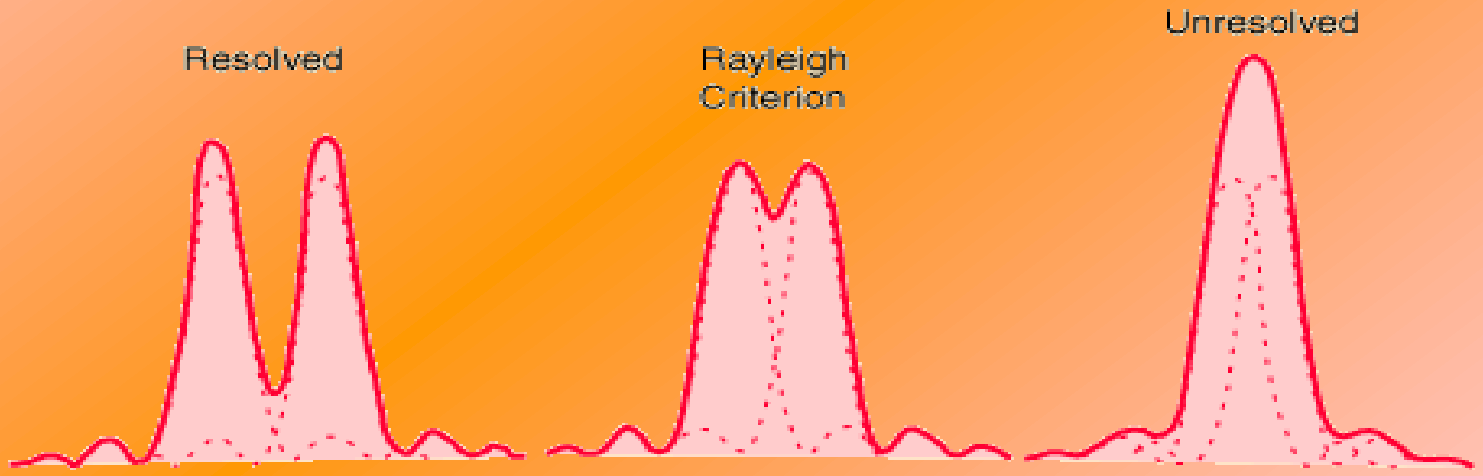
10^6x, 2 nm

20,000, 10 nm (3-d)

Resolution: The Rayleigh criteria

How well can you resolve two point objects?





A single light-emitting spot will be smeared out, no matter how small the spot is, because of the wavelength of light to ~ $\lambda/2$.

Point-Spread Function (PSF)

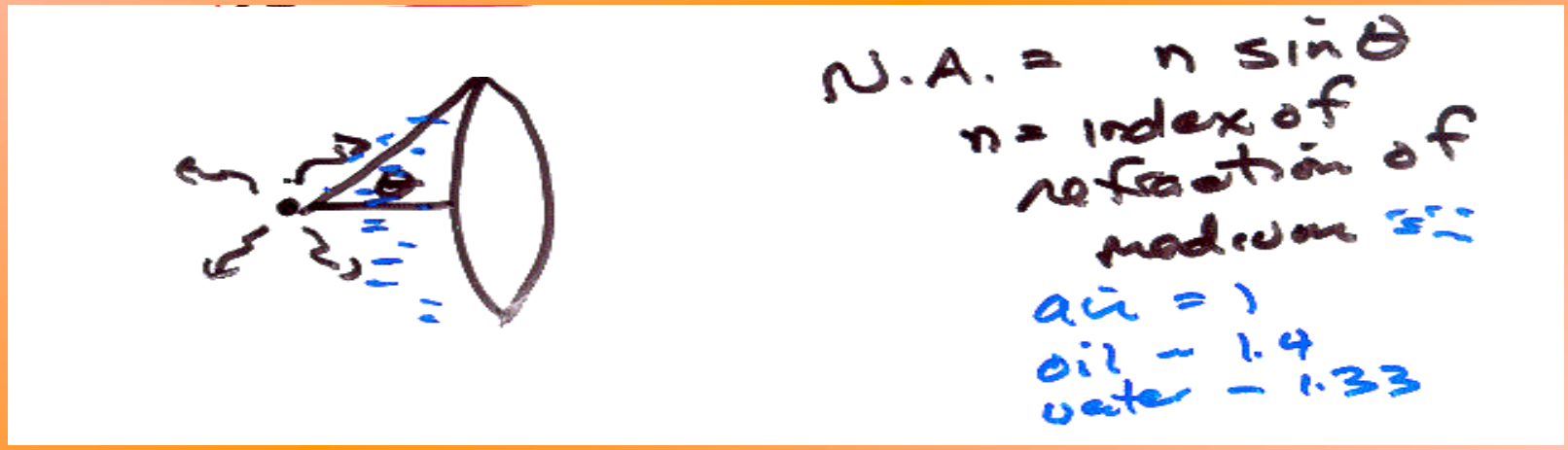
What determines (ultimate, i.e. best) resolution of technique··· microscope, eye, etc.?

[2 parts]

1. Primarily λ (wavelength).

   Why? Uncertainty principle (Will show).

2. Collection Angle/focal length/ Numerical Aperture



Resolution ≈ # λ/N.A.

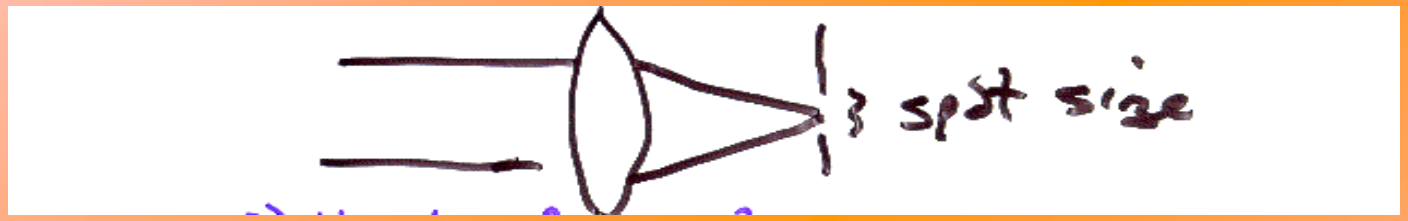
= a factor = ½ (details not important)

Resolution ≈ λ/[2N.A.]

Why is resolution $\lambda/2$ N.A. (N.A. = $n\sin\theta$)

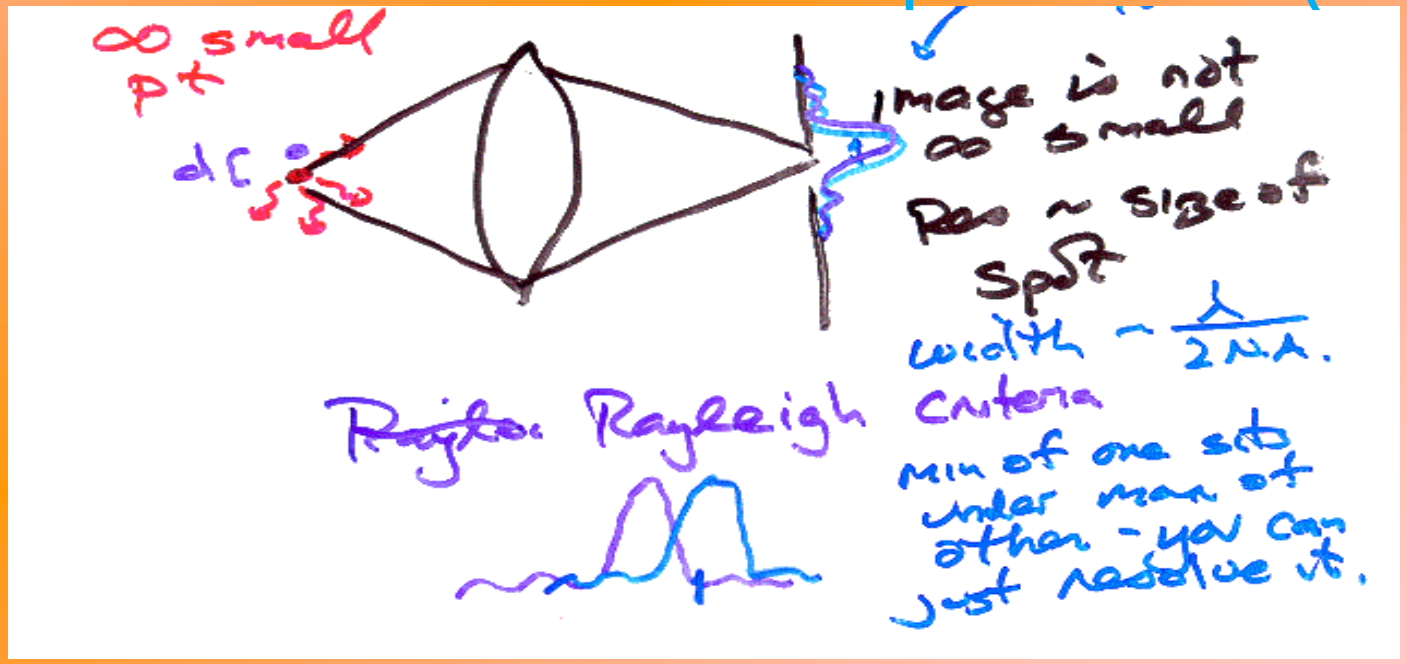
Resolution:

1) how small of a spot of excitation light can you make (scanning microscope)



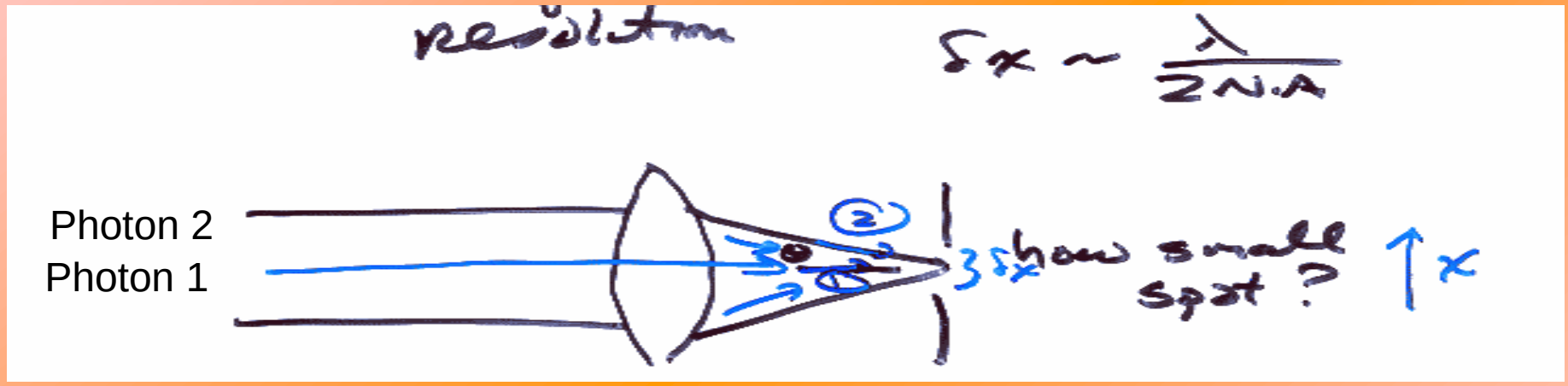
2) how big of a spot an infinitely small object makes

Point Spread Function (PSF)



Calculating optimal diffraction-limited resolution.

What is uncertainty principle applied here?



Photon 1: $p = p\,\hat{y}$

Photon 2: $p_x = p \sin\theta$: $p_y = p \cos\theta$

$\Delta p_x = p \sin\theta - (- p \sin\theta)$

$= 2p \sin\theta$

Calculating resolution (con't)

$\Delta p \Delta x = h/2\pi$

$2p\sin\theta\ \Delta x = h/2\pi$

$p = h/\lambda$

$\left(\frac{2\pi \sin\theta}{\lambda}\right)(\Delta x) \sim \pi = \frac{\pi}{2\pi}$

resolution: Δx $\frac{\lambda}{4\pi \sin\theta}$

within a factor $\frac{\lambda}{\sin\theta}$

Wavelength at screen

… need n.

Where does n come in?

Calculating resolution (con't) : "n", index of refraction

Coverslip (glass)
(thin, n= 1.33)

object

Fill with oil (n ~ 1.5)

where air = 1

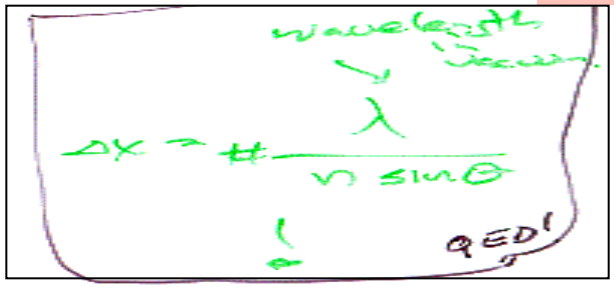
$v = \lambda f$ for any wave

n = index of refraction = $\frac{c}{v}$ → speed of light in vac.
→ speed of light in medium of index n

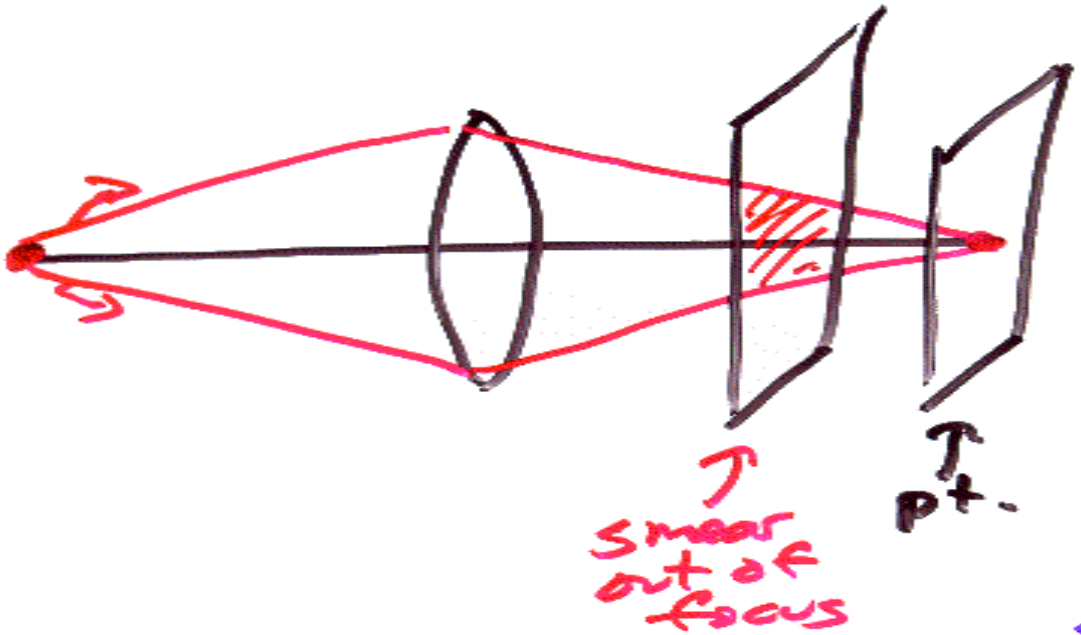
slowed light down
if speed → speed ↓

either λ gets smaller or f gets smaller
which one? → ans. $\lambda \rightarrow \frac{\lambda}{n}$

(Homework)

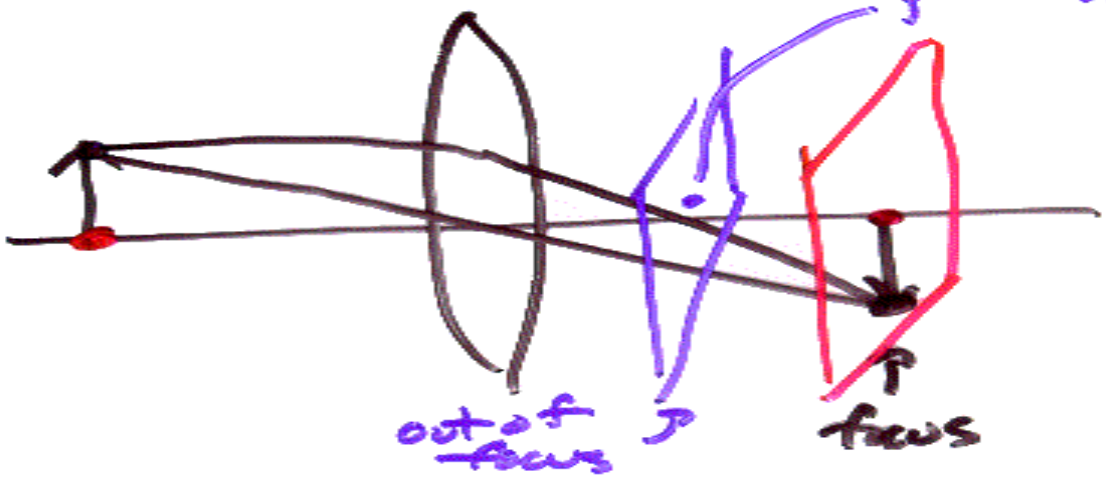


Sidelight: what does it mean for something to be in focus?



pt on screen has light from many diff. parts of object

each pt on object gets imaged to one pt in image plane



Bottom line:

$$\text{Resolution} \approx \lambda/[2NA]$$

$NA \approx 1$ $\lambda \approx 500$ nm (green)

(for visible light)

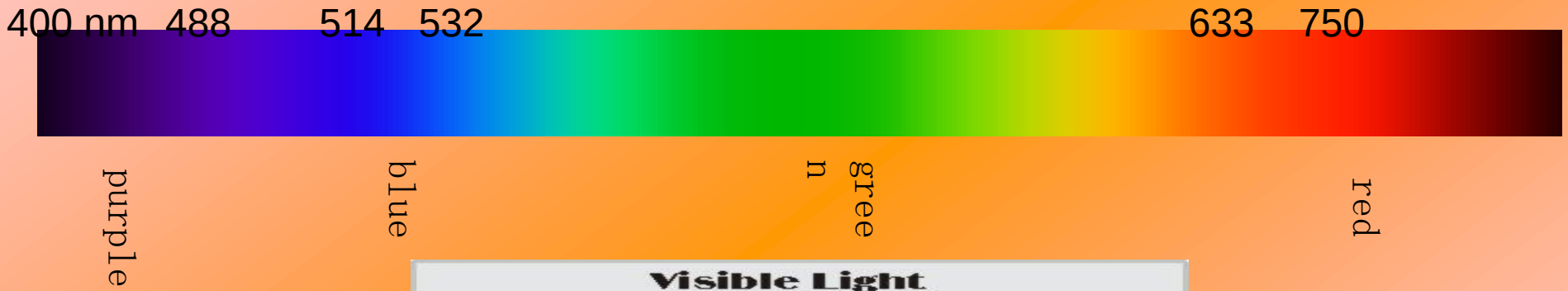
Resolution ≈ 250 nm

Wavelength & Resolution

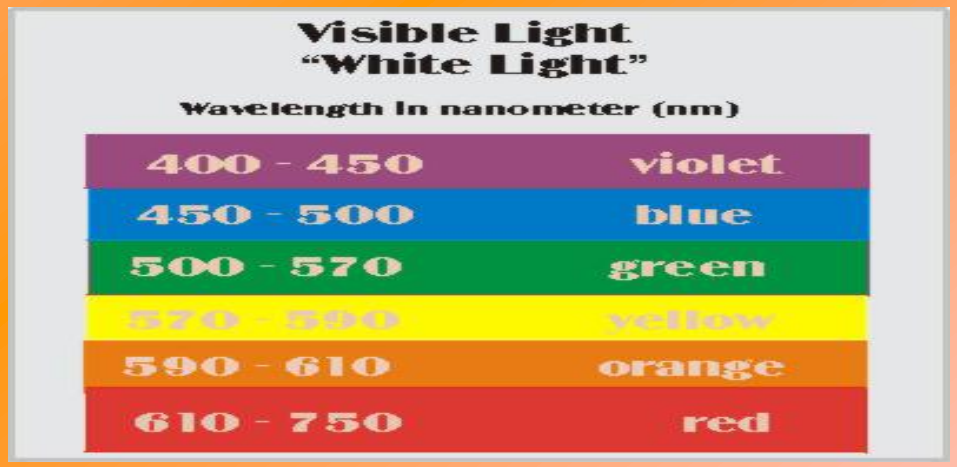
$\lambda_{visible}$=≈ 400-700 nm

λ/2 N.A.: air= λ/2: oil= λ/(2)(1.4)

λ = 500 nm: Best resolution 200-250 nm



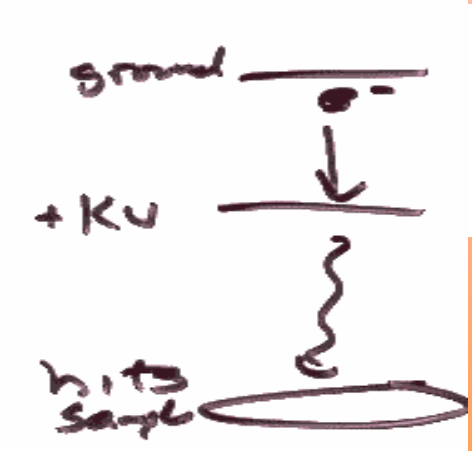
Short λ



Long λ

Modern day optical microscopes are highly optimized– perfect diffraction limited. (Electron microscopes are 1000's of times worse.)

λ of electrons



(Who was famous guy who got Nobel prize in 1929 for the "wave nature of electrons"?

Debroglie

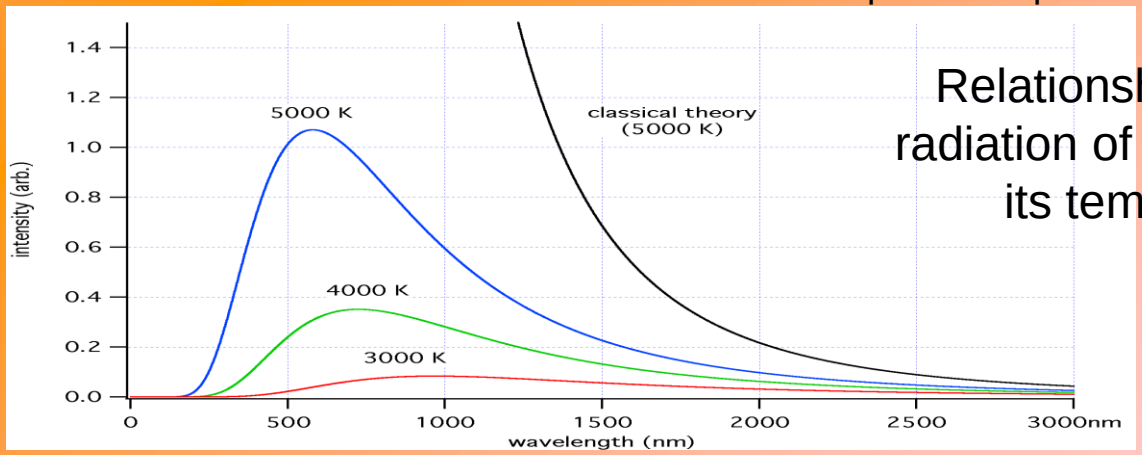
What relationship between wavelength, λ, and energy, E, and momentum, p, does this correspond to?

$E = h\nu = hc/\lambda$; $p = h/\lambda$

Where does Planck's constant come from?

The Planck constant came from law of black body radiation: that the electromagnetic radiation emitted by a black body could be modeled as a set of harmonic oscillators with **quantized** energy of the form: $E = h\nu$

http://en.wikipedia.org/wiki/Black-body



Relationship between radiation of an object and its temperature

Resolution of Electron Microscope

Given electron 100 KeV,
(typical upper-value for electron microscope)
what is λ?

(h =6.63 × 10^{-34} J-sec = 4.1 × 10^{-15} eV-sec)

E_{100kV} = 0.004 nm (really short!)

In reality, because not perfect electron lenses, resolution is ~1 nm.

E.M. are far from ideal.

Noise

Why can't you see starlight in the day?

(The stars are just as bright during the day as at night.)

You have a "bright" background (sun)...

which has a lot of noise.

If you have N photons, then you have $\sqrt{N}$ noise.

(This is important to remember!)

Example: Sun puts out a 10^6 photons/sec. Noise = 10^3 photons/sec
Therefore: if star puts out 10^3 photons/sec,
can just barely "see it" with Signal/Noise =1
(Really want to "see it" with S/N of at least a few >2-5))

Example of Noise con’t

Let’s say star puts out 100 photon/sec.
(It turns out you (your eye) can see about 1 photon!!)

S/N day?

At night?

Fluorescence vs standard light Microscopy

Biophysics 101

Essentials of fluorescence

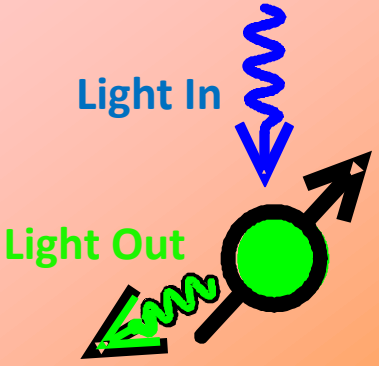
Basic Fluorescence
Single molecule detection methods
(confocal, two-photon, TIRF)
Imaging (FIONA, etc…)

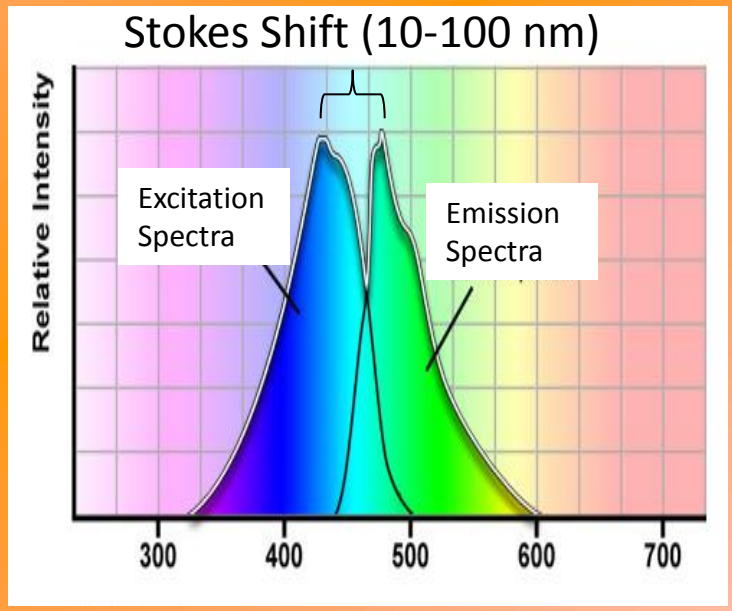
(Later)

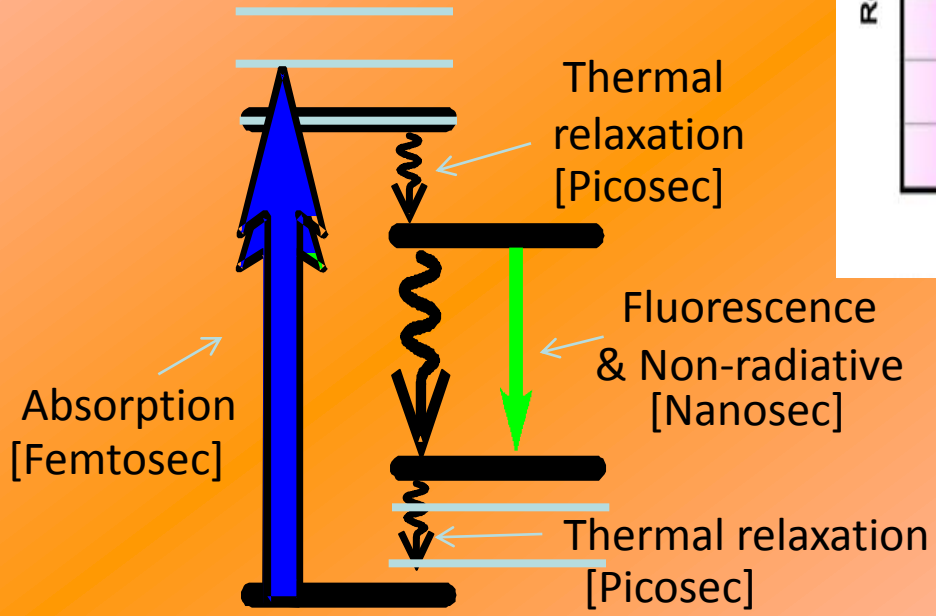
FRET
(Polarization, FCS)

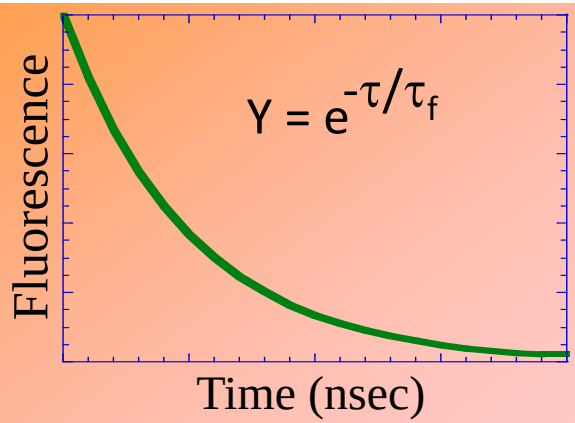
Basics of fluorescence

Shine light in, gets absorbed, reemits at longer wavelength



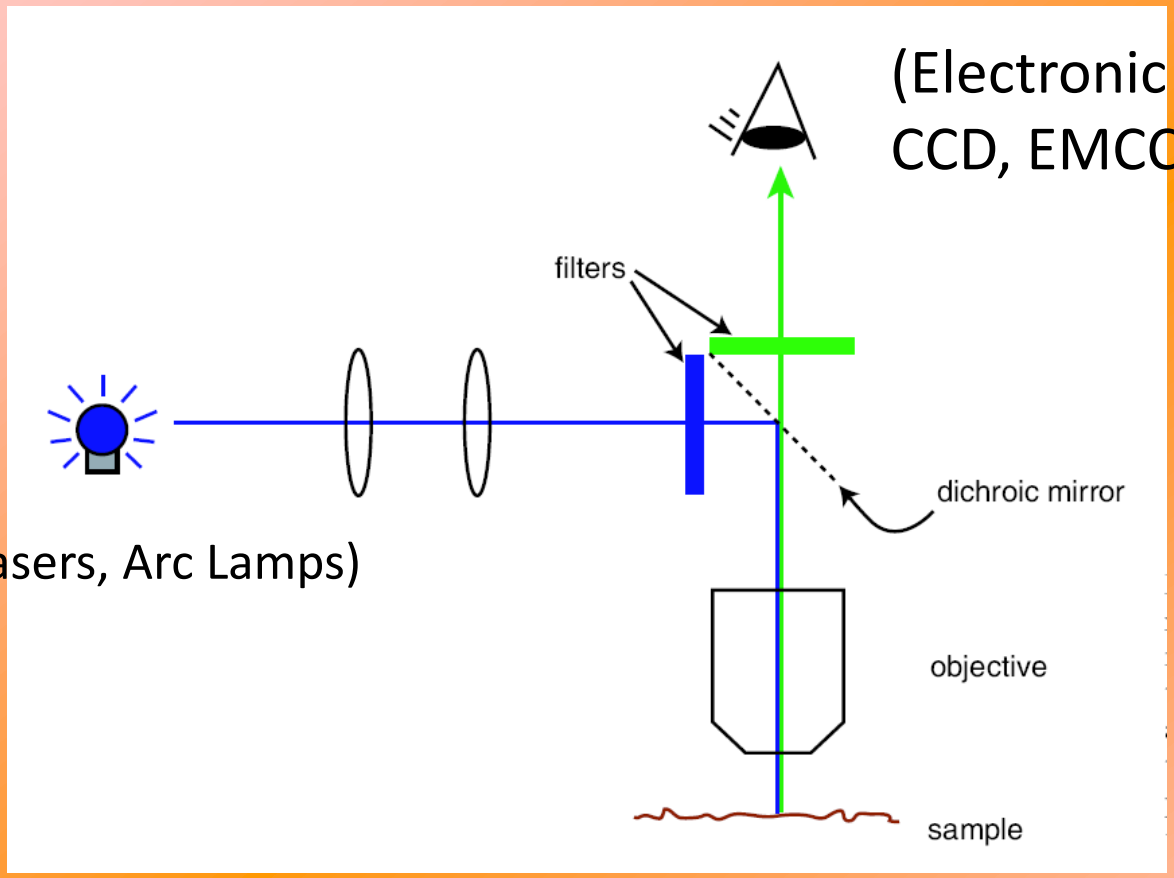






Photobleaching Important: Dye emits $10^5 \rightarrow 10^7$ photons, then dies!

Basic Set-up of Fluorescence Microscope

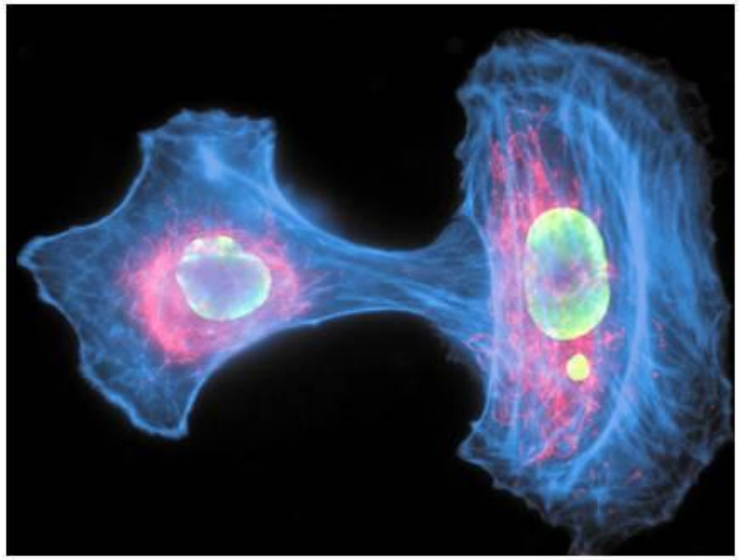


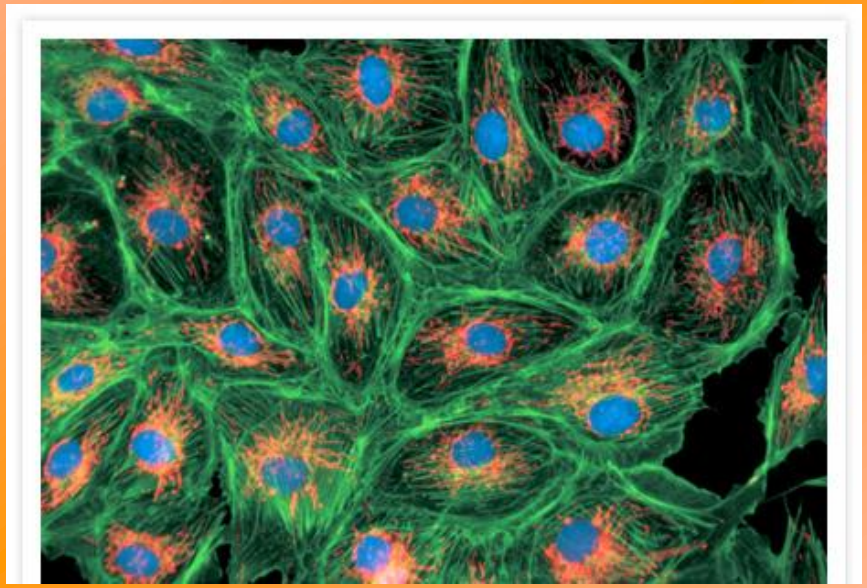
Nikon, Zeiss, Olympus, Leica—Microscope Manufacturer

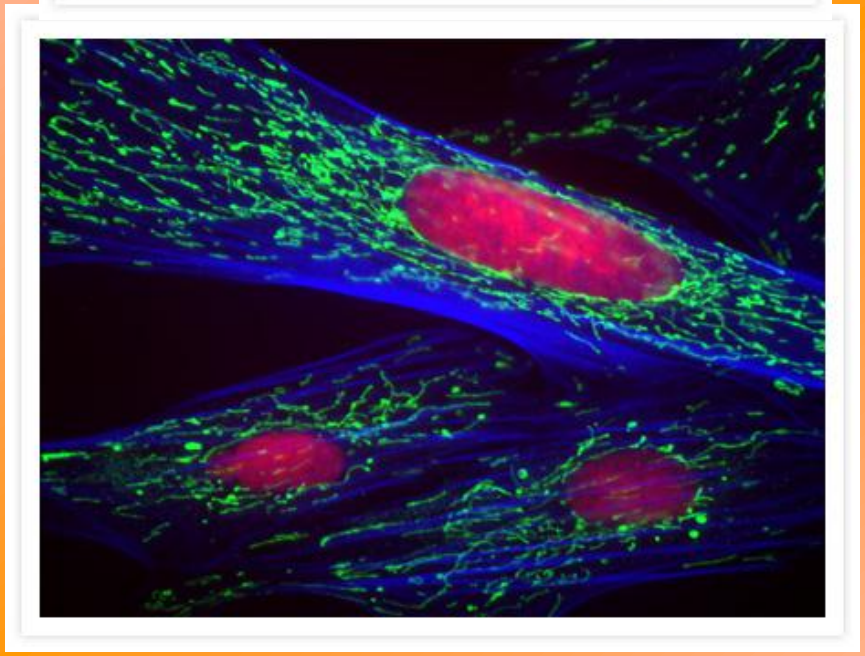
Andor, Hamamatsu, Princeton Instruments, other…make EMCCDs

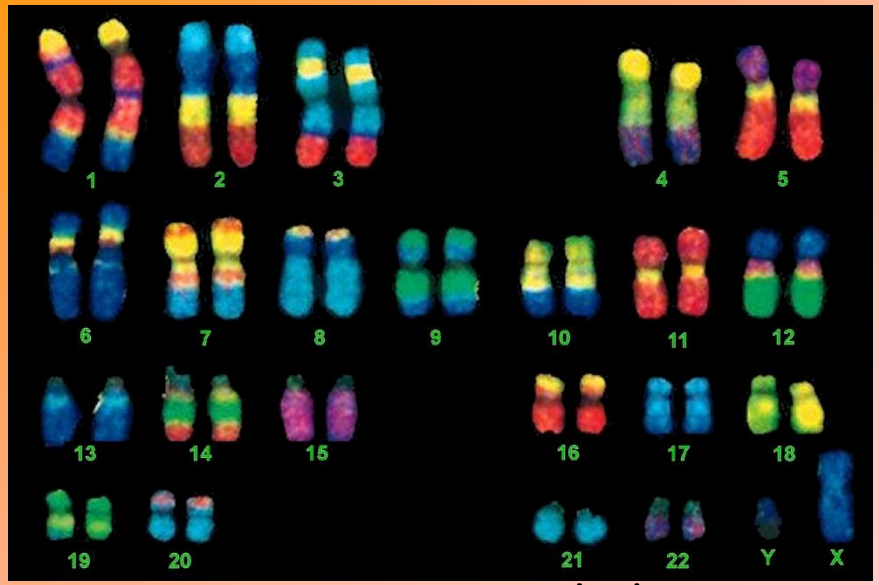
Semwogerere & Weeks, Encyclopedia of Biomaterials and Biomedical Engineering, 2005

You can get beautiful pictures









www.invitrogen.com

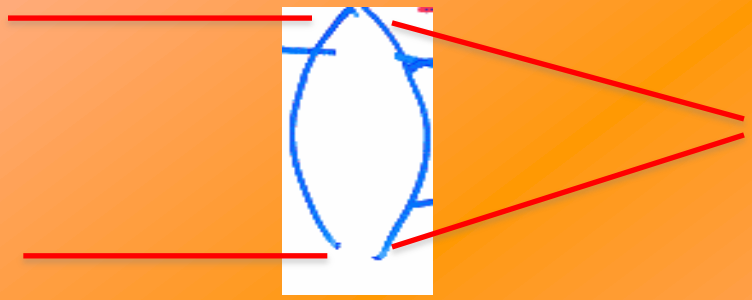
Fluorescence vs. standard light Microscopy

Which is more sensitive?

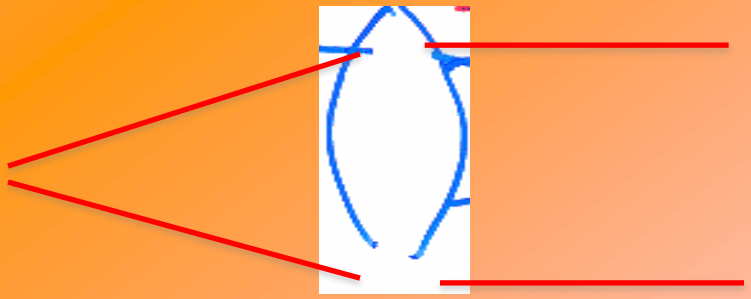
Answer: Fluorescence…no background!

Wide-field and Point illumination

Point illumination: Come into objective with parallel illumination.



Widefield illumination: want to have parallel light hitting sample. Shine light at back-focal plane.



Back focal length

Class evaluation

1. What was the most interesting thing you learned in class today?

2. What are you confused about?

3. Related to today's subject, what would you like to know more about?

4. Any helpful comments.

Answer, and turn in at the end of class.