NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Optical Microscopy

Eli Rothenberg
Department of Biochemistry and Molecular Pharmacology
NYU School of Medicine
Eli.Rothenberg@nyumc.org

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Outline

- Conventional Light Microscopy

Brightfield, phase & interference contrast

- Fluorescence Microscopy
- Confocal and Two-Photon Microscopy
- TIRF Microscopy
- Advances In Fluorescence Microscopy

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Microscopy Resources

- Web:

www.microscopyU.com (Nikon)
Micro.magnet.fsu.edu
www.olympusmicro.com (Olympus)

- Papers (will be uploaded to course website)

Lecture slides are based on the above resources…
Additional resources (Molecular probes/Invitrogen)

Some notes and slides adapted/modified from Prof. Aryeh Weiss (BIU@Israel)

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

What is the Physical Nature of Light?
What is Fluorescence?

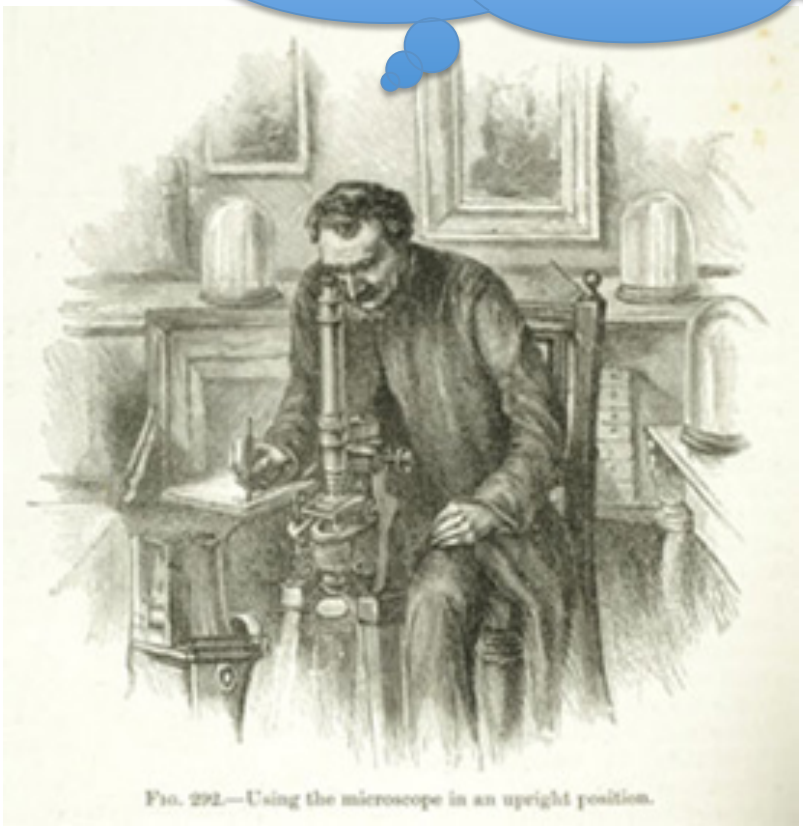
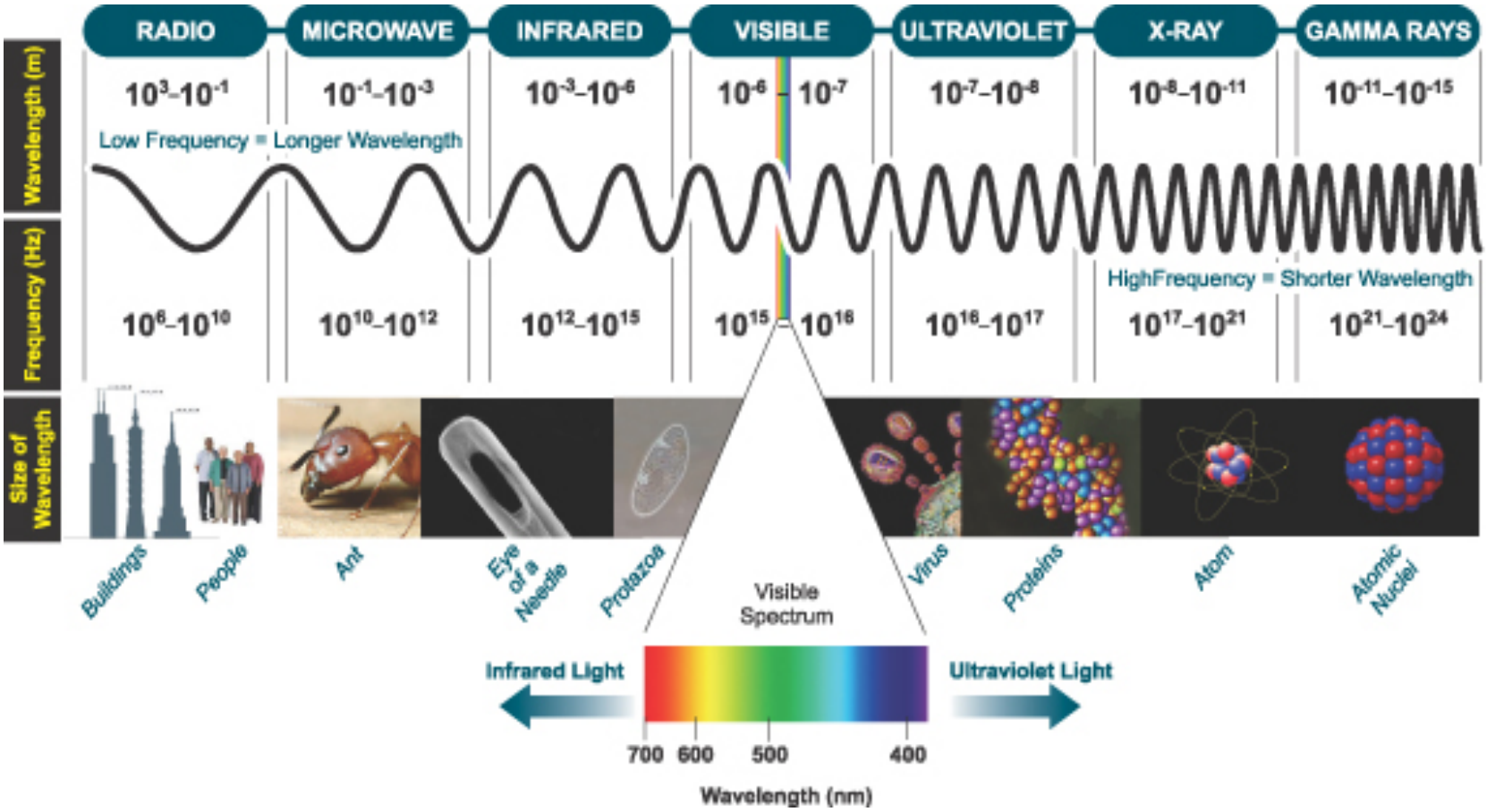


FIG. 292.—Using the microscope in an upright position.



Andor Technology

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Descriptions for the Properties of Light

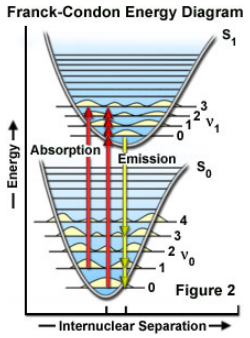
Most Basic: Ray diagrams.

Classical: Electromagnetic waves.

$$\frac{\partial^2 \mathbf{E}}{\partial t^2} - {c_0}^2 \cdot \nabla^2 \mathbf{E} = 0$$

$$\frac{\partial^2 \mathbf{B}}{\partial t^2} - {c_0}^2 \cdot \nabla^2 \mathbf{B} = 0$$

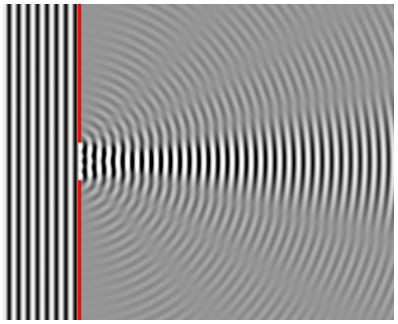
Light-Matter Interaction: Quantum Entities – Photons.



NYU School of Medicine
NYU LANGONE MEDICAL CENTER

The Duality of Light: What is the Physical Nature of Light?

Light behaves like… Waves
(Diffraction, Interference, refraction)



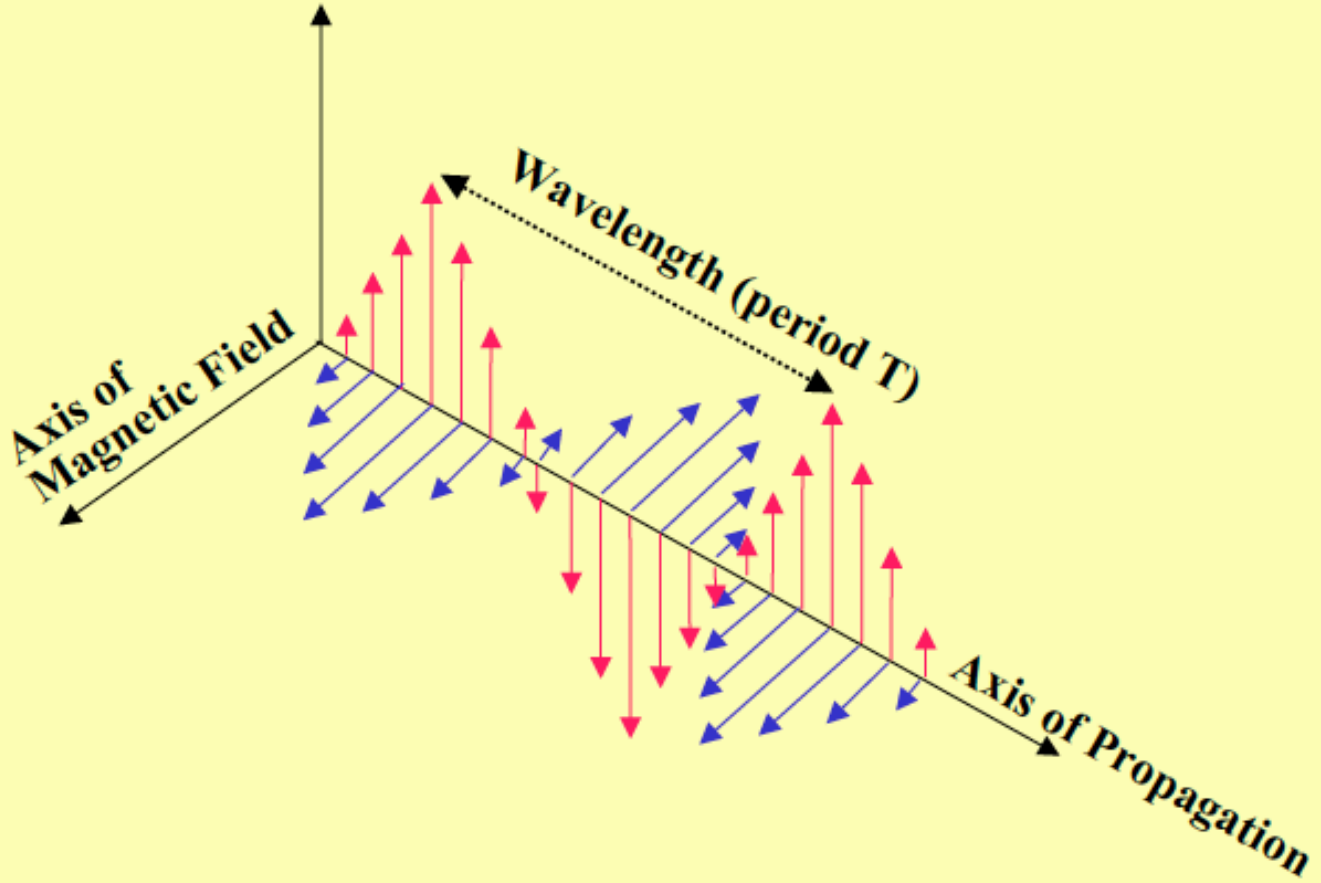
Newton, Huygens, Fresnel, Young, Maxwell

Maxwell's Equations
(Electromagnetic Waves)

$$\frac{\partial^2 \mathbf{E}}{\partial t^2} - {c_0}^2 \cdot \nabla^2 \mathbf{E} = 0$$

$$\frac{\partial^2 \mathbf{B}}{\partial t^2} - {c_0}^2 \cdot \nabla^2 \mathbf{B} = 0$$

ELECTROMAGNETIC WAVE



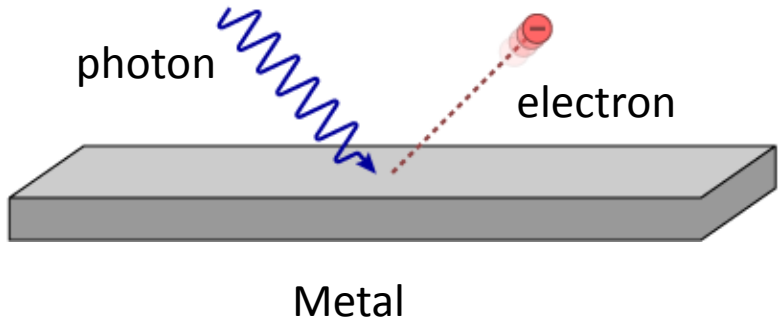
NYU School of Medicine
NYU LANGONE MEDICAL CENTER

The Duality of Light: What is the Physical Nature of Light?

Light behaves like…. Particles
(Discrete Quantities of Energy- light quantas or photons)

Hertz, Thomson, Lenard, Einstein

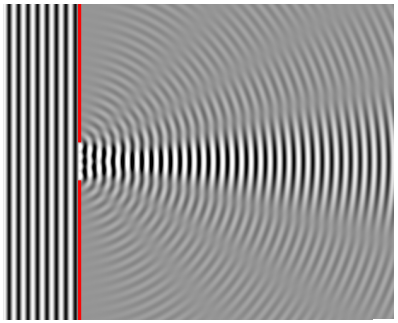
The Photoelectric Effect

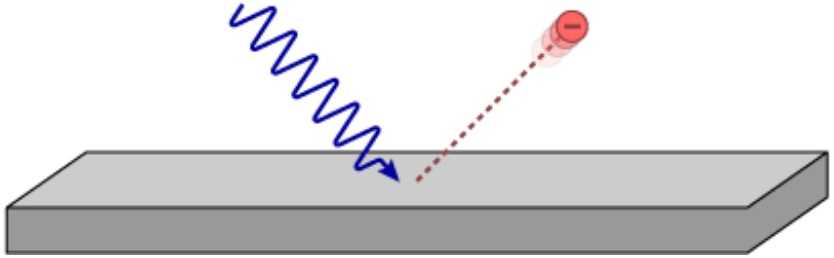


NYU School of Medicine
NYU LANGONE MEDICAL CENTER

The Duality of Light

Light has a duality, wave AND particle





"Once and for all I want to know what I'm paying for. When the electric company tells me whether light is a wave or a particle I'll write my check."

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

The Duality of Light

Equations that describe the properties of light:

Wave equations: (frequency, amplitude).

$\Psi(x,t)=A*exp(i(\kappa x+\omega t)$
$\kappa=2\pi/\lambda$
$\omega=2\pi\nu$

Energy of Photons:

$$E= \quad h\nu = hc/\lambda$$

ν-*frequency*
λ-*wavelength*
c-speed of light

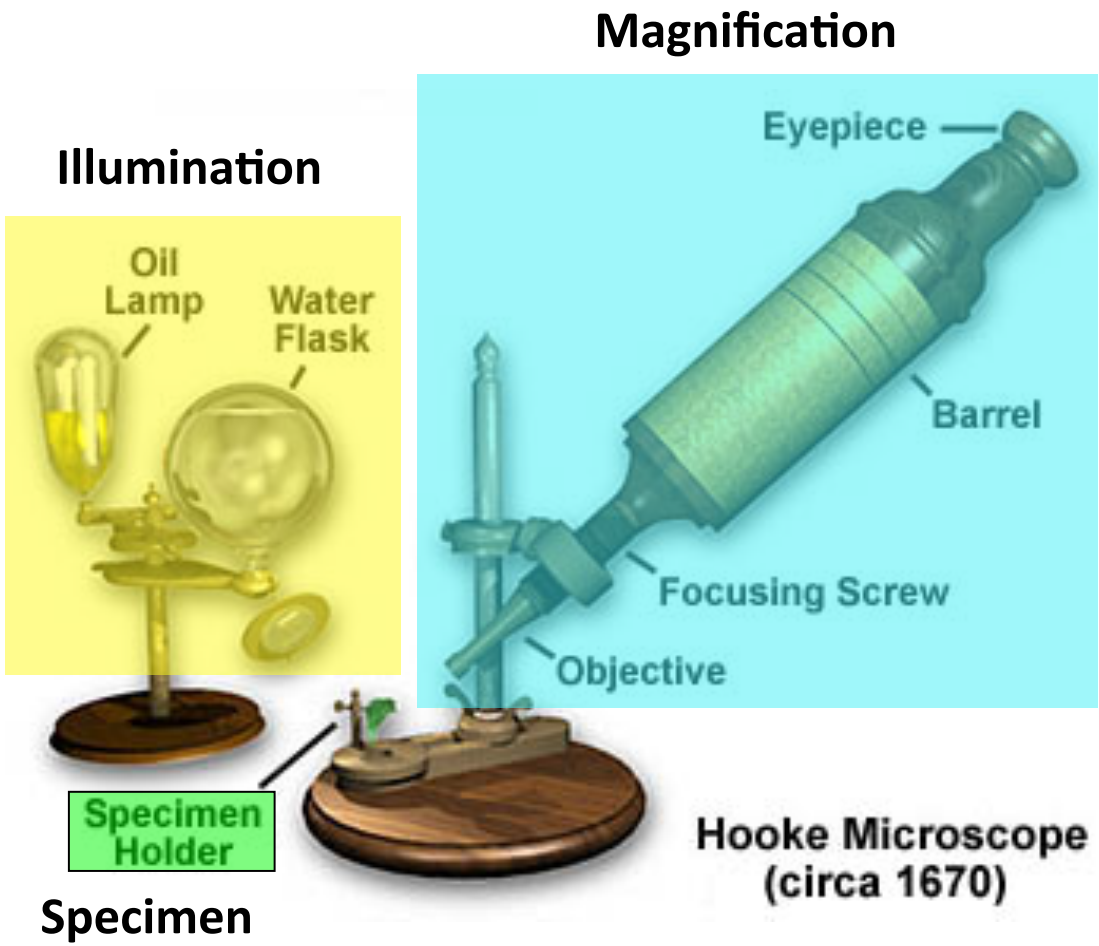
NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Conventional Light Microscopy

Bright field, phase & Interference contrast

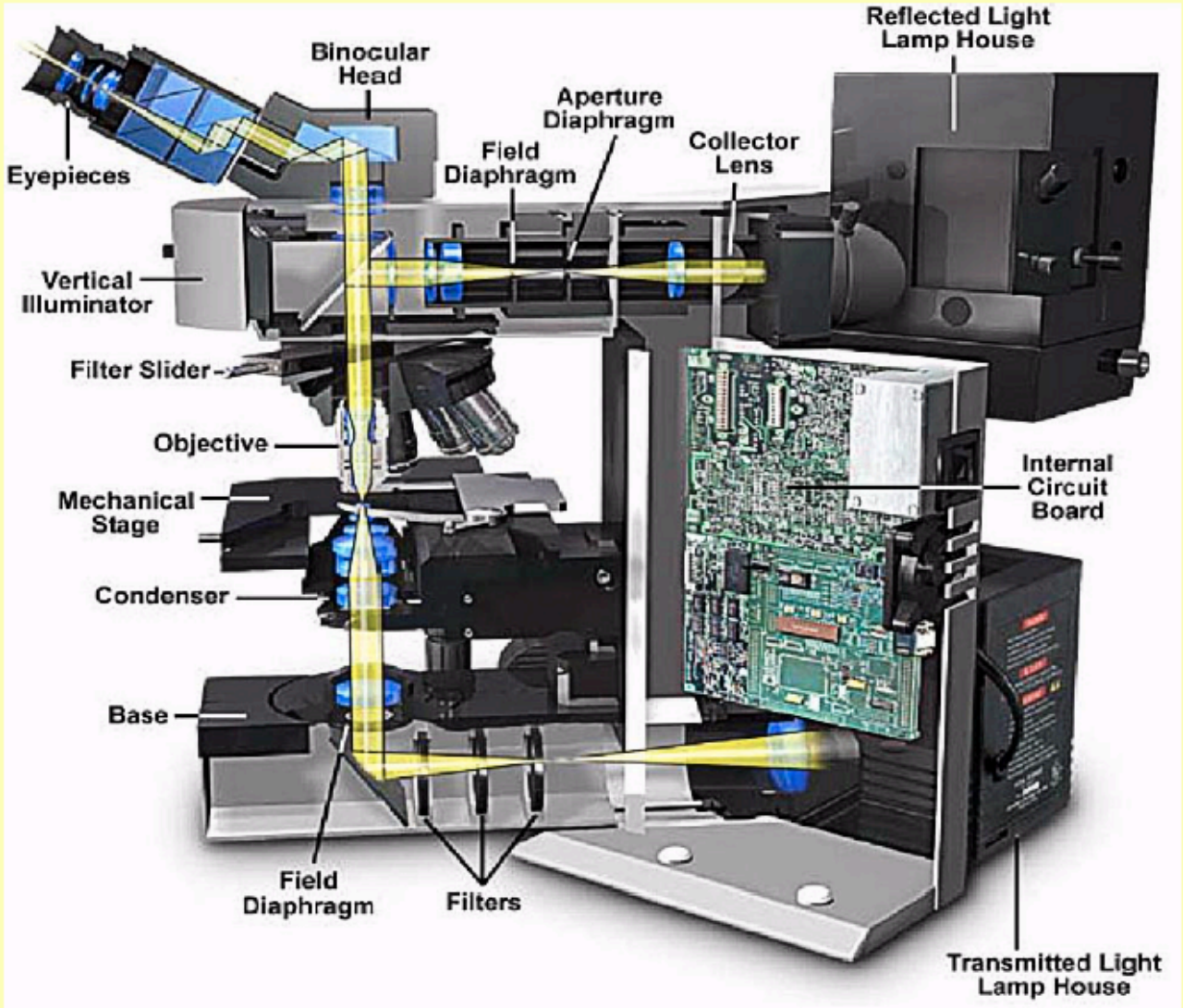
NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Basic Anatomy of Light Microscope

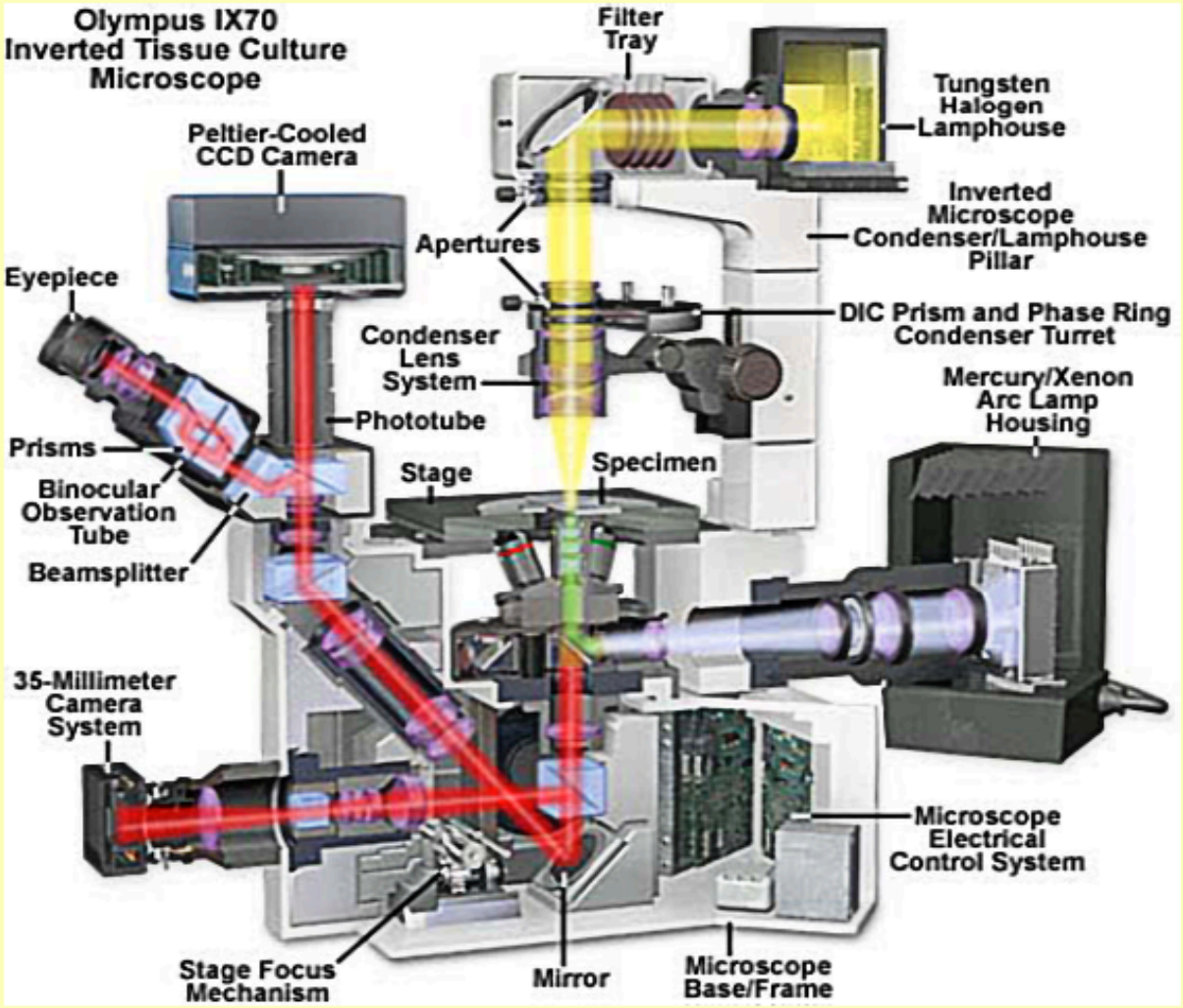


(with modification http://micro.magnet.fsu.edu)

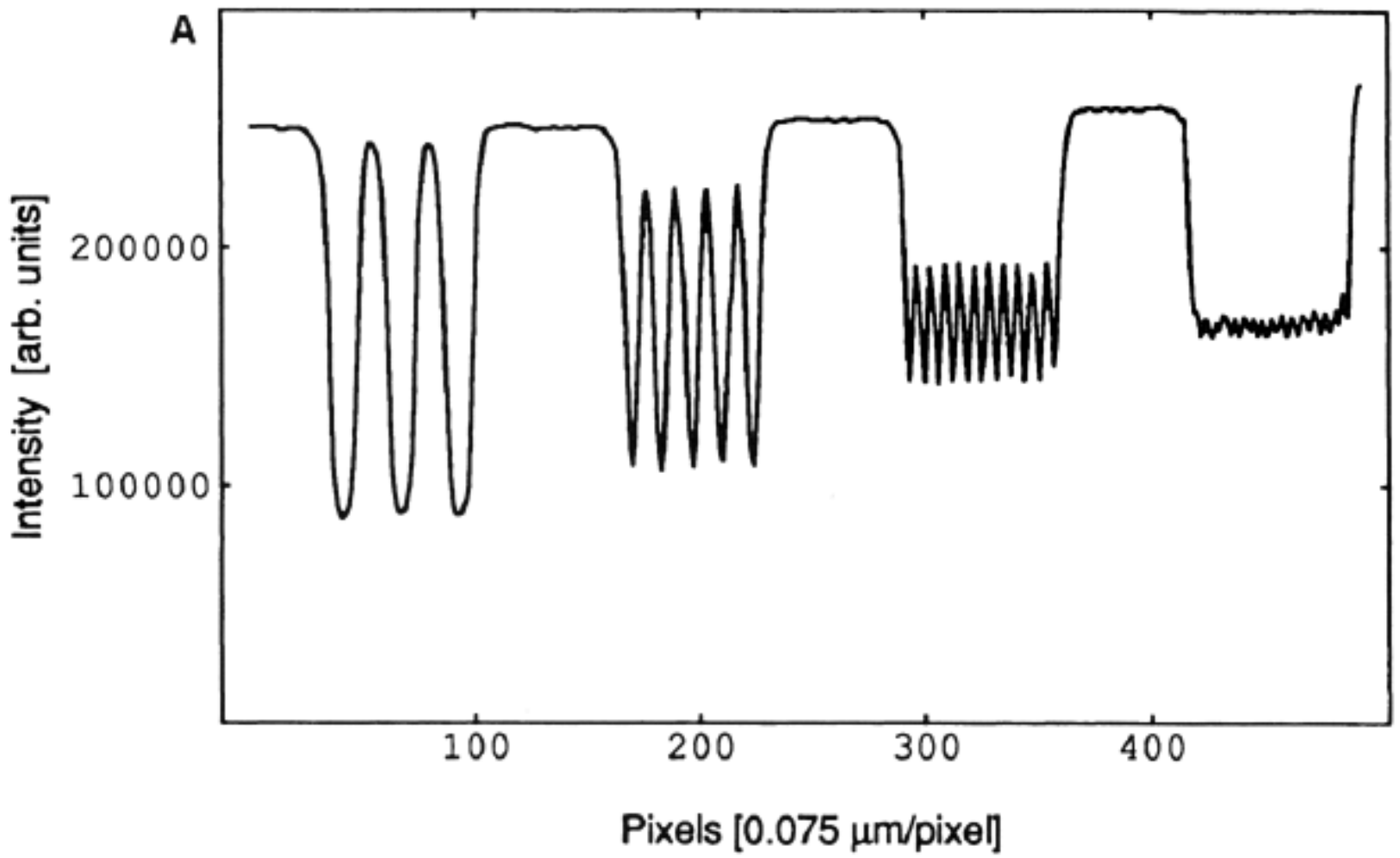
UPRIGHT MICROSCOPE



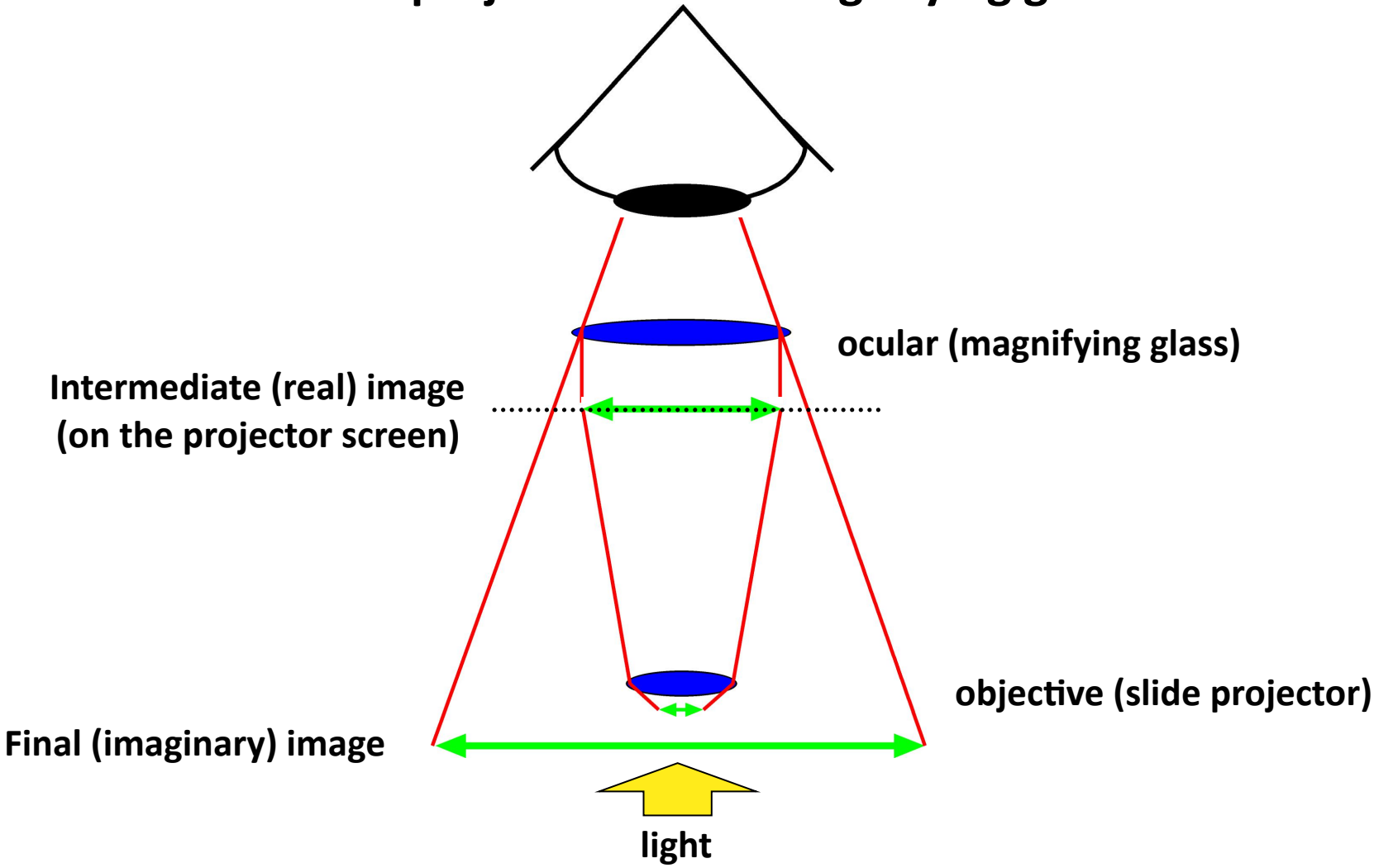
INVERTED MICROSCOPE



WHAT IS CONTRAST



A light microscope is a combination of a slide projector with a magnifying glass

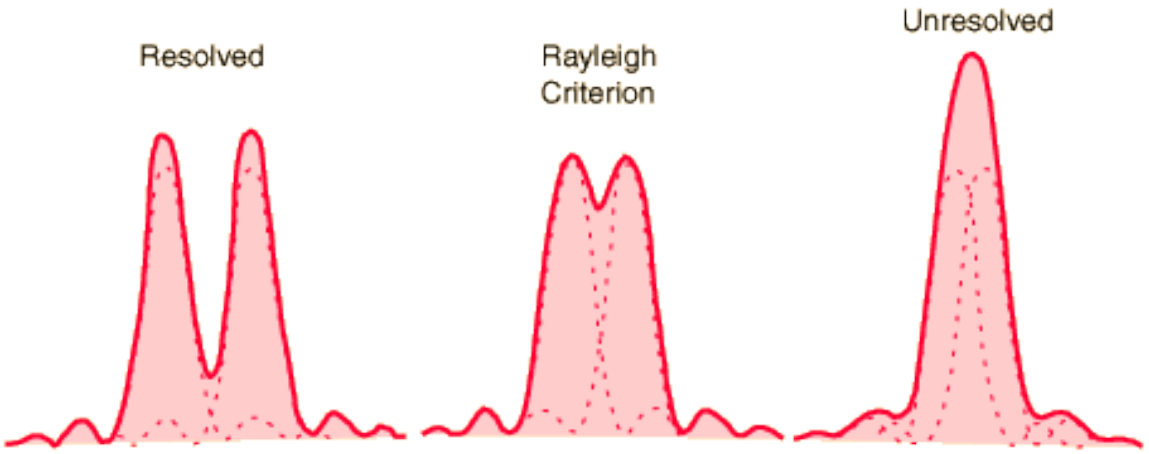


Total magnification = $M_{objective}$ x M_{ocular}

Rayleigh Criterion-
Diffraction limit of light

Or capability of resolving adjacent objects in a microscope

P.S.F - Point Spread Function



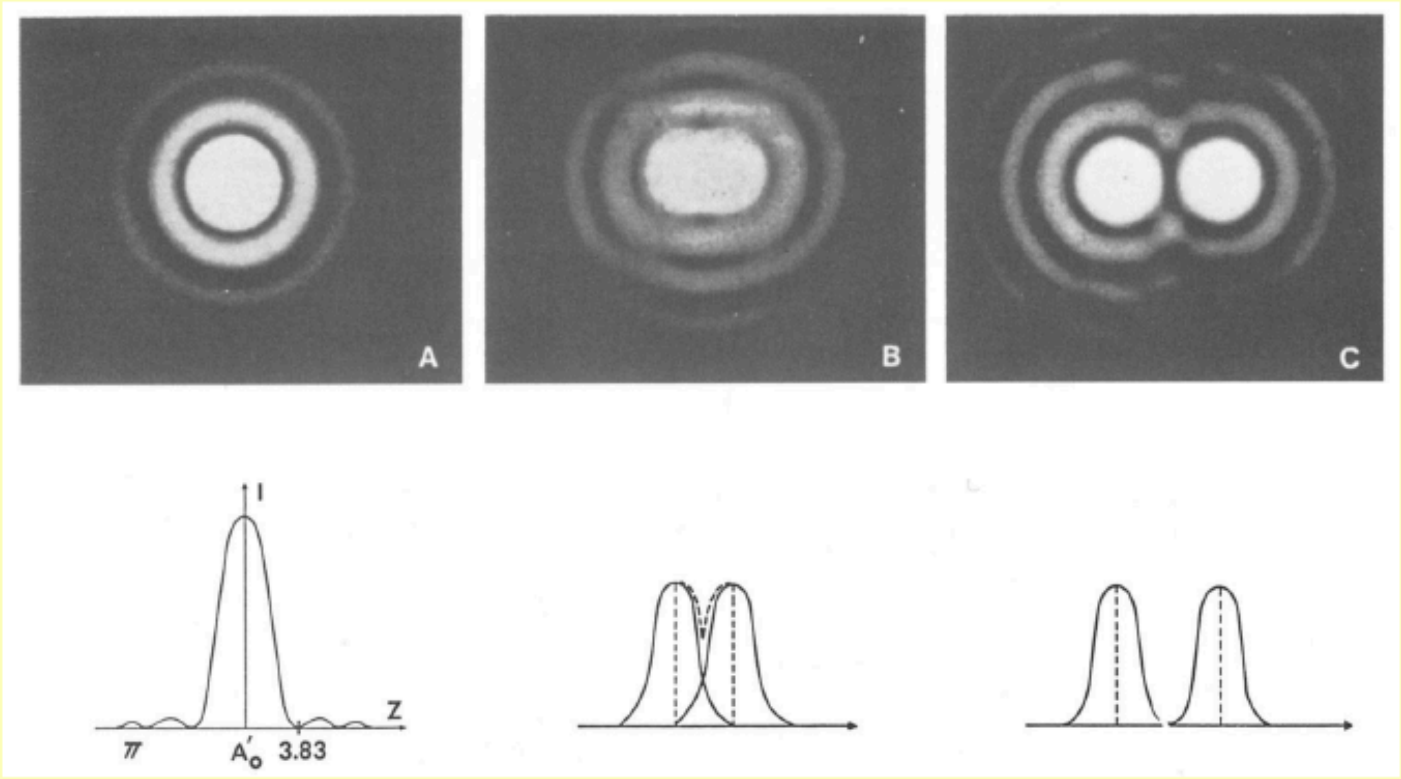
Lord Rayleigh (John Strutt)
(1842-1919)

$$R = \frac{0.61\lambda}{\mathrm{NA}}$$

$$\mathrm{NA} = n \sin\theta$$

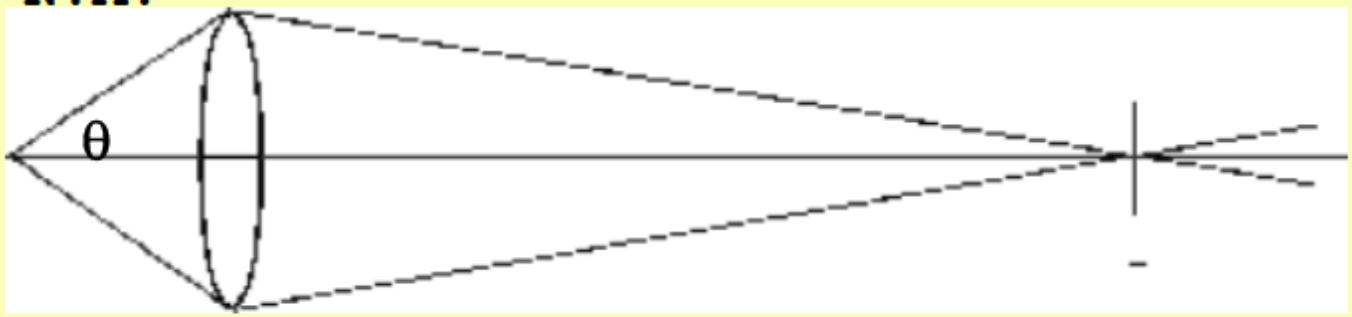
Angular collection ability of an objective lens

DIFFRACTION LIMITED IMAGING

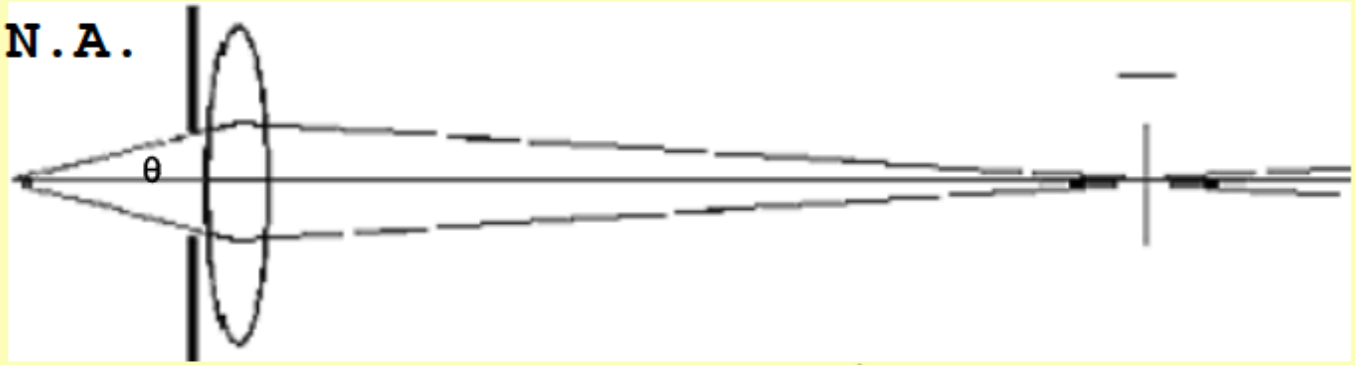


NUMERICAL APERTURE

High N.A.



Low N.A.



$$N.A. = n \sin(\theta)$$

RAYLEIGH CRITERION

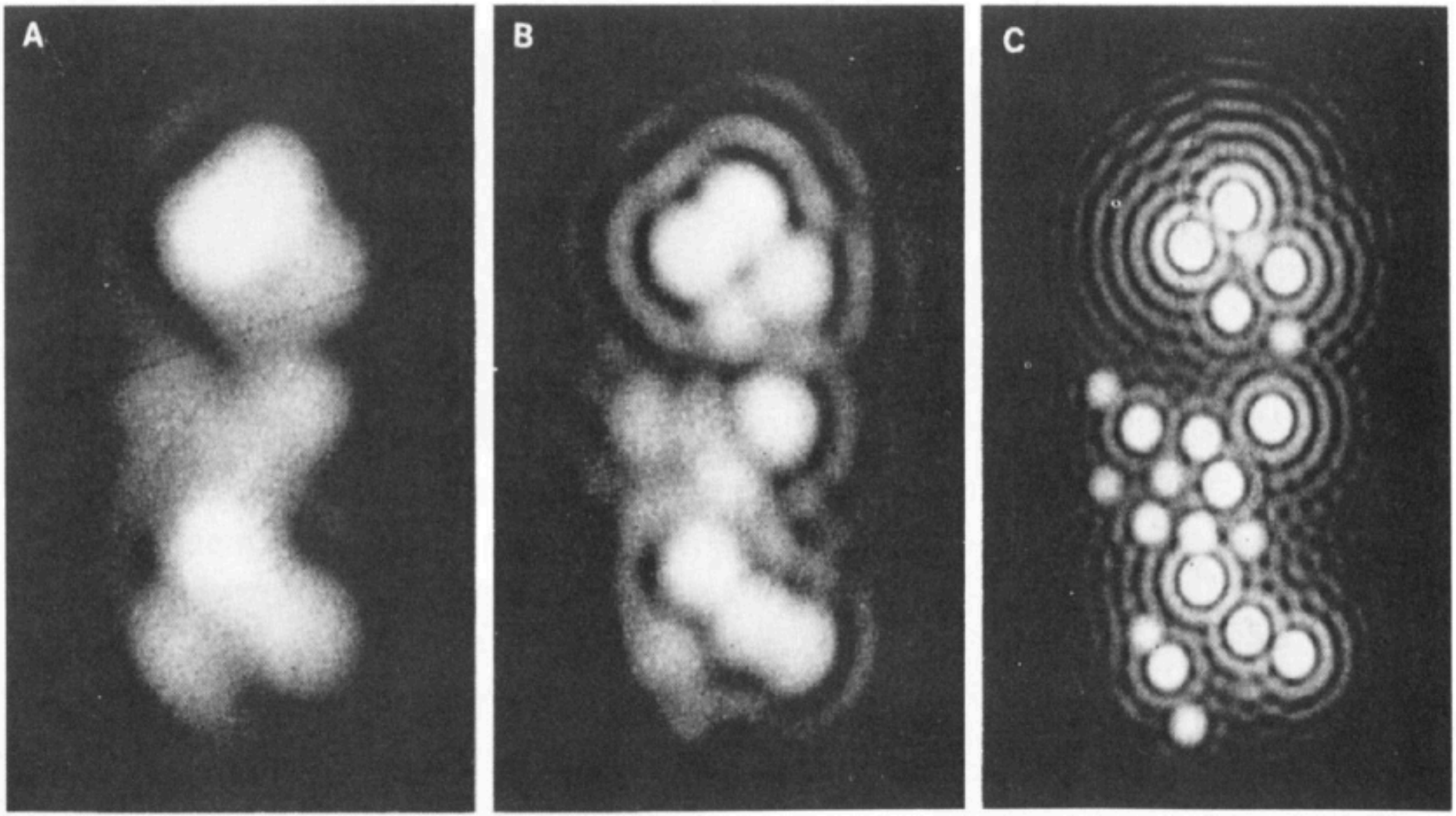
$$r = 0.61\lambda/NA$$

$$NA = n \sin(\theta)$$

For high NA objectives (0.4-1.4)

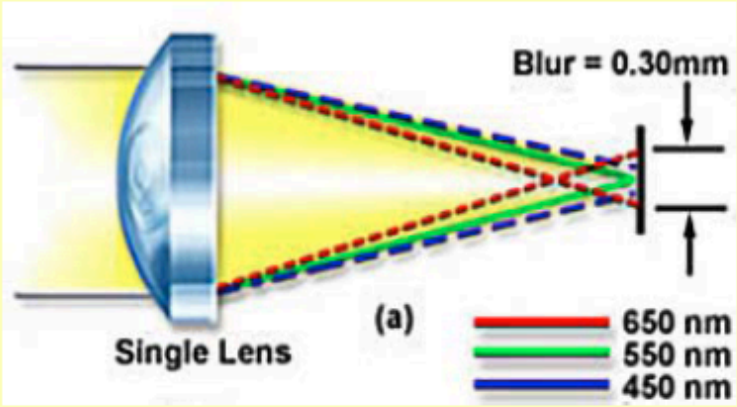
$$1.5\lambda > r > 0.44\lambda$$

EFFECT OF NA ON RESOLUTION

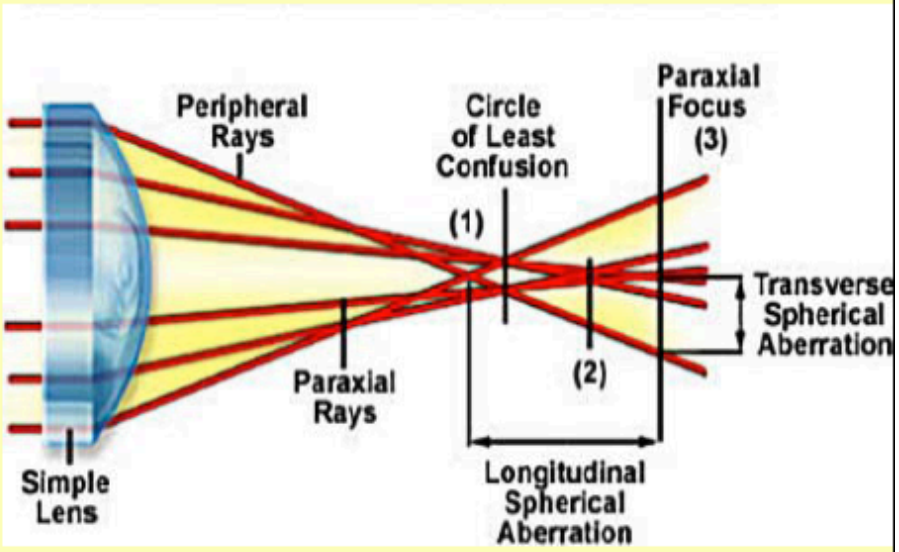


ABERRATIONS

- **CHROMATIC ABERRATION**
  - due to n(λ)



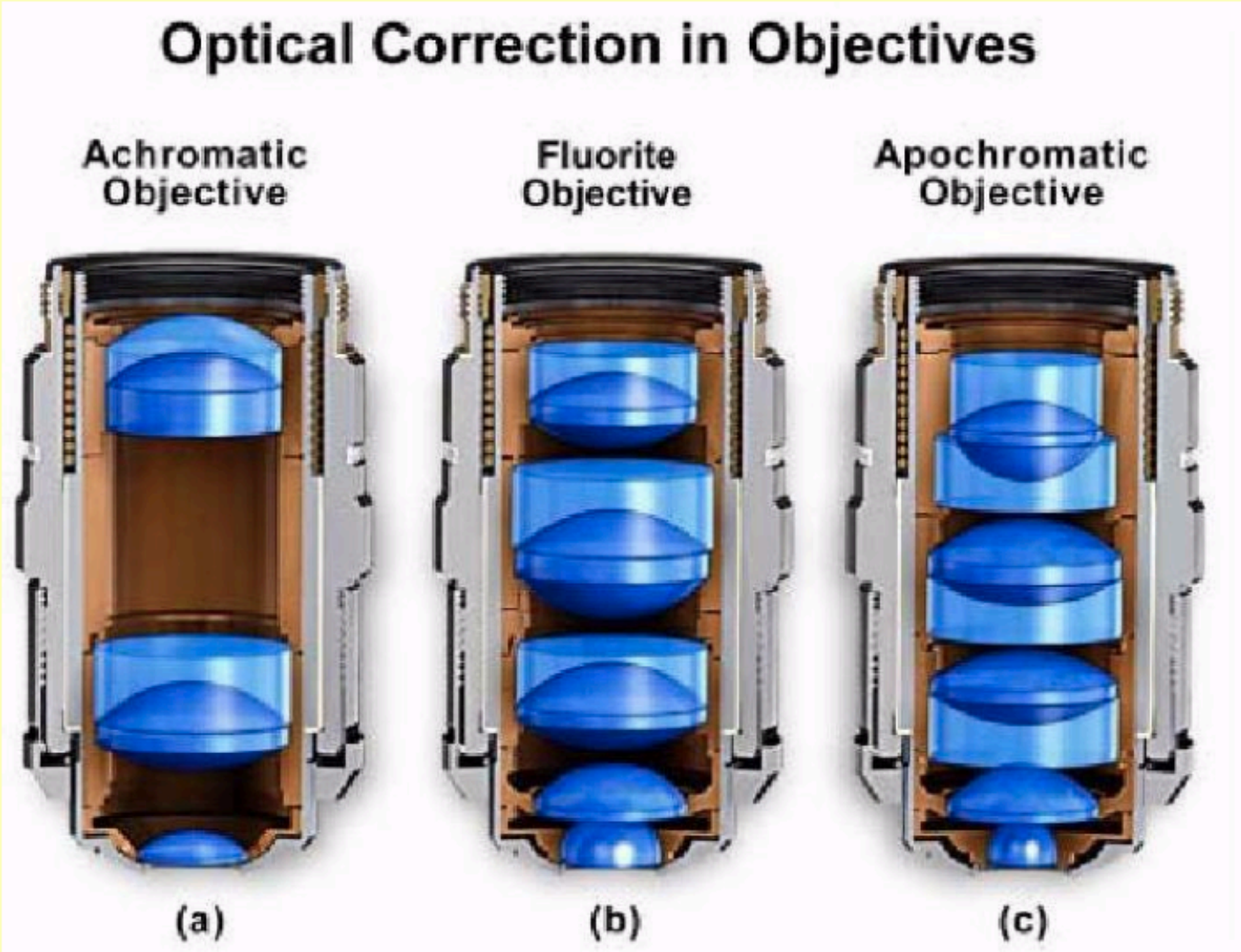
- **SPHERICAL ABERRATION**
  - paraxial approx.



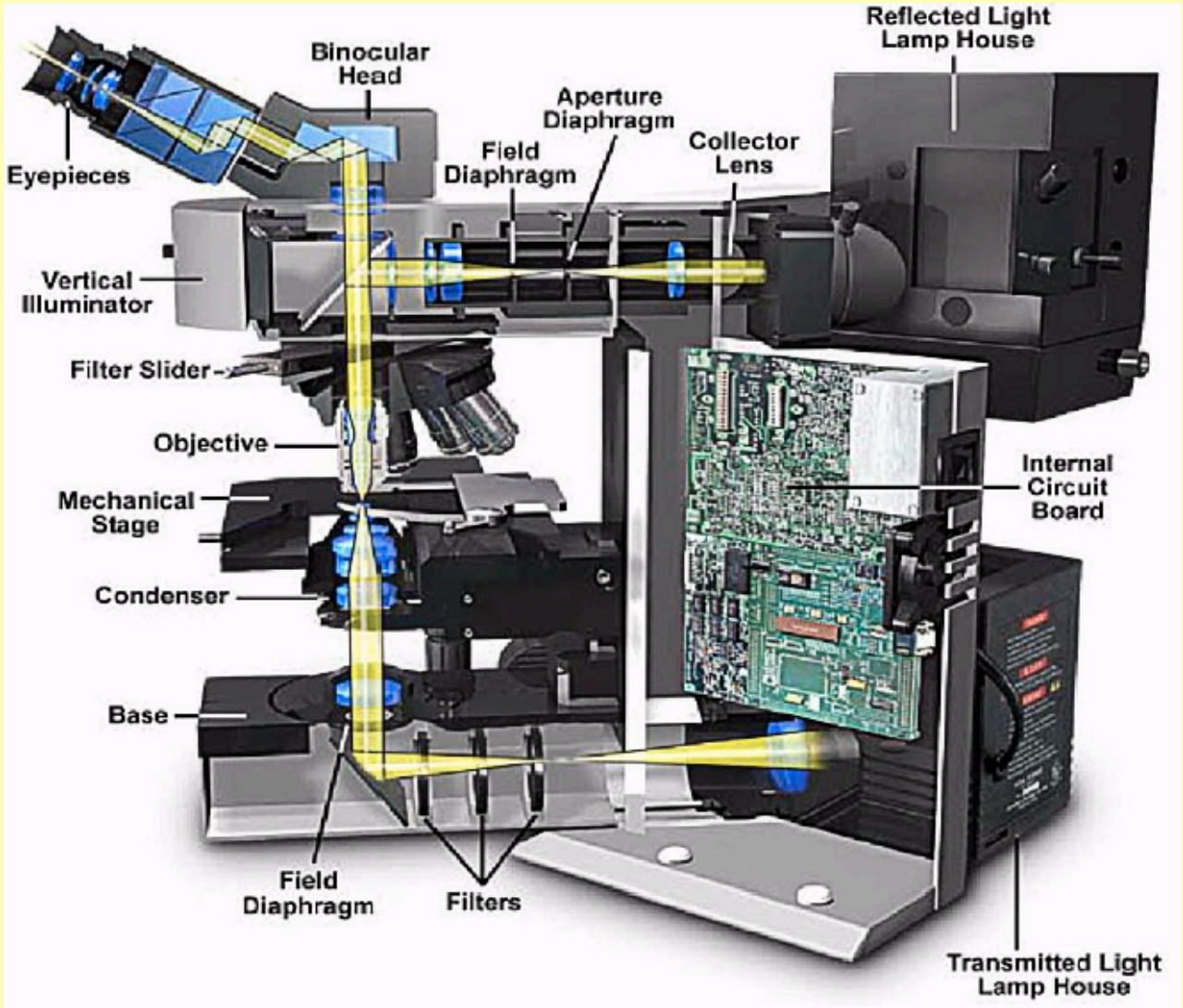
OBJECTIVES


Objective Specifications
Manufacturer
Flat-Field Correction
Linear Magnification
Specialized Optical Properties
Tube Length
Cover Slip Thickness
Screw Thread
Aberration Correction
Numerical Aperture
Immersion Medium
Working Distance
Color Code
Finger Grip
Spring-Loaded Front Lens
OLYMPUS
Plan Apo
60X/1.40 Oil
DIC H
∞/0.17 WD 0.21

WHY SO EXPENSIVE?



UPRIGHT MICROSCOPE



GENERATION OF CONTRAST

- Darkfield
- Rheinberg illumination
- Phase contrast microscopy
- DIC (Nomarski)

Using Properties of Light for Better Imaging

Algae

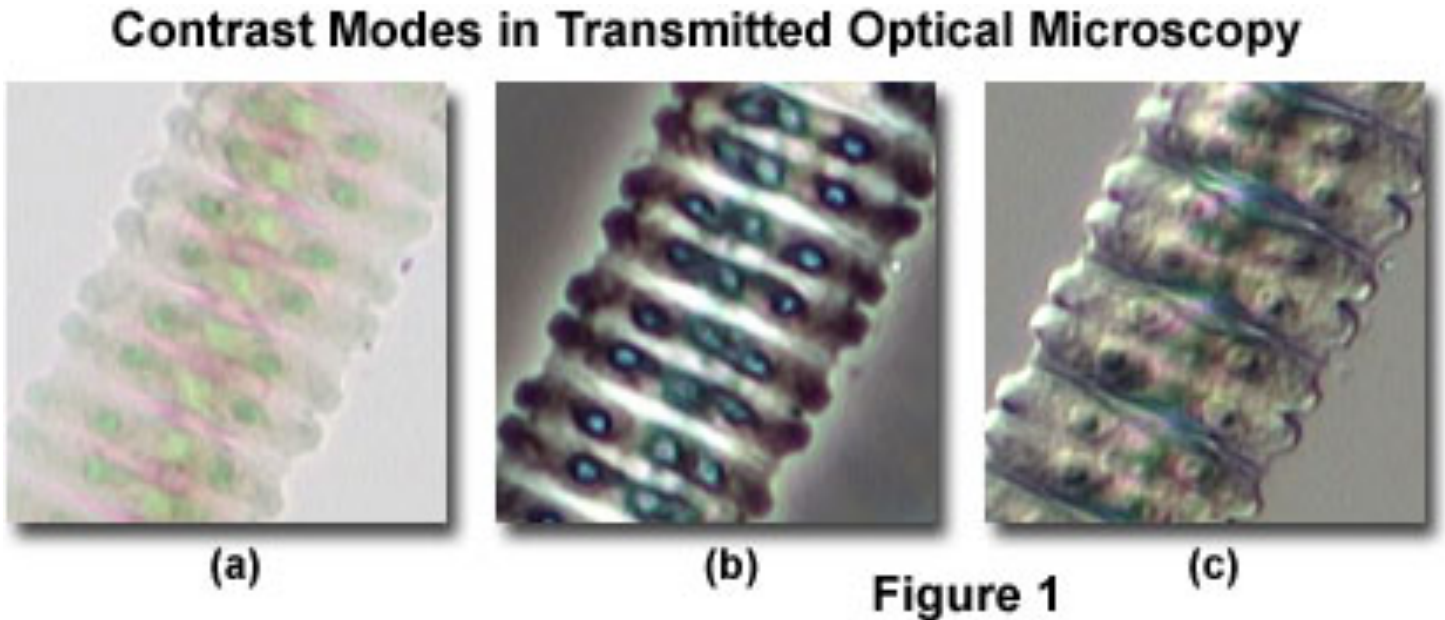


Figure 1

Bright field

phase

DIC

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Phase Contrast Microscope Configuration

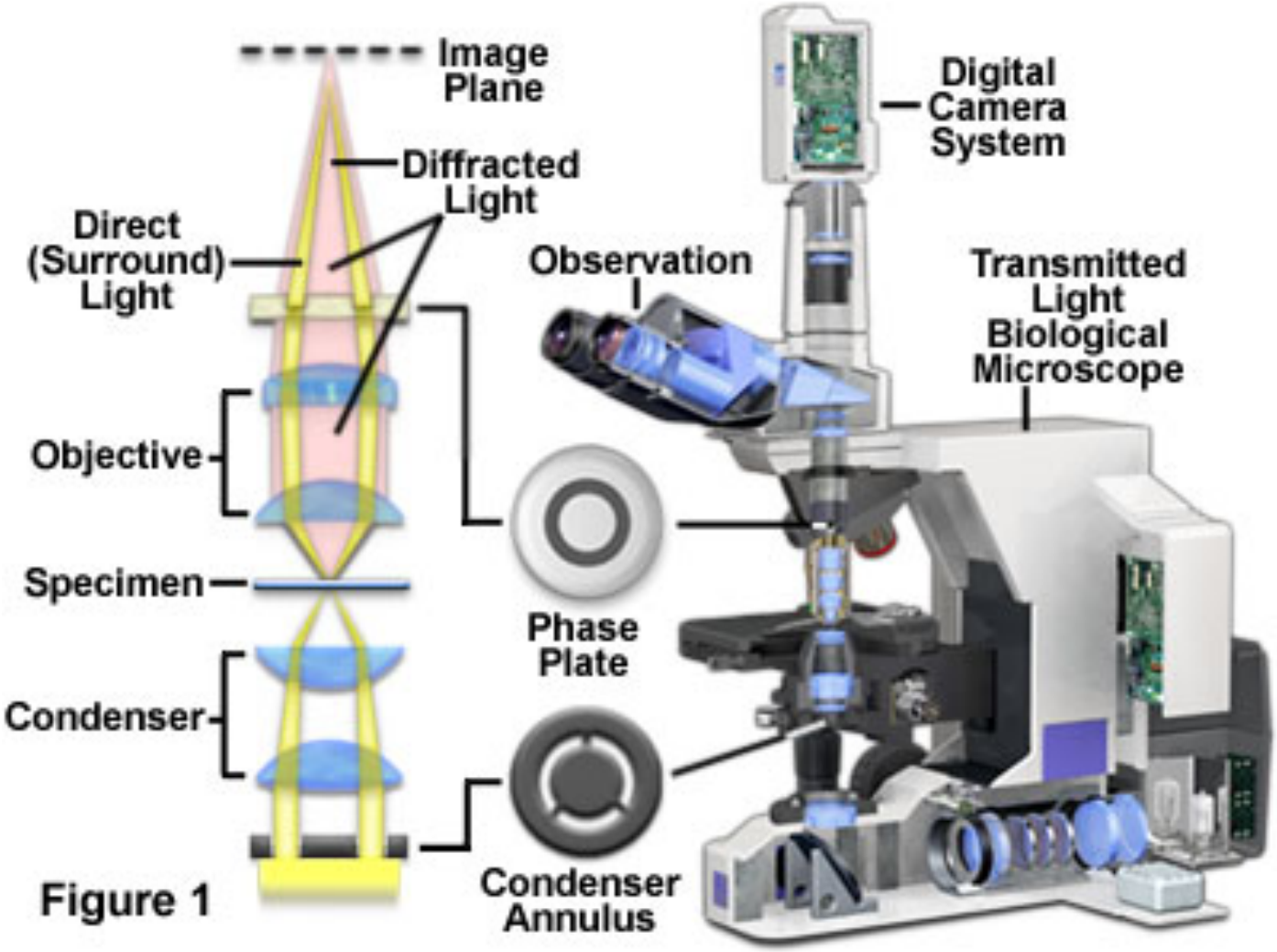
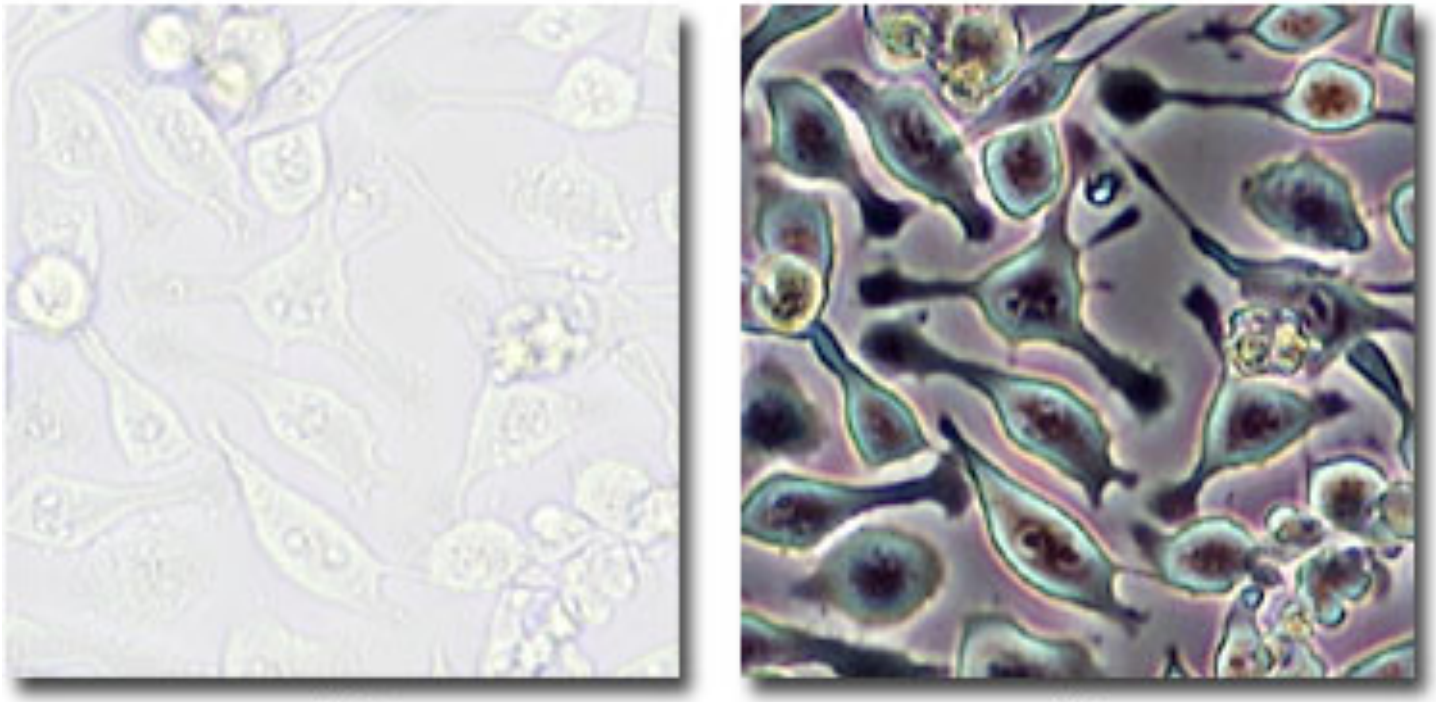


Figure 1

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Living Cells in Brightfield and Phase Contrast



(a) (b)

Figure 2

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

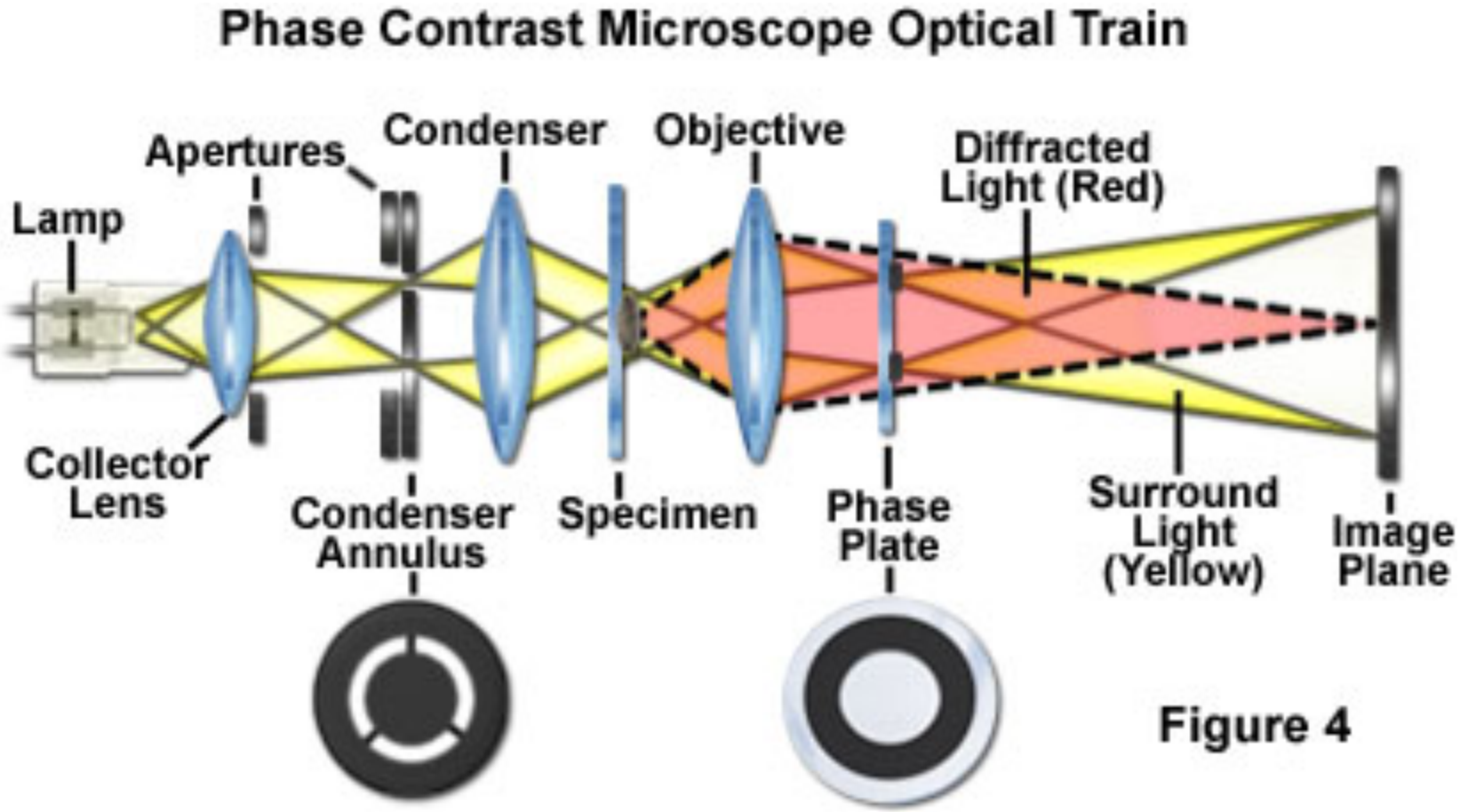
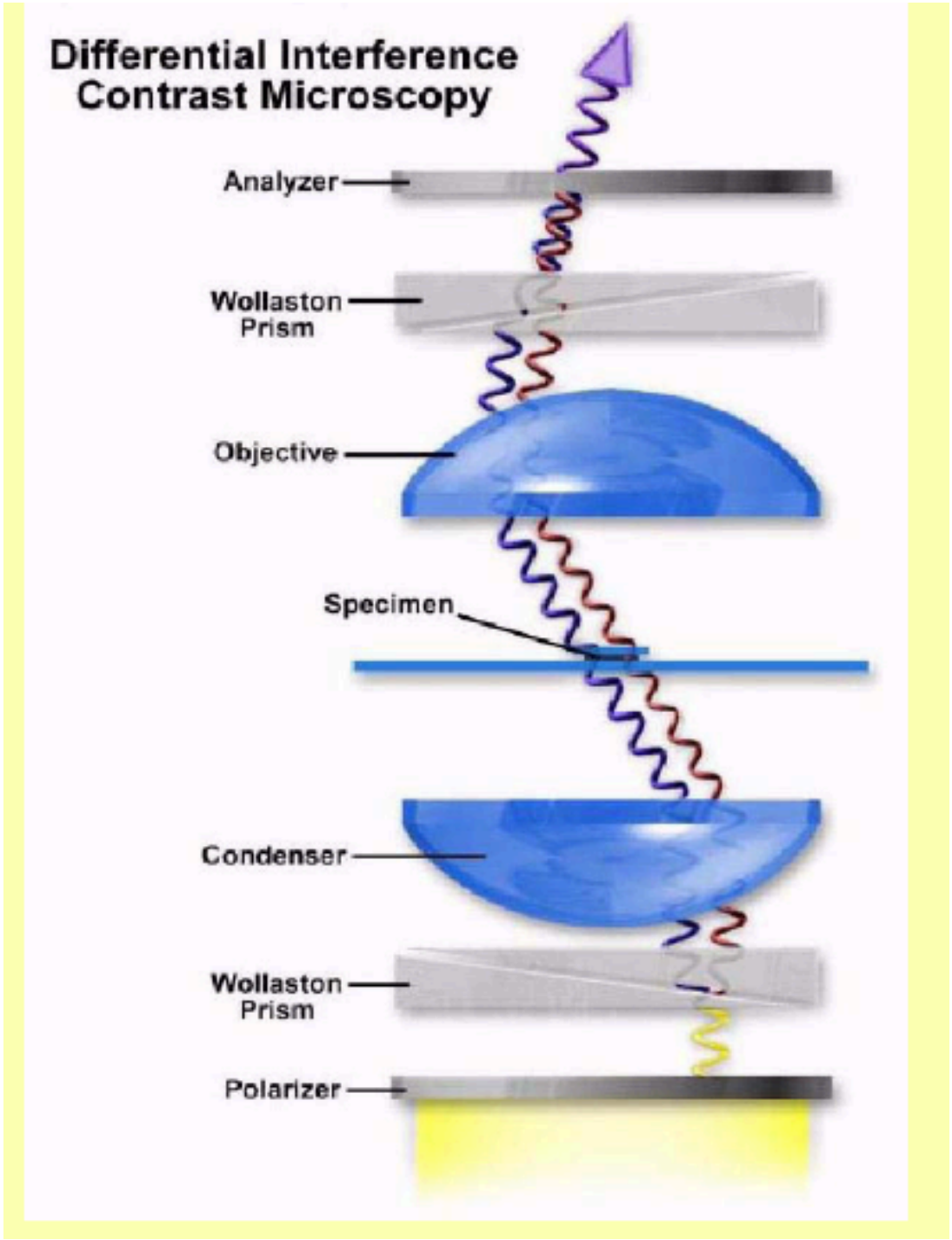
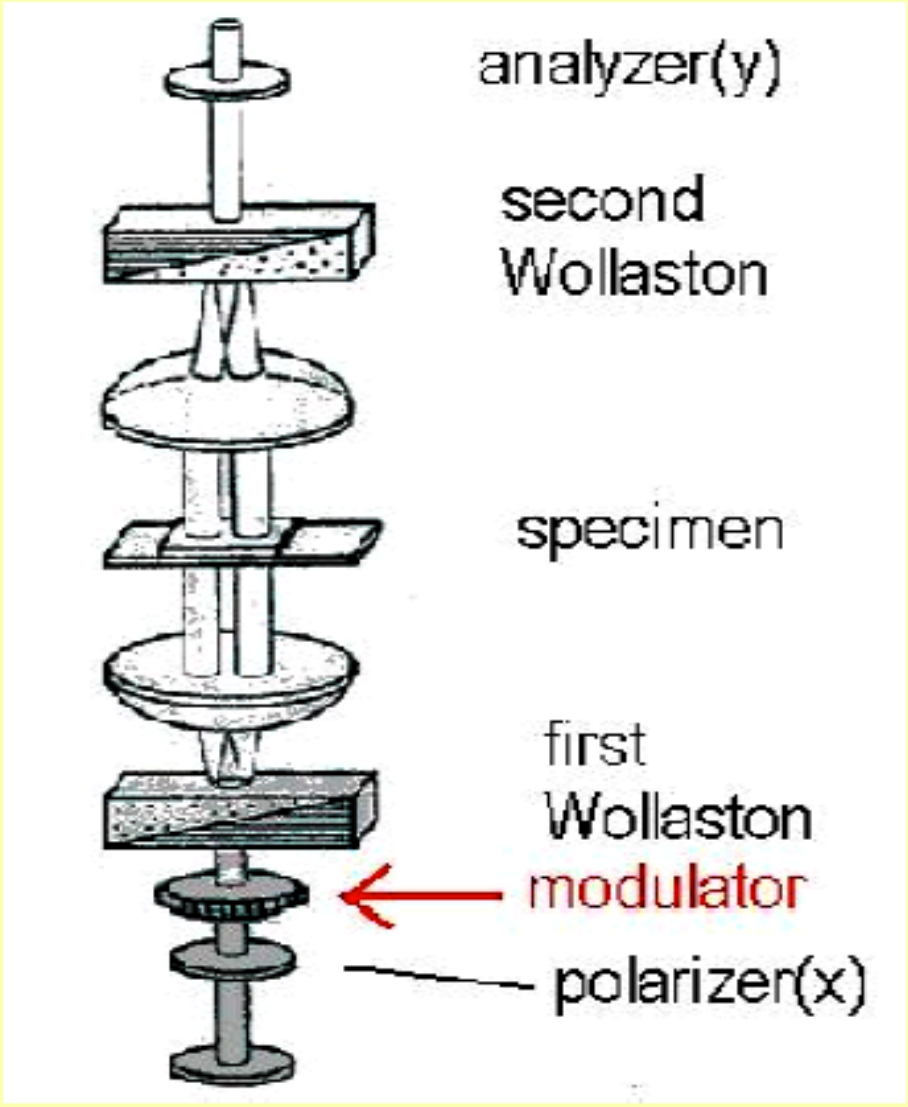


Figure 4


Differential Interference Contrast Microscopy
Analyzer
Wollaston Prism
Objective
Specimen
Condenser
Wollaston Prism
Polarizer



Holzwarth, Webb, Kubinski, and Allen,
J. Microscopy, p*249-254* (1997)

Algae

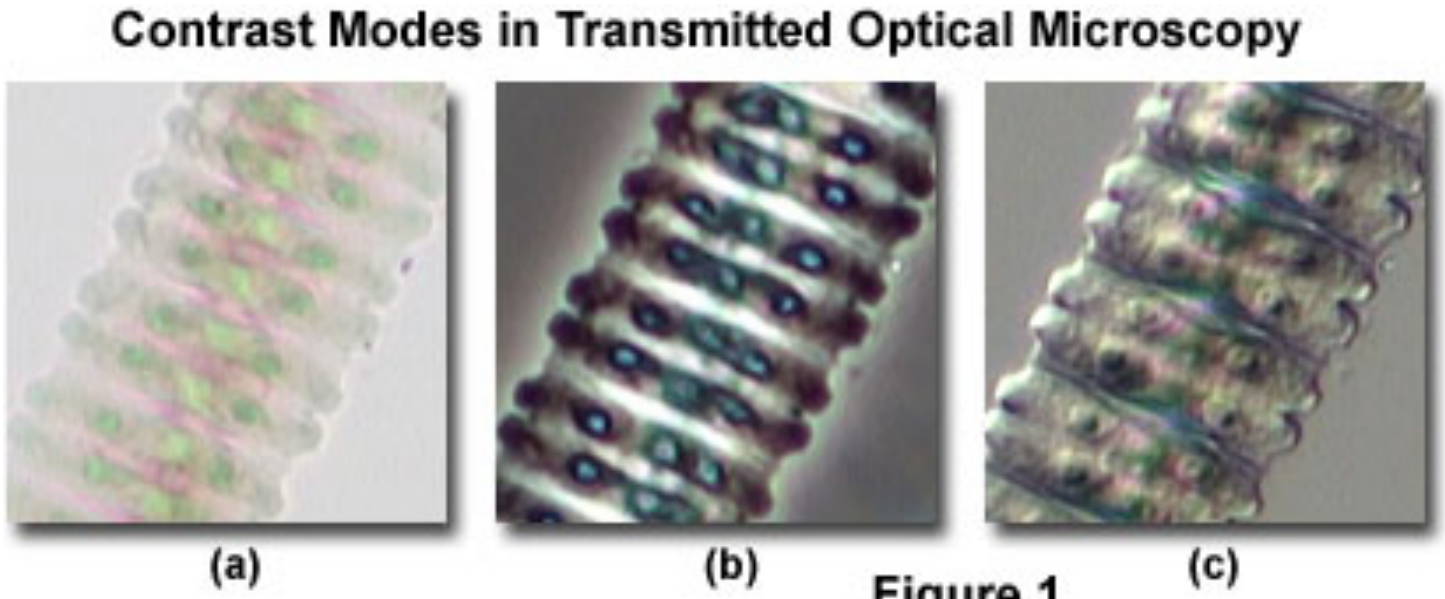


Figure 1

Bright field

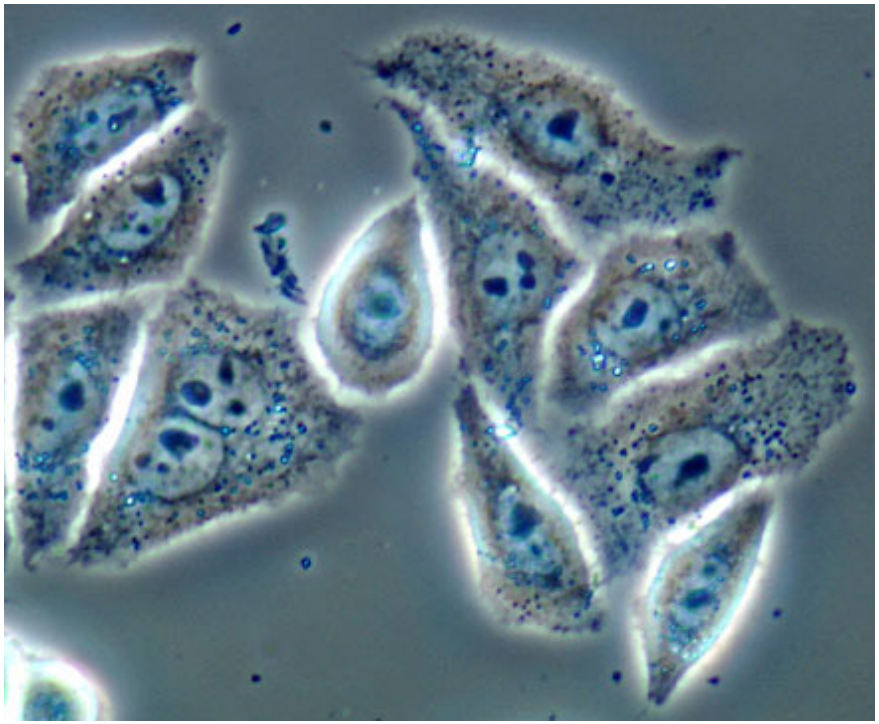
phase

DIC

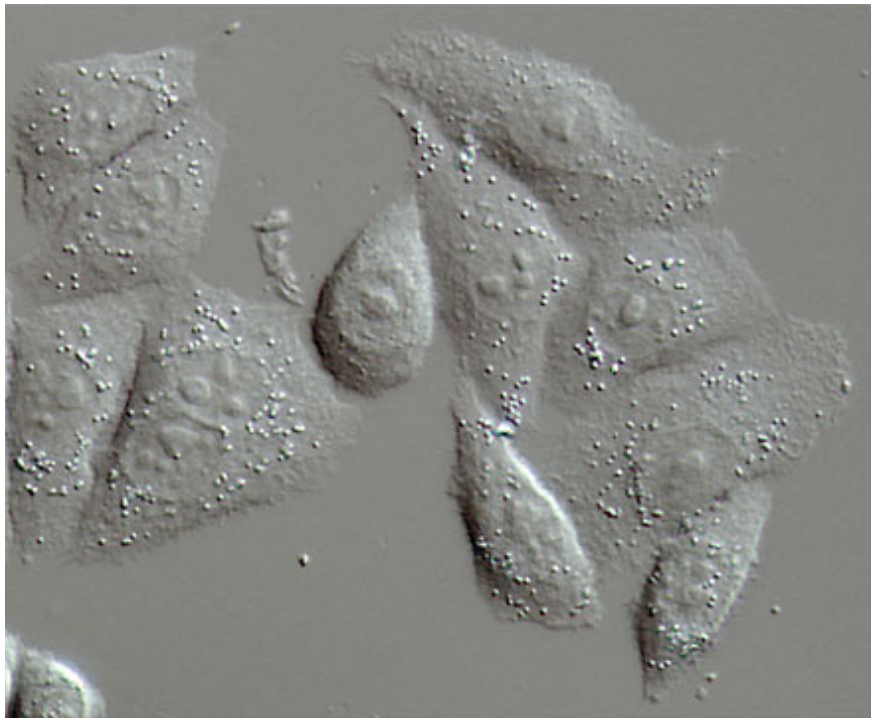
NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Phase vs DIC

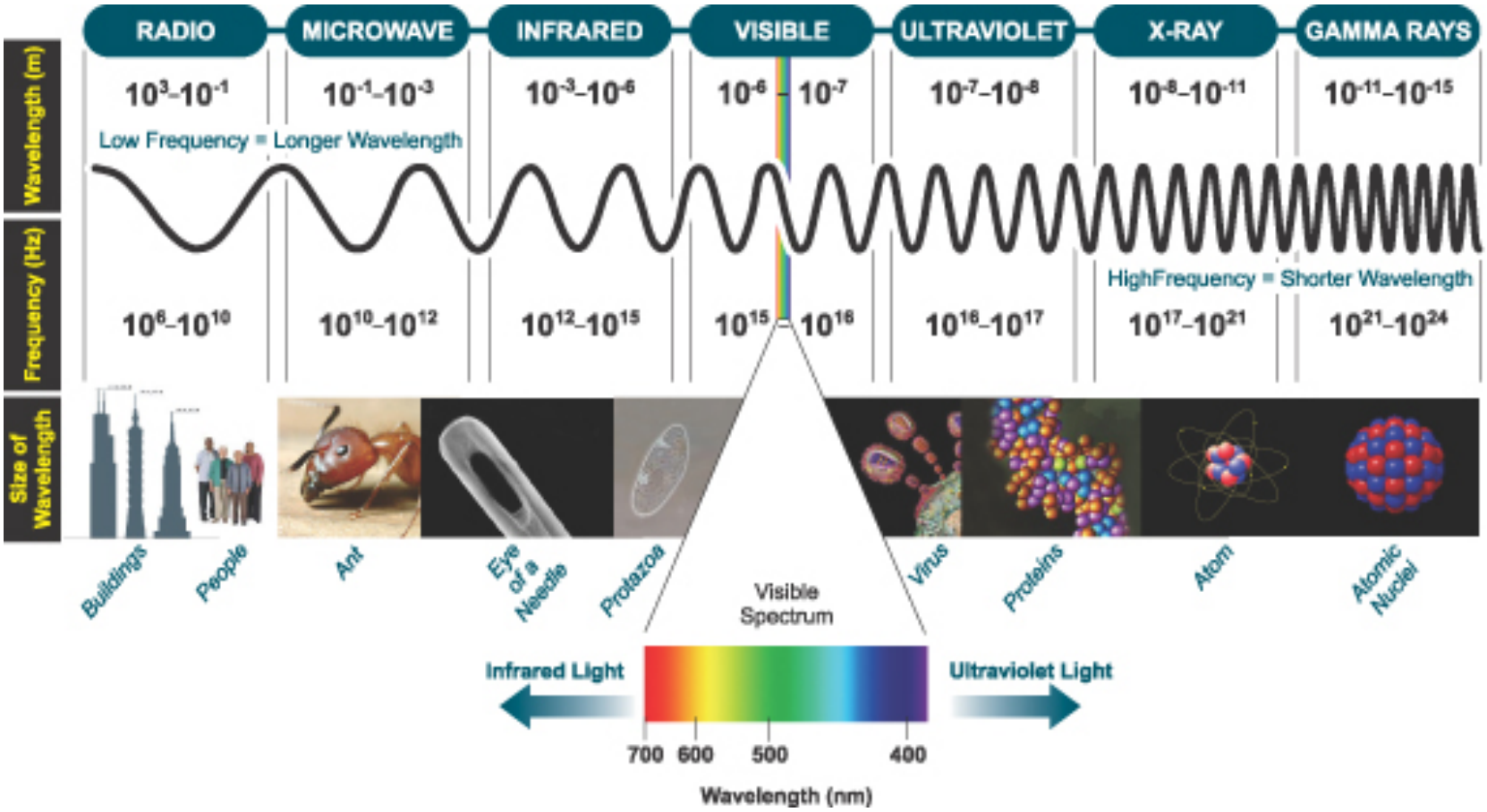
HeLa Cells



phase



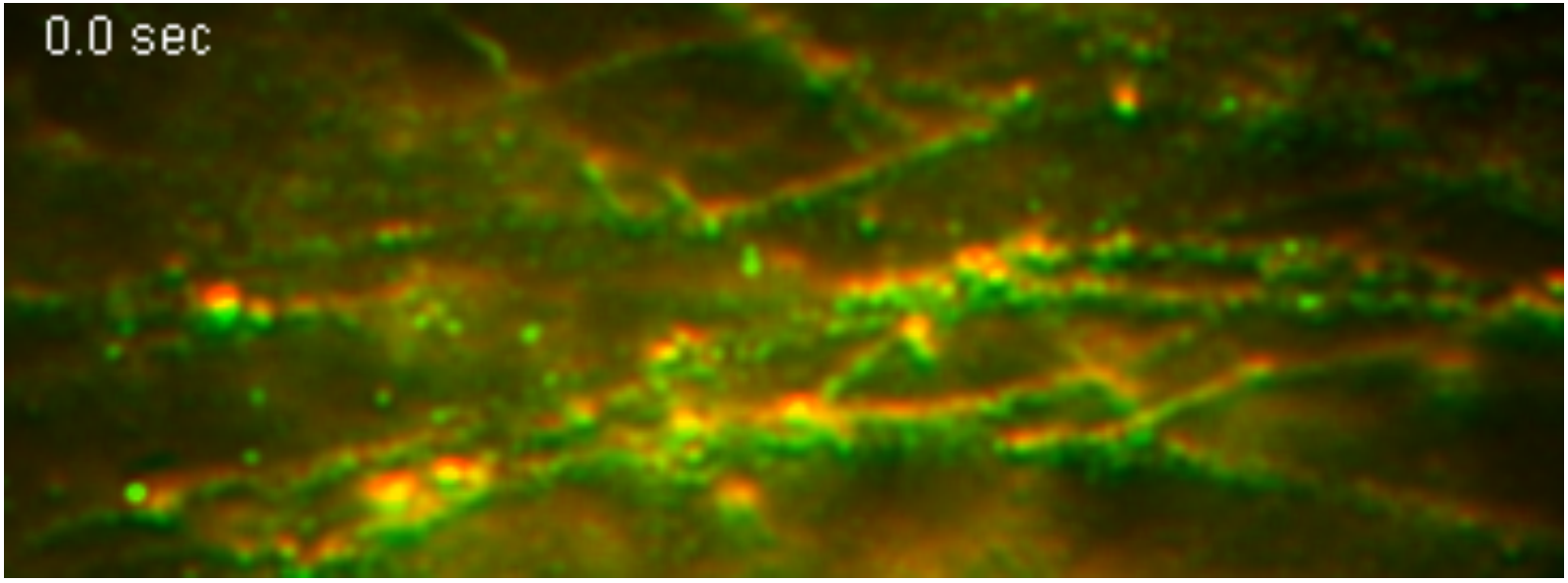
DIC



Andor Technology

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Fluorescence Microscopy



Rat Neuron

Why Fluorescence Microscopy

Cells ~ 40-100 µm (10^{-6} m)

Inside the cell:

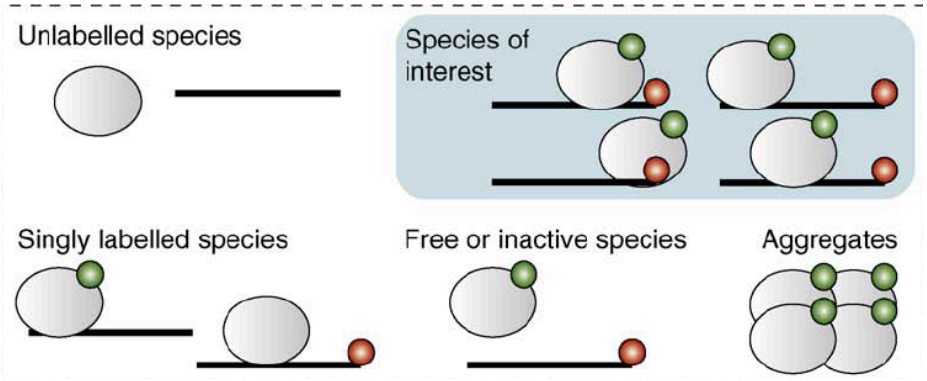
Proteins and nucleic acids – nanometer and angstrom scale (10^{-9}-10^{-10} m).

Seeing biology as it happens – light microscopy (Visualizing Small Objects)

Problem: Small objects will not scatter light

Solution: Fluorescence microscopy

Instead of looking directly we attach to the biomolecules of interest a florescent molecule (Tag)



Trends in Biochemical Sciences (2009)

Fluorescent Molecules:

- **Fluorophores/Fluorochromes/Dye molecules**
- **Fluorescent Proteins (Bioluminescence)**
- **Quantum-Dots**

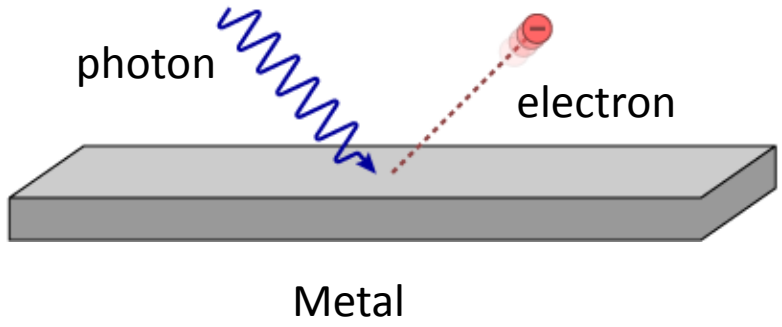
NYU School of Medicine
NYU LANGONE MEDICAL CENTER

The Duality of Light: What is the Physical Nature of Light?

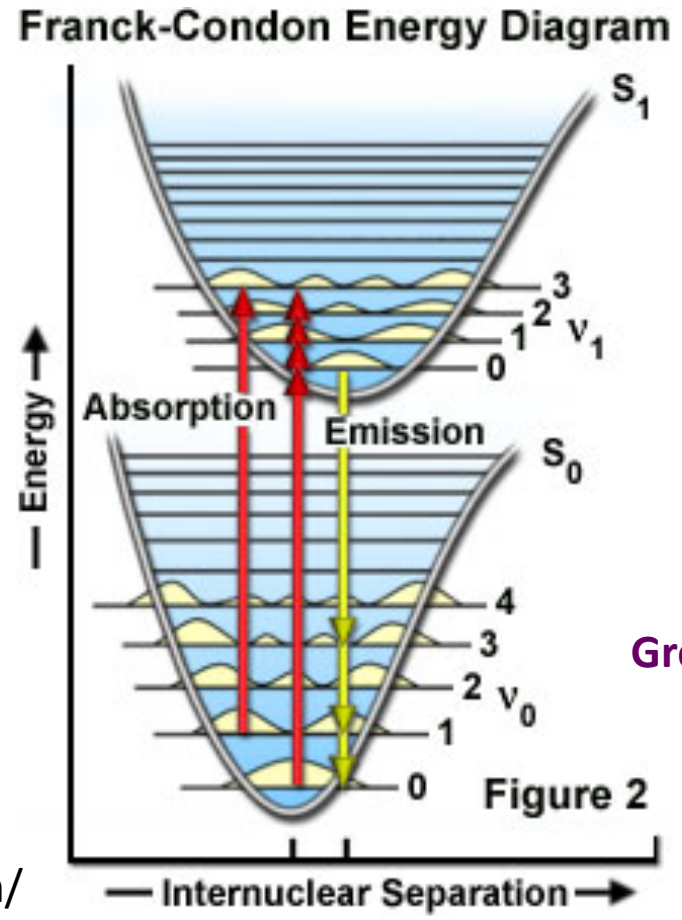
Light behaves like…. Particles
(Discrete Quantities of Energy- light quantas or photons)

Hertz, Thomson, Lenard, Einstein

The Photoelectric Effect



Fluorescence



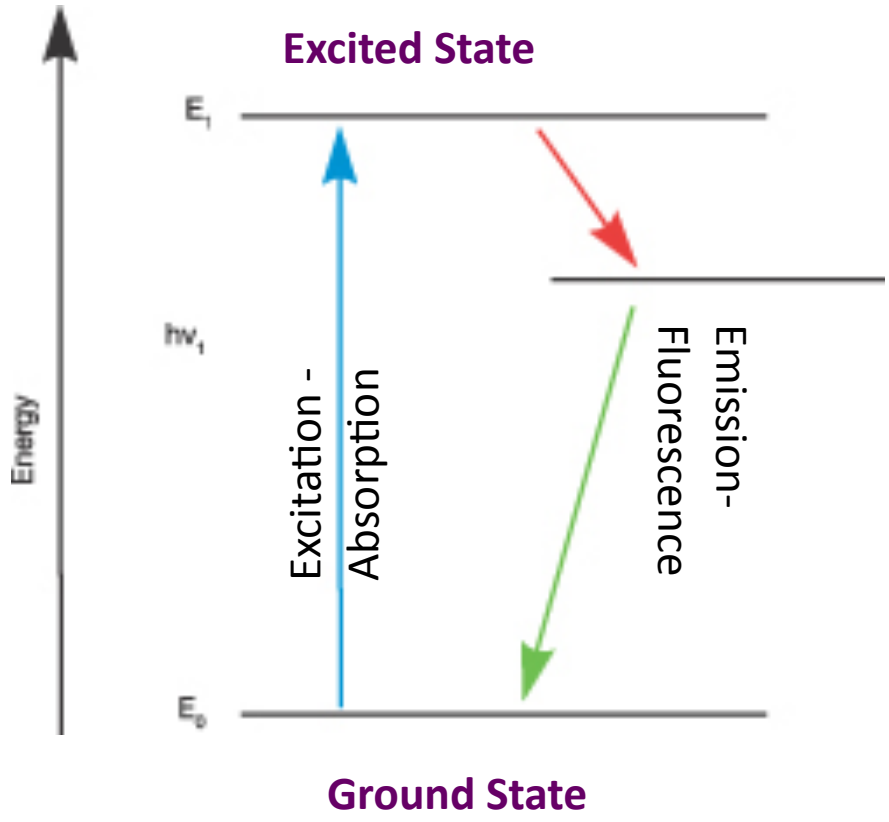
Excited State

Ground State

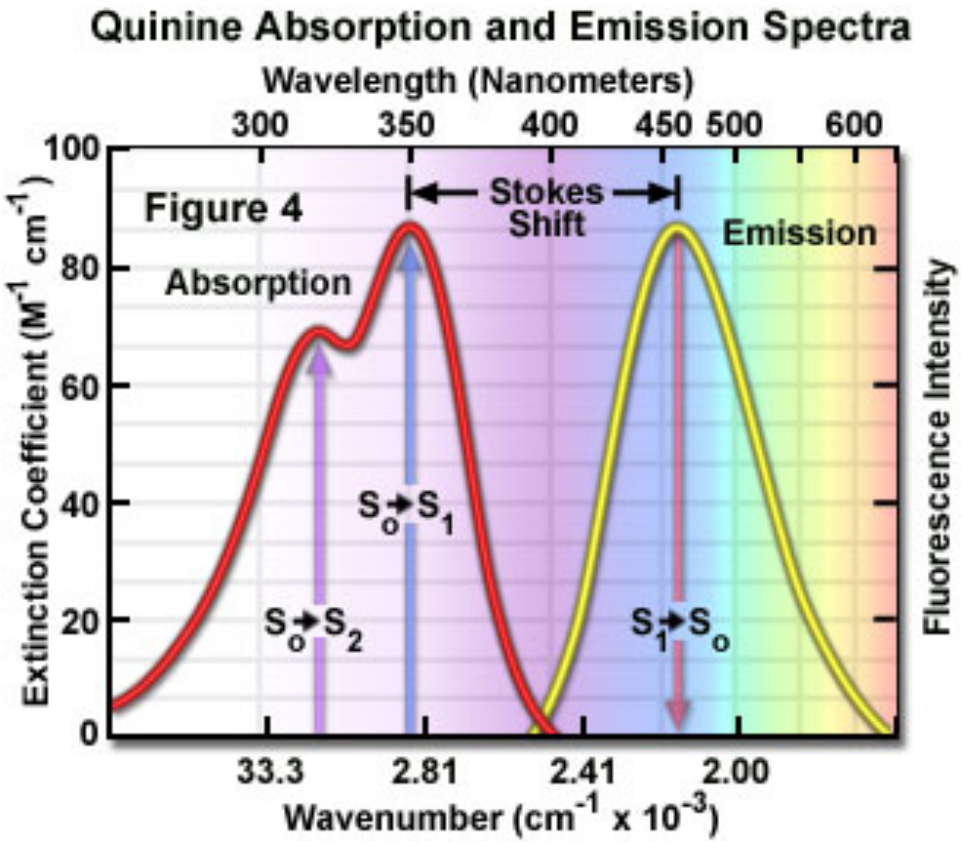
http://www.olympusmicro.com/

Olympus Microscopy Resource Center

Fluorescence



Fluorescence



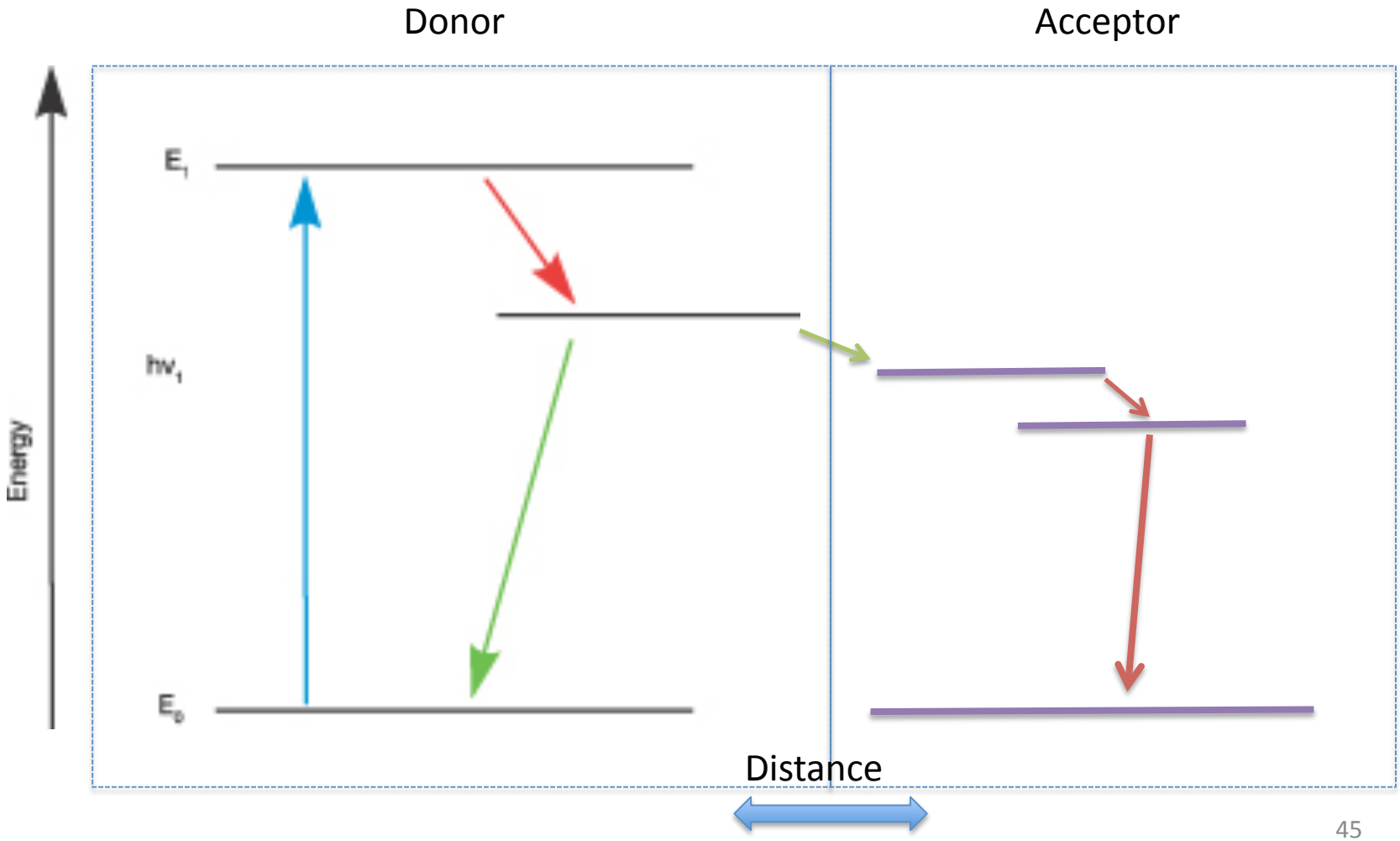
Stokes Shift – Fluorescence Microscopy

Olympus Microscopy Resource Center

Fluorescence

Absorption/Excitation	D+$h\nu 1$	→	D*
Fluorescence	D*	→	D+$h\nu 2$
Energy Transfer	D*+A	→	D+A+$h\nu 3$
Electron Transfer	D*+Q	→	D+Q-
Non-Radiative Decay	D*	→	D+heat

Fluorescence Resonance Energy Transfer (FRET)



Fluorescence Resonance Energy Transfer (FRET) "Spectroscopic Ruler"

$2\ nm < R < 8\ nm$

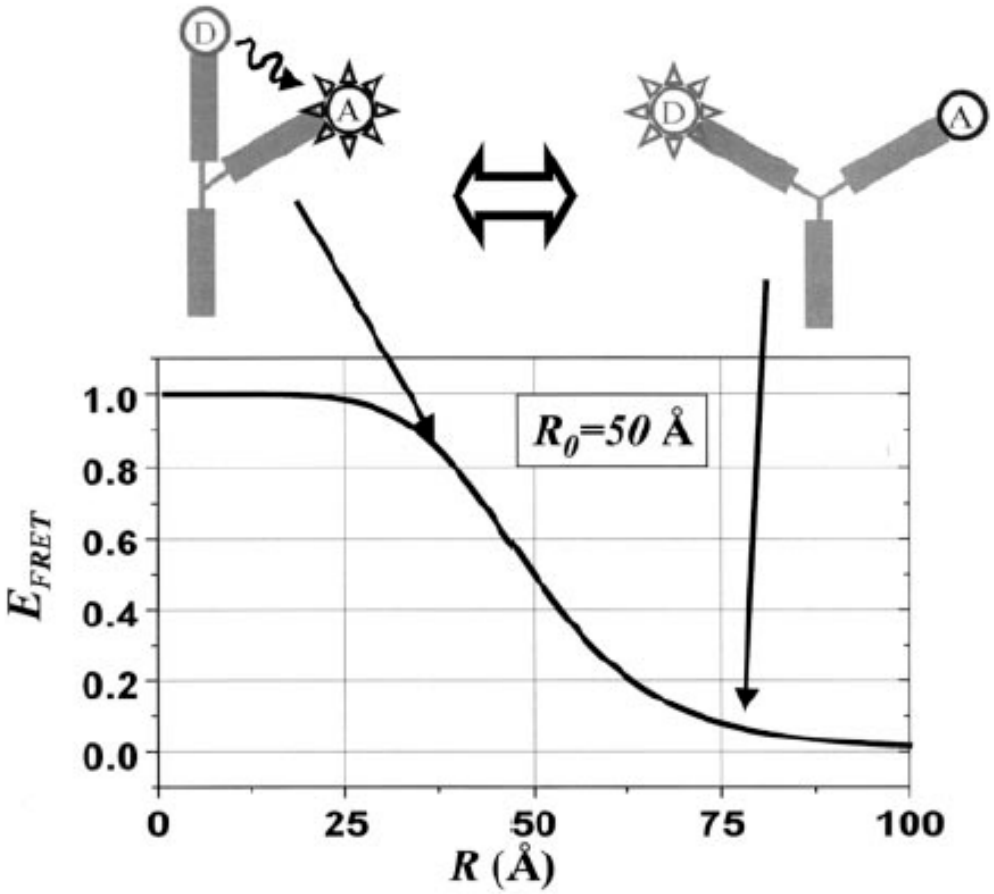
$E_{FRET} = I_A/(I_A+I_D)$

$E_{FRET} = (1+(R/R_0)^6)^{-1}$

$R/R_0 = ((1/E_{FRET})-1)^{1/6}$

R_0(Cy3-Cy5)= 6.3 nm

MEASURE DISTANCE CHANGES OVER SMALL SCALES



Increased Acceptor Signal = Decreased Donor Signal = High Transfer (FRET) Efficiency = Shorter Distance

Fluorescent Molecules:

•**Fluorophores/Fluorochromes/Dye molecules** (Alexa, Atto, CyDye, YO-PRO etc.)
Advantages: Huge selection, small, specific, bright.
Disadvantages: Introduction into cells.

•**Fluorescent Proteins** (Bioluminescence)
Advantages: genetically encoded, growing selection, specificity.
Disadvantages: Stability, size, brightness.

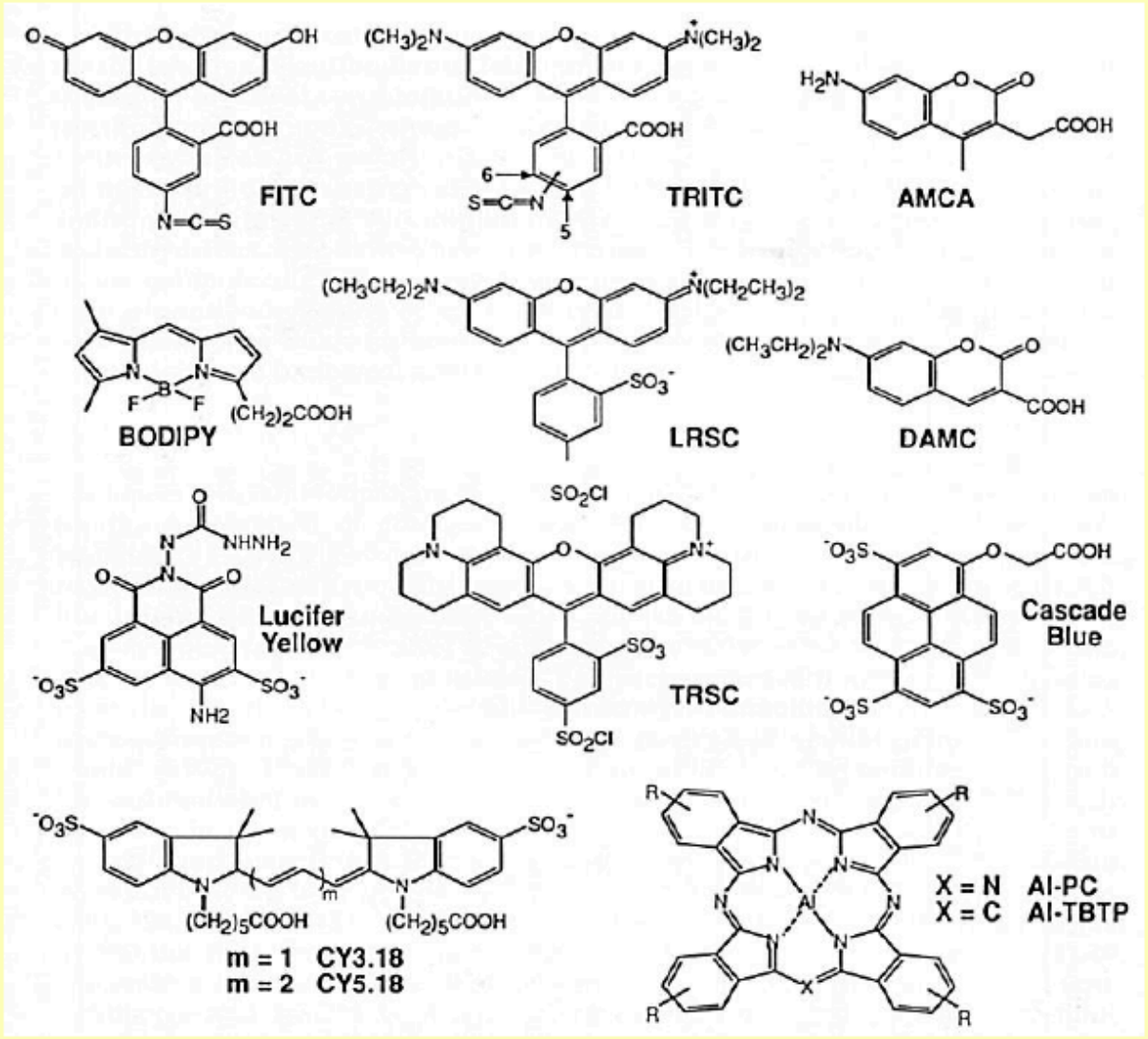
•**Quantum-Dots**
Advantages: extremely bright and stable, wide selection, continuous excitation.
Disadvantages: Very large, hard to conjugate.

TWO IMPORTANT PROPERTIES

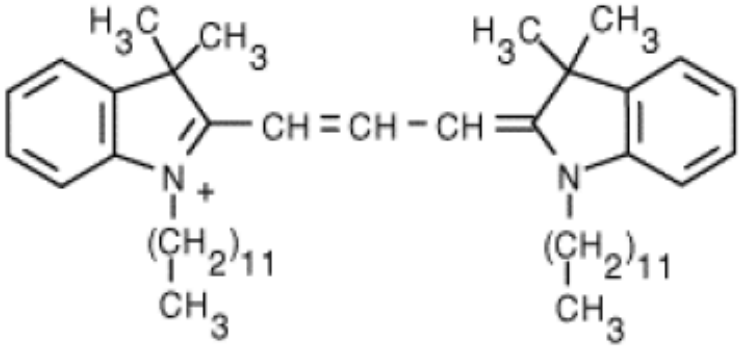
- Quantum efficiency:
  photons emitted/photons absorbed

- Photobleaching efficiency:
  probability of bleaching/photon absorbed

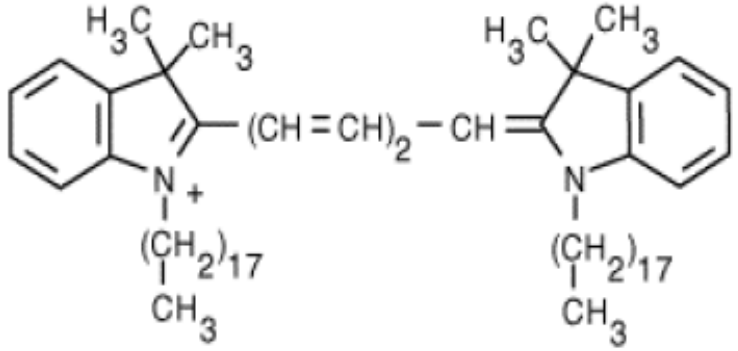
SOME COMMON FLUOROPHORES



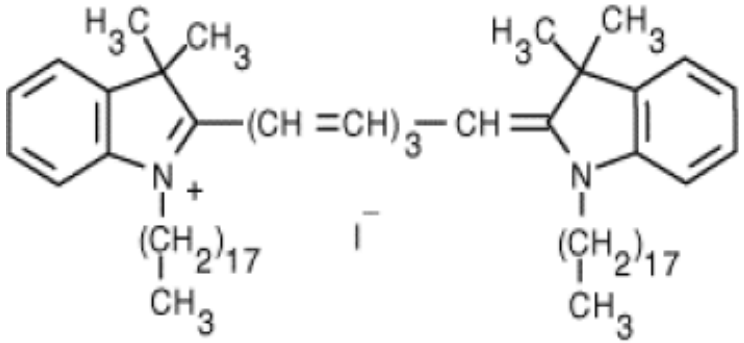
DYES COME IN FAMILIES



Abs = 549nm, Em = 565nm



Abs = 650nm, Em = 670nm



Abs = 748nm, Em = 780nm

THINGS DYES CAN SENSE

- Ca, Mg, Na, pH
- DNA, RNA, together or separately
- Potential (fast and slow dyes)
- Lipid vs aqueous surroundings
- Temperature
- Viscosity
- Each other

Fluorescent Proteins

Tagging with GFP

The available fluorescent proteins:

BFP

ECFP

EGFP

PAGFP

EYFP & related

dsRED

DHCred

Other choices

Where to tag?

N-terminal

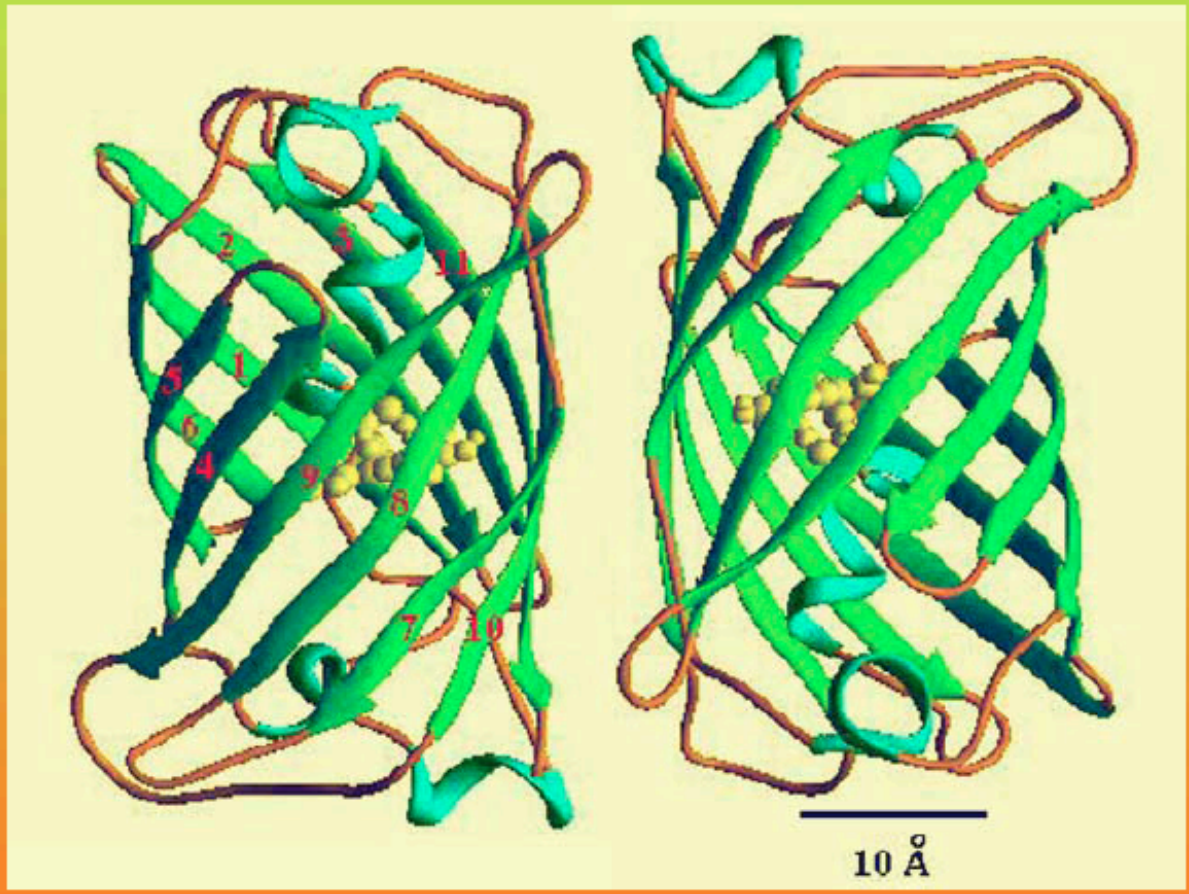
C-terminal

Localization signals

Other considerations:

Localization

function



Fluorescent Proteins

Tagging with GFP

The available fluorescent proteins:

BFP

ECFP

EGFP

PAGFP

EYFP & related dsRED

DHCred

Other choices

Where to tag?

N-terminal

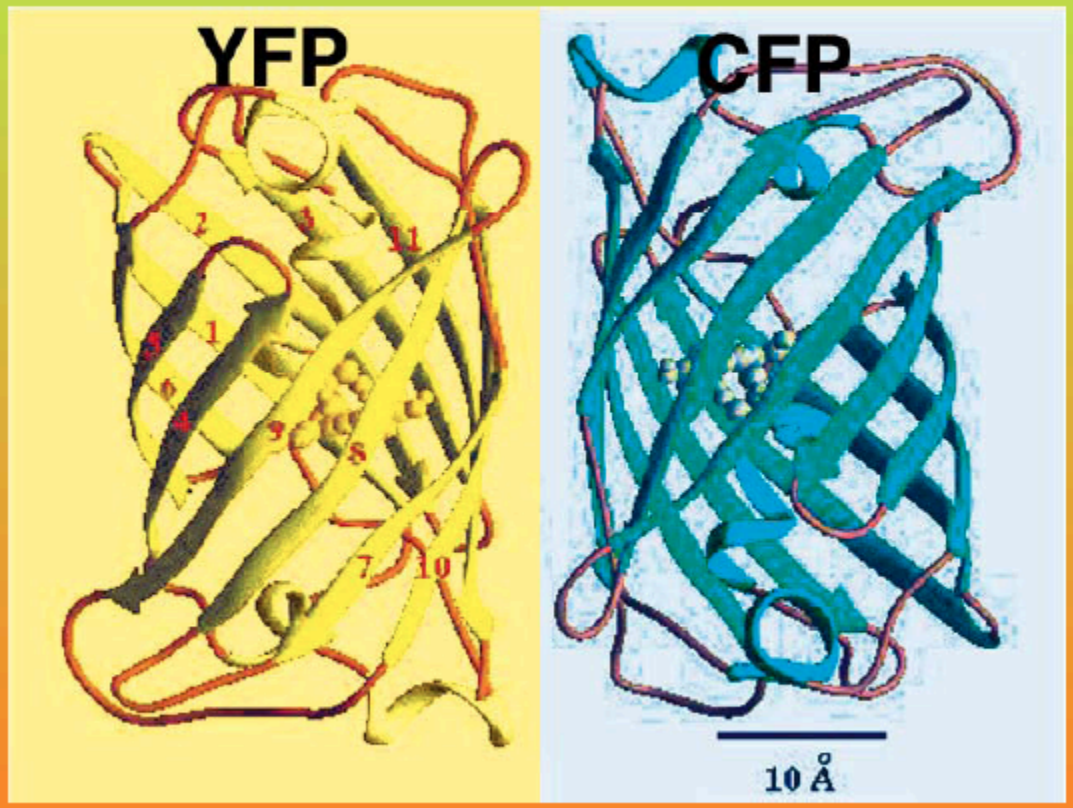
C-terminal

Localization signals

Other considerations:

Localization

function



Fluorescent Proteins

Tagging with GFP

The available fluorescent proteins:

BFP

ECFP

EGFP

PAGFP

EYFP & related

dsRED

DHCred

Other choices

Where to tag?

N-terminal

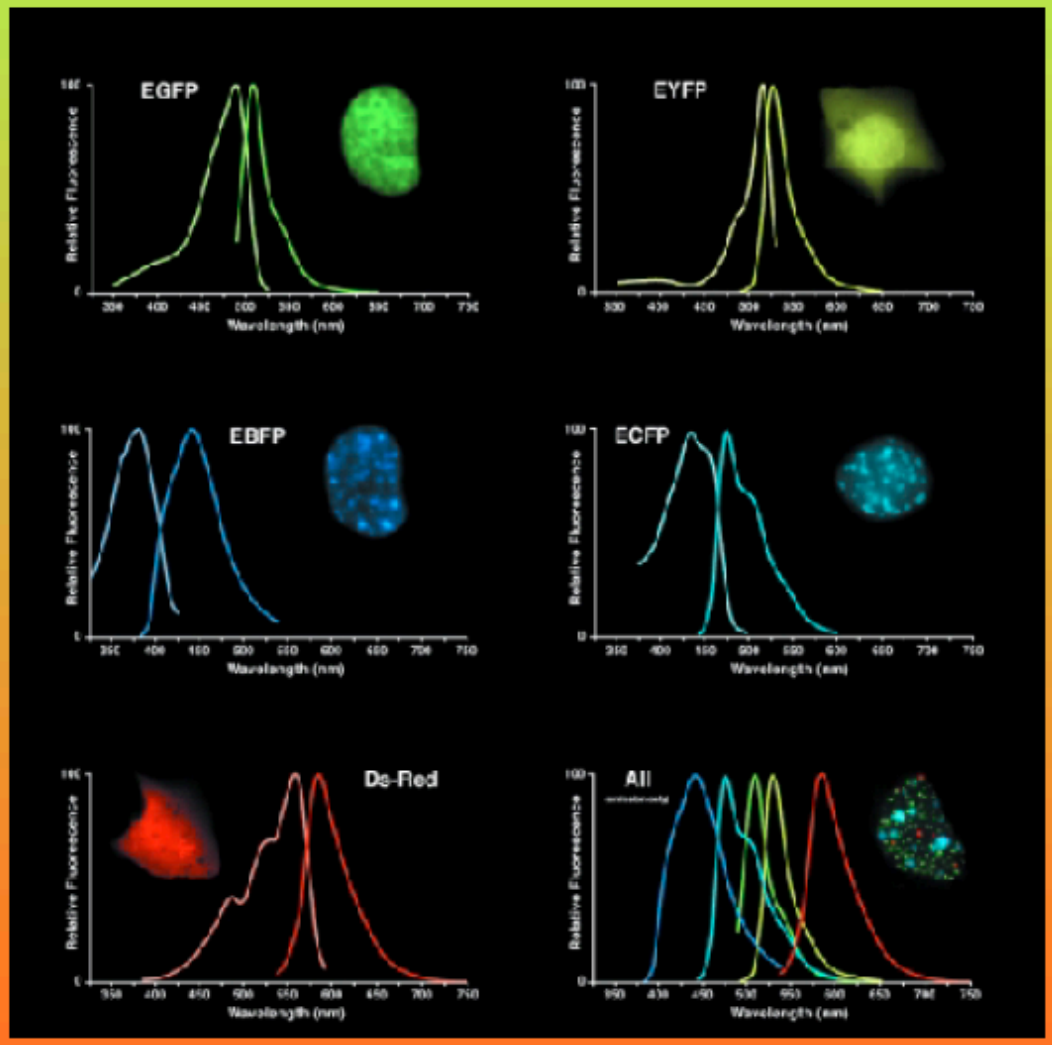
C-terminal

Localization signals

Other considerations:

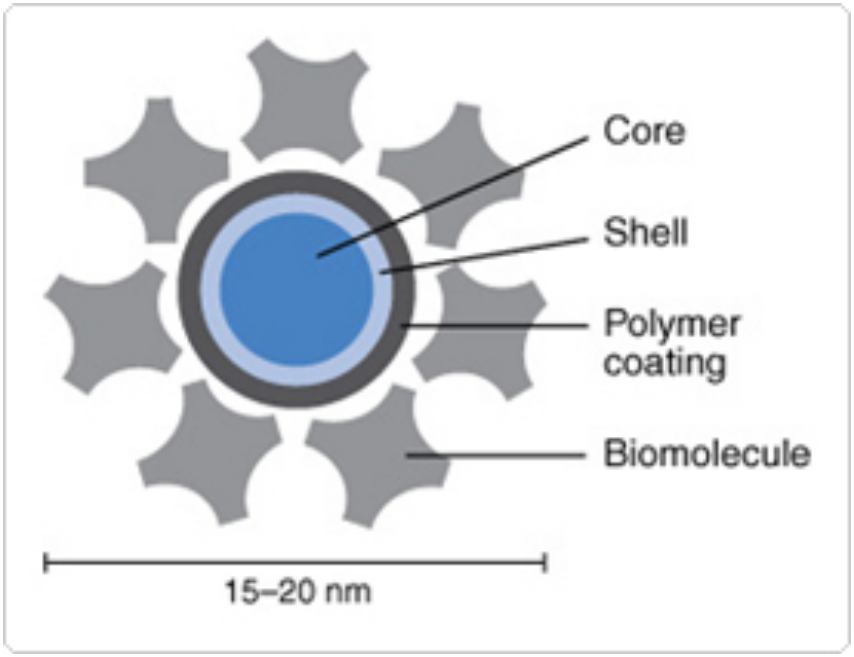
Localization

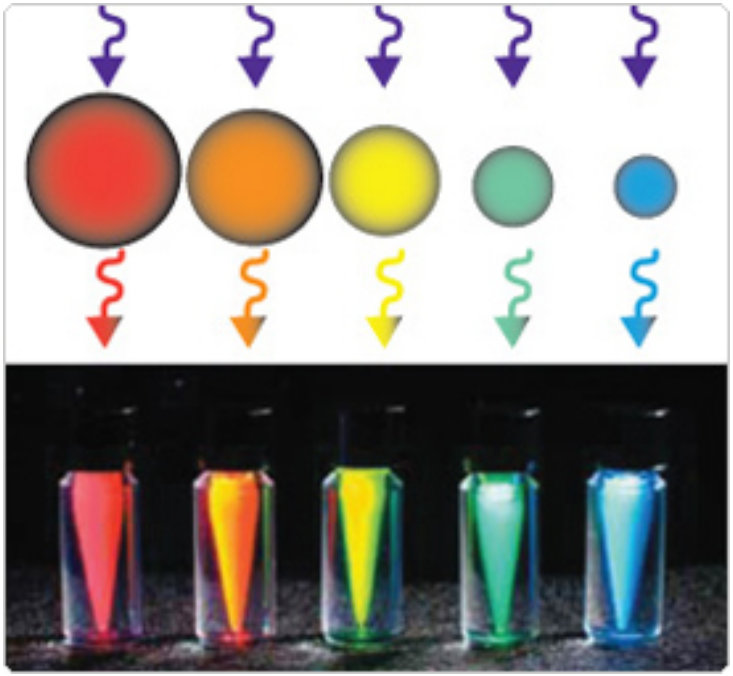
function



NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Quantum-Dots

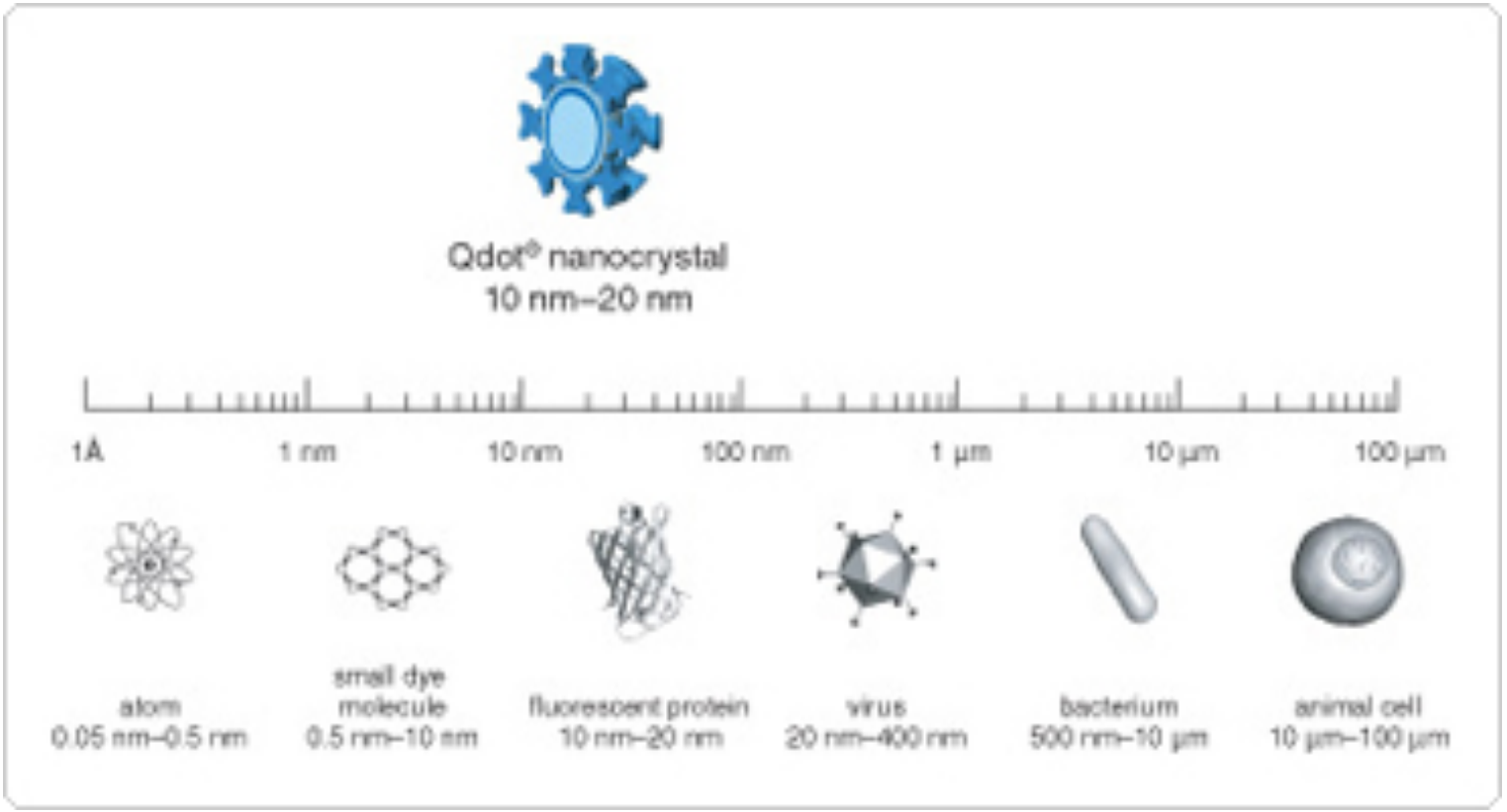




Core-Shell Semiconductor Nanocrystals (CdSe/ZnS)
Quantum-Confinement: Absorption and Emission is dependent upon size.
Due to the number of atoms- Many energy levels=continuous absorption/excitation.

NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Quantum-Dots



Labeling Biological Targets

Fixed Cells

Fluorescent In-Situ Hybridization (FISH)
uses target (epitope) specific antibody
(primary) to mark the target, and another
fluorescently labeled antibody (secondary)
which is specific against the first antibody

Live- Cells

Fluorescent-Proteins.

Genetically coded tags.

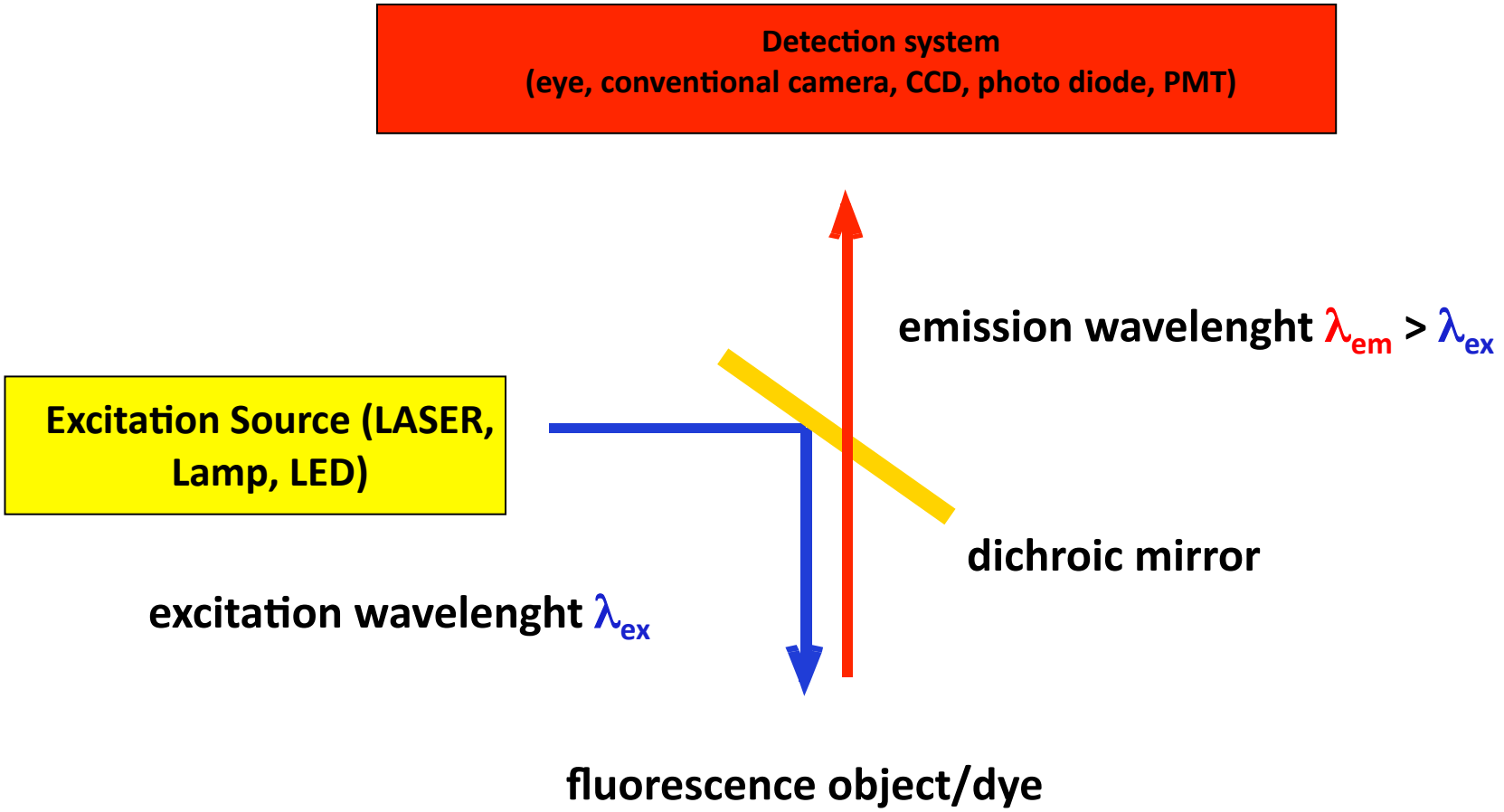
Cellular deliver of of dyes: Membrane permeable
Chemically/electrically induced cell uptake.
Microinjection

Chemically specific Dyes.
Membrane Dyes, DNA, RNA intercalating dyes,
mitochondria dyes.

INSTRUMENTATION

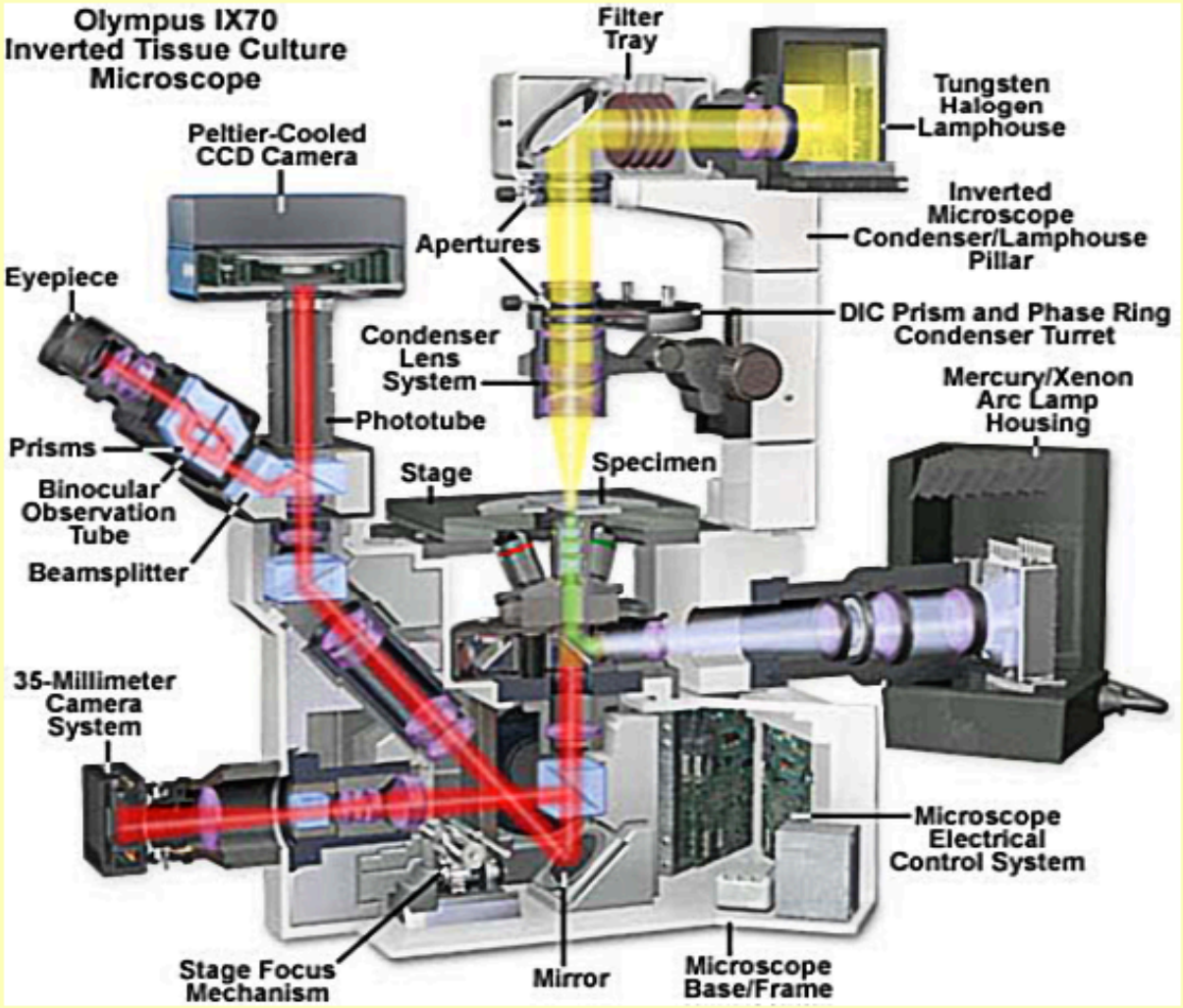
- Excitation sources
- Filter sets
- Objectives
- Detection

Fluorescence Microscopy



Excitation: Shine Light at one wavelength and get fluorescence at another (lower energy)

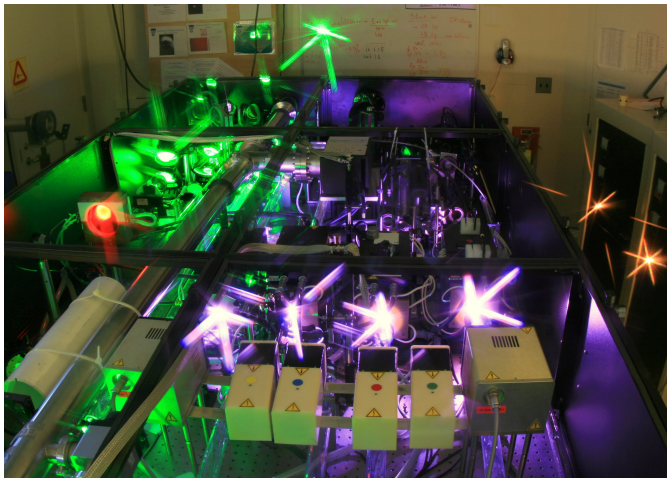
INVERTED MICROSCOPE



NYU School of Medicine
NYU LANGONE MEDICAL CENTER

Fluorescence Microscopy

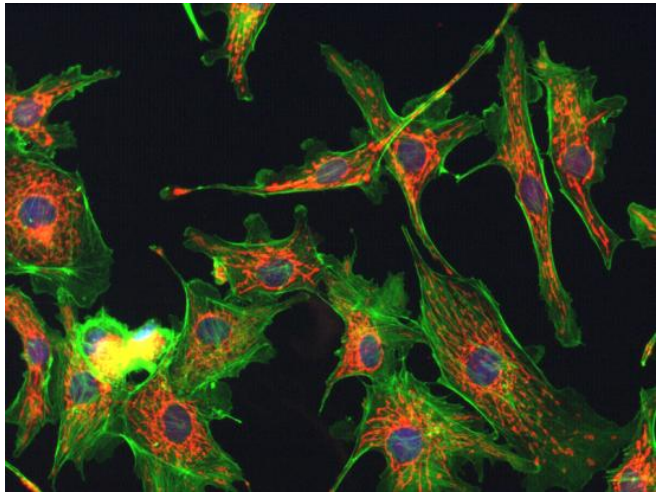
Excitation Source: Lasers, LEDs (narrow wavelength), Lamps



Fluorophores (fluorescent molecules):

Quantum-Yield (probability of absorbed photon resulting in an emitted photon)

Absorption cross section (probability of absorbing a photon or how many photons we need to excite the molecule)



Excitation Sources

<u>**Arc lamps**</u>

Xenon
Mercury

<u>**Laser types**</u>

UV  **IR**

Argon	**351 364 457 477 488 514**
Blue diode	**405 440**
Helium-Cadmium	**354 442**
Krypton-Argon	**488 569 647**
Green Helium-Neon	**543**
Yellow Helium-Neon	**594**
Orange Helium-Neon	**612**
Red Helium-Neon	**633**
Red diode	**635 650**
Ti:Sapphire	**720-980**

FILTER CUBES

Figure 6

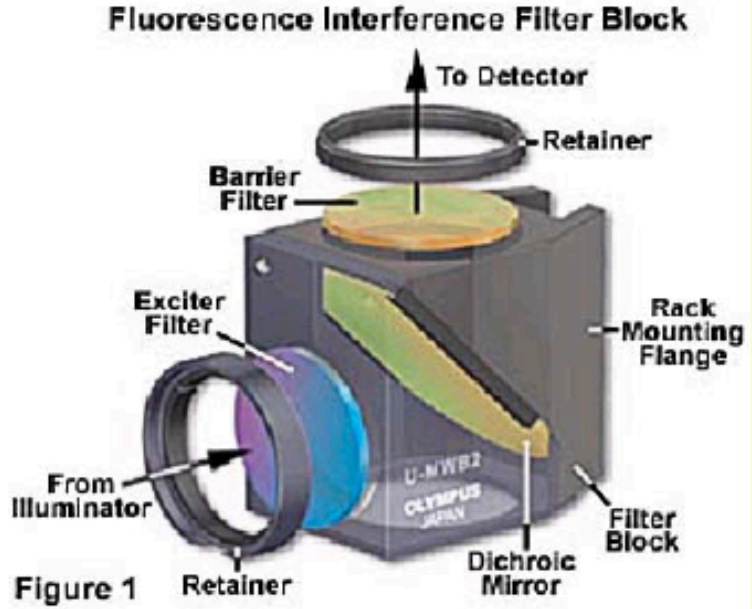
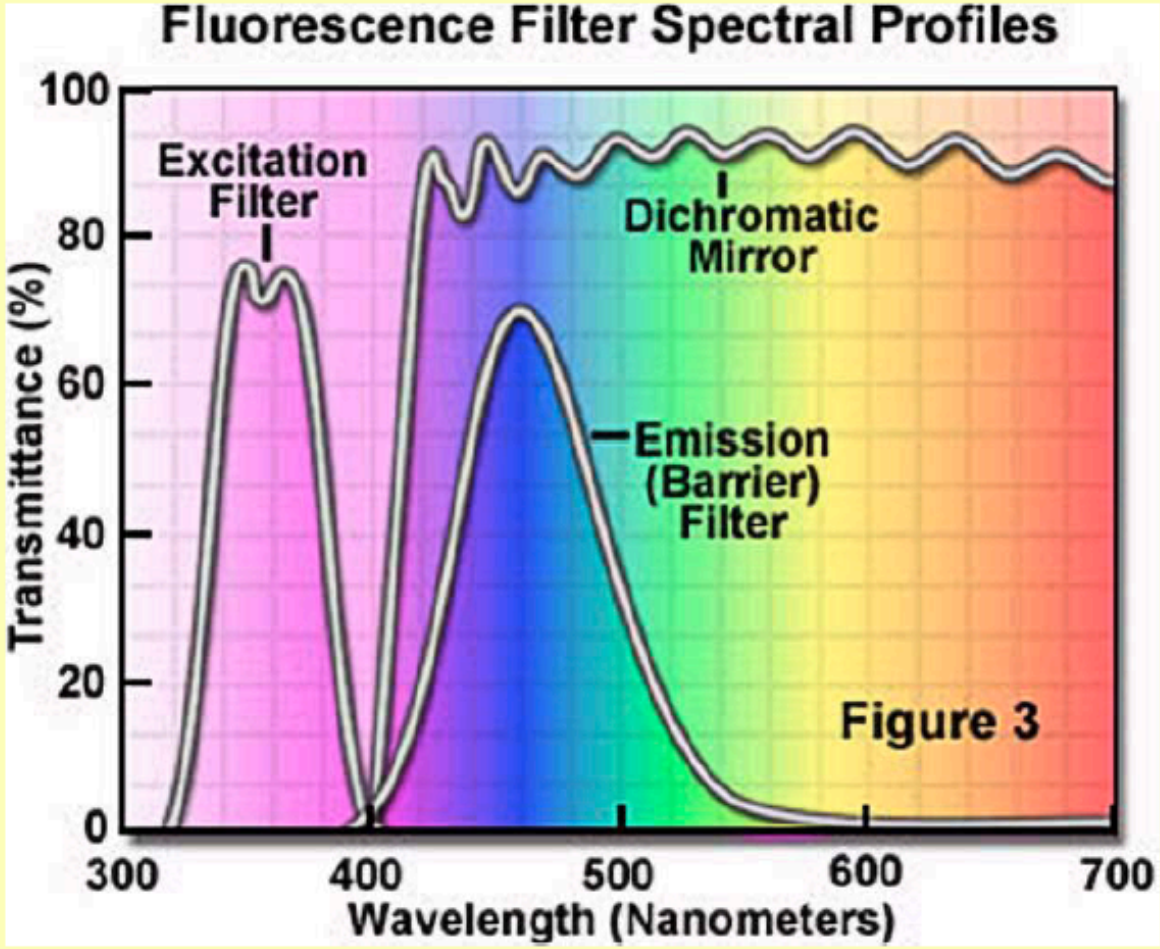


Figure 1

FILTER TRANSMISSION CURVES

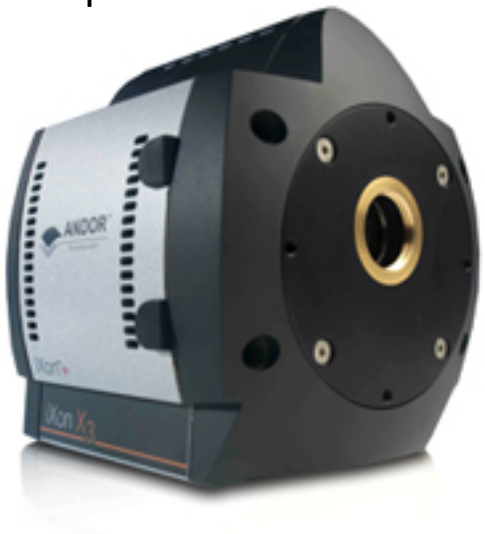


Fluorescence Microscopy

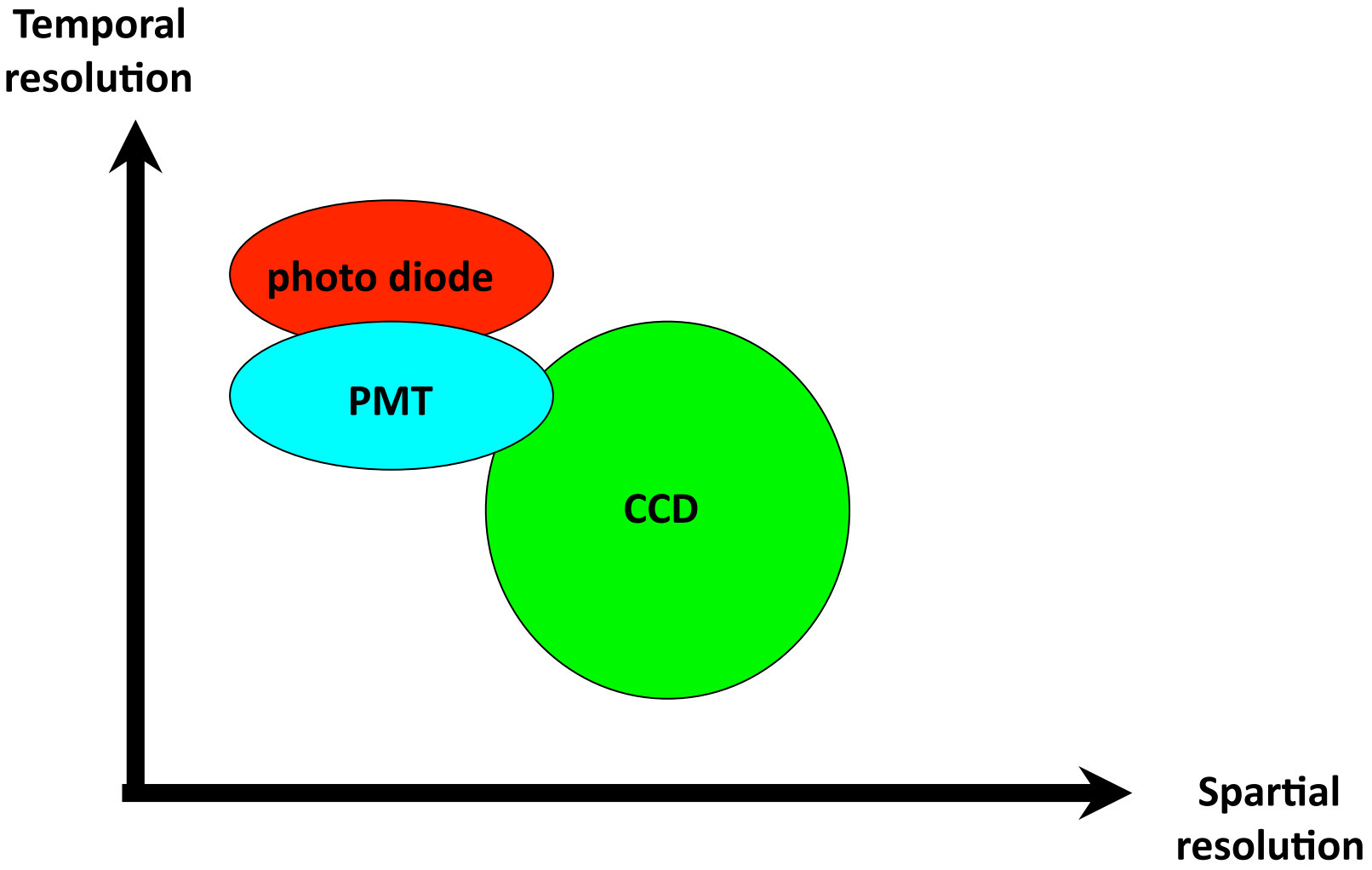
Microscope Objective
(collecting the fluorescence from sample):
-High magnification (63x, 100x)
-High Numerical Aperture (N.A. > 1.4)

Detectors
EMCCD camera
(electron multiplying Charge-Couple Device):

-High Quantum Efficiency (QE) – sensitivity to light
-Fast Acquisition rates



Fluorescence detector systems ...



OPTIMIZE DETECTION

Improve QE

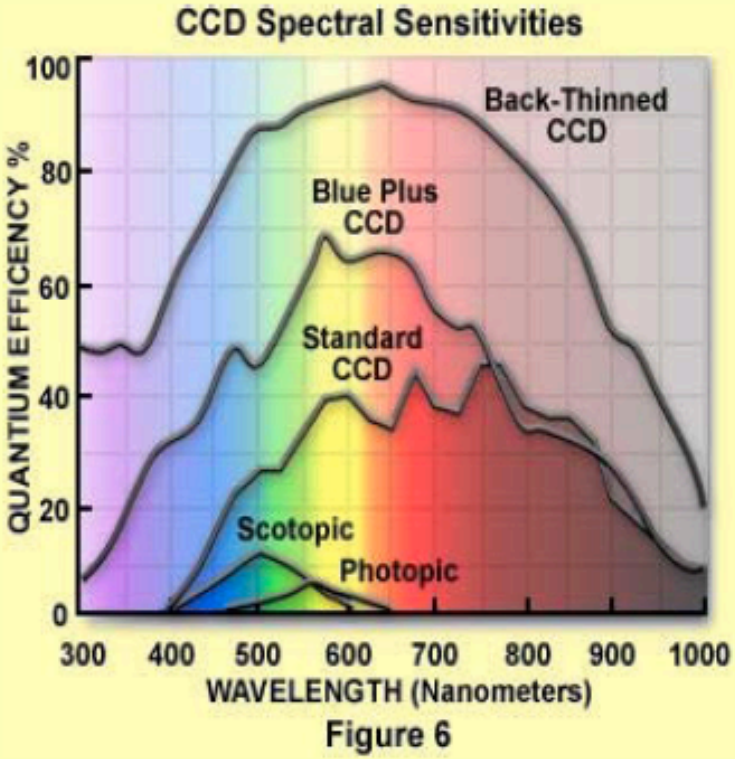
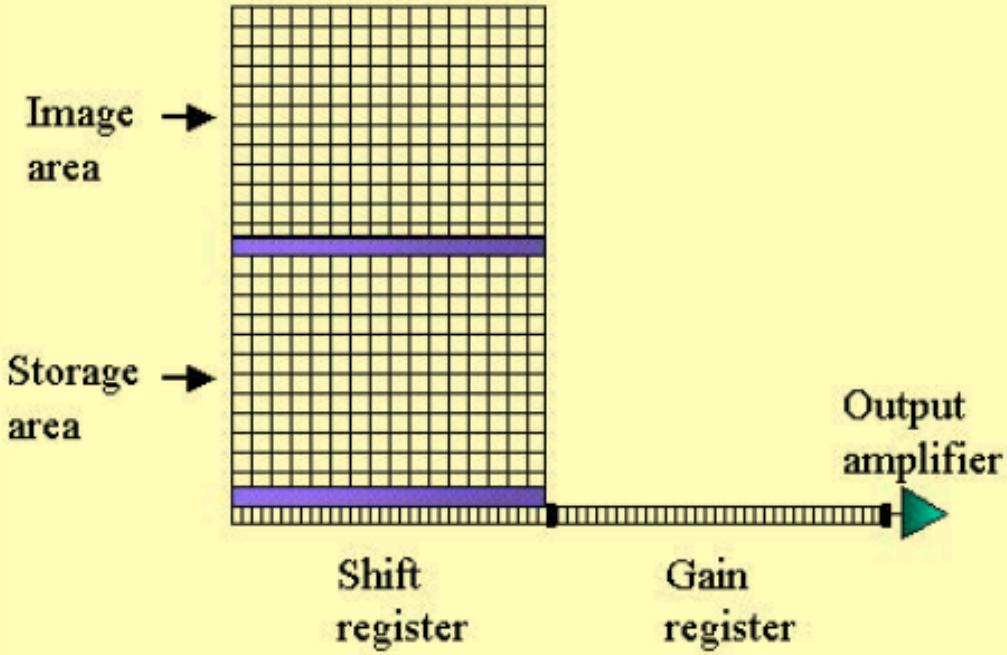


Figure 6

Reduce readout noise



Disadvantageous of widefield microscopy

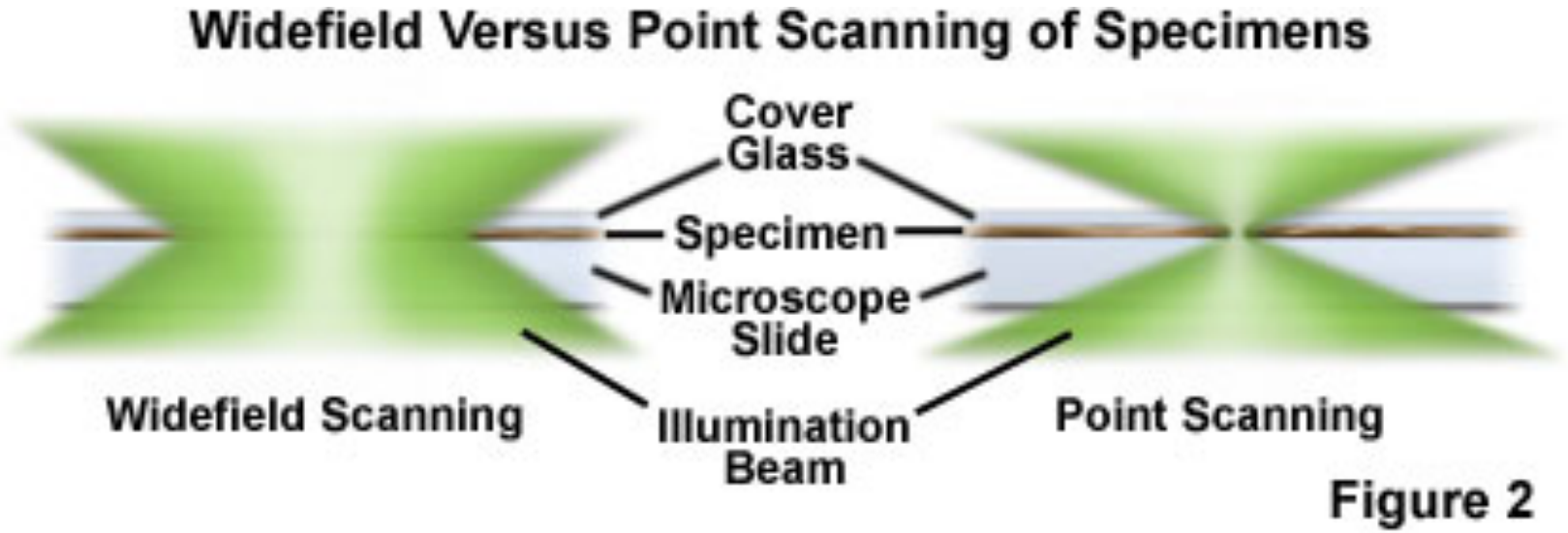


Figure 2

EFFECT OF NA ON RESOLUTION