Introduction to Light Microscopy

Paul S. Maddox

" You can observe a lot by watching ”

-Yogi Berra

Reference materials

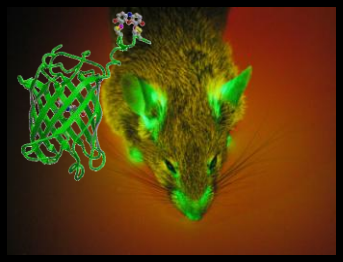
- Fundamentals of light microscopy and electronic imaging
  - Douglas B. Murphy, 2001, Wiley-Liss Inc.
    - A very good introductory text
- Video microscopy, 2nd edition
  - Shinya Inoue and Kenneth R. Spring, 1997, Plenum Pub.
    - More advanced and excellent reference book
- Proper alignment and adjustment of the light microscope
  - E. D. Salmon and Julie C. Canman
    - Just the basics
- Molecular Expressions webpage
  - http://micro.magnet.fsu.edu/primer/
    - Lots of useful info/tutorials
    - Where I stole lots of figures for this talk

History of microscopy

1595: The first compound microscope built by Zacharias Janssen

1910: Leitz builds first "photo-microscope"

Video microscopy developed early 1980s (MBL)



1994: GFP used to tag proteins in living cells

1955: Nomarski invents Differential Interference Contrast (DIC) microscopy

1600 1700 1800 1900 2000 2010

1680: Antoni van Leeuwenhoek awarded fellowship in the Royal Society for his advances in microscopy

1934: Frits Zernike invents phase contrast microscopy

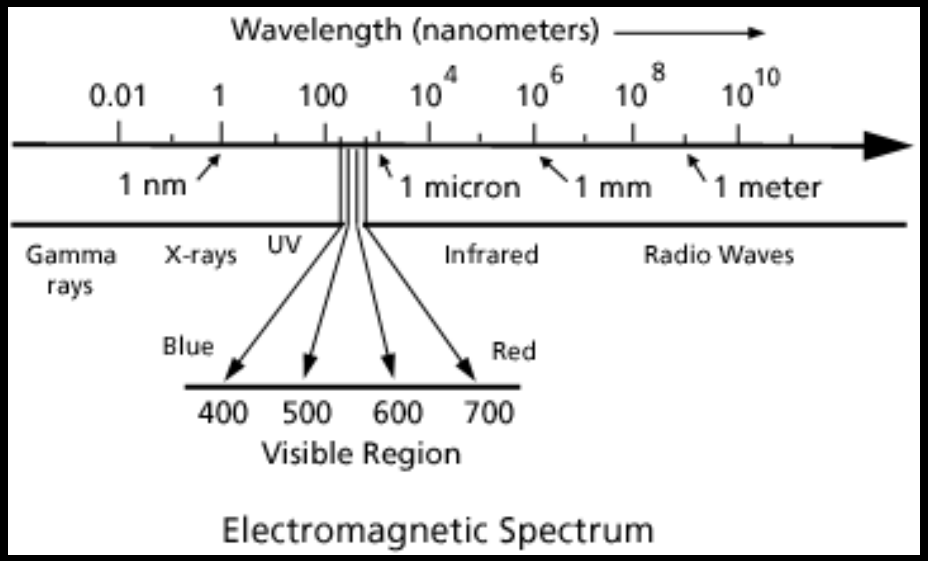
1960: Ziess introduces the "Universal" model

Super-Resolution light Microscopy

Images taken from:
Molecular Expression and Tsien Lab (UCSD) web pages

Quick facts

- Visible wavelengths ~400 to 700 nm

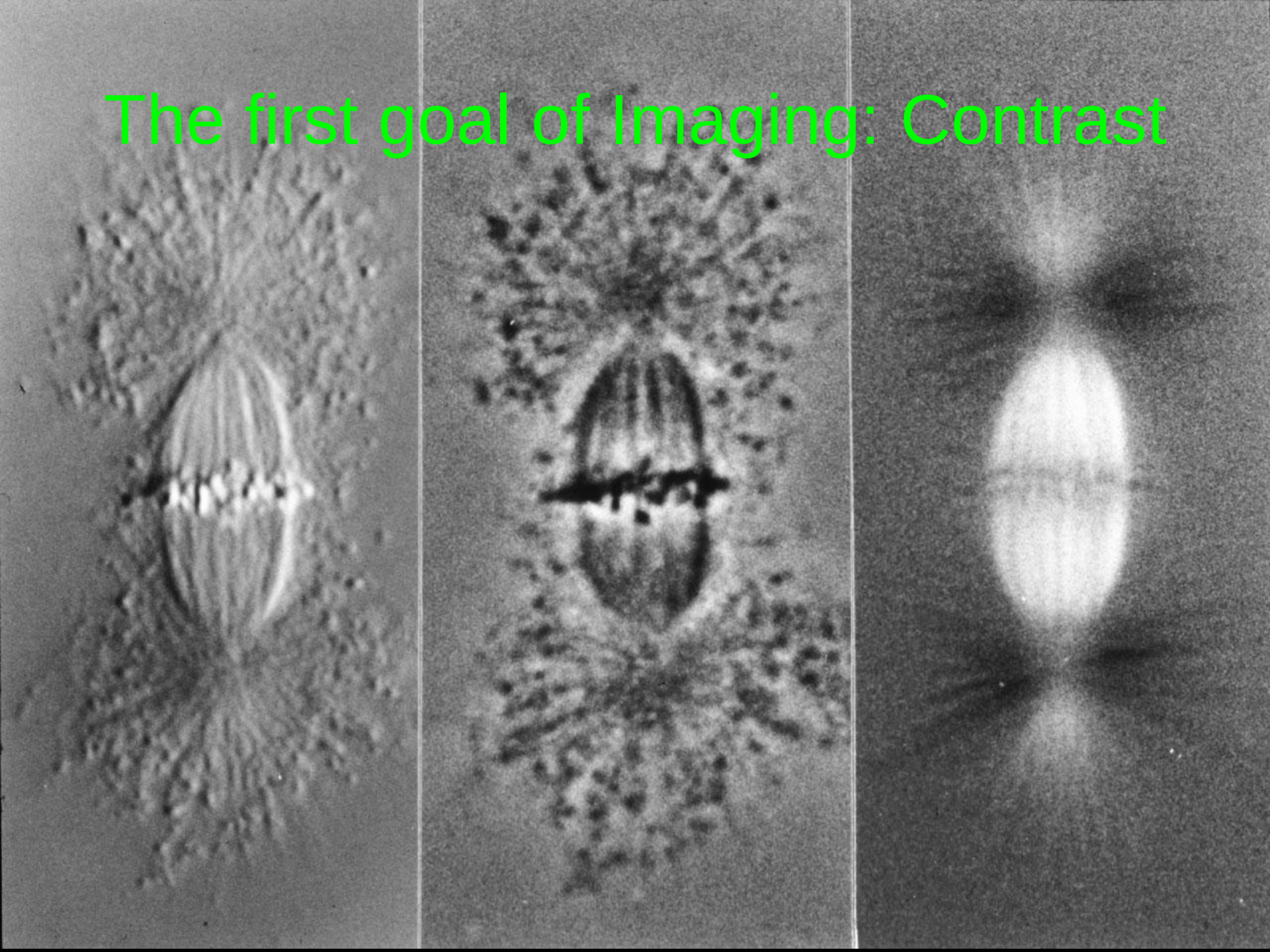


- Human eye most sensitive to ~530 nm (greenish)

More quick facts

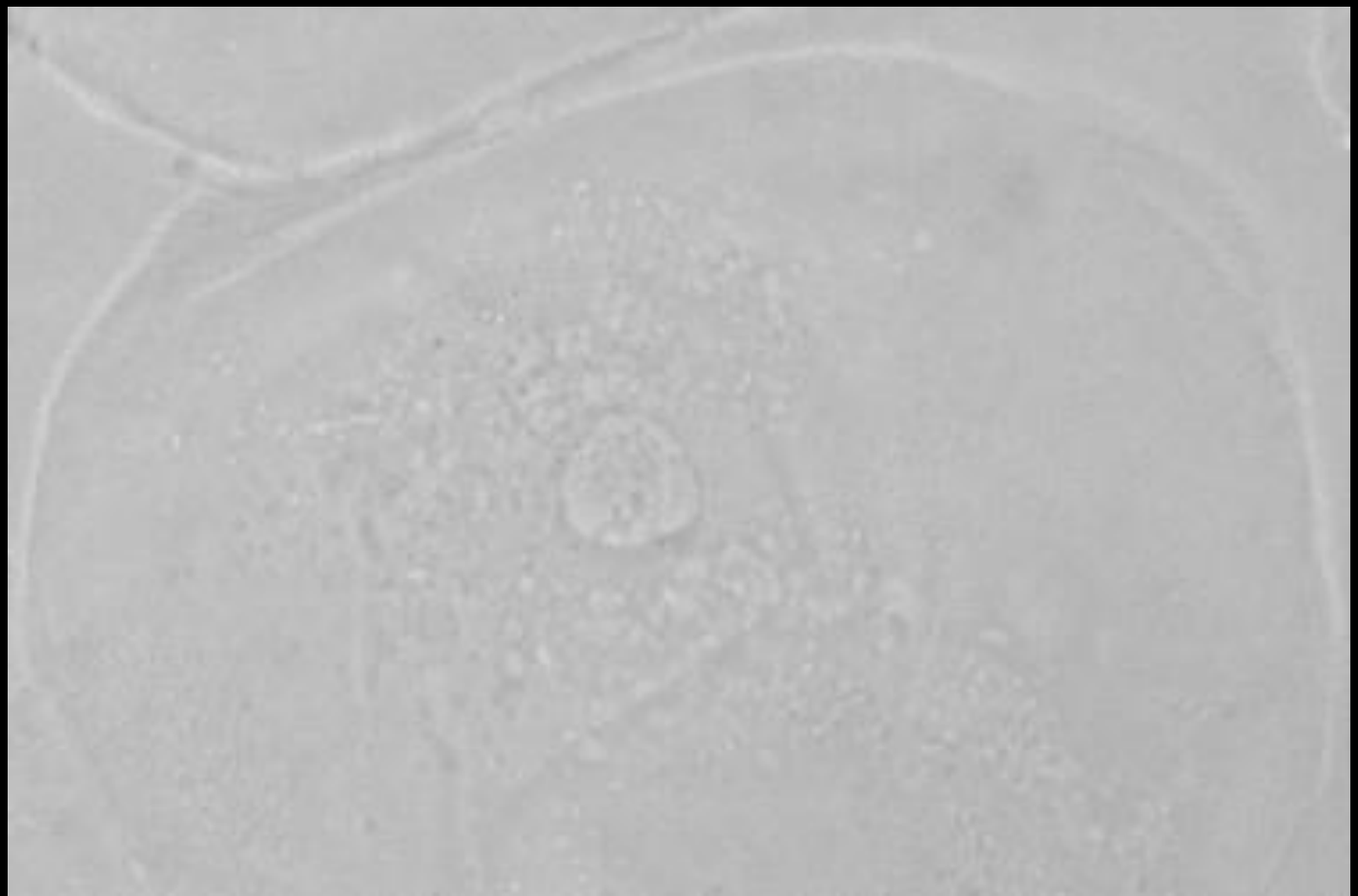
- Biological specimens are *generally* larger than the wavelength of visible light
  - *Xenopus* Egg 1.000 mm
  - Human hair 0.150 mm
  - Sea urchin Egg 0.060 mm
  - Typical somatic cell 0.020 mm
  - Typical nucleus 0.010 mm
  - Typical vesicle 0.001 mm
  - Microtubule (diameter) 0.000025 mm

The first goal of Imaging: Contrast



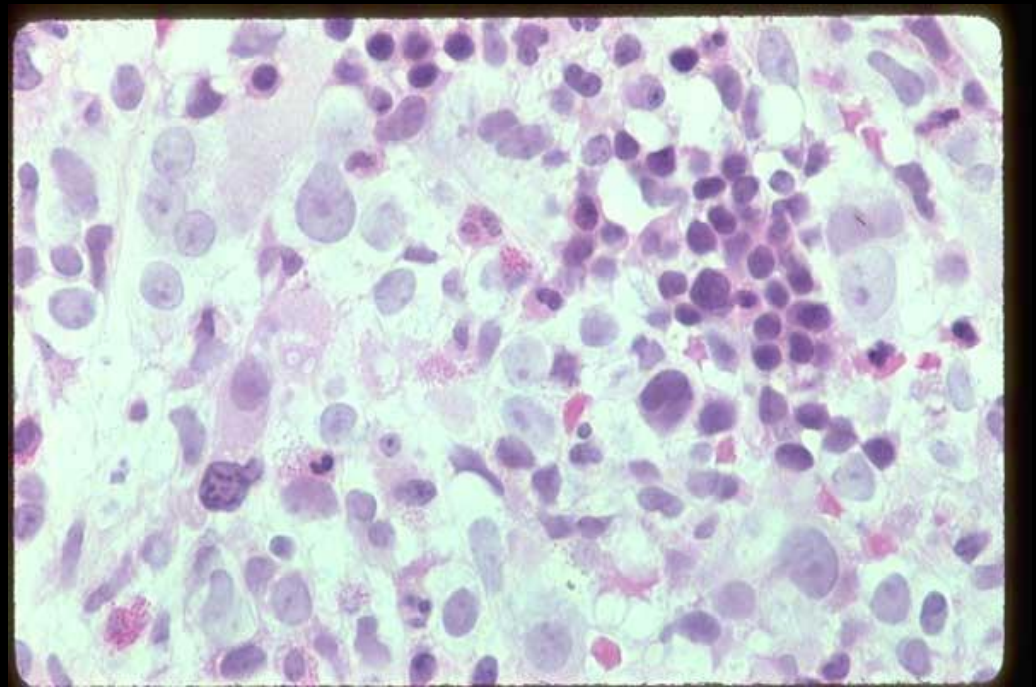
Contrast: Biological specimens are (generally) optically clear

- Cells are mostly (~70%) water

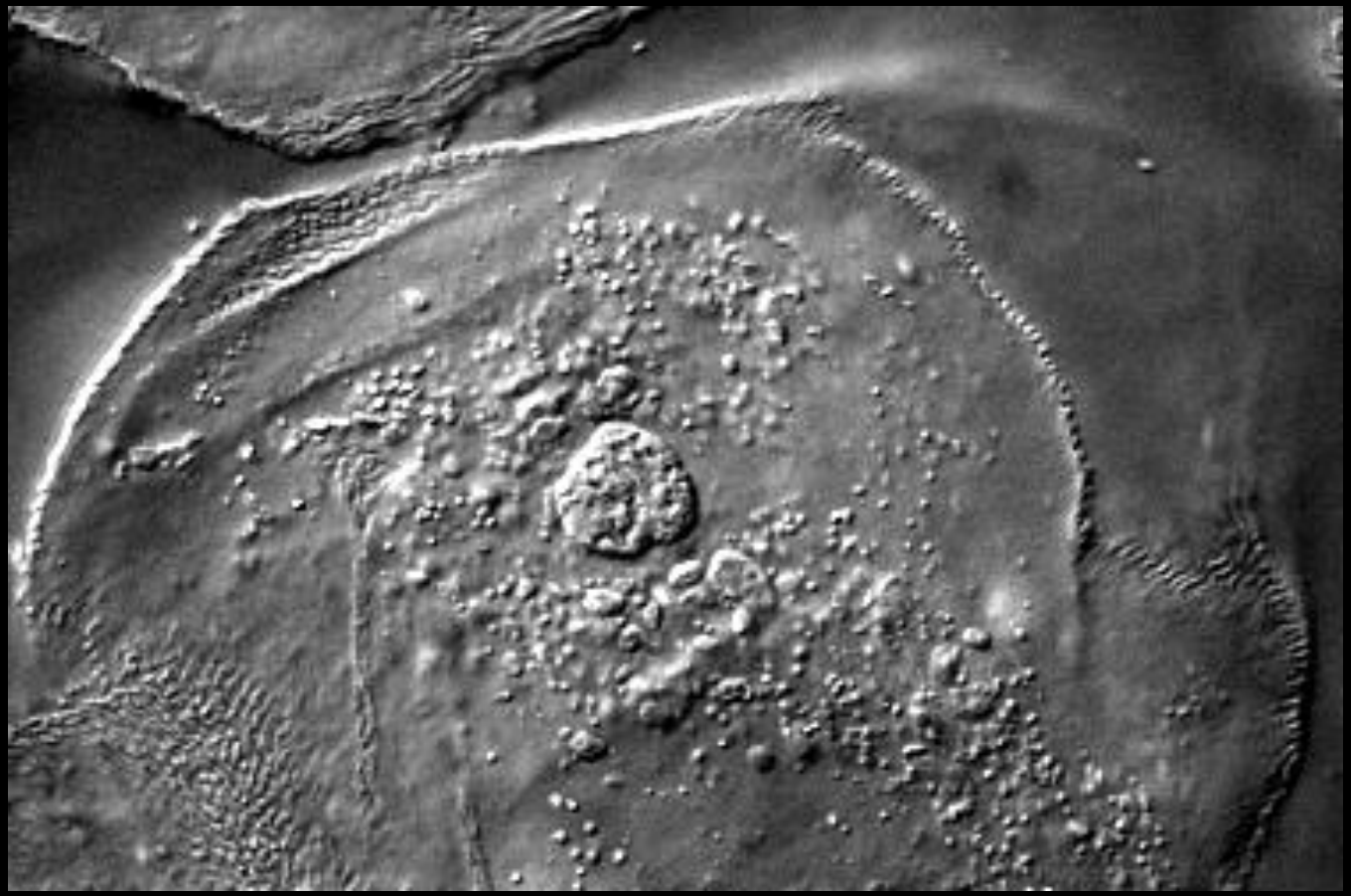


Contrast: Staining

- Hematoxylin and Eosin (H & E)
  - Hematoxylin stains negatively charged nucleic acids (nuclei & ribosomes) blue. Eosin stains proteins pink.



Contrast: No staining



Enhancing Contrast

- Light interacts with matter
  - Light travels slower in some materials
- Light interacts with Light
  - Constructive interference
  - Destructive interference
- Use interference to make contrast

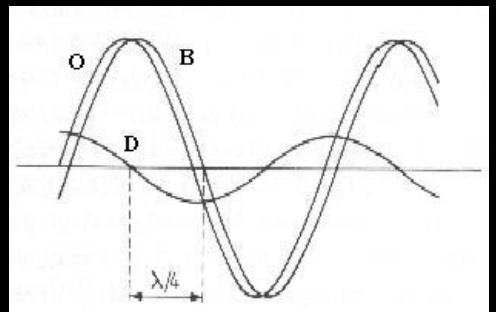


Fig. 47. Effect of phase-object

Basic Optics

- Definitions
- Spherical and Plane wavefronts
- Interference
- Refraction
- Diffraction
- Resolution

Light propagates as a oscillating, sinusoidal, transverse electromagnetic wave:

Electric field is most important for biological specimens; magnetic field is ignored

(Note: x,y,z spatial coordinates)

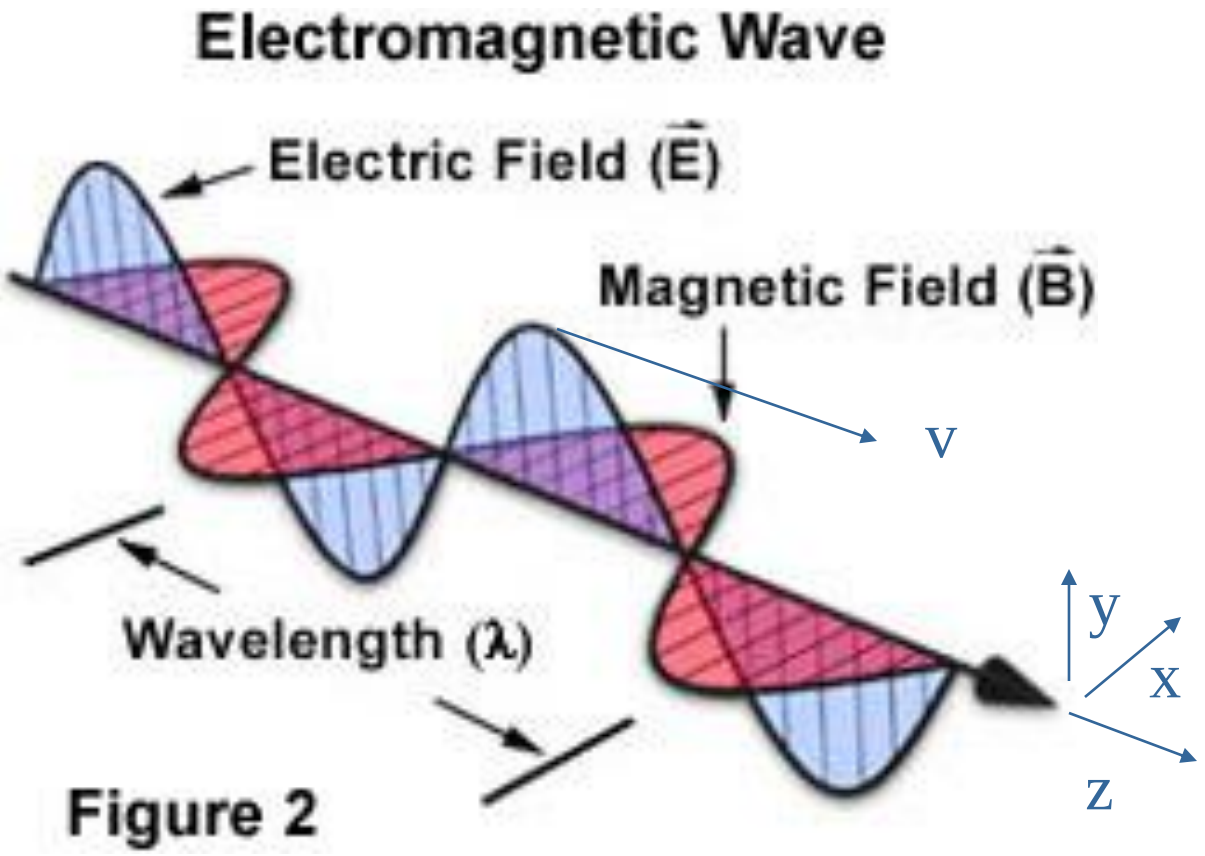
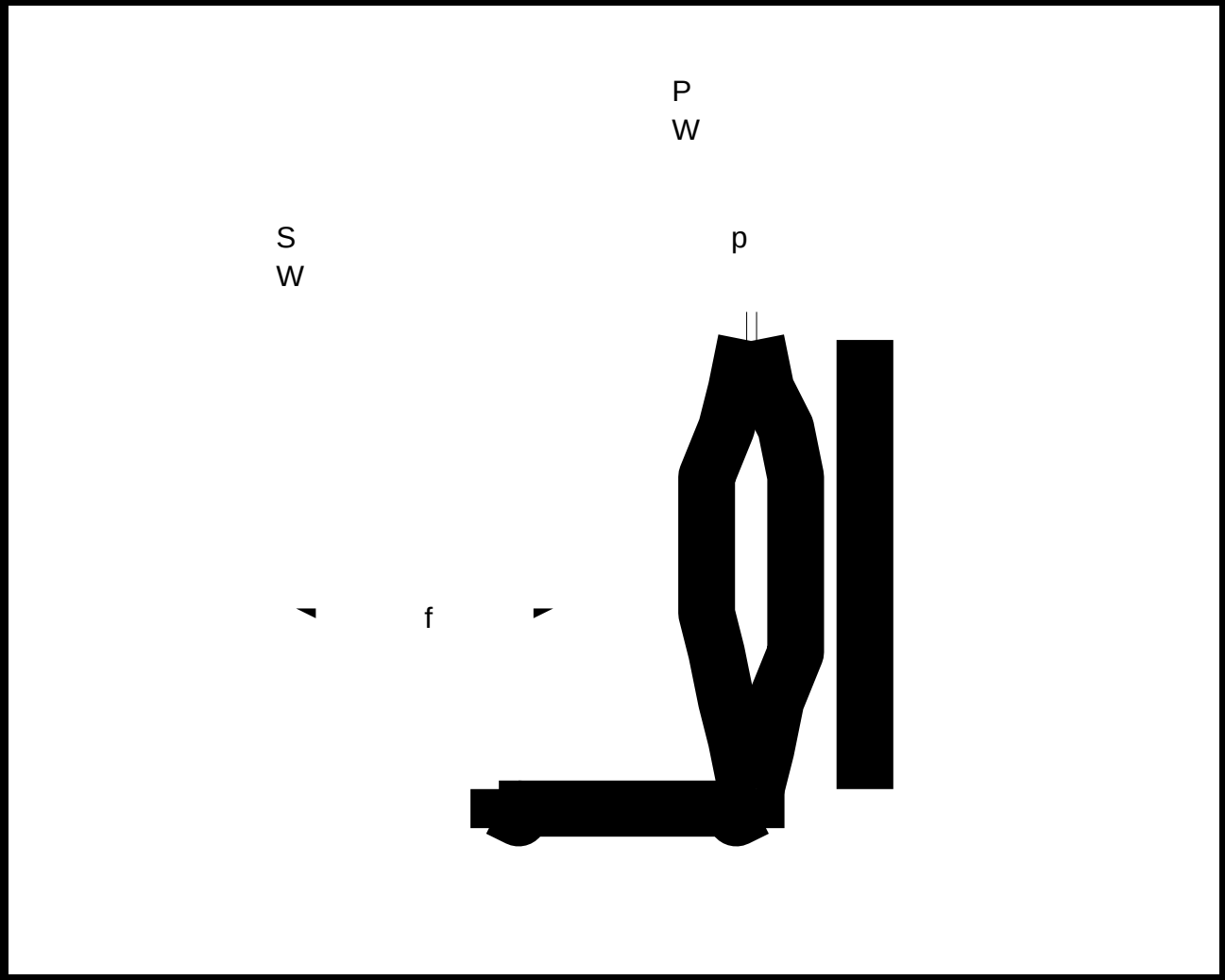
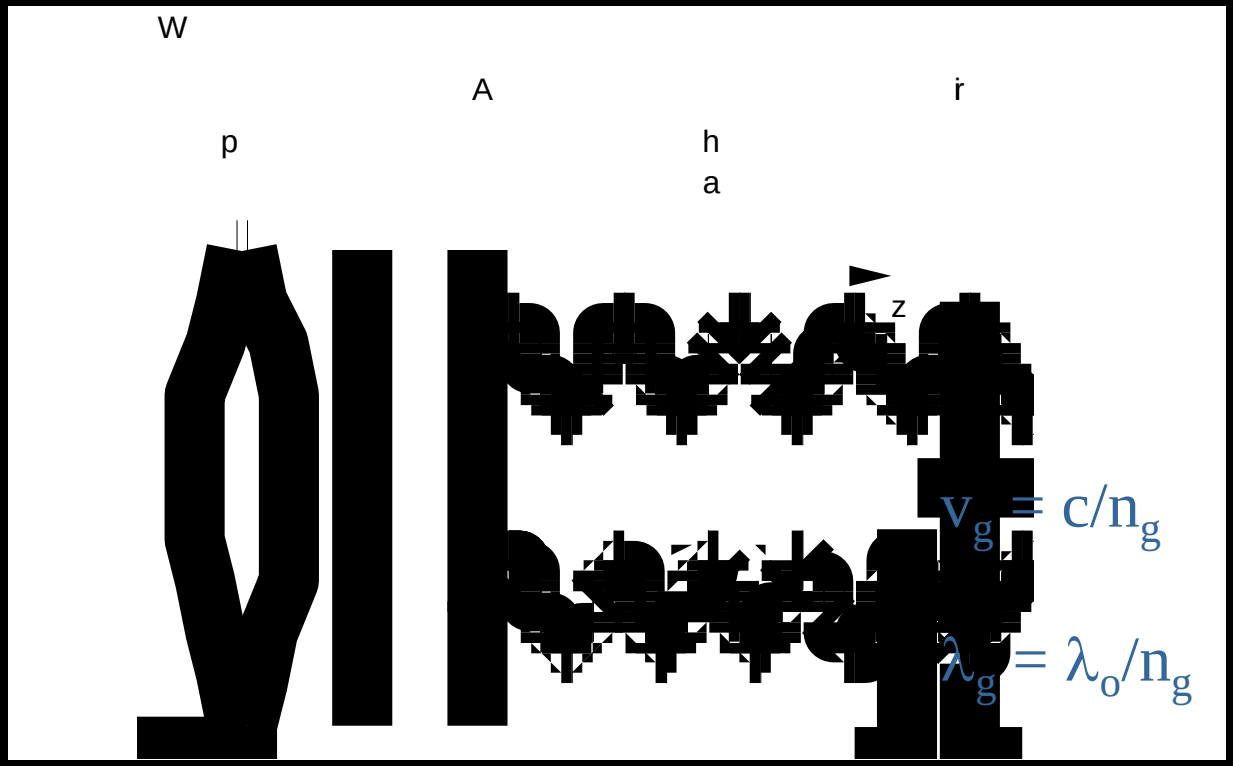


Figure 2

Plane wave fronts are produced by placing a point source of light at the front focal point of a converging lens



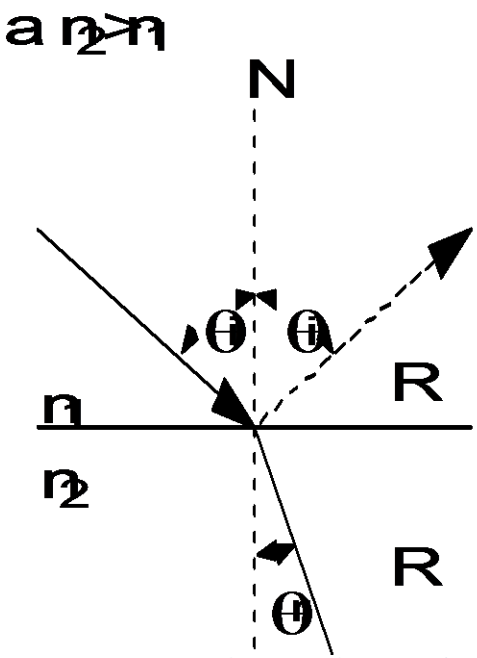
Wavefronts can become out of phase if they travel through different optical paths: Here the wave passing through glass is “retarded” relative to the one in air. The “optical path difference” is OPD = $(n_{glass}-n_{air})*d$, where d is the depth of the glass.

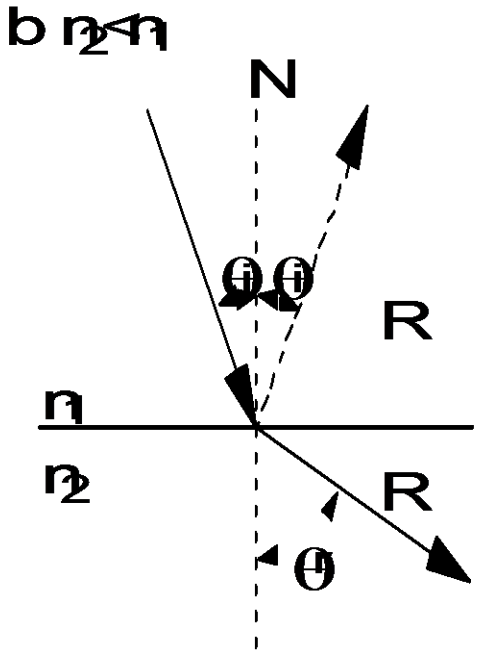


Refractive Index (n)

- Light moves in straight lines through homogenous materials
- The refractive index of media X=(speed of light in a vacuum)/(speed of light in media X)
- Refractive index of some common materials
  - Air = 1
  - Water = 1.33
  - Cytoplasm = 1.38
  - Protein = 1.5
  - Nucleus = 1.4
  - Oil = 1.515
  - Glass = 1.52

Light does not move in straight lines at refractive index boundaries





Snell's Law of Refraction

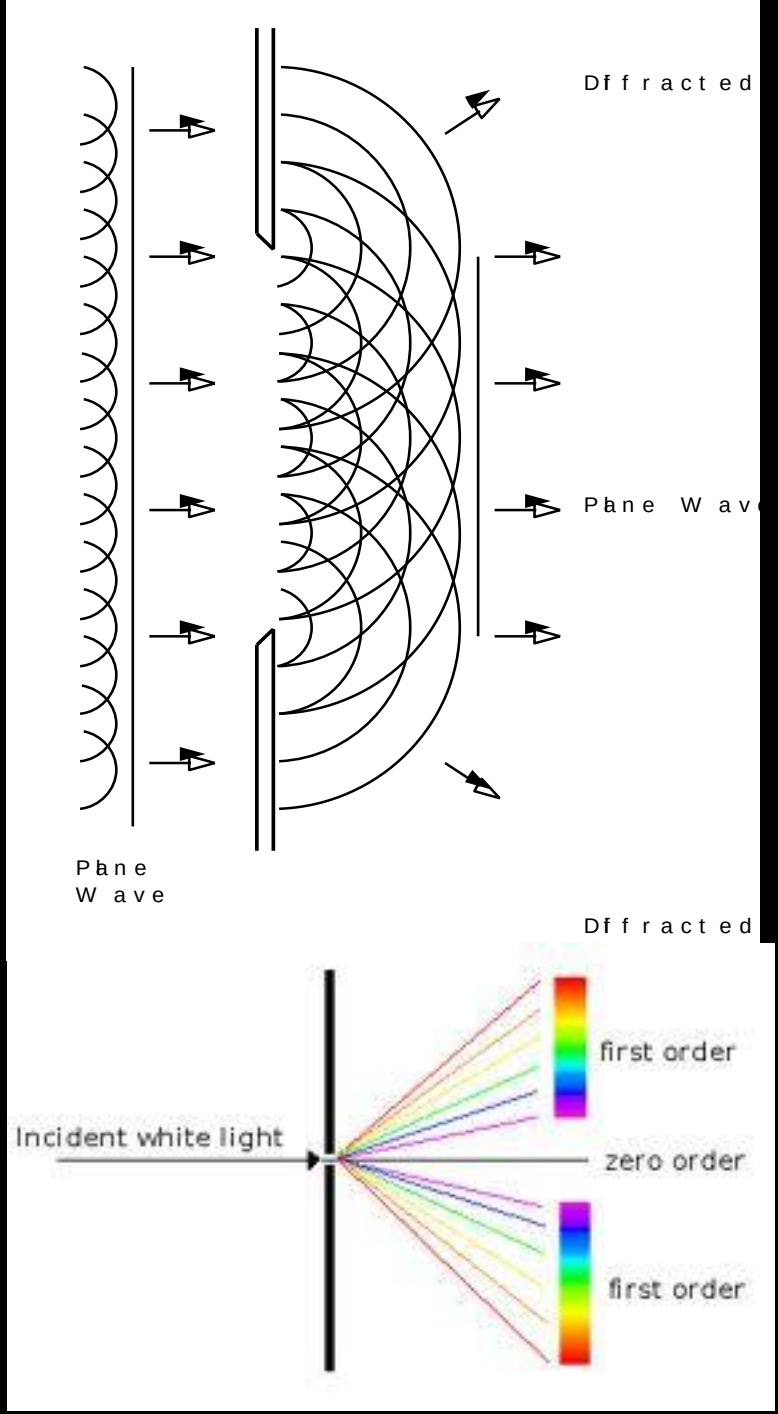
$n_1 \sin(\Theta_i) = n_2 \sin(\Theta_r)$

Diffraction

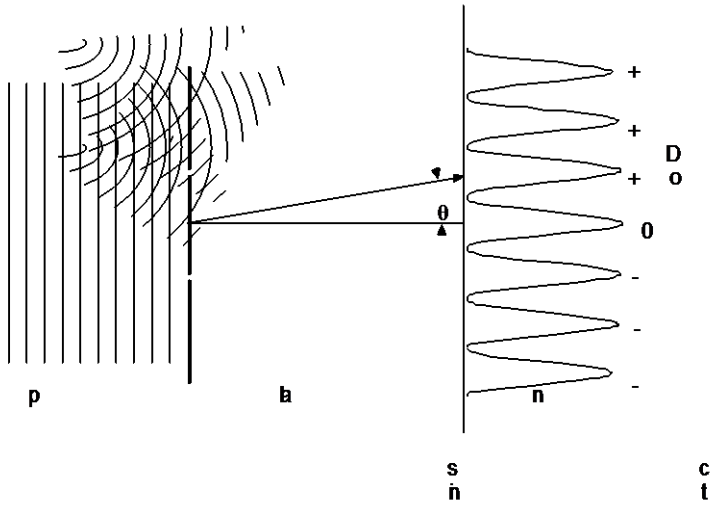
Huygen's principle can be used to analyze diffraction of light

- "Every point on a wavefront acts as a source of secondary wavelets, and the position of the wavefront at any time may be found by drawing the envelope of the secondary wavelets at that time"

In the center of the slit, the new wavefront is a plane wave; at the edges, the secondary wavelets spread out as spherical waves (diffracted light)

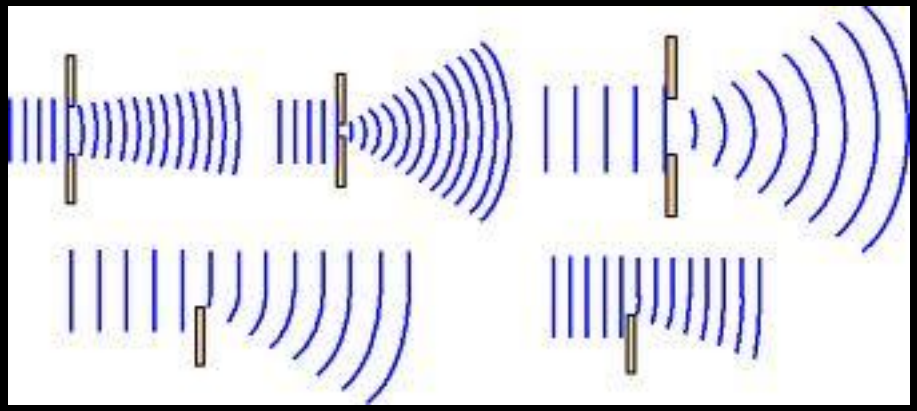


Diffraction from gratings and grids produces an interference pattern: Young's (1801) demonstration of the wave nature of light:



"Diffraction pattern"

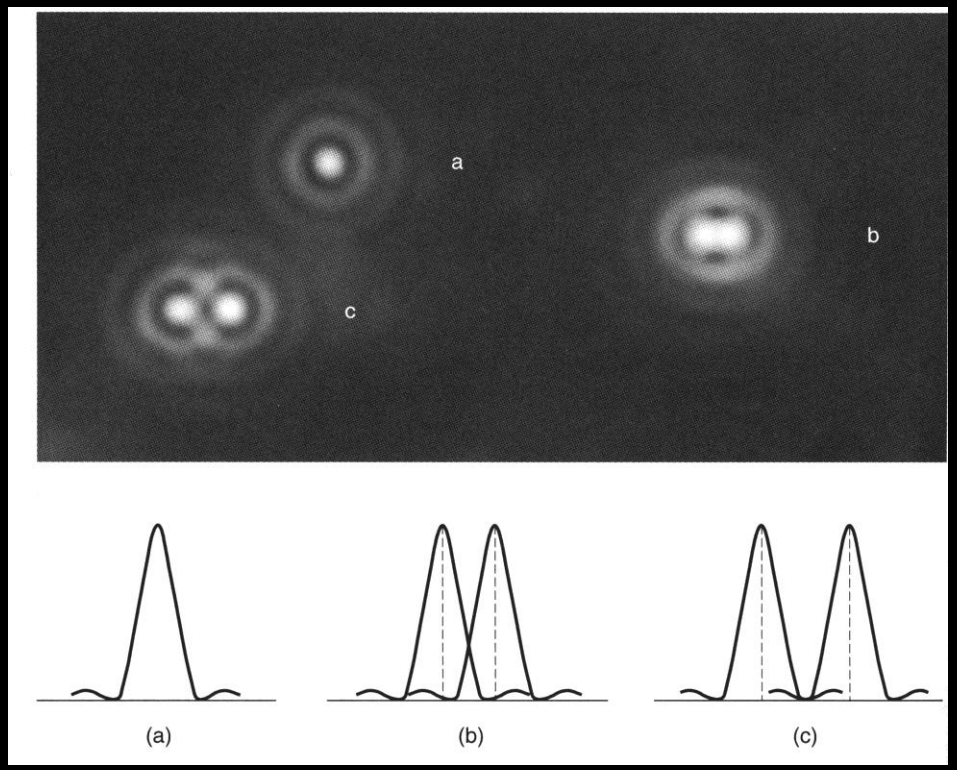
Some other aspects of diffraction



- Transparent objects diffract light, but most of the light passes through undiffracted (ie zero order, want to attenuate this in transmitted microscopy)
- Most objects generate complex diffraction patterns because their structural detail contains complex spatial frequencies
- The diffraction pattern of an object is equivalent to a Fourier transform of the specimen

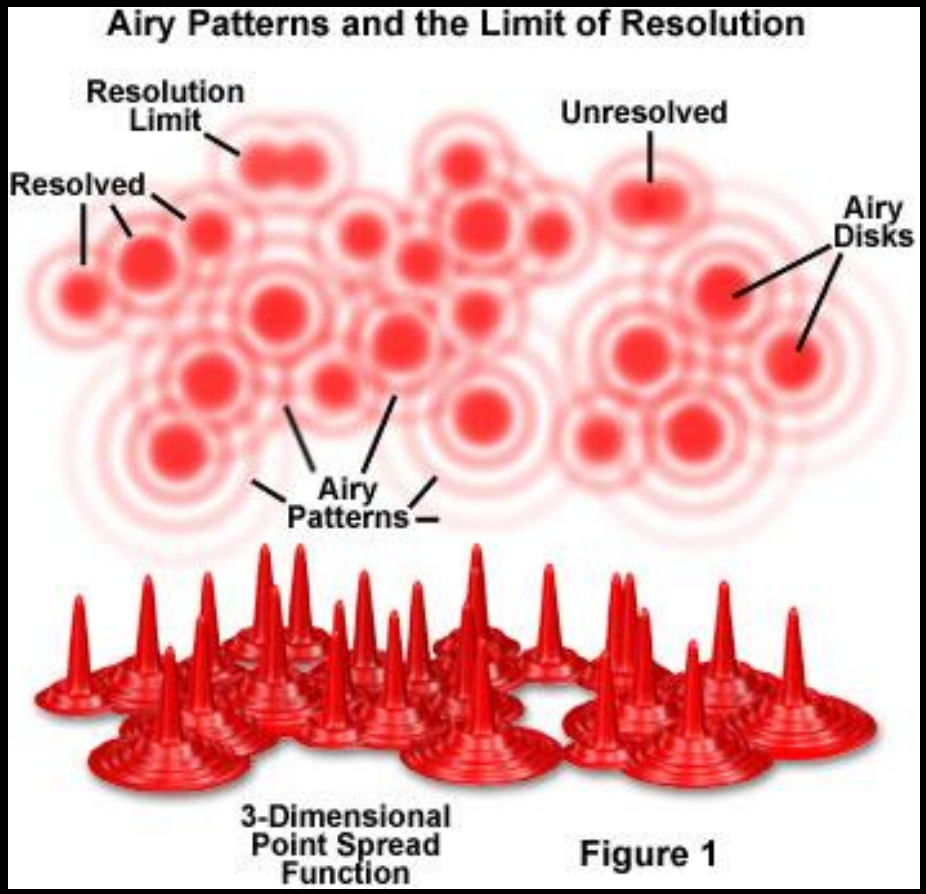
A point source of light creates a Diffraction Pattern called an Airy Disk at the focal plane

- Occurs in plane (x,y) and on axis (z)
  - Spreading is more dramatic (3 to 4x) on axis (z)
- Directly proportional to resolution



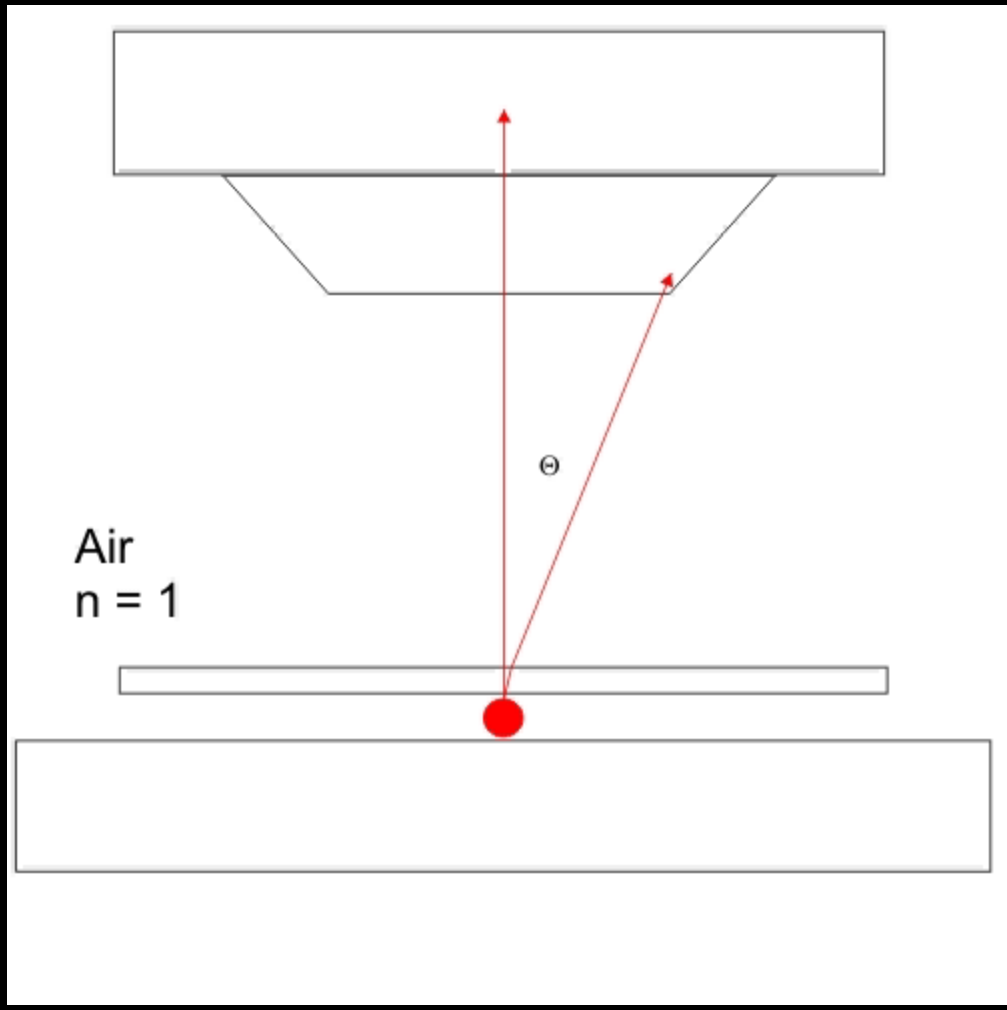
How much separation is required for resolution?

- R = 1.22 λ /(NAcond + NAobj)
  - For Fluorescence… R = 0.61 λ / NAobj
  - For NA 1.40… R = λ / 2.29
- Rule of thumb:
  - Distance between two objects is greater than half the imaging λ

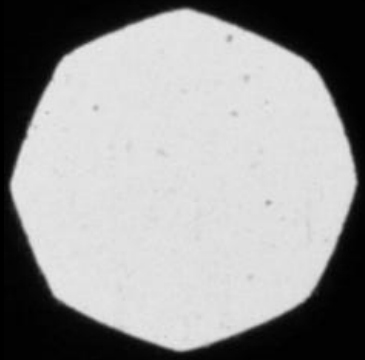


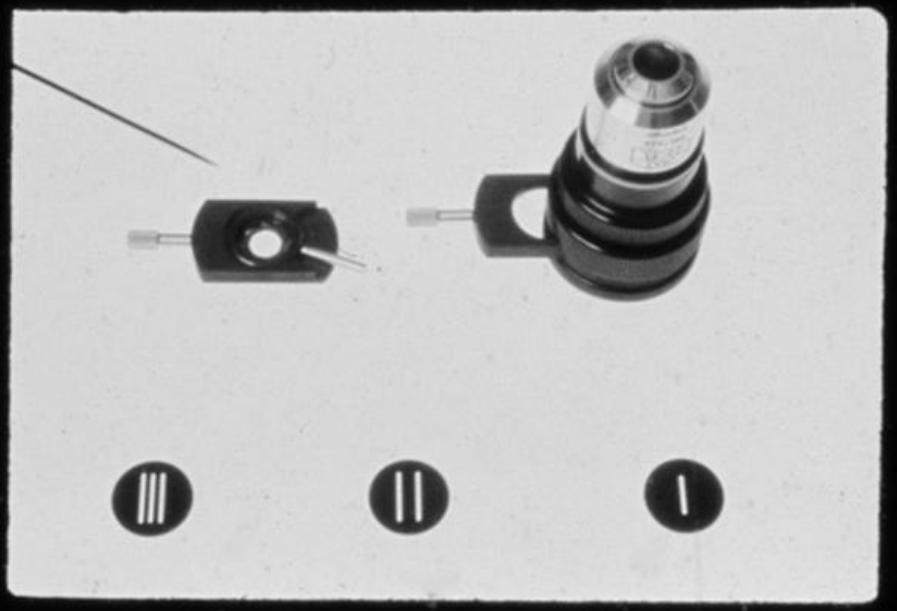
Numerical Aperture

- NA = n(sinθ)
- Increase n with immersion fluids to increase NA
- Because more light is collected, intensity goes UP with NA^2



Diffraction and Resolution



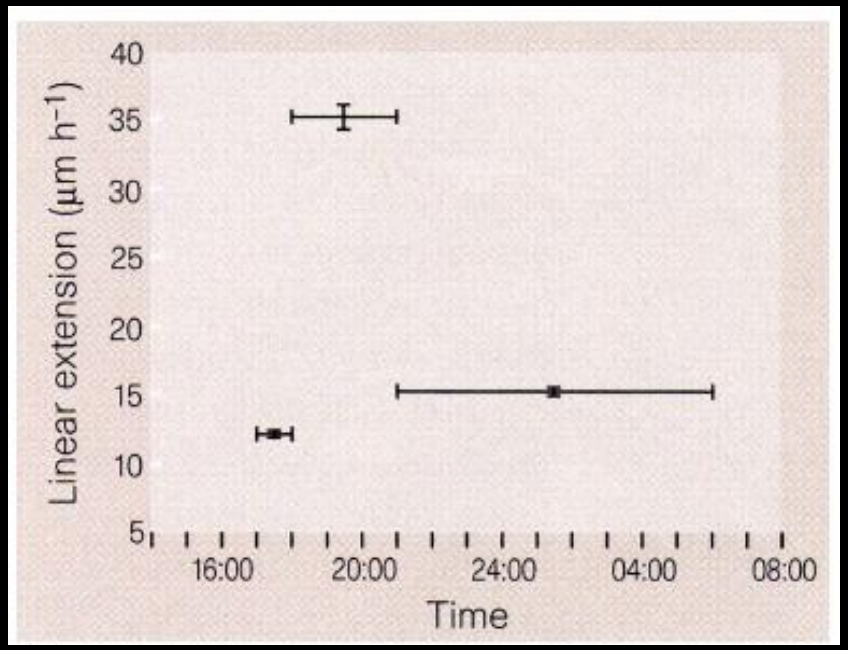


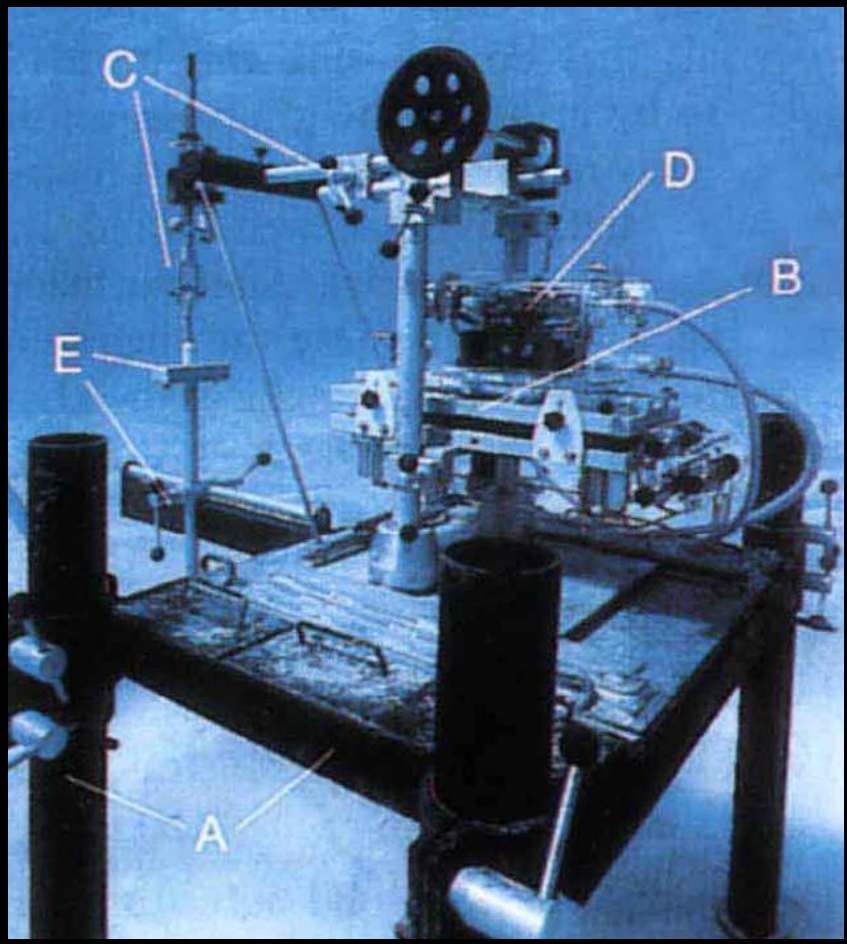
http://www.guycox.com/diffkit/diffkit.htm

Using Diffraction

Laser measurements of coral growth

NATURE | VOL 386 | 6 MARCH 1997





Optical aberrations

- Light passing through the microscope makes several media transitions
  - Each transition refraction and or reflection occurs
  - Of axis light focuses somewhat differently than light on axis
  - Some aberrations are color dependent
    - Different wavelengths behave differently at each transition

Corrections require more lens elements

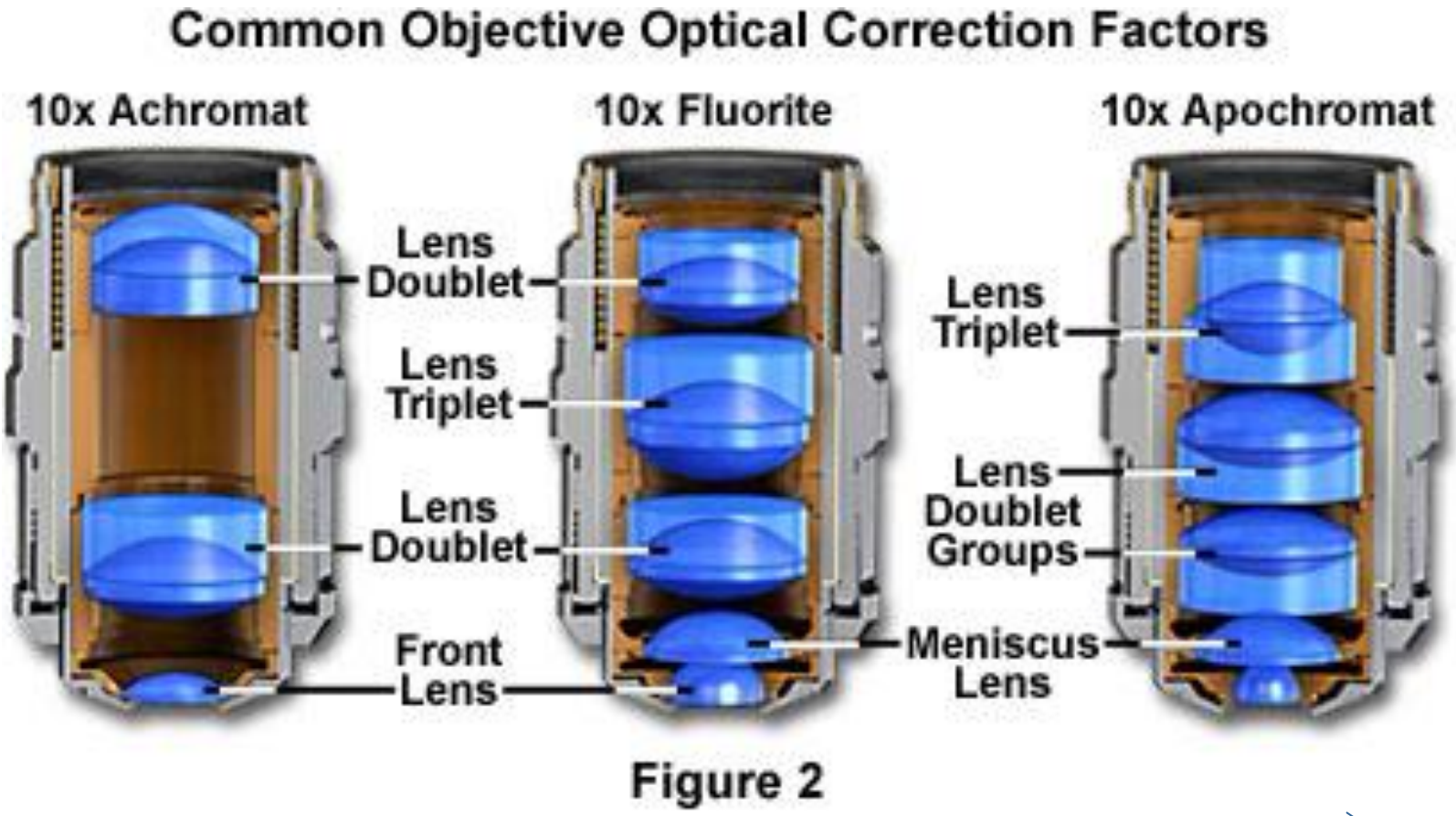


Figure 2

Chromatic and Mono-Chromatic Corrections

Objective Immersion Type

• Dry	(no marking)
• Water (direct)	W.WI
• Water (coverglass)	W Korr
• Glycerol	G, Gly
• Oil	Oil, Oel
• Multi-immersion	Imm (Water, glycerol, oil)

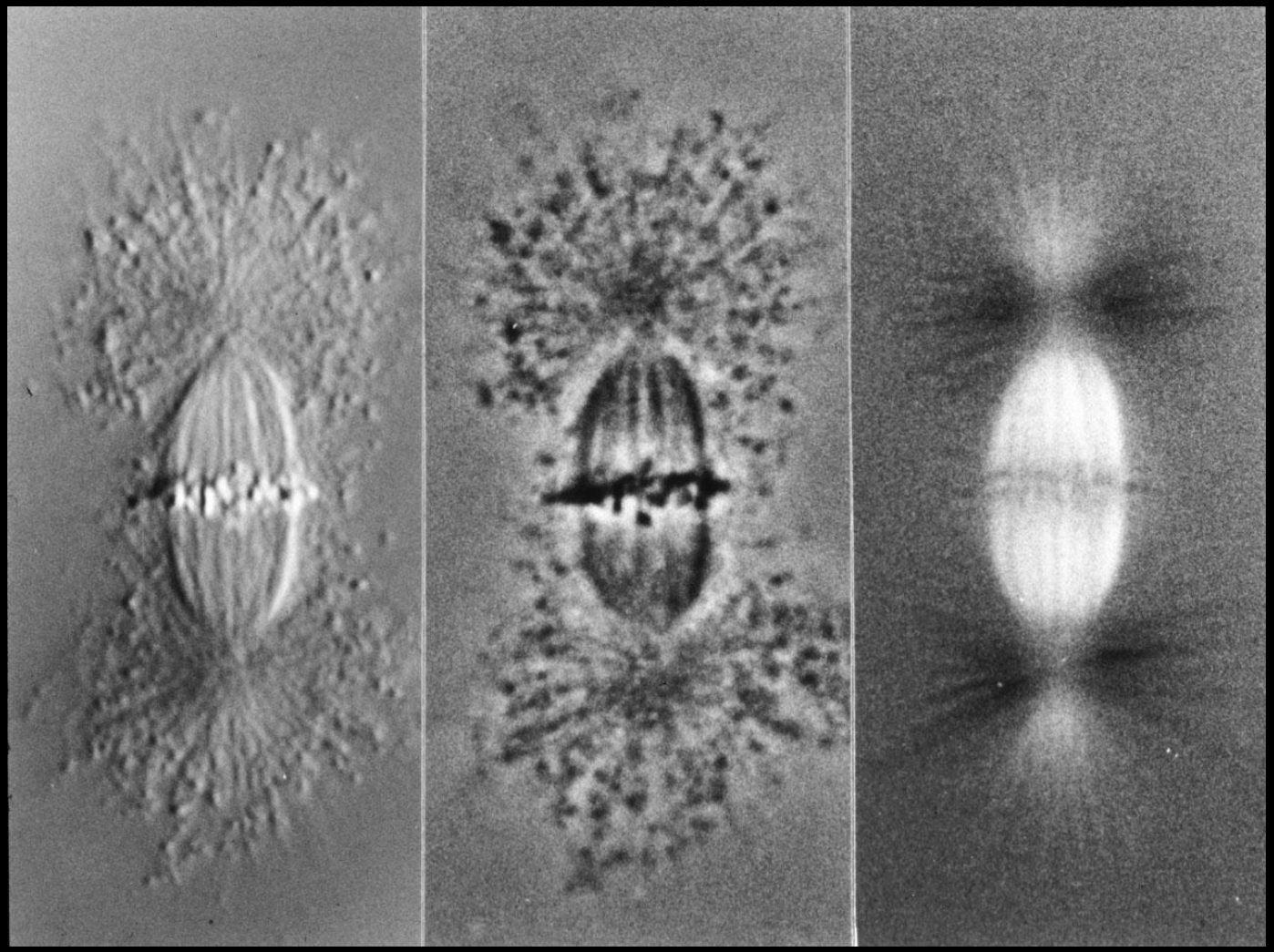
Cover Slip Thickness

- # 0: 0.1-0.13 mm
- # 1: 0.13-0.17 mm
- # 1.5: 0.15-0.20 mm; 0.17 mm for Dry Obj.
- # 2: 0.17-0.25 mm
- # 3: 0.25-0.5 mm

Objective Specifications

- Magnification
- Numerical Aperture (NA)
  - NA = n (sinθ)
- Working distance
  - 0.22 means lens can focus 220 microns PAST the coverslip
- Corrections
  - Infinity corrected
  - Should use a #1.5 CS (170 microns thick)

Transmitted light

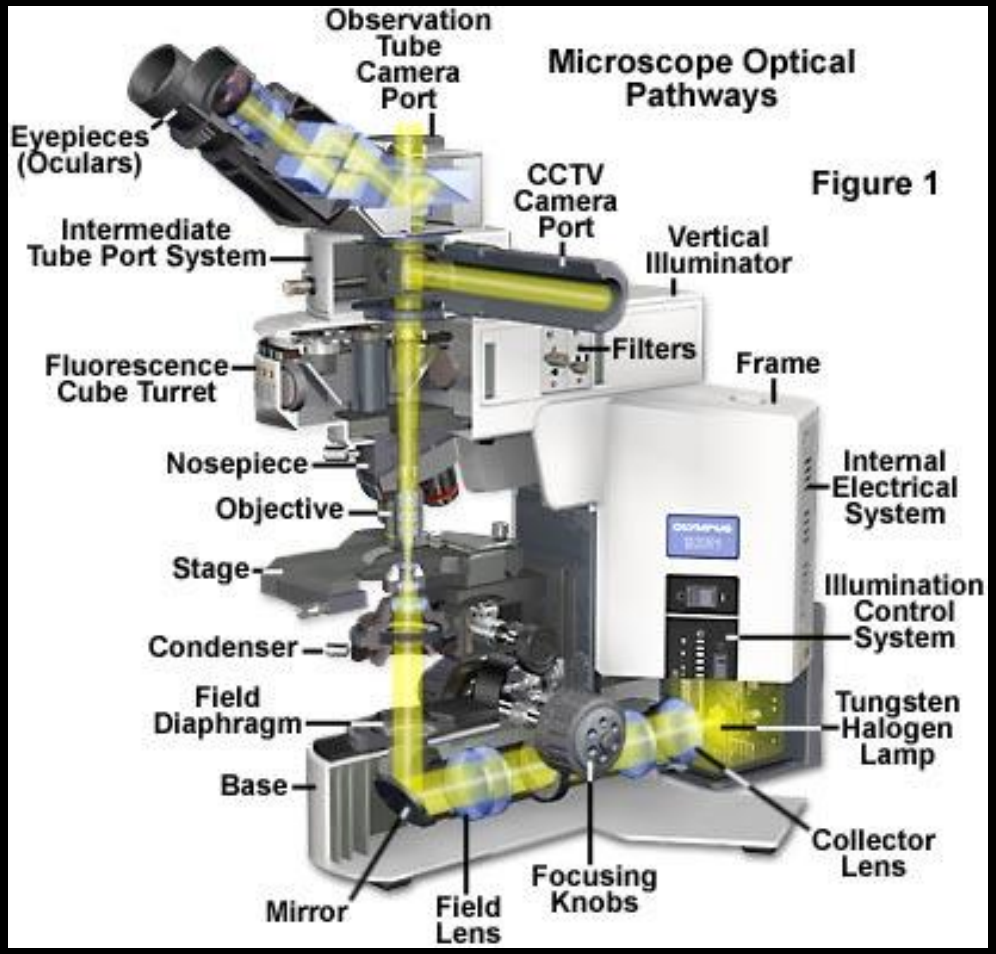


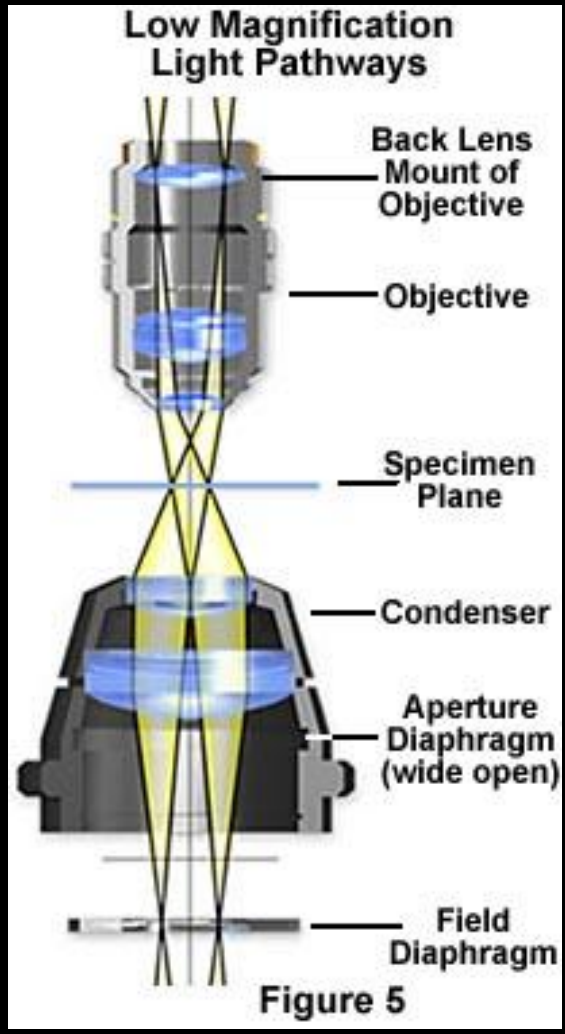
E. D. Salmon

Transmitted light

- Light is passed through the specimen
  - Biological tissue generally transparent
  - Light interacts with the specimen
- Various types of contrast enhancement
  - Phase contrast, Polarized Light, DIC
  - Hoffman, Darkfield

Transmitted light is generally a "Bright-Field" technique

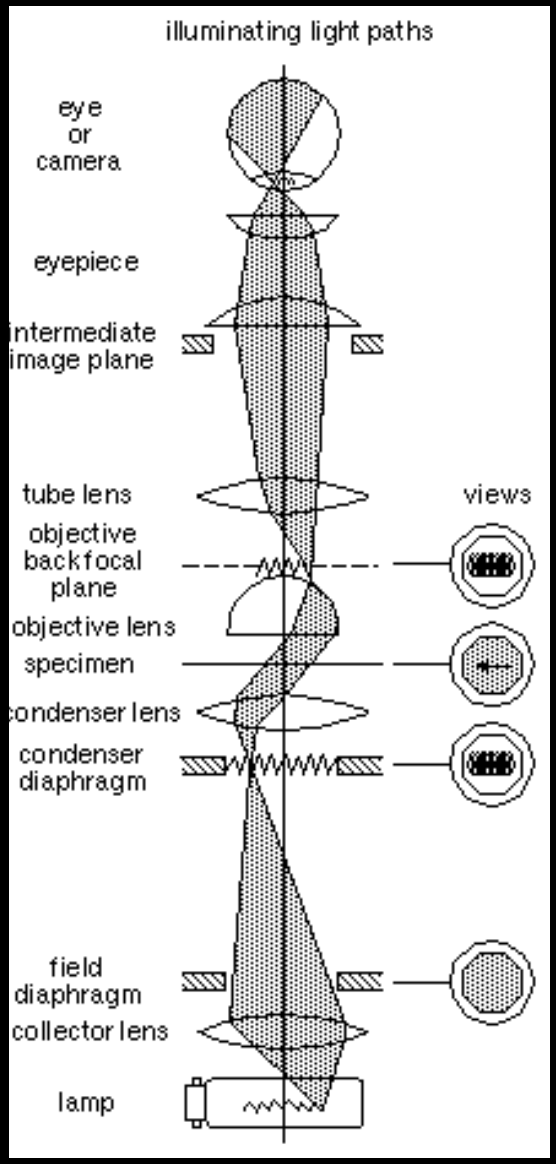




Two types of illumination

- Critical
  - Focus the light source directly on the specimen
  - Only illuminates a part of the field of view
  - High intensity applications only (VeDIC, confocal)
- Köhler
  - Light source out of focus at specimen
  - Even field of illumination
  - Less intensity per unit area
- Criminal
  - Anything else…

Köhler Illumination

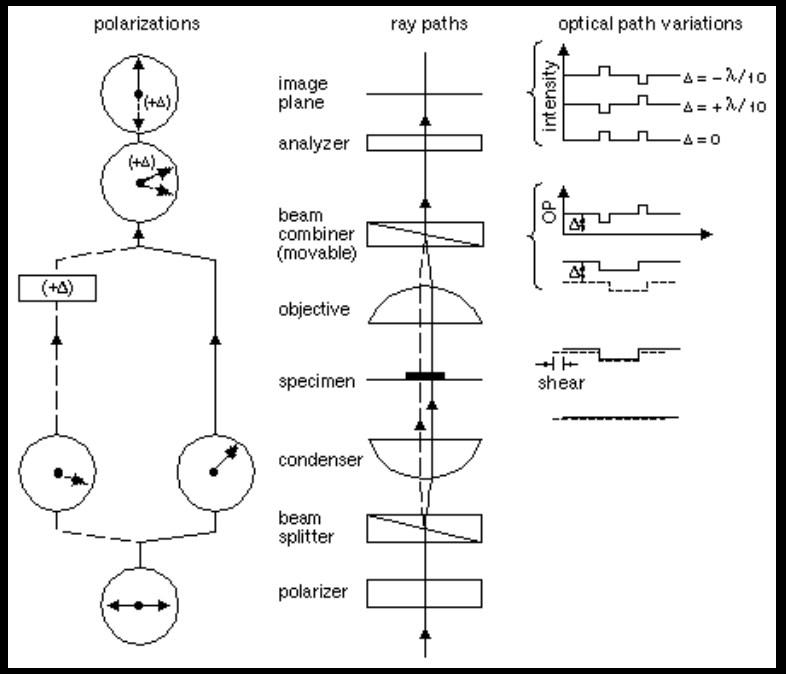


Using Field and Aperture Diaphragms: Kohler Illumination

- Focus on specimen
- Focus condenser until field diaphragm is in the same plane as the specimen
- Center the field diaphragm
- OPEN the aperture diaphragm
  - Closing lowers the NA of the condenser (only useful for darkfield)

DIC

- Differential Interference Contrast
- Invented by Nomarski in 1955
- Thin optical section (good for thick things)



Video Enhanced DIC Microscopy of Mitosis in Newt Lung Cells (Taricha granulosa)

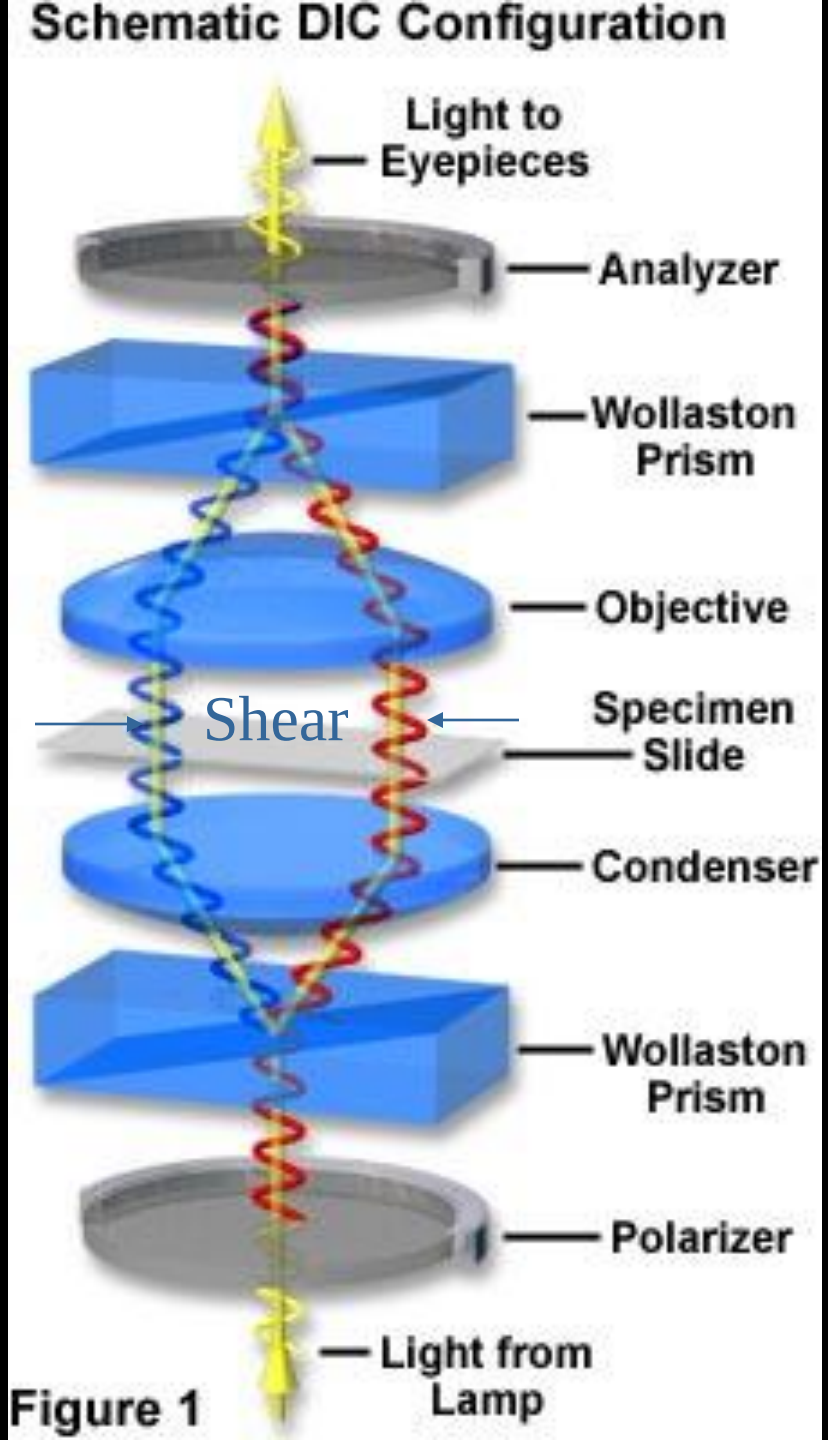
Victoria Skeen,
Robert Skibbens, and
E. D. Salmon

University of North Carolina at Chapel Hill

(see Skibbens et al., 1993, J. Cell biol. 122:859-875)

Frame Time = HR:MIN:SEC

The DIC Microscope Is a Dual-Beam Interferometer Made with Polarization Optics



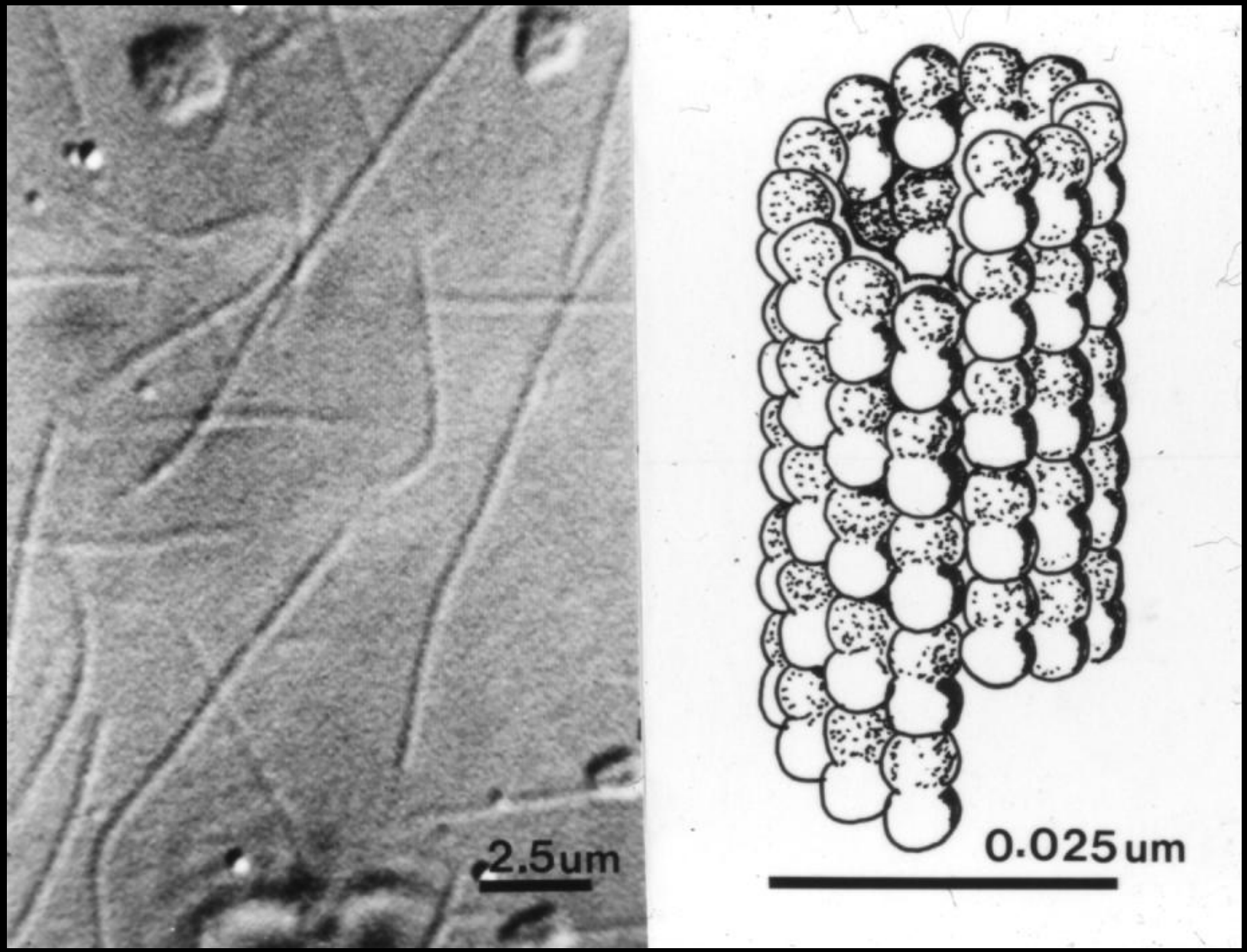
Microscope Alignment For DIC

1. Achieve Koehler illumination
2. Align for Polarization Microscopy: Polarizer E-W, Analyzer Crossed
3. Rotate Condenser Turret to Select DIC Prism to Match Objective
4. Use Correct Objective DIC Prism
5. Adjust Compensation for maximum Contrast of Specimen Detail of Interest

Features of a DIC Image

- Contrast is directional
  - maximum in one direction and minimum in the orthogonal direction
- Contrast highlights edges
  - uniform areas have brightness of background
- Each point in object is represented by two overlapping Airy disks
  - one brighter and one darker than background
- “Shear”
  - The direction of Airy disk separation
  - The distance of Airy Disks separation
    - typically ½ radius of a single Airy Disk

Manifestation of the features



High Resolution VE-DIC Image of Microtubules

Practical Information; DIC

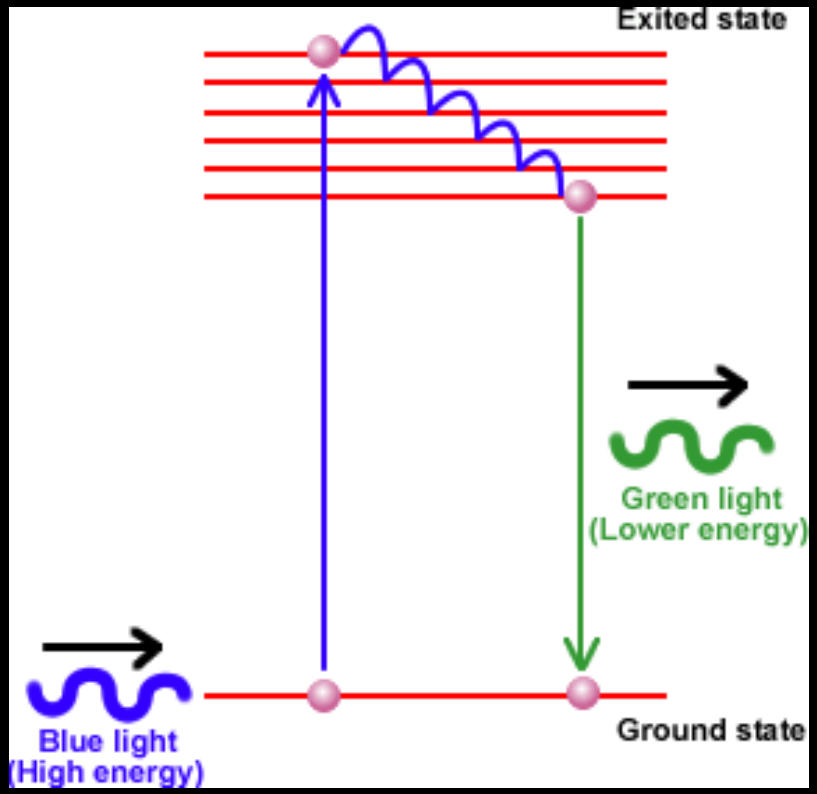
- Need to use optics that will not affect polarization
  - Strain in lenses affects polarization
- Specimen preparation
  - Plastics (poly-blahblah) really affect polarization
- Enhancements can used to image sub-resolution particles
  - Single microtubule imaging (detection, not resolution)

Fluorescence

- Absorption and subsequent re-radiation of light by organic and inorganic molecules
- Uses a different (“Epi”) light path, generally not transmitted
- Very useful for study of a single species of molecules (specificity)
- Power of genetically encoded fluorescent proteins

Fluorescence

- Excite with short wavelength (high energy)
- Emit longer wavelength (lower energy)
- Fluorophores are characterized by:
  - Extinction Coefficient (absorption for a given concentration)
  - Quantum Efficiency (# of photons emitted/# of photons absorbed)
- Environmental indicators (DAPI, FURA, etc)



Chemical and Biological fluorophores

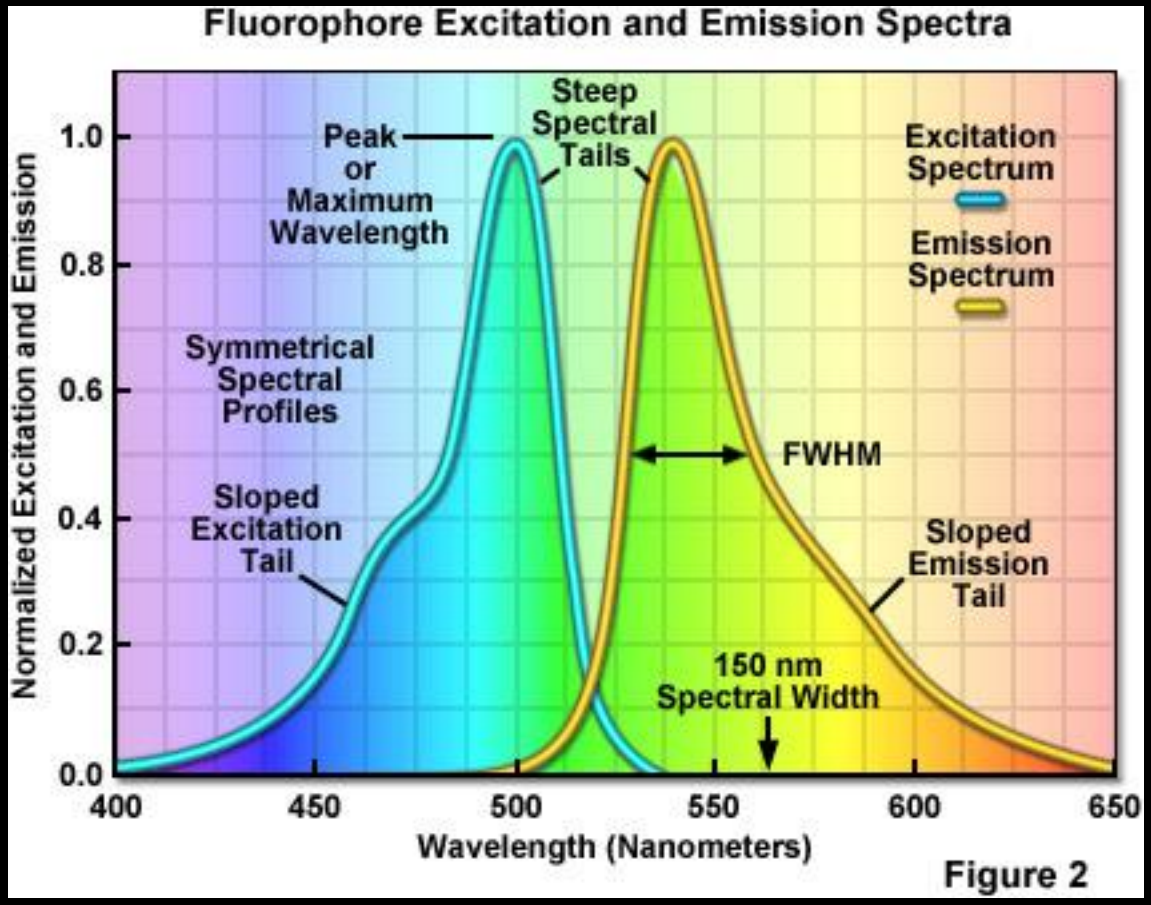
- FITC (494/518)
- Rhodamine (530/555)
- Cy-5 (650/670)
- Green (and other colors) Fluorescent Protein GFP
- Complete lists online at various sites

Illumination Alignment in Epi-Fluorescence

- Same as Transmitted light
- Critical
  - Project the image of the bulb (arc) on the specimen plane
  - Bright but uneven field illumination
- Kohler
  - Bulb out of focus on the specimen plane
  - Even illumination
    - Important for quantifying

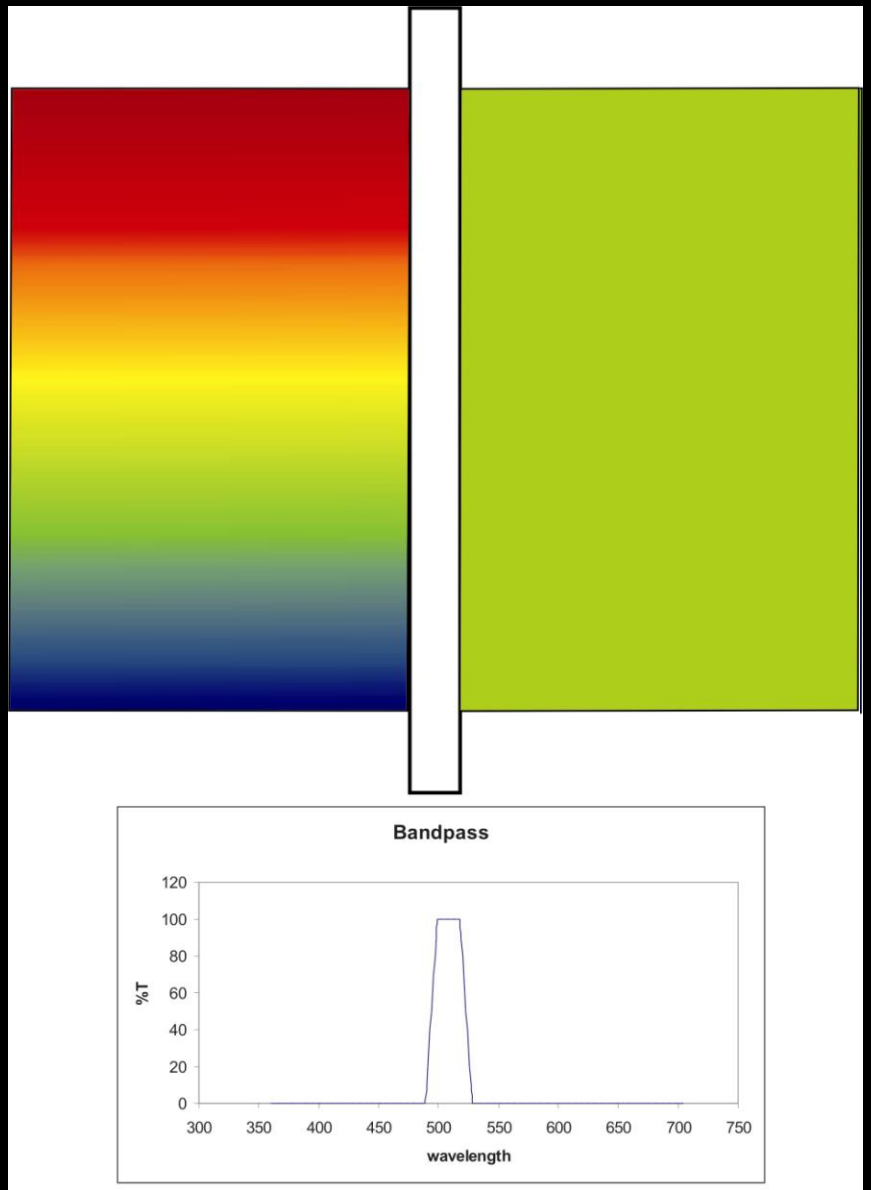
Epi-light path

Detecting fluorophore emission



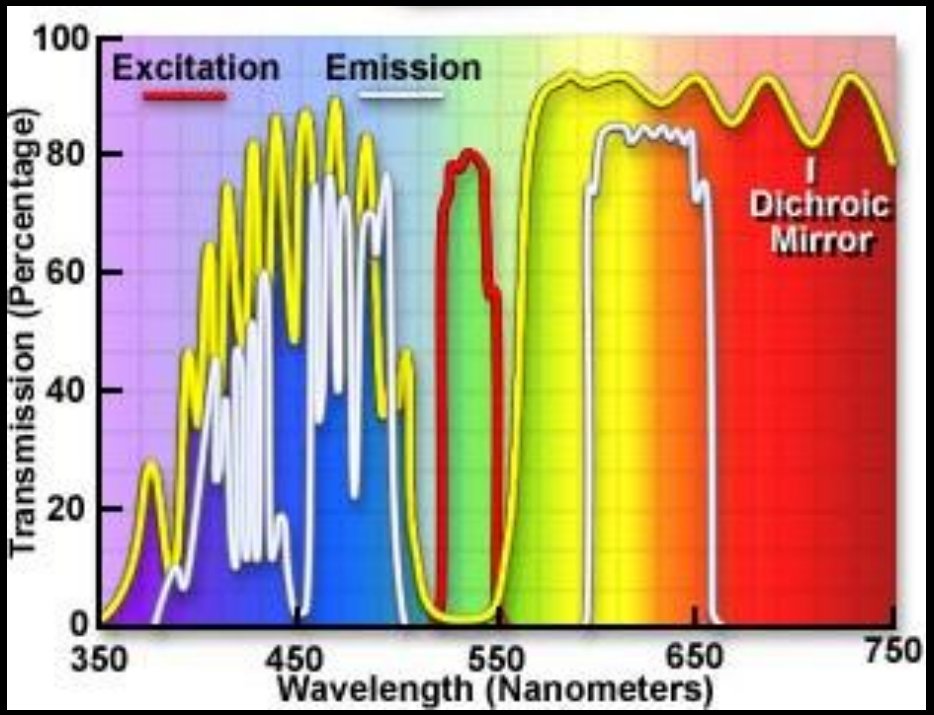
Types of filters

- Short pass
- Long pass
- Band pass



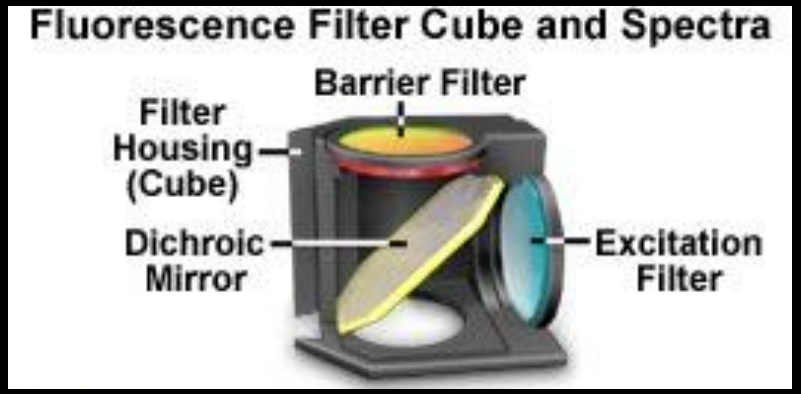
Dichroic Mirrors

- Reflect only specific wavelengths; transmit others
- Spectral response sensitive to angle



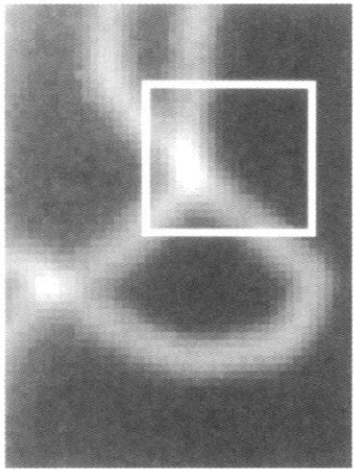
Filter Sets

- Excitation Filter
  - Usually a single Band Pass
- Dichroic mirror
  - Reflects excitation light
  - Transmits emission light
- Emission Filter
  - Band or Long pass
  - Eliminates excitation light
    - Orders of magnitude more intense
    - Stray light bouncing around can get through



Detectors

Digital Images are made up of numbers



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64	90	132	167	210	214	180	115	71	37	36	31	13	15	9	8	15	6	0	5	-14	4	12
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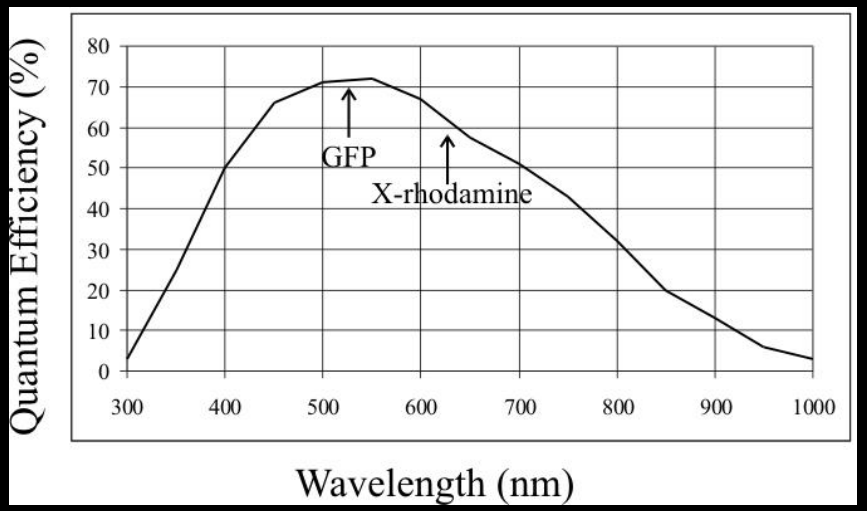
103	108	134	180	201	233	185	115	55	38	26	25	15	20	18	6	2	2	1	4	-3	-13	0
142	132	161	216	238	223	160	90	59	45	17	10	9	13	10	11	4	-9	5	2	7	0	5
172	162	175	231	239	238	155	88	48	28	24	17	15	13	0	14	0	11	-3	4	9	0	-10
226	219	230	260	265	236	161	92	43	31	31	11	5	11	7	13	19	9	18	-11	-9	-2	8
234	247	256	302	311	253	174	97	48	27	12	15	7	7	0	16	8	5	3	-4	0	-6	4
260	263	297	346	349	303	196	126	65	27	30	24	3	6	7	1	12	3	9	0	-2	-13	2
244	293	340	388	399	321	223	130	74	29	24	30	17	4	3	11	0	8	7	-3	-2	-2	-2
209	273	359	423	436	365	264	141	80	57	32	45	13	3	18	8	-7	0	-6	4	-1	-2	-3
176	253	342	430	443	394	291	161	86	59	37	23	18	5	0	7	8	11	1	-3	13	-5	-2
152	218	311	425	470	420	325	208	111	66	52	29	28	9	4	7	8	4	-7	11	-18	-13	-2
129	199	294	413	469	441	384	257	148	111	69	34	20	20	6	3	15	4	-2	-6	-3	-10	9
140	206	294	385	439	442	365	310	223	157	114	76	45	28	9	21	5	15	-4	-13	0	-5	-1
173	233	309	354	392	375	333	303	261	214	135	92	51	47	18	12	13	12	20	-9	4	1	15
221	278	300	321	306	293	286	279	250	231	184	142	108	67	41	18	13	5	8	-8	0	7	5
267	302	291	244	228	211	201	215	241	227	205	184	136	110	68	51	26	11	8	3	0	8	-3
284	279	257	202	133	129	137	151	183	213	209	188	187	155	109	69	49	26	25	8	8	18	-4
275	248	191	143	95	85	87	98	122	166	184	192	206	194	176	135	98	50	44	19	21	0	1

General Info on CCDs

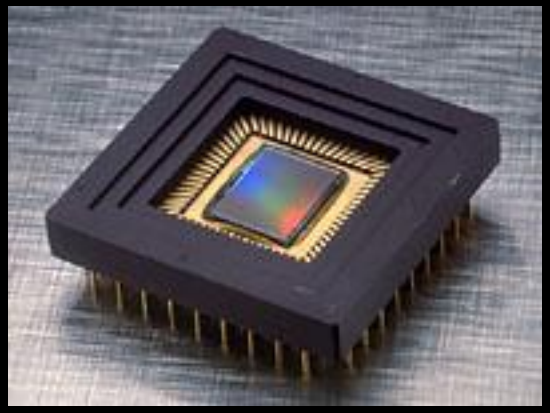
- Charge Coupled Device
- Silicon chip divided into a grid of pixels
- Pixels are electric “wells”
- Photons are converted to electrons when they impact wells
- Wells can hold “X” number of electrons
- Each well is read into the computer separately
- The Dynamic Range is the number of electrons per well / read noise

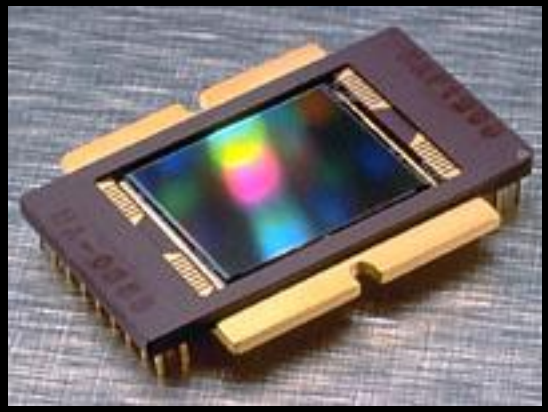
General Info on CCDs

- Different CCDs have different Quantum Efficiency (QE)
  - Think of QE as a probability factor
  - QE of 50% means 5 out of 10 photons that hit the chip will create an electron
  - QE changes at different wavelengths

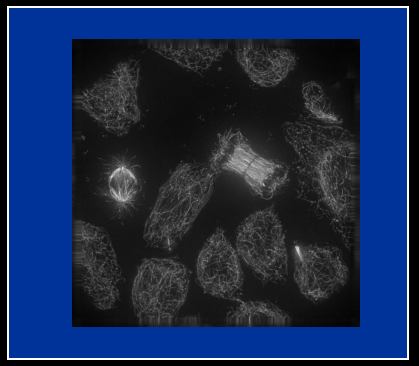


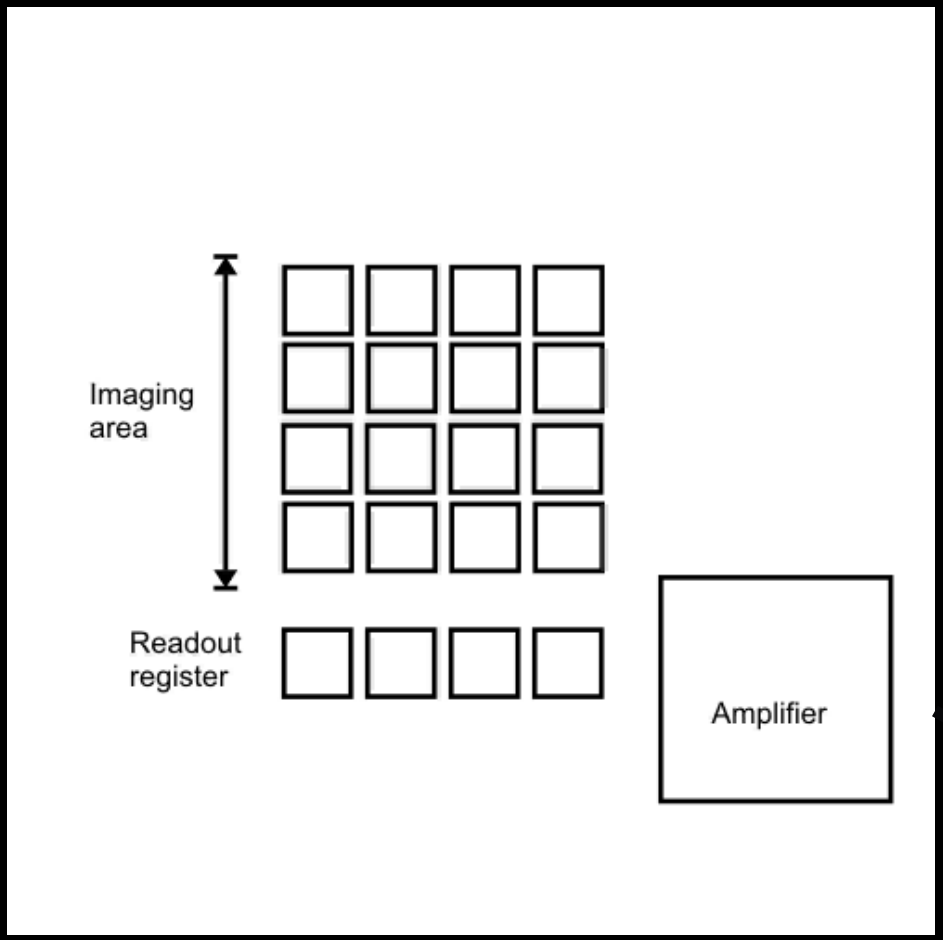
How do CCDs work?





How do CCDs work?





Computer

Full Well Capacity

- Pixel wells hold a limited number of electrons
- Full Well Capacity is this limit
- Exposure to light past the limit will not result in more signal

Readout

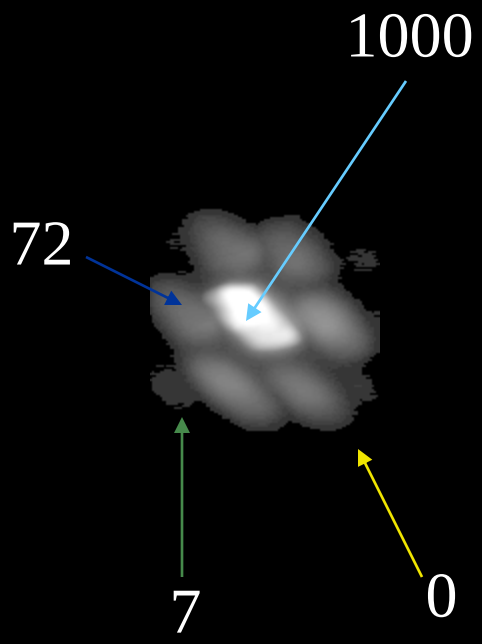
- Each pixel is read out one at a time
- The Rate of readout determines the “speed” of the camera
- 1MHz camera reads out 1,000,000 pixels/second (Typical CCD size)
- Increased readout speeds lead to more noise

CCD Bit depth

- Bit depth is determined by the number of electrons/gray value
- If Full Well Capacity is 1000 electrons, then the camera will likely be 8 bits (every 4 electrons will be one gray value)
- If Full Well Capacity is 100,000 electrons the camera can be up to 16bits

CCDs are good for quantitative measurements

- Linear
  - If 10 photons = 5 electrons
    1000 photons = 500 electrons
- Large bit-depth
  - 12 bits = 4096 gray values
  - 14 bits = ~16000 gray values
  - 16 bits = ~64000 gray values



Sensitivity and CCDs

- High QE = more signal
- High noise means you have to get more signal to detect something
- Sensitivity = signal/noise

Noise

- Shot noise
  - Random fluctuations in the photon population
- Dark current
  - Noise caused by spontaneous electron formation/accumulation in the wells (usually due to heat)
- Readout noise
  - Grainy noise you see when you expose the chip with no light

Dark Current noise and Cooling

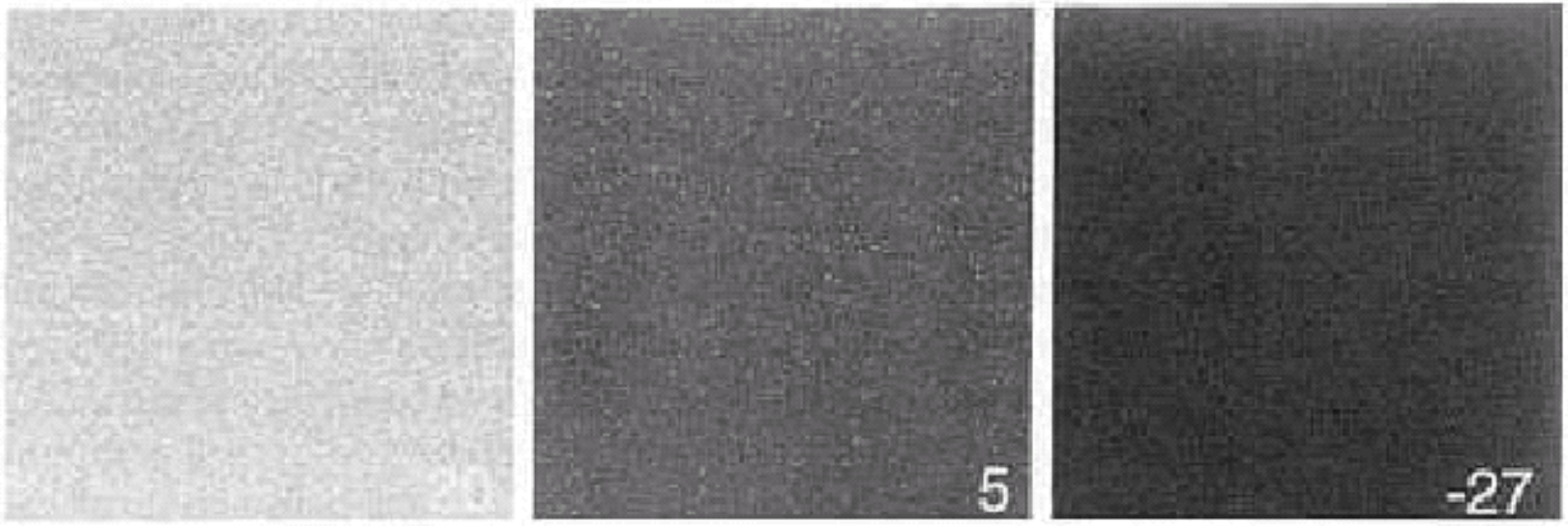
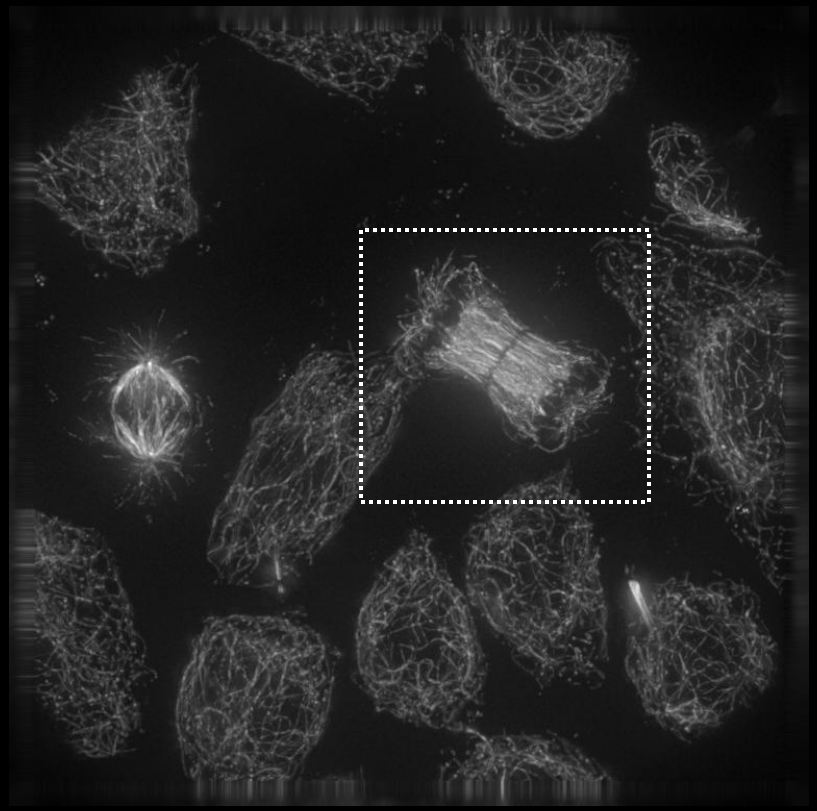


Fig. 8 Dark images from the C4880 camera as a function of temperature of the CCD chip. Exposures were for 5 sec and temperature in degrees centigrade is indicated on each frame.

Attributes of most CCDs

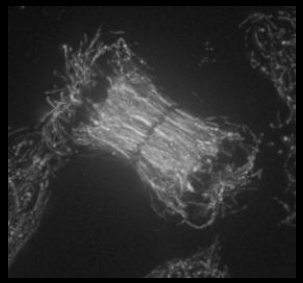
- Can “sub-array”
  - Read pixels only in a certain area
  - Speeds up transfer (fewer pixels)
- Binning
  - Increases intensity by a factor of 4 without increasing noise
  - Lowers resolution 2 fold in x and y
  - Speeds up transfer (fewer pixels)

Sub-array



1,000,000 pixels

1 Second at 1MHz

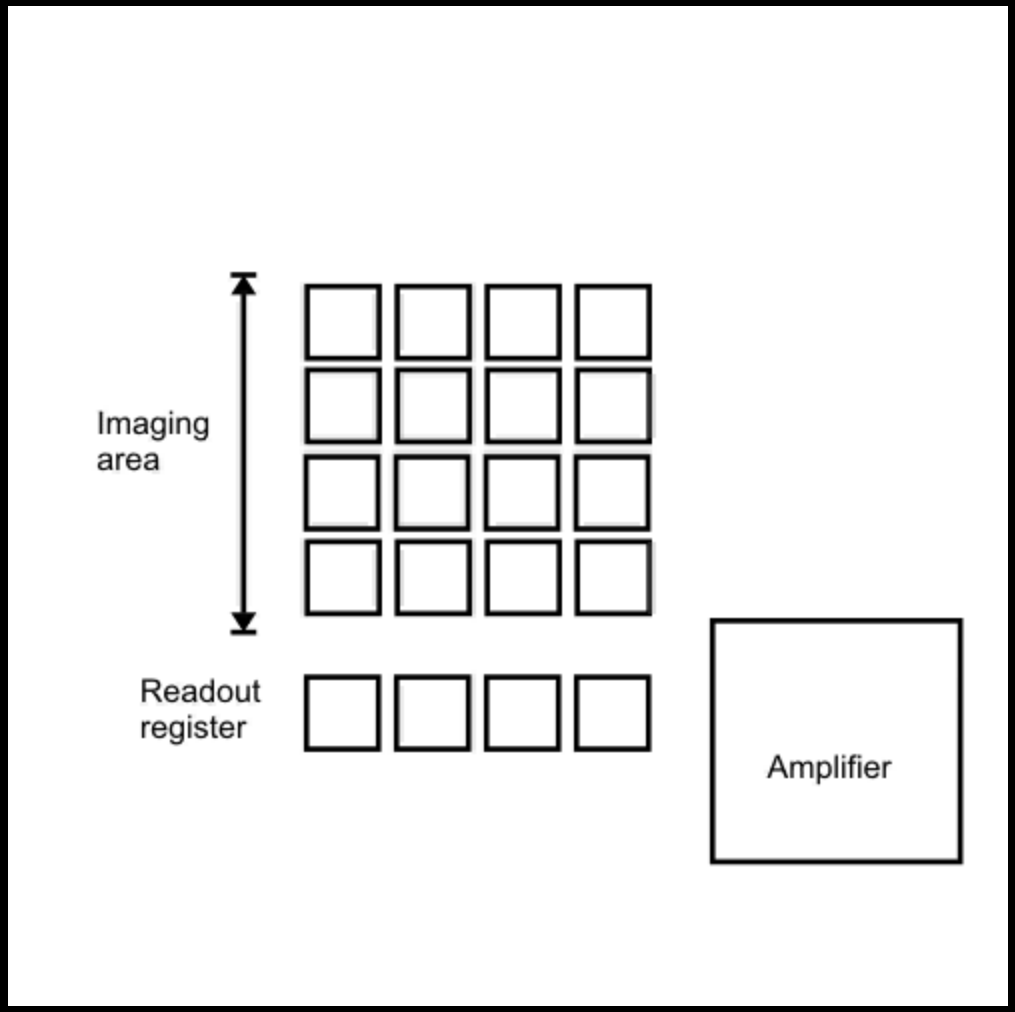


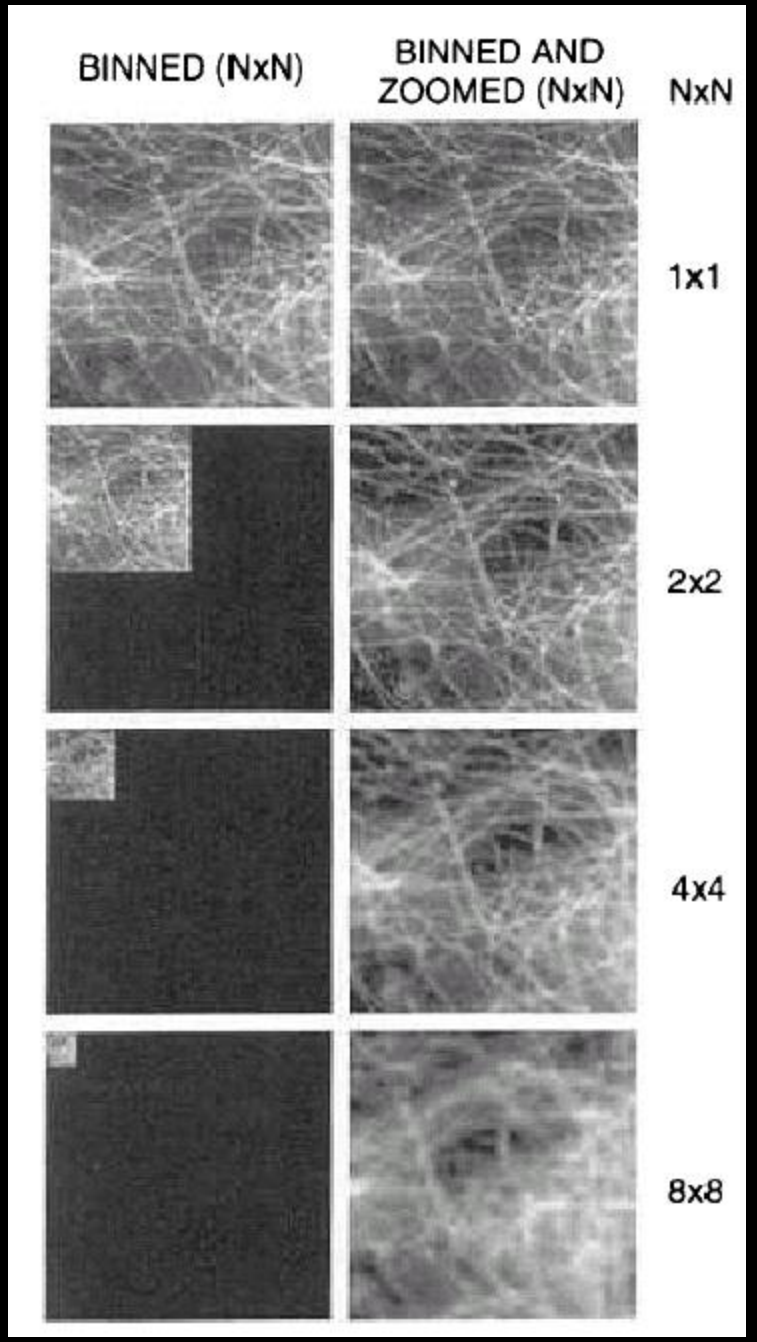
~200,000 pixels

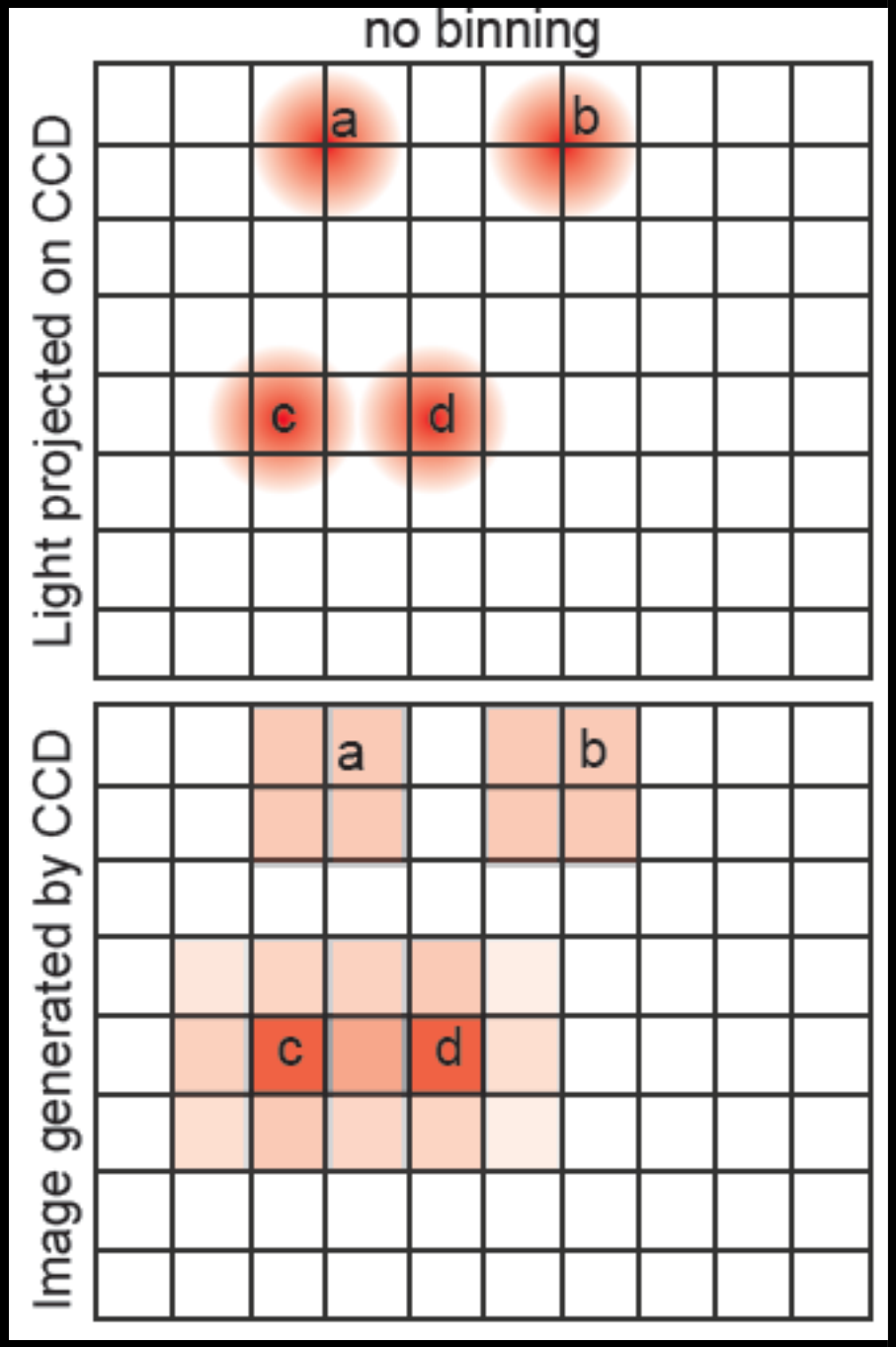
0.2 Seconds at 1MHz

Faster Image transfer

Binning


Imaging
area
Readout
register
Amplifier


BINNED (NxN)
BINNED AND
ZOOMED (NxN)
NxN
1x1
2x2
4x4
8x8


no binning
Light projected on CCD
a
b
c
d
Image generated by CCD
a
b
c
d

Magnification and Detector Resolution

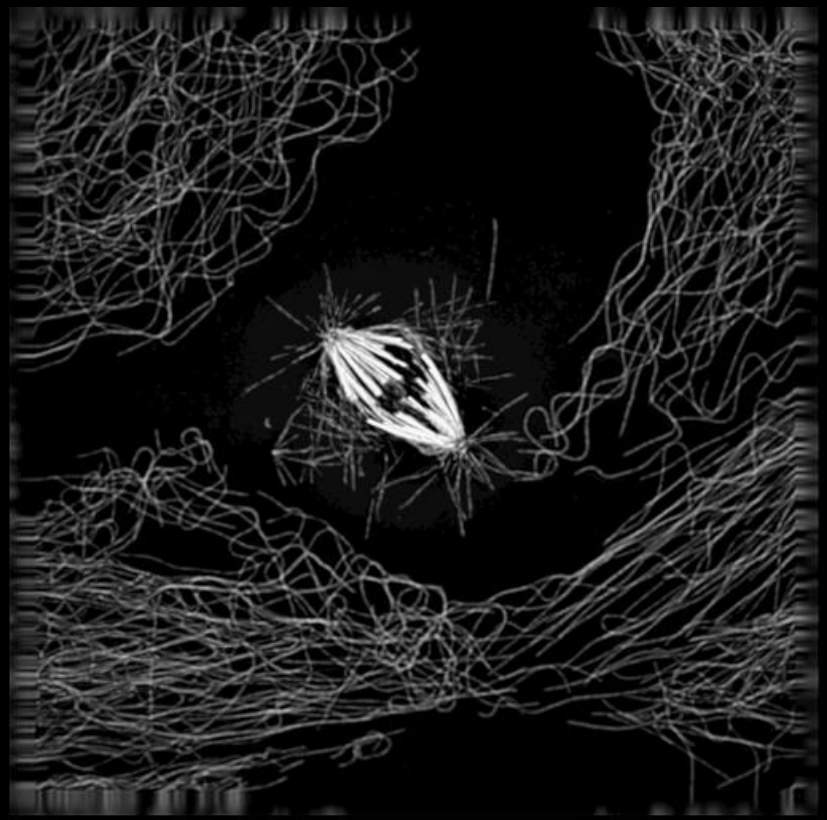
- Need enough mag to match the detector
  - The Nyquist criterion requires a sampling interval equal to twice the highest specimen spatial frequency
  - Microscope Magnification = (3*Pixel-width)/resolution = (3*6.7 μm) / 0.27 = 74.4x
- But intensity of light goes down (by 1/mag^2) with increased mag

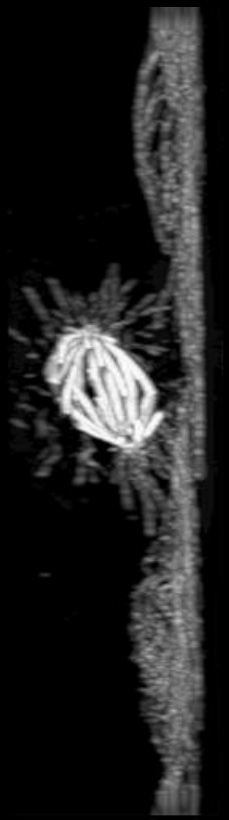
Modifications/Specializations

- Deconvultion
- Confocal
- Super-resolution
- Various assays
  - FRAP, FLIP, FRET, etc…

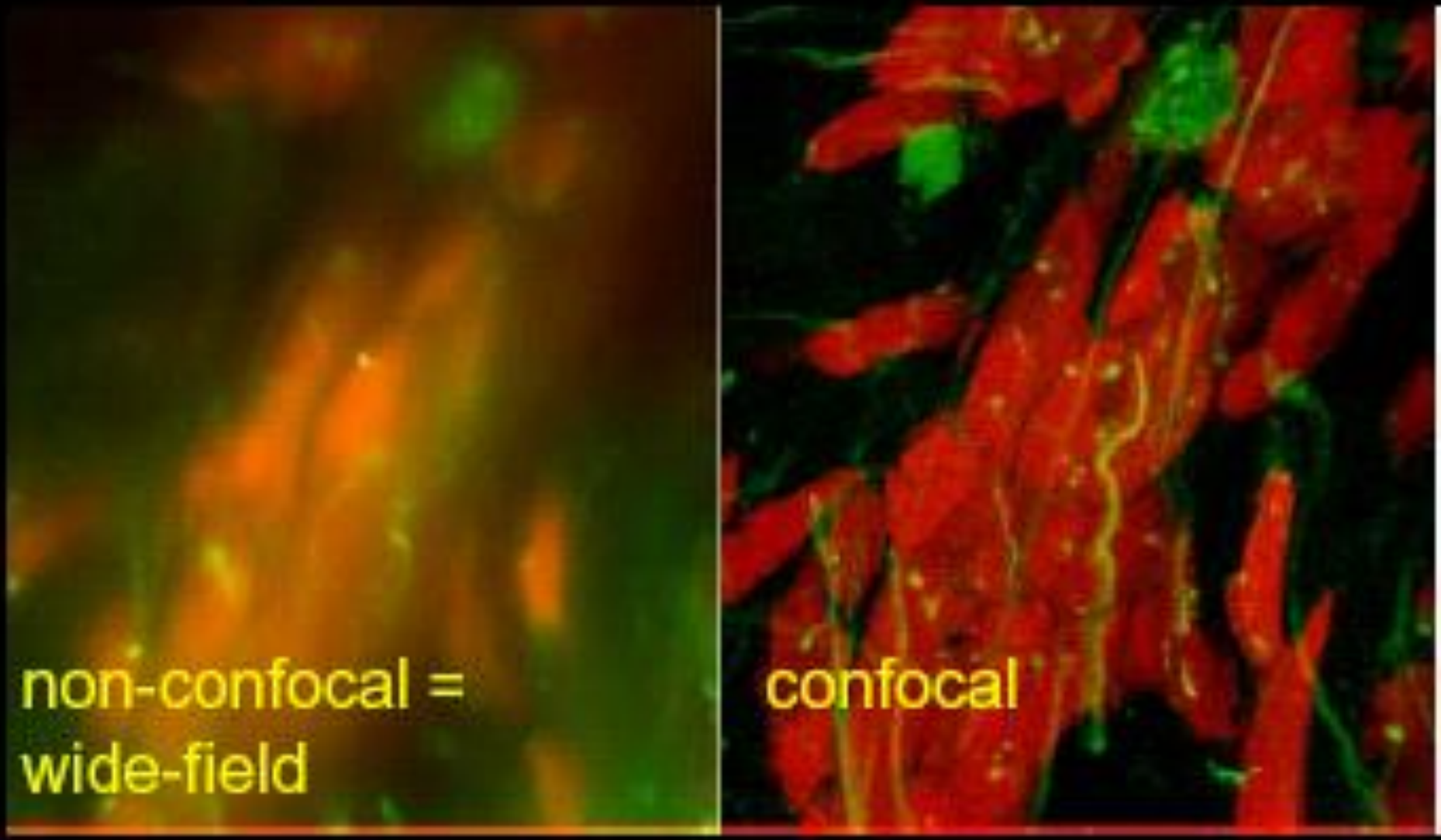
Deconvolution

- A point source of light spreads out by diffraction and refraction in the microscope
- You can “de-spread” the light with math
- Increases resolution
- See http://www.api.com/ for details

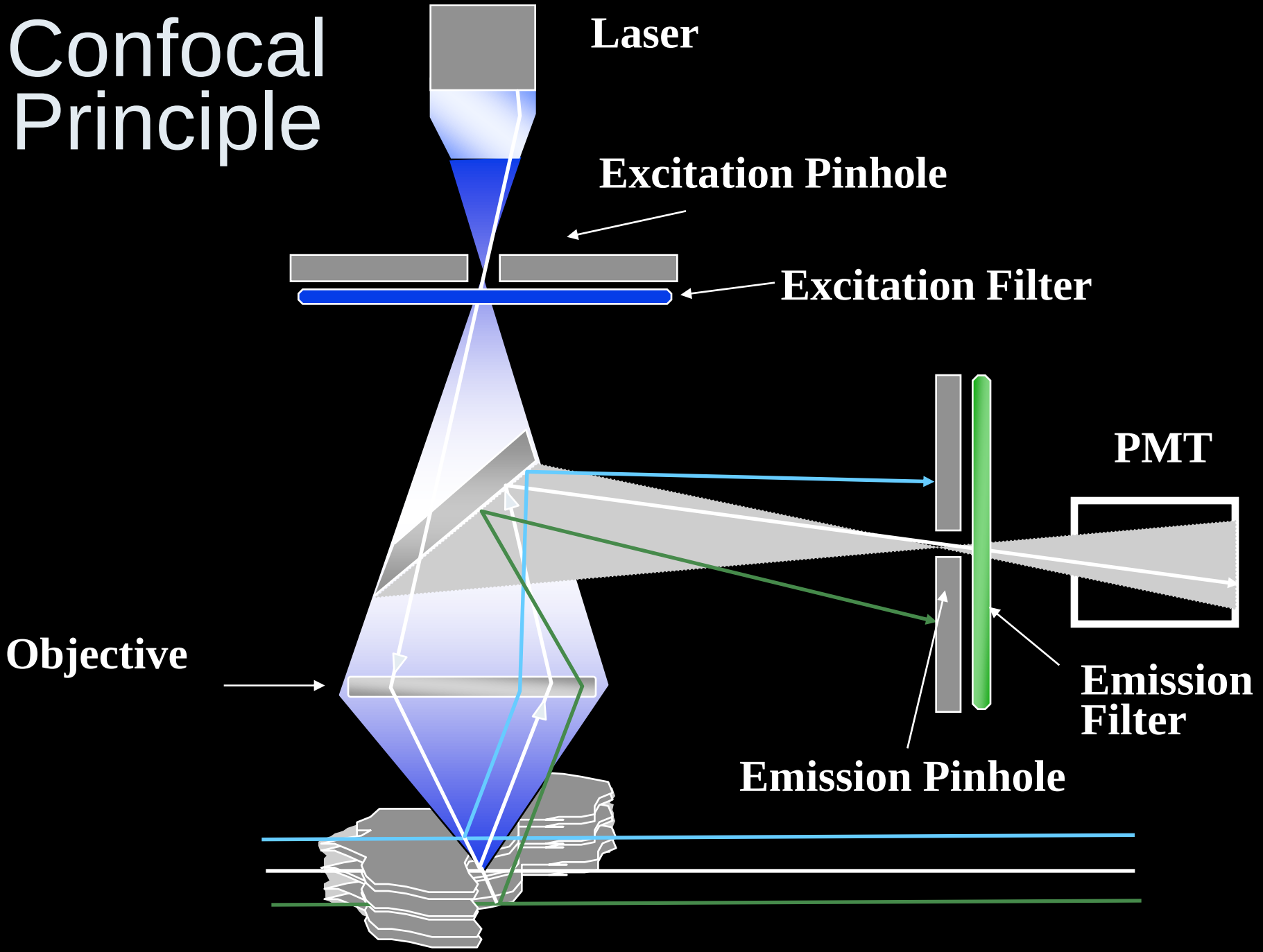




Confocal Sectioning



Confocal Principle



Laser Confocal Scanning Microscopy

- The laser beam excites a point on the specimen. It also inadvertently excites other points on the specimen.
- Only the In-Focus emission light is allowed to be detected by the PMT.
- The Light detected by the PMT is associated to a pixel (picture element) on the monitor.
- The laser beam then moves to the the next point and another pixel is collected.

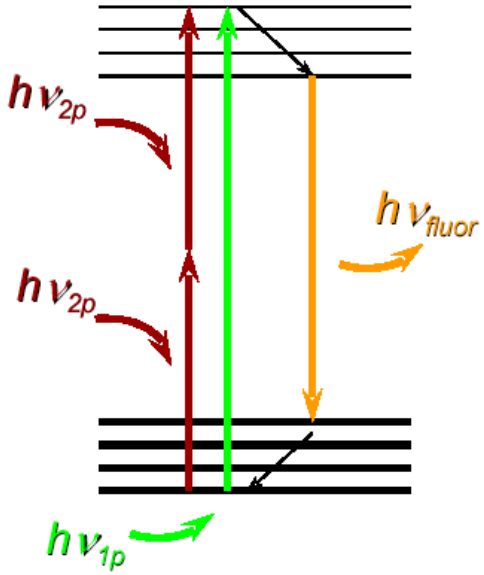
Confocal Pinhole

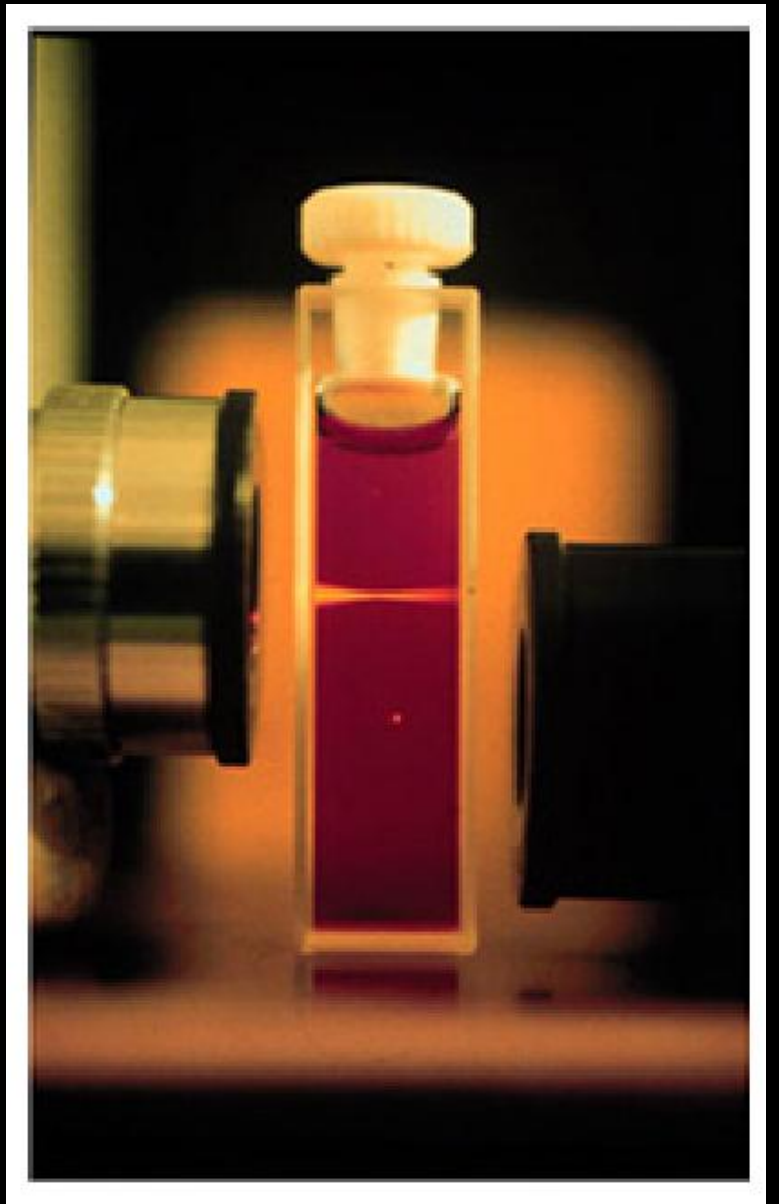
Size	Result on the Intensity of the Image	Result on the Resolution of the Image
Increase opening	Increase Intensity	decrease resolution
Decrease Opening	Decrease intensity	increase resolution

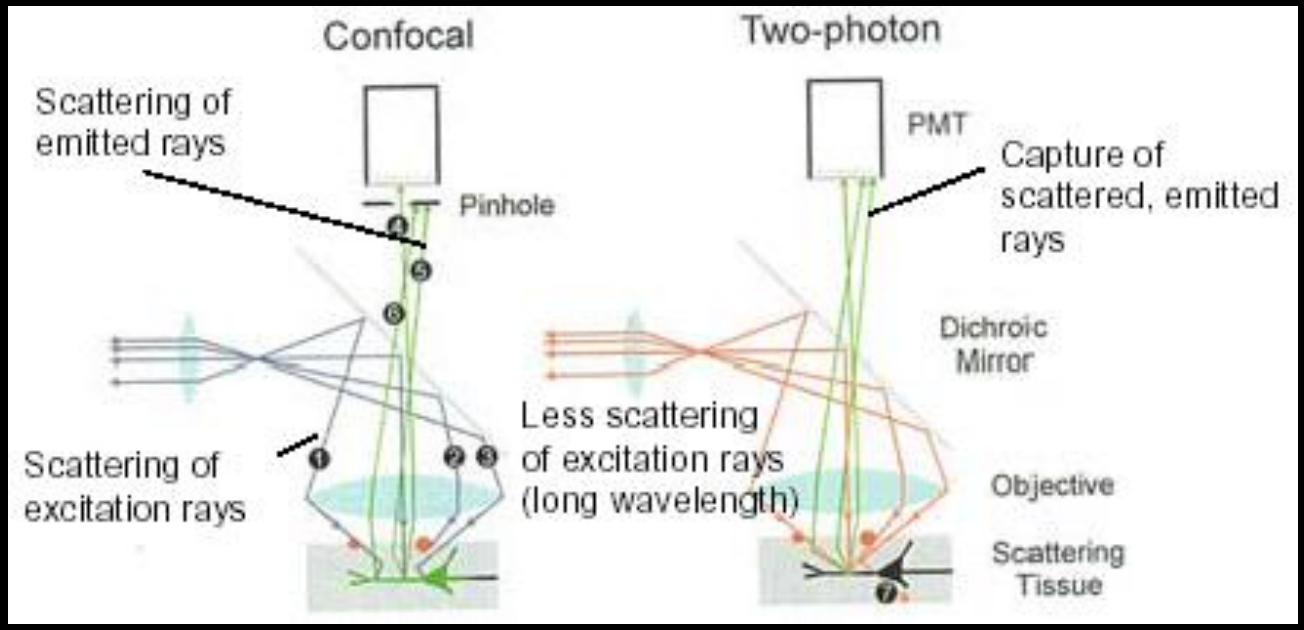
Multi-photon

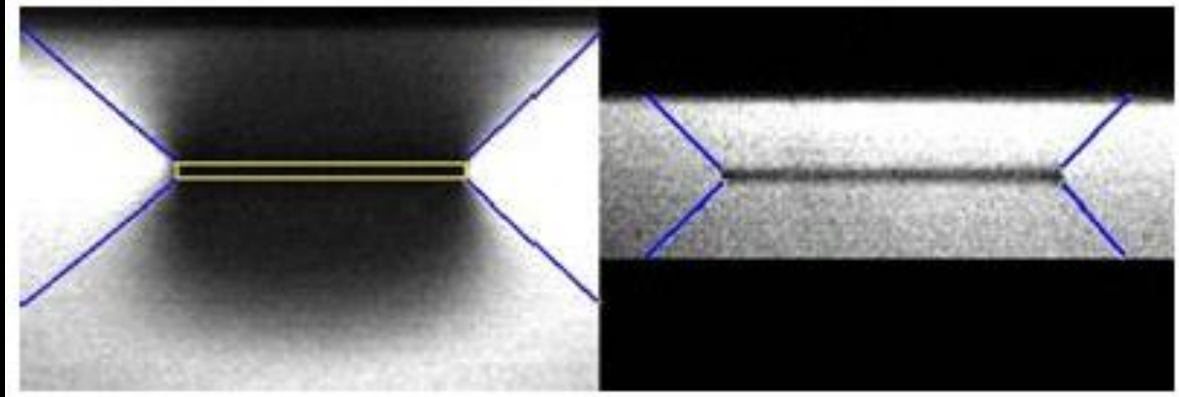
Two-photon excitation of fluorescence

- Excitation by simultaneous absorption of two photons
- Excitation in NIR
- High peak power laser required
- Other possibilities: stepwise excitation, three photon excitation



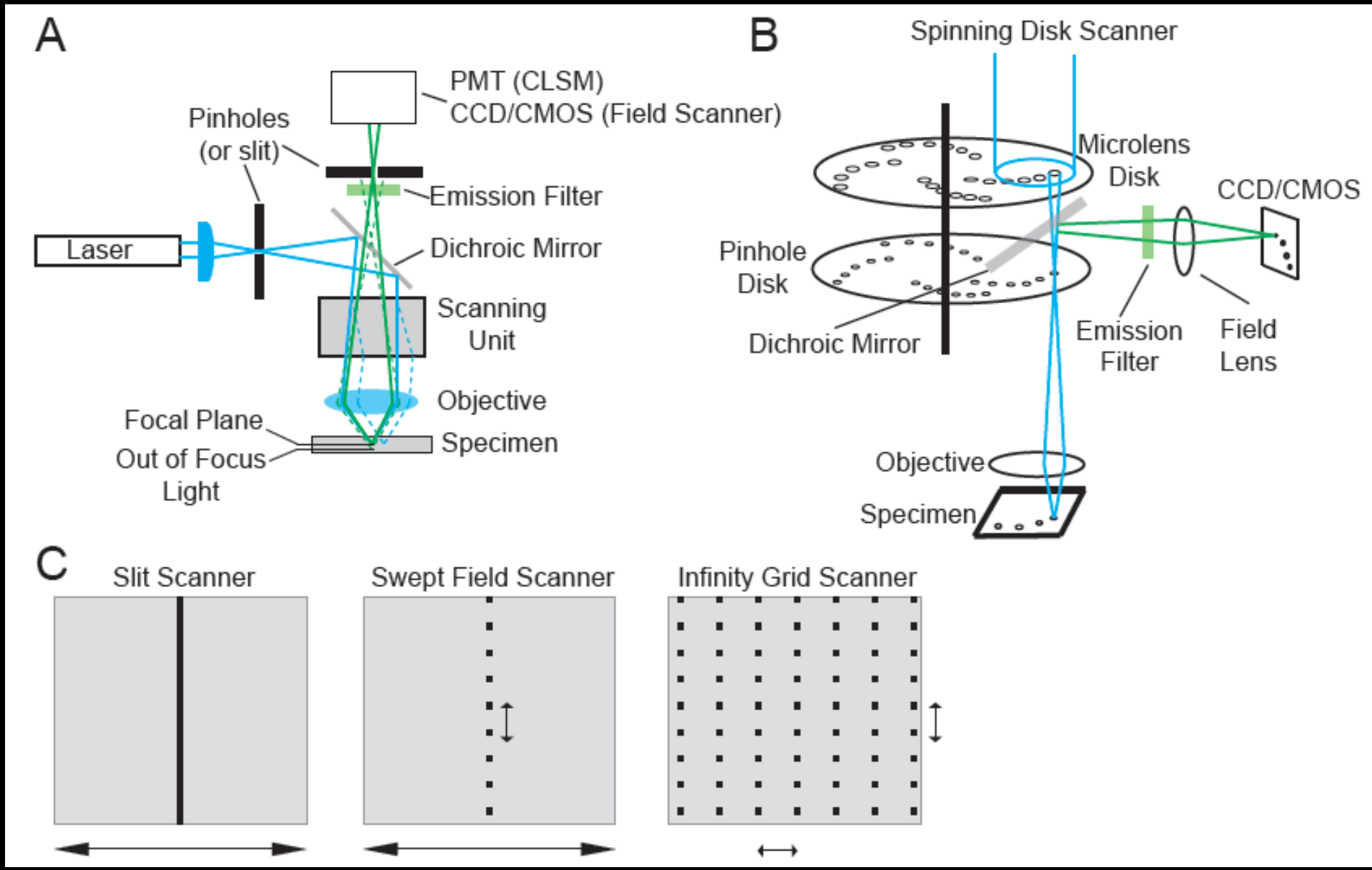



Confocal
Two-photon
Scattering of emitted rays
Pinhole
PMT
Capture of scattered, emitted rays
Dichroic Mirror
Less scattering of excitation rays (long wavelength)
Scattering of excitation rays
Objective
Scattering Tissue


Conventional confocal
2-photon excitation

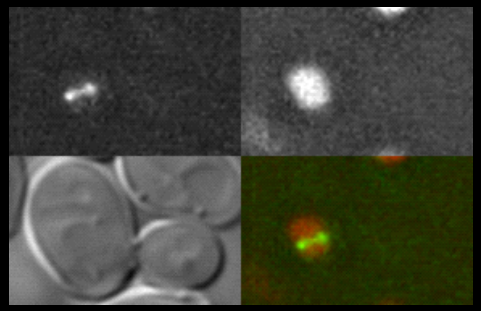
Multi-Pinhole and Slit-Scanning Confocals

- Spinning Disk
- Slit Scanners
- Pinhole sweeping

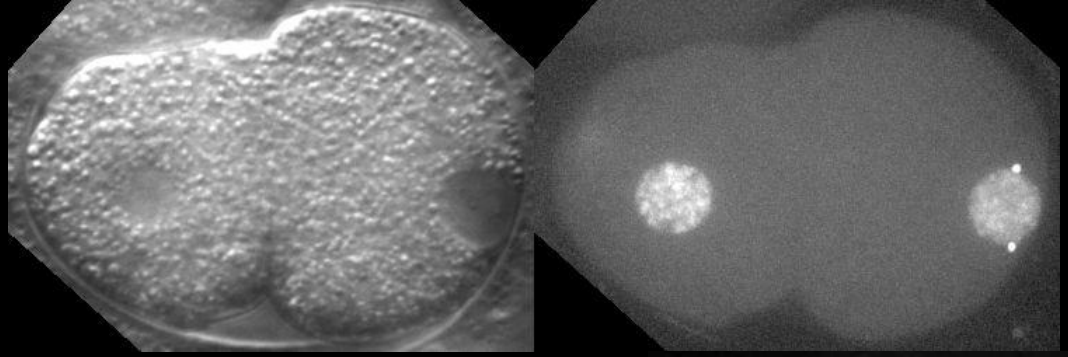

A
PMT (CLSM)
CCD/CMOS (Field Scanner)
Pinholes
(or slit)
Emission Filter
Laser
Dichroic Mirror
Scanning
Unit
Objective
Focal Plane
Out of Focus
Light
Specimen
B
Spinning Disk Scanner
Microlens
Disk
CCD/CMOS
Pinhole
Disk
Dichroic Mirror
Emission
Filter
Field
Lens
Objective
Specimen
C
Slit Scanner
Swept Field Scanner
Infinity Grid Scanner

Super-Resolution

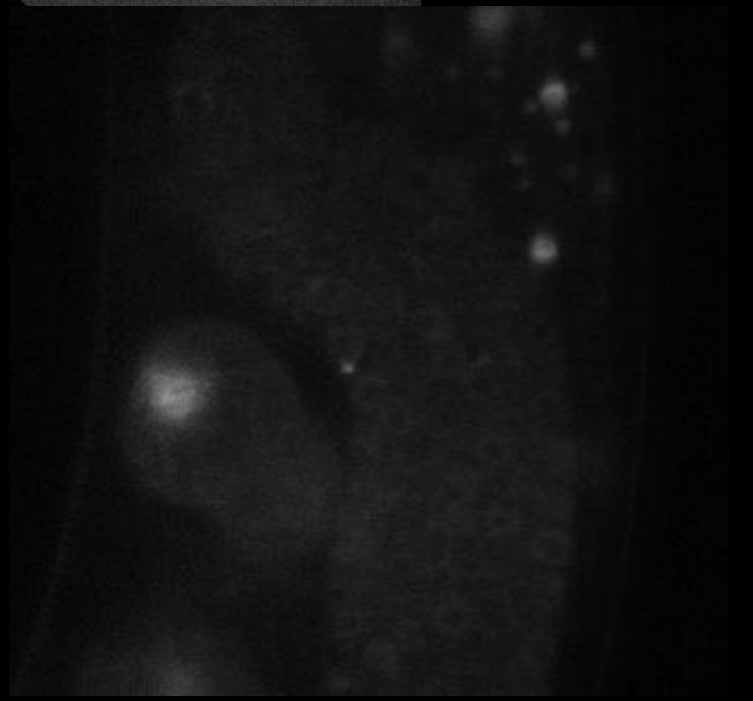
- X, Y resolution to between 20-100nm
- Z resolution to 100nm
- Structured Illumination (SIM)
- Photo-Activation (PALM)
- Stimulated Emission (STED)

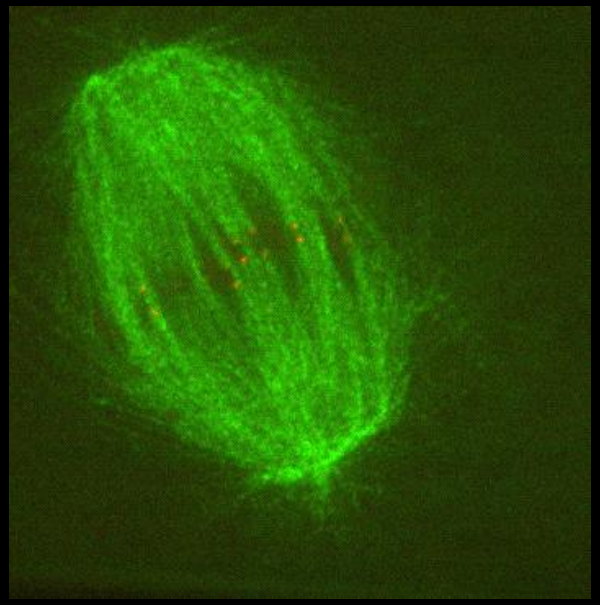


Budding Yeast



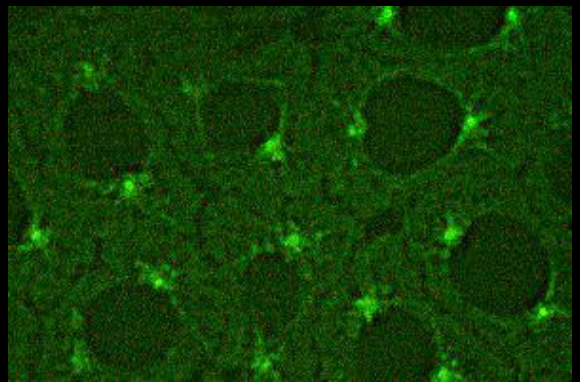
C. elegans

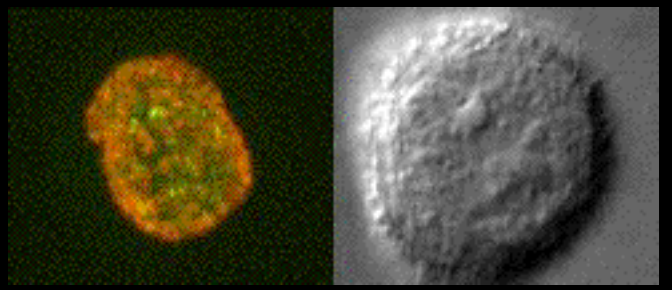




Xenopus Extract

Drosophila





HeLa, Human Fibroblast

Paul S. Maddox

- Phone: 1-919-843-8637
- Email: pmaddox@unc.edu

- Microscopy course:
  - Montréal Light Microscopy Course (mlmc.ca)

Super Resolution Light Microscopy

What is super resolution?

- Ability to resolve objects which are below the resolution physically defined by the optical system
- What does that mean???
  - A point of light will make a smaller point spread function
  - Two small objects close to one another will be better separated
  - Possible to generate molecular structure

Why not just use Electron Microscopy?

- To date, EM is a fixed cell technique
- Classic EM fixing staining protocols damage many cellular structures
  - Physical sectioning with a knife disrupts organelles
  - Negative staining can be “harsh”
- Intermediate voltage EM tomography is better
  - Still fixed
  - Rapid freezing preserves structure and physical sections are thicker
  - There are really 2 of these in the world which are available for use…(Colorado and Albany NY)

Gains of Super Resolution

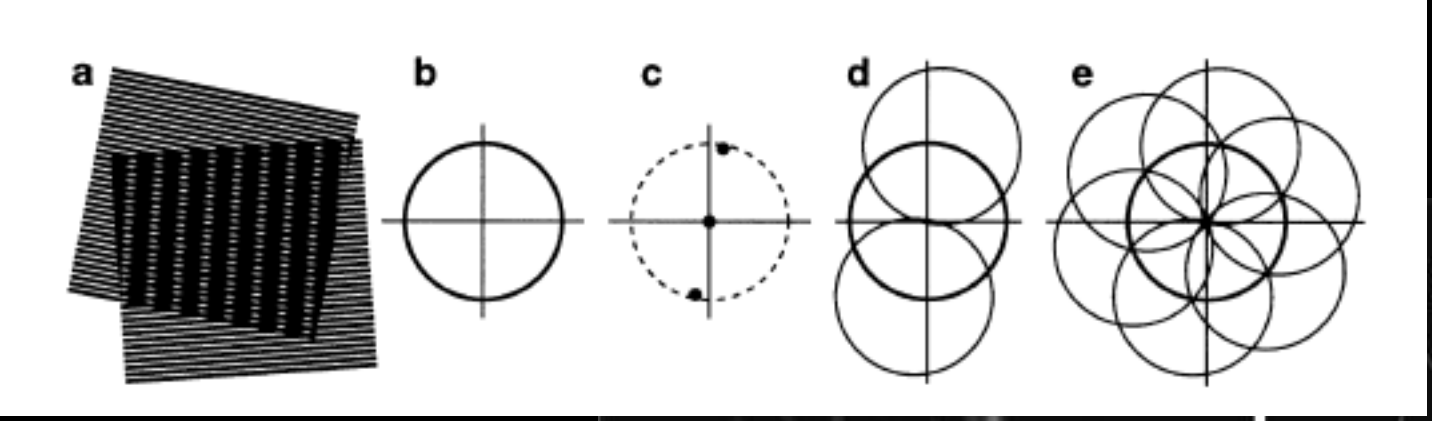
- Close to molecular resolution at high mag/NA
  - Typically less than 100nm in x, y (2-3 fold more resolution)
  - Can be as small as 10nm! (10-20 fold more resolution!!!)
- Can use on living cells
- Structural preservation better than EM
- Sample prep faster, less specialized than EM

Types of Super Resolution

- Structured Illumination Microscopy (SIM)
- Photo-Activation Localization Microscopy (PALM)
- Stochastic Optical Reconstruction Microscopy (STORM)
- Stimulated Emission Depletion Microscopy (STED)
- Near-Field Scanning Optical Microscopy (NSOM)

Structured Illumination Microscopy (SIM)

- Illuminate with a grating (usually generated by a laser)
  - Image of stripes on your field of view
- Rotate the grating and take many pictures (15-25 per color per focal plane)
  - Lots of bleaching
  - Lots of time
  - Conventional fluors (up to 4 stains in one sample)!
- Effect is to increase the NA of the lens
  - Grating illumination increases the frequency range of diffracted light gathered by the lens



M. G. L. GUSTAFSSON
Journal of Microscopy, 2000

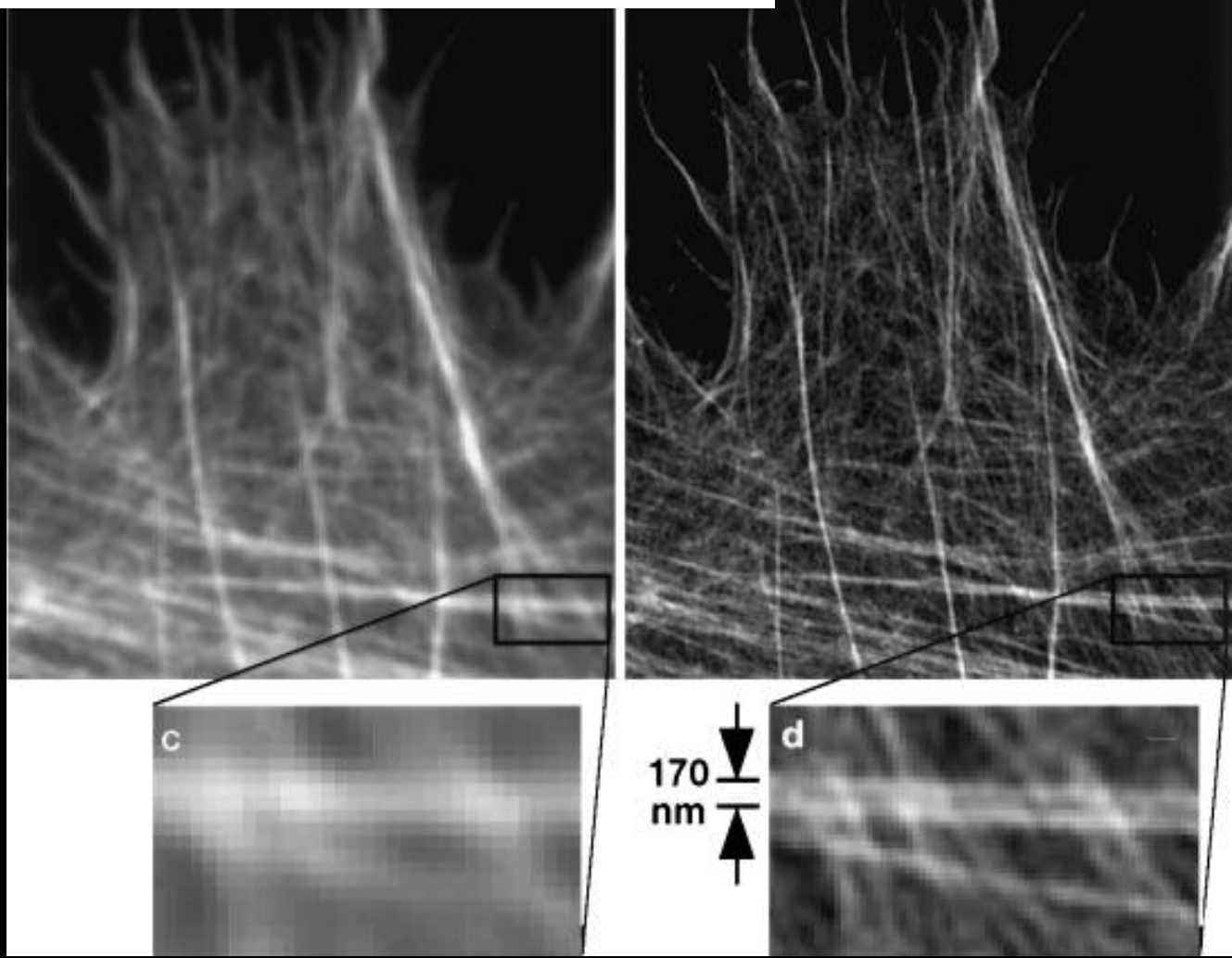
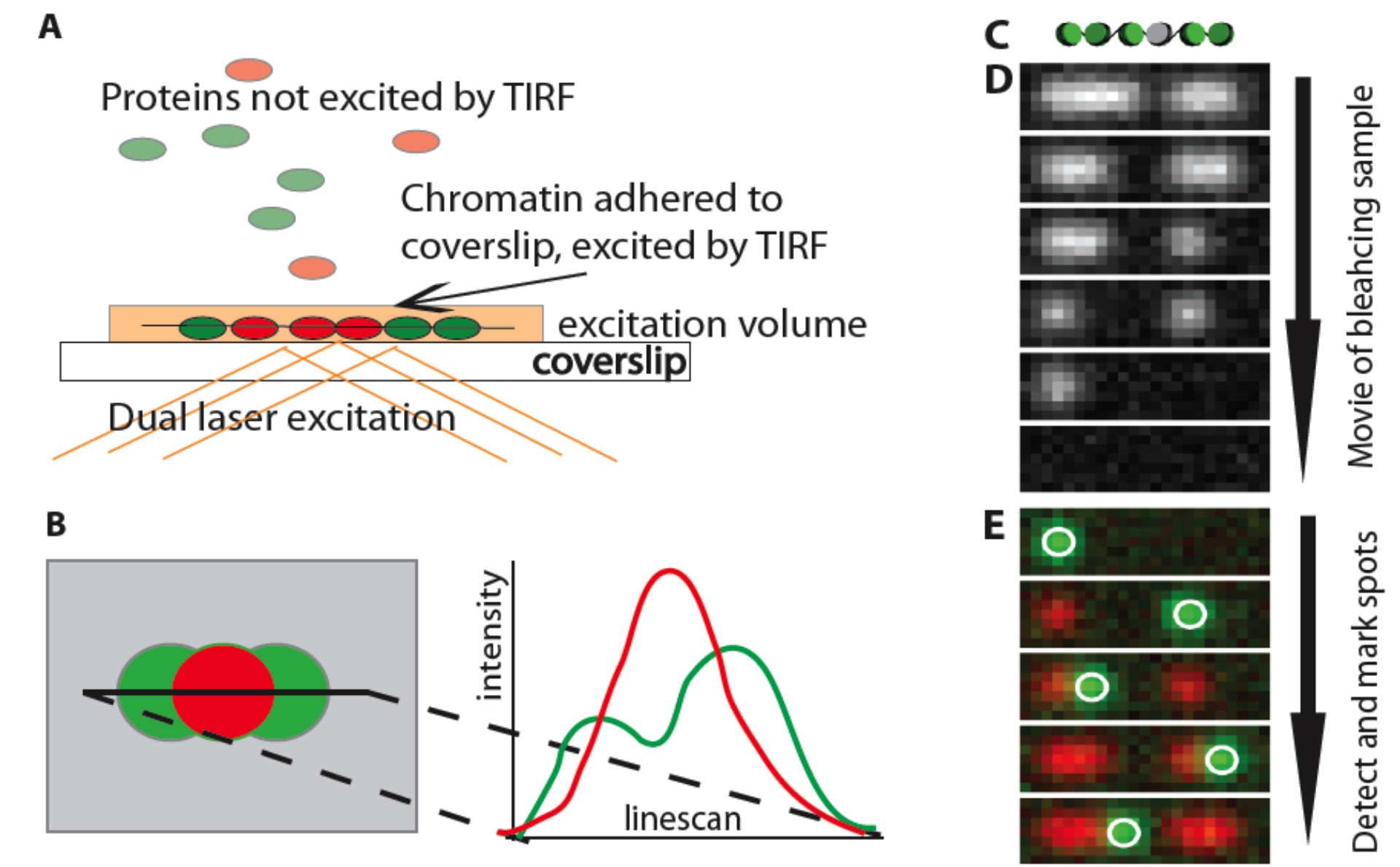
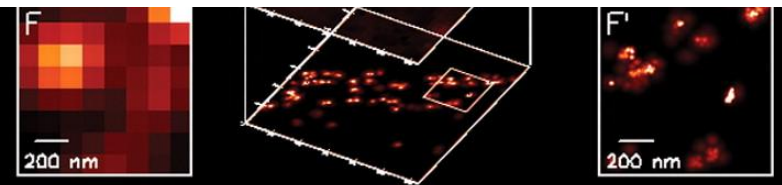


Photo-Activation Localization Microscopy (PALM)

- Randomly photo-activate molecules labeling a structure one molecule at a time
  - Takes a long time to collect an image (10 min to hours)
  - Have to use fluors which are photo-activatable
- Each activation event generates a new PSF in the image
- Each new PSF can be very accurately localized (10-20nm)
  - Highest resolution to date!
- At this point, imaging is restricted to the coverslip surface (TIRF, 100nm deep into sample)
- If you have a very low number of fluorophores, can be used in photobleaching also (Reverse of PALM)



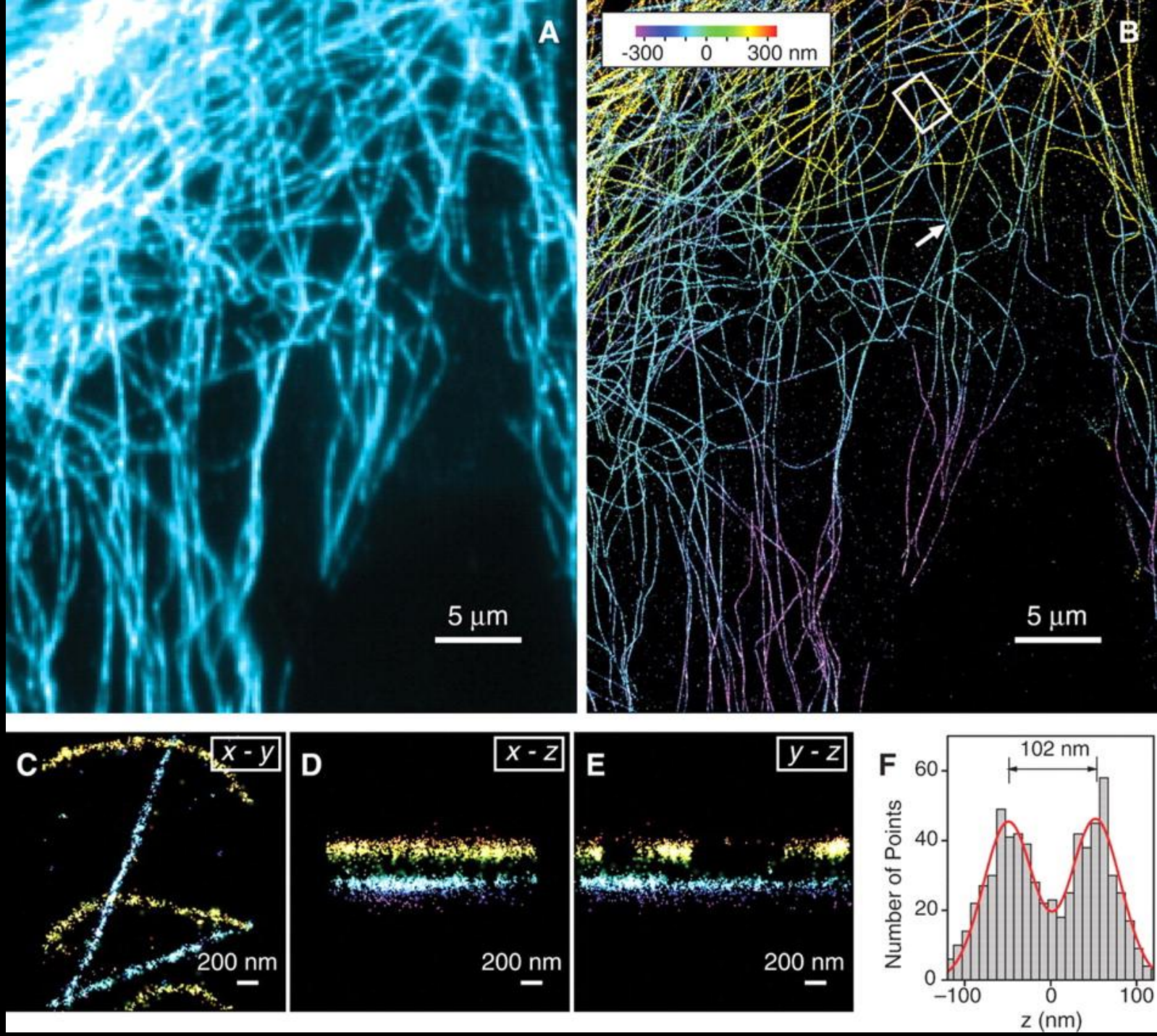


Betzig et al., Science 2006

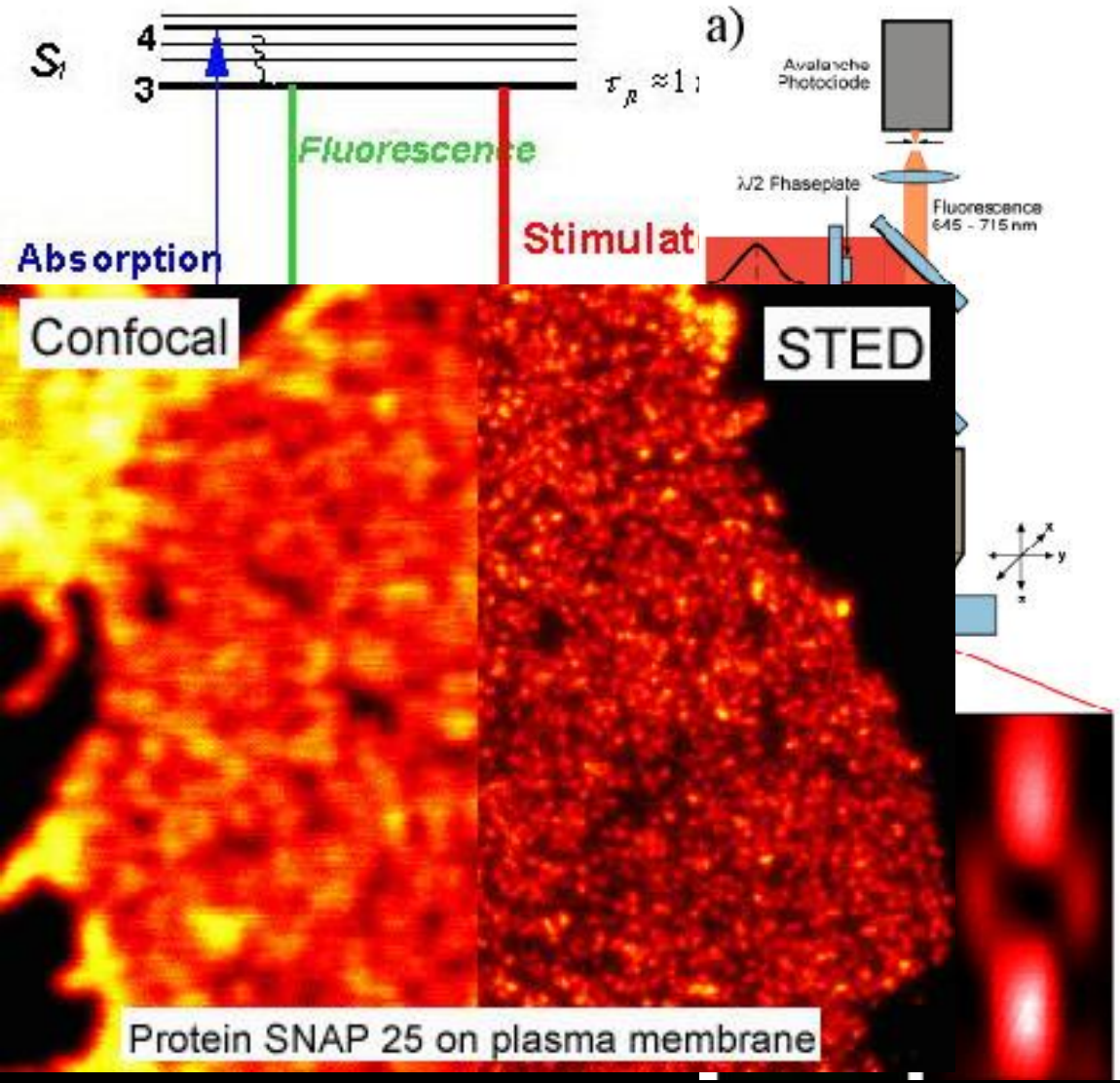
Stochastic Optical Reconstruction Microscopy (STORM)

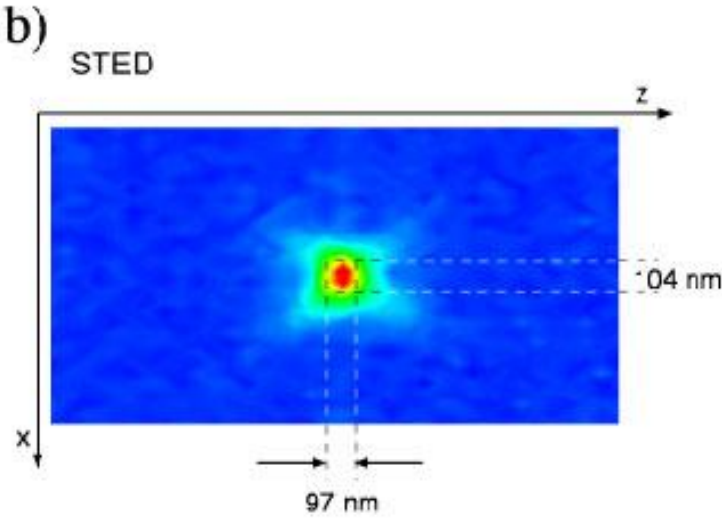
- Similar principle to PALM
- Can be used in 3D (not limited to coverslip surface)
- Photoswitchable cyanine dyes (Cy5, Cy5.5, Cy7, and Alexa 647) that can be reversibly cycled between a fluorescent and a dark state by light of different wavelengths
  - Not Photo-Activation

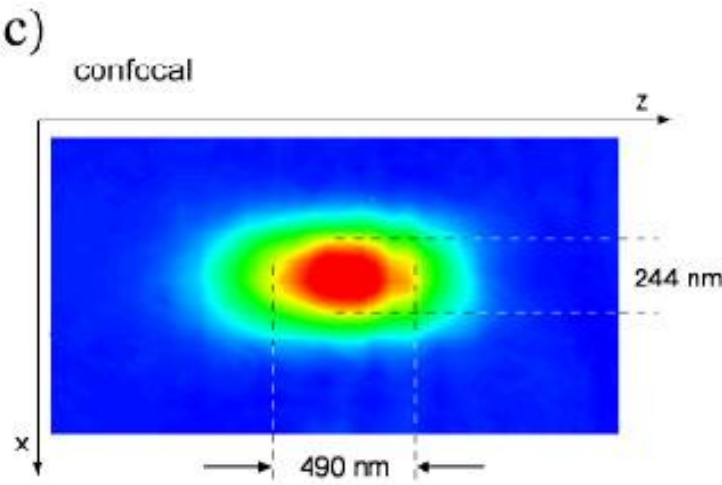
Huang et al.,
Science, 2008



Stimulated Emission Depletion Microscopy (STED)







Hell, S. W. and J. Wichmann (1994). Opt. Lett. **19(11): 780-782.**

Stimulated Emission Depletion Microscopy (STED)

- Two types, Pulsed or continuous wave (CW) laser
- Pulsed type has higher resolution
  - 40nm
  - Requires special fluors
  - Greater than one color is difficult
- CW type is more practical, less resolution
  - 100nm
  - Can be used with GFP
  - Multi-color is easier
  - Cheaper…

Pitfalls

- How do you really know that what you are seeing by super resolution is a real structure?
  - Need EM to confirm?
- Very Expensive at this point
- So far, only high Magnification
  - Small field of view
- Files can be extremely large