

Introduction to imaging: Laser camera Action!



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Various modes of imaging:

Using EM waves

Using electrons

Using near field probes

SEM



Optical microscopes

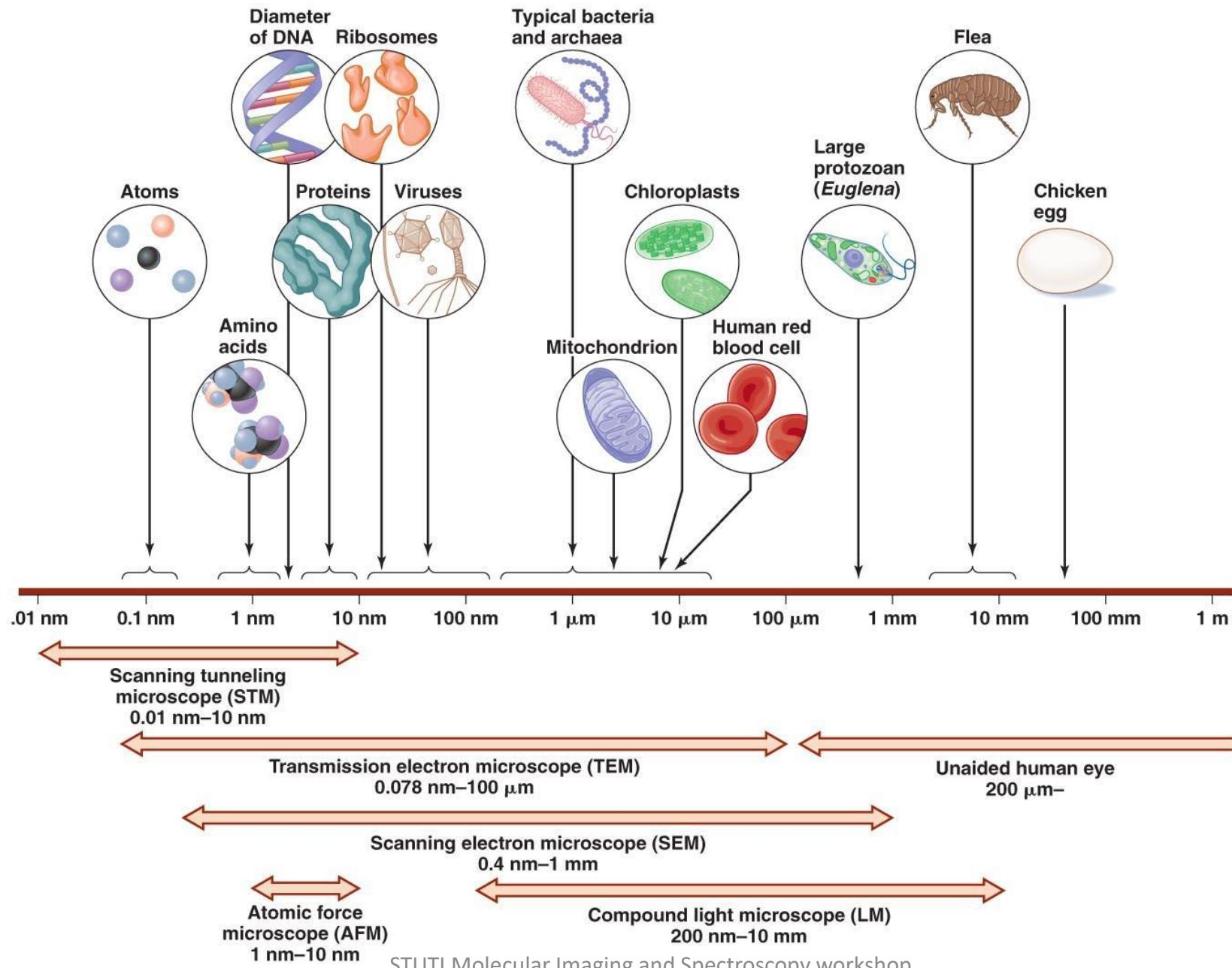


AFM



TEM





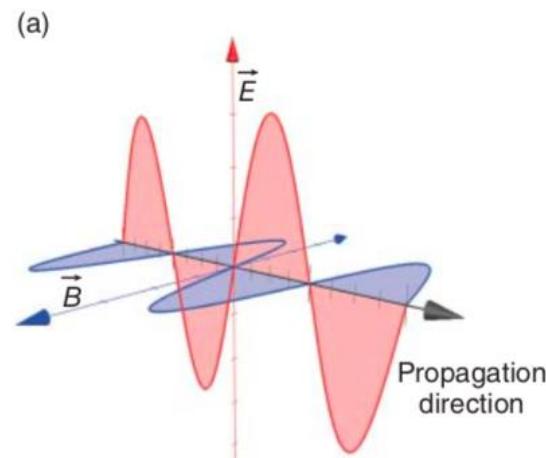


Figure 1.3 Sketch of a linearly polarized electromagnetic wave. (a) Wave with electric and magnetic field components, \vec{E} and \vec{B} . (b) Temporal oscillation at a fixed place in space. (c) Still image of the wave.

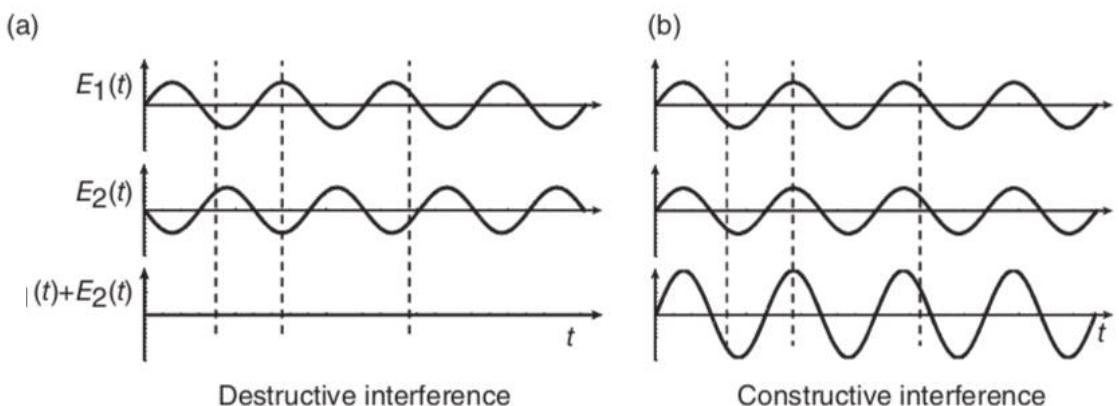
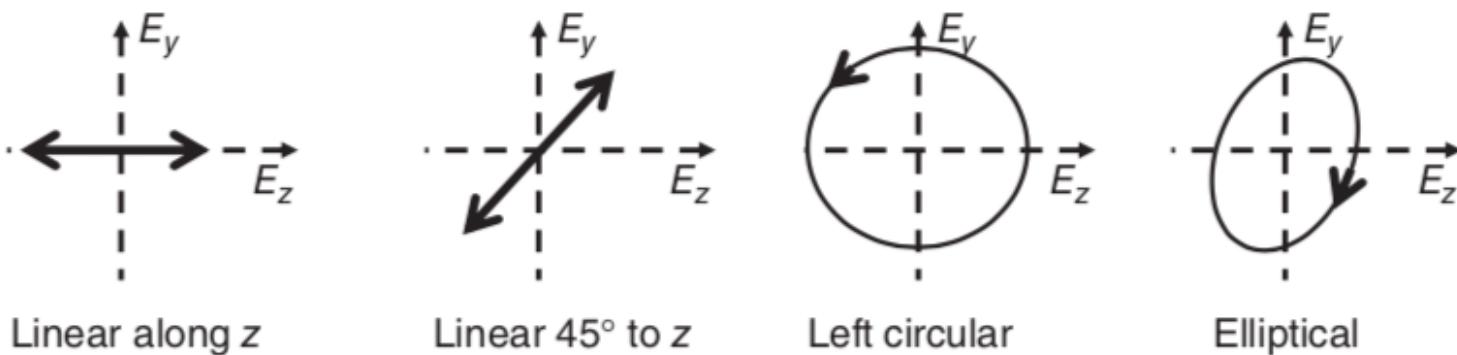
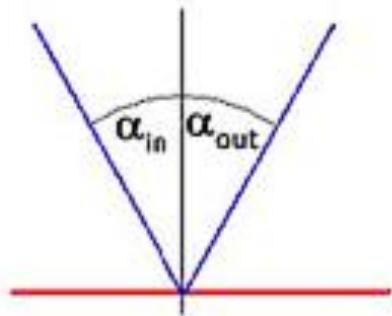


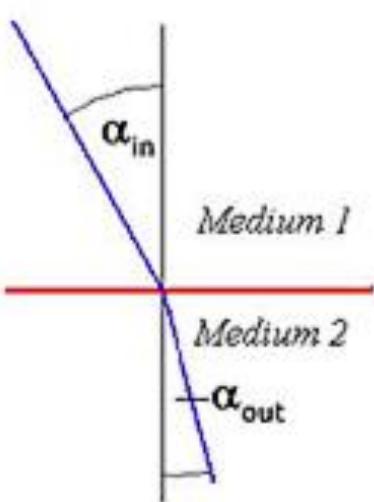
Figure 1.2 Destructive and constructive interference. (a) Two waves cancel each other if the electric fields of two interfering light waves $E_1(t)$ and $E_2(t)$ are always of opposite value, that is, they have a phase shift of π . (b) Constructive interference occurs if the two waves are completely in phase.



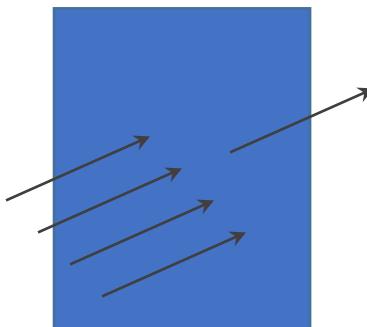
Reflection



Transmission

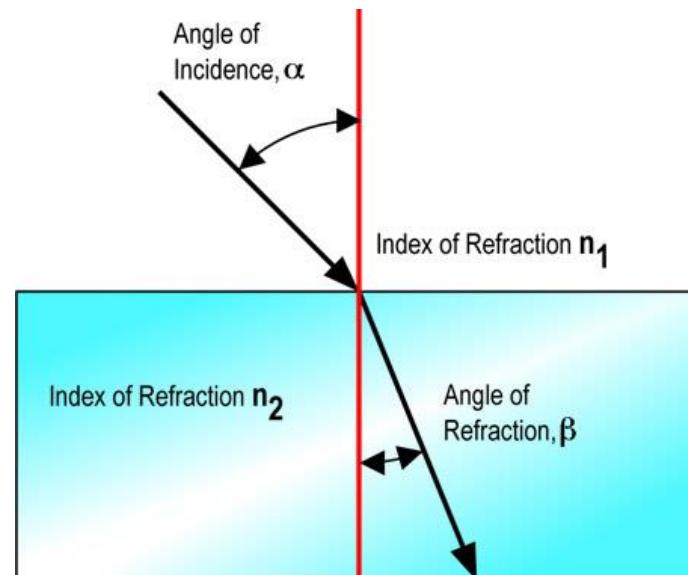


Absorption



Refraction:

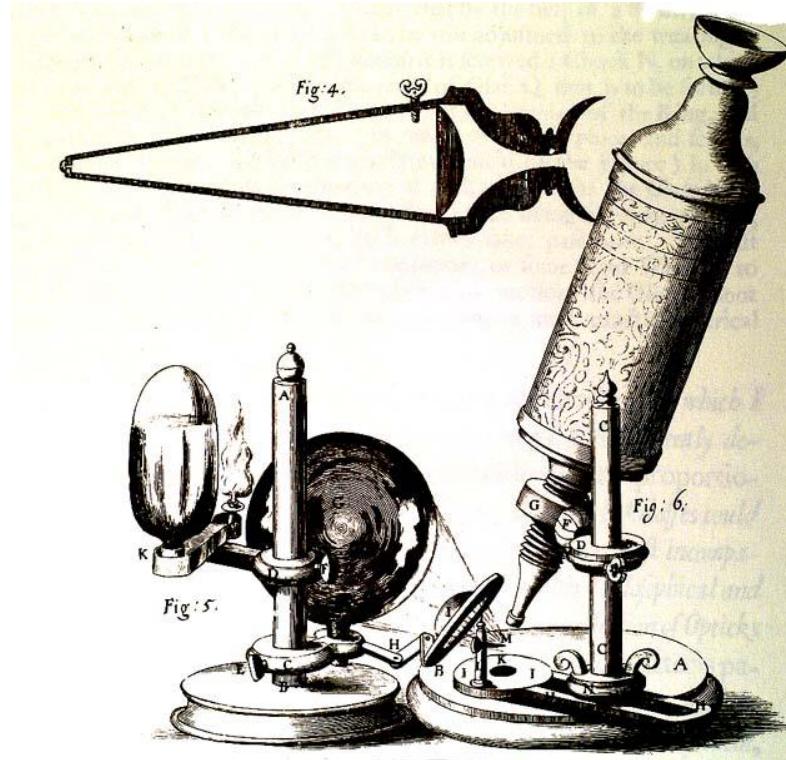
light travels at different speeds through different media (ex = air, water, glass)



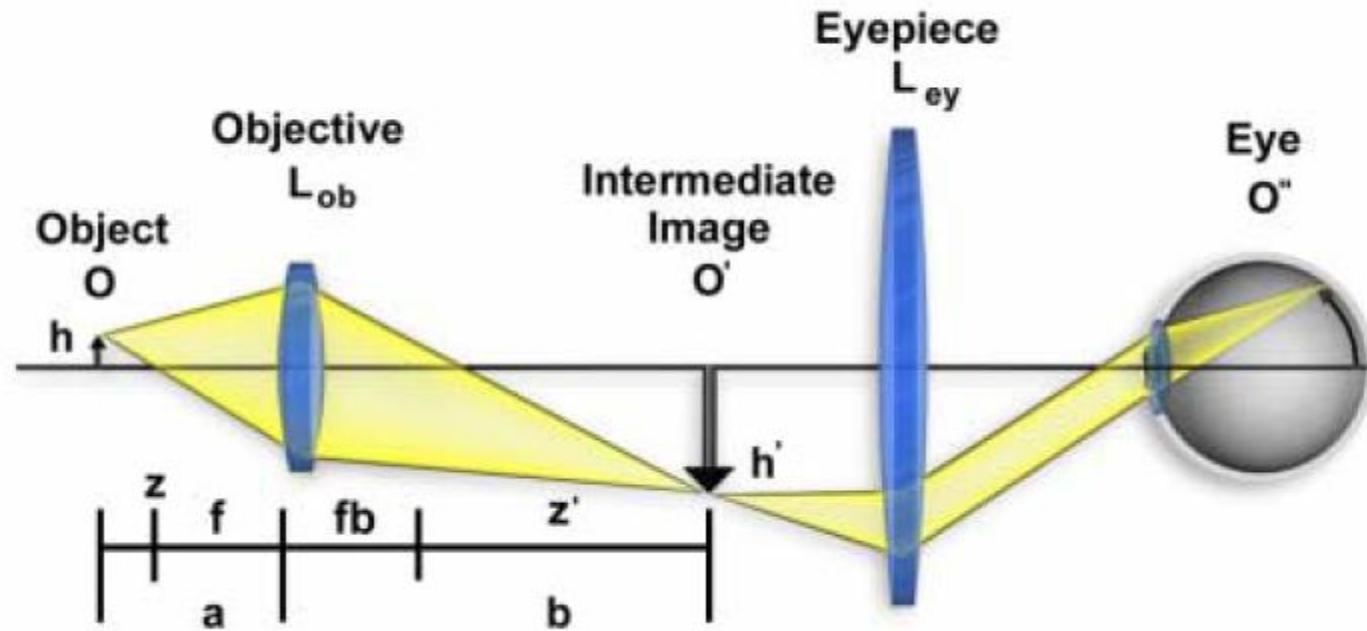
$$n_1 \sin \alpha = n_2 \sin \beta$$

17th Century

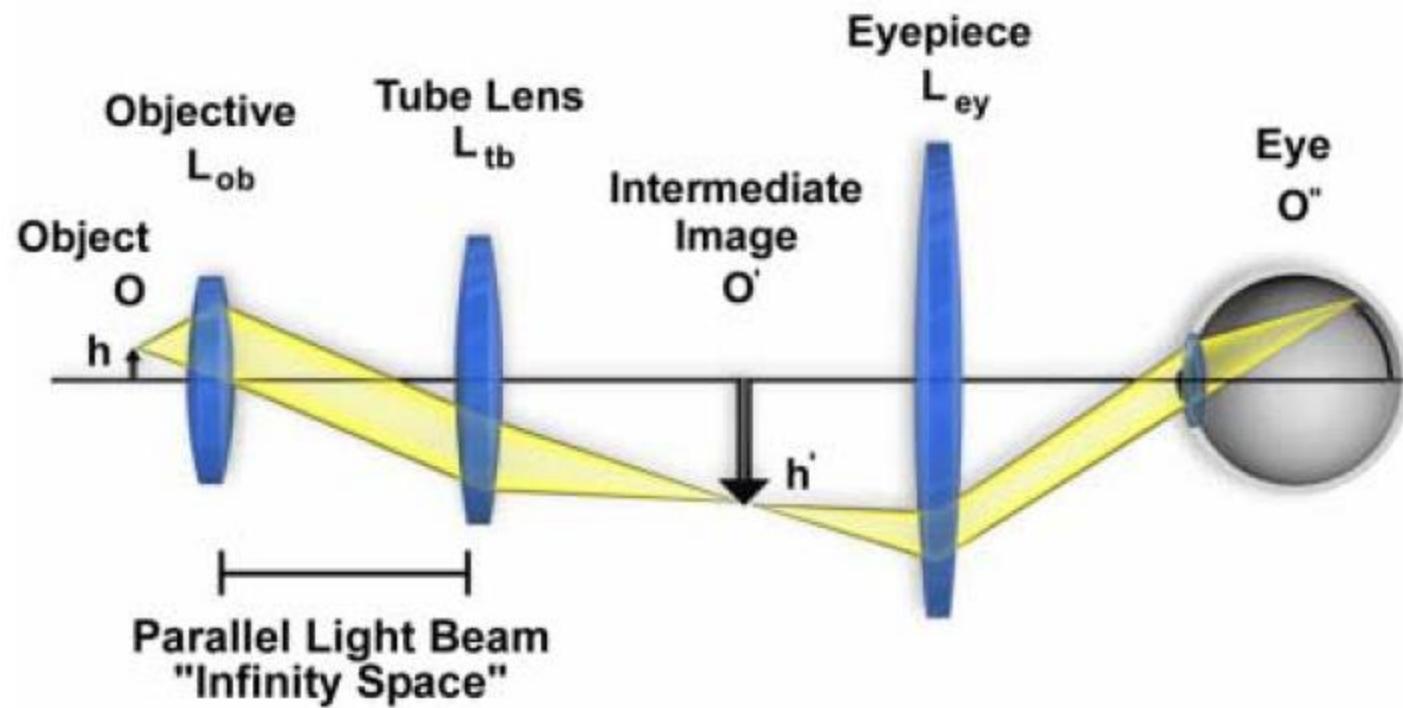
- Robert Hooke's microscope, published in 1665, is considered to be the progenitor of the modern compound microscope. Parts were made of wood and brass, with tubes of leather covered pasteboard.



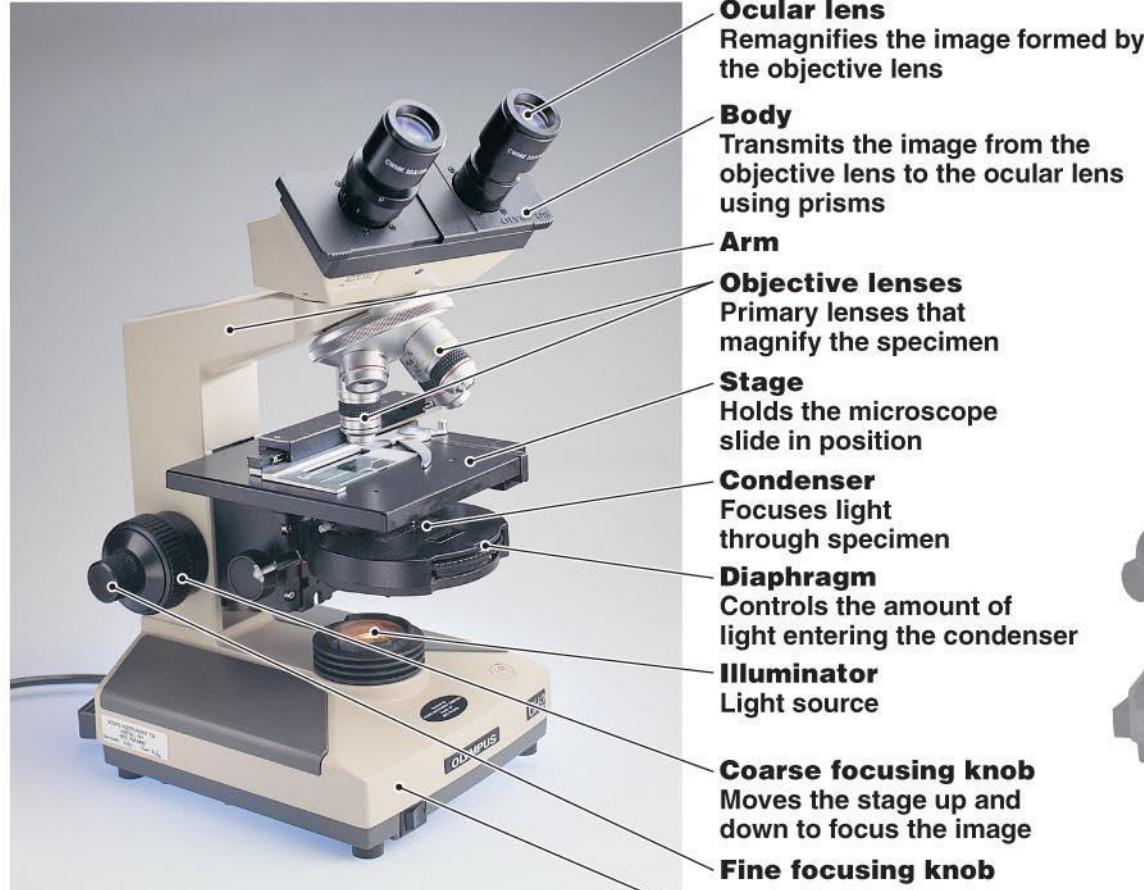
Finite-Tube Length Microscope Ray Paths



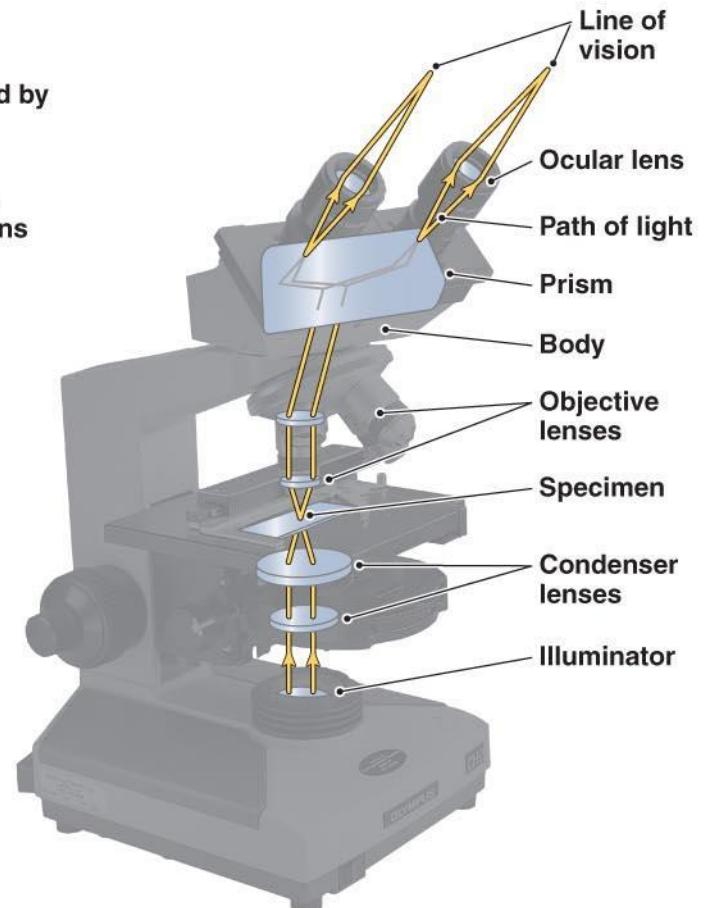
Infinity-Corrected Microscope Ray Paths



A bright-field, compound light microscope



(a)



(b)

Aberration Corrections

Reduce the resolution of microscope

Two primary causes of non-ideal lens action:

- Spherical (geometrical) aberration – related to the spherical nature of the lens
- Chromatic aberration – arise from variations in the refractive indices of the wide range of frequencies in visible light

Spherical Aberration
(Monochromatic Light)

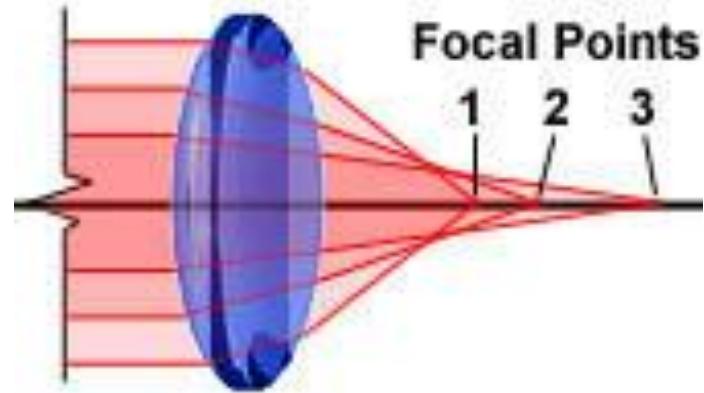
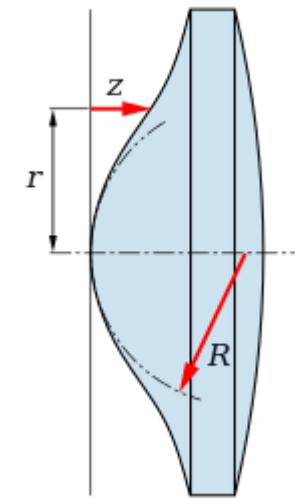


Figure 2

Aspheric lens



Chromatic Aberration

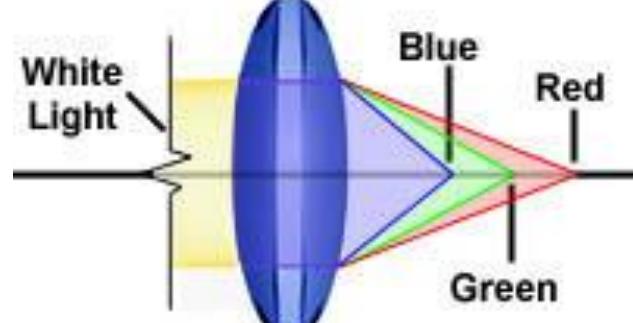


Figure 3

Achromat Doublet

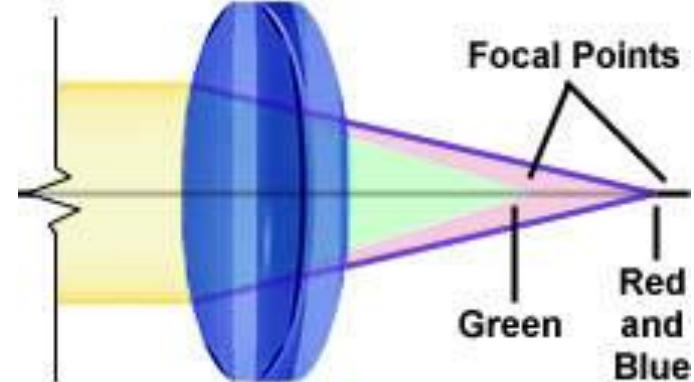
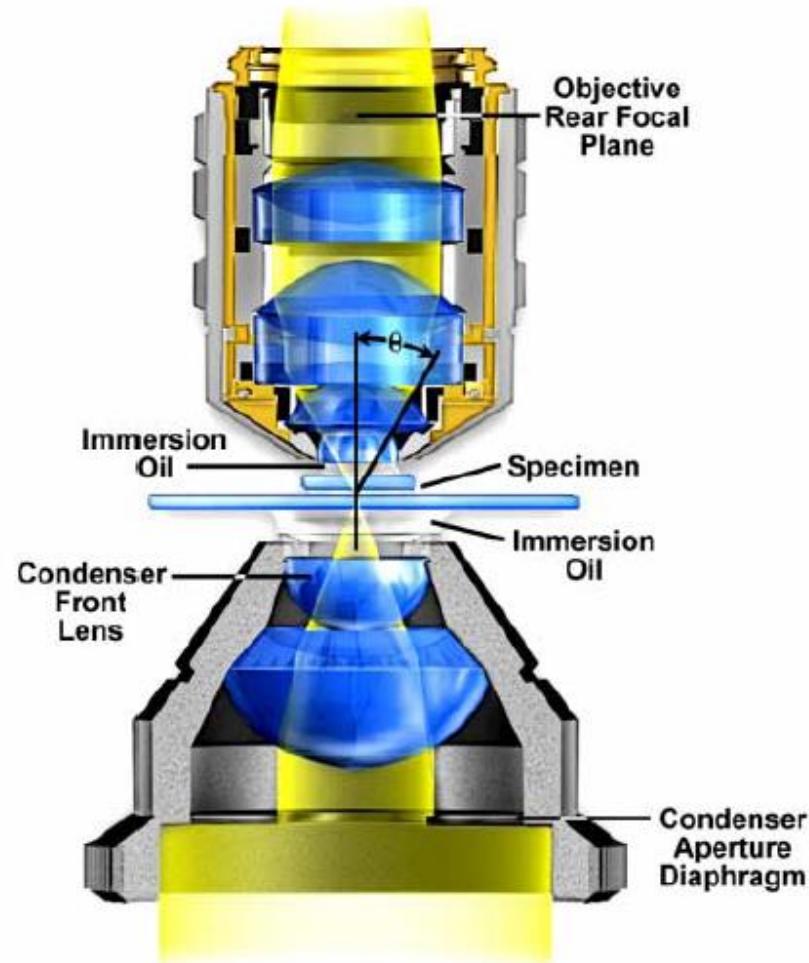
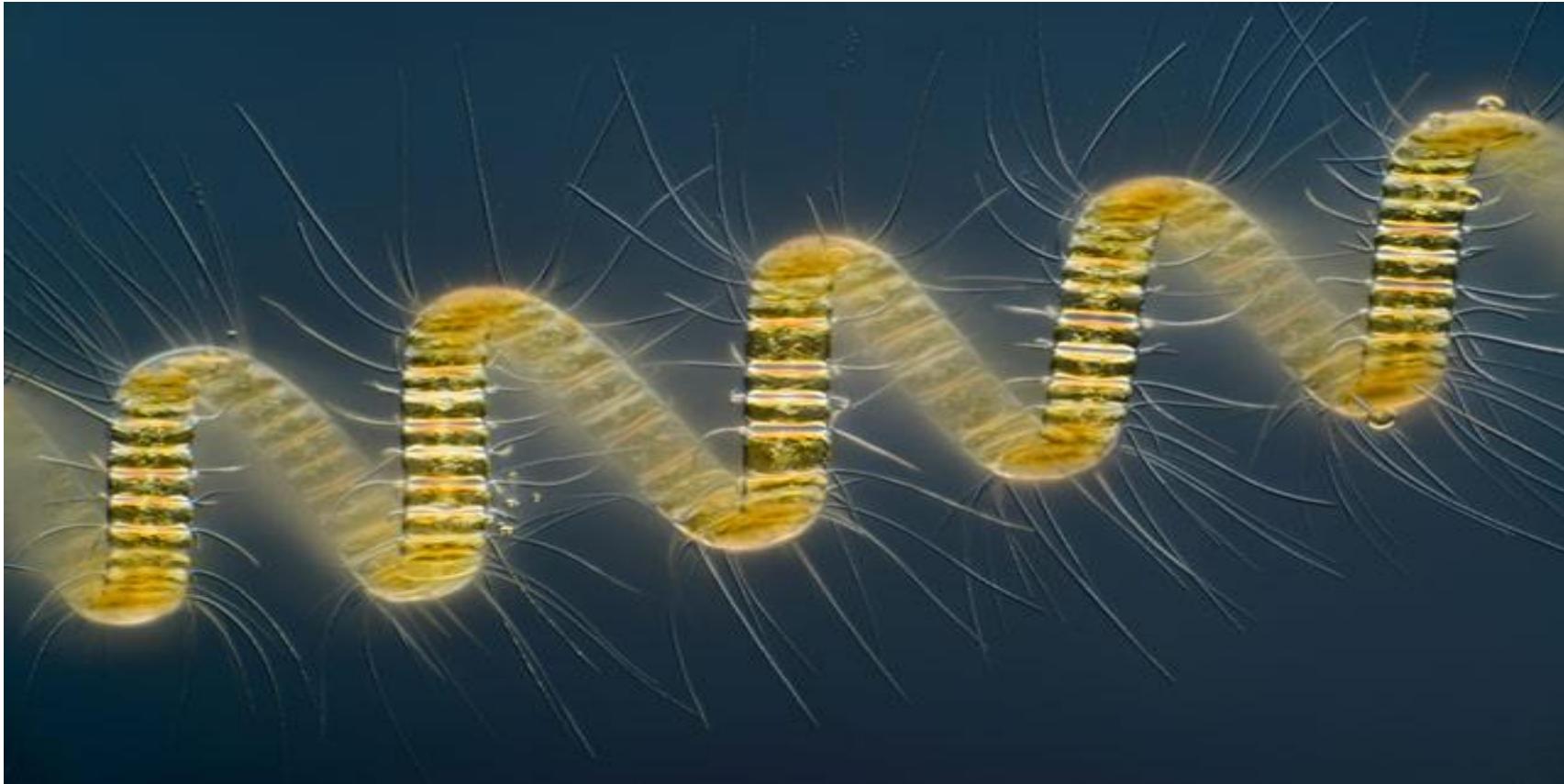


Figure 4

Abbe Condenser/Objective Combination

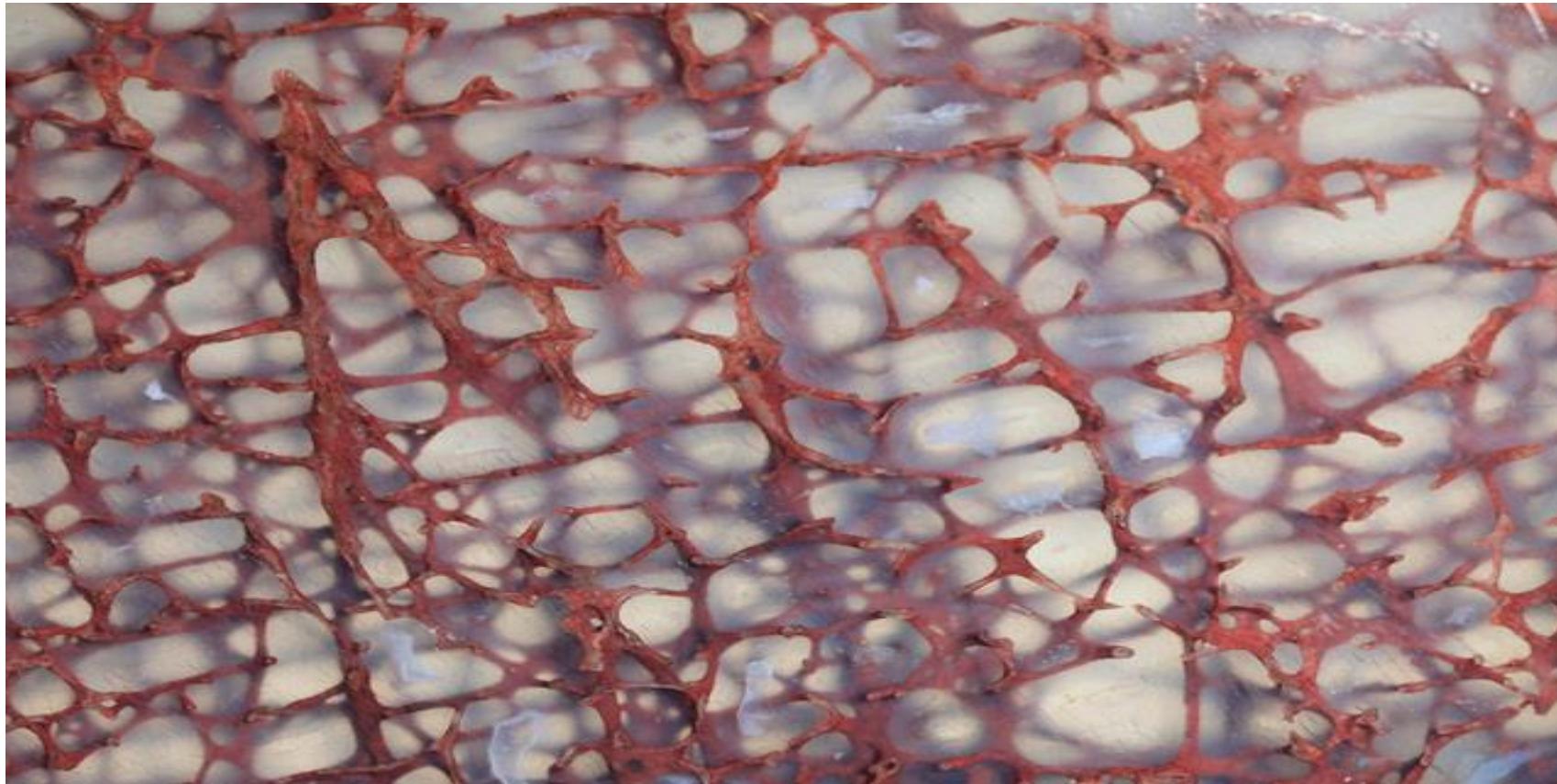




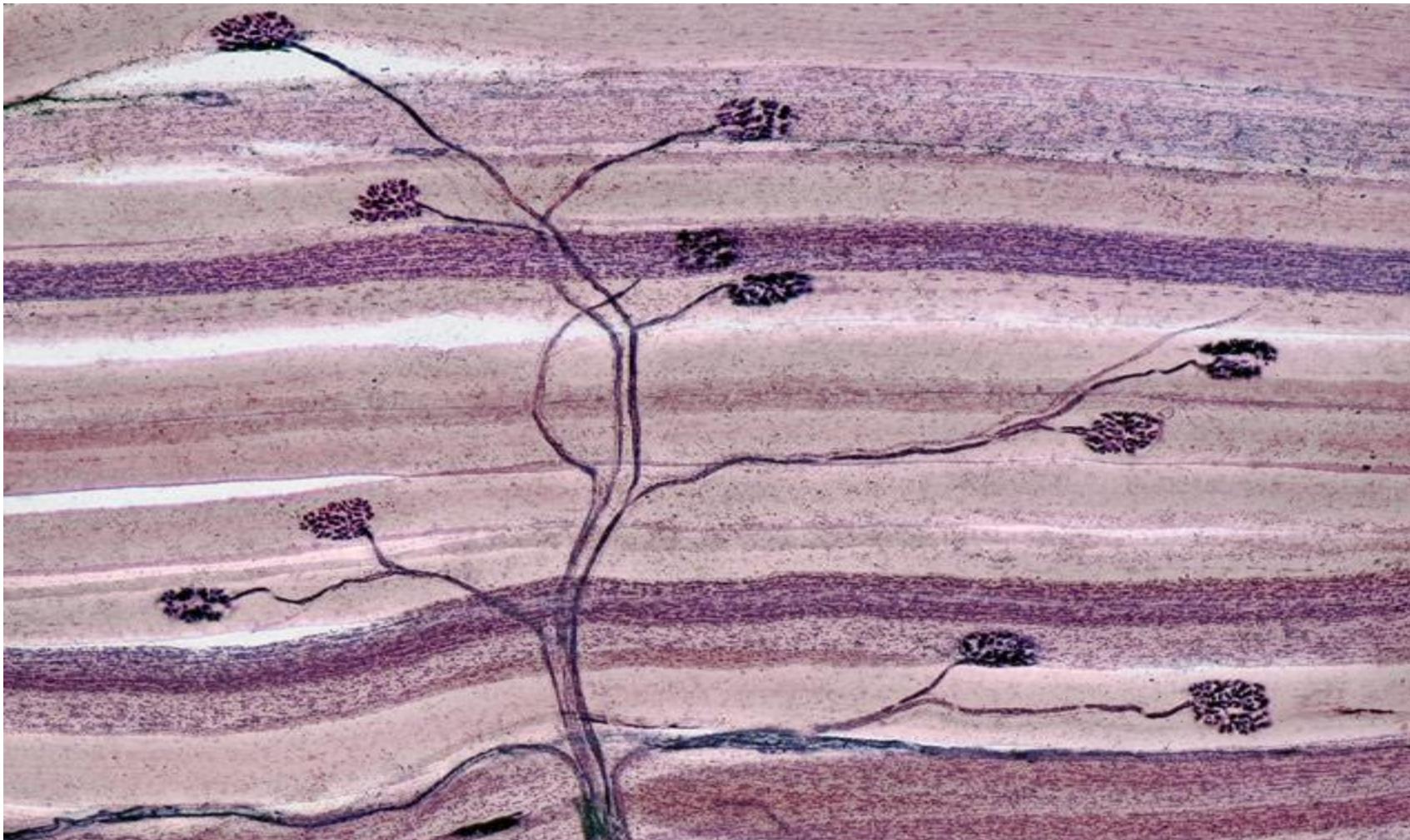
Chaetoceros debilis (marine [diatom](#)), a colonial plankton organism
(250x)



Paramecium sp. showing the nucleus, mouth and water
expulsion vacuoles (40x)



Thin section of a dinosaur bone preserved in clear agate (10x)



12-08-2022

Nerve and muscle thin section (40x)

STU Molecular Imaging and Spectroscopy workshop



Closterium, a common genera of green algae (chlorophyta) found in lakes and ponds around the Puget Sound region (400x)



Vorticella sp. (protozoa) (20x)



Cacoxenite ([mineral](#)) from La Paloma Mine, Spain (18x)

Modern day objectives

Objective Specifications

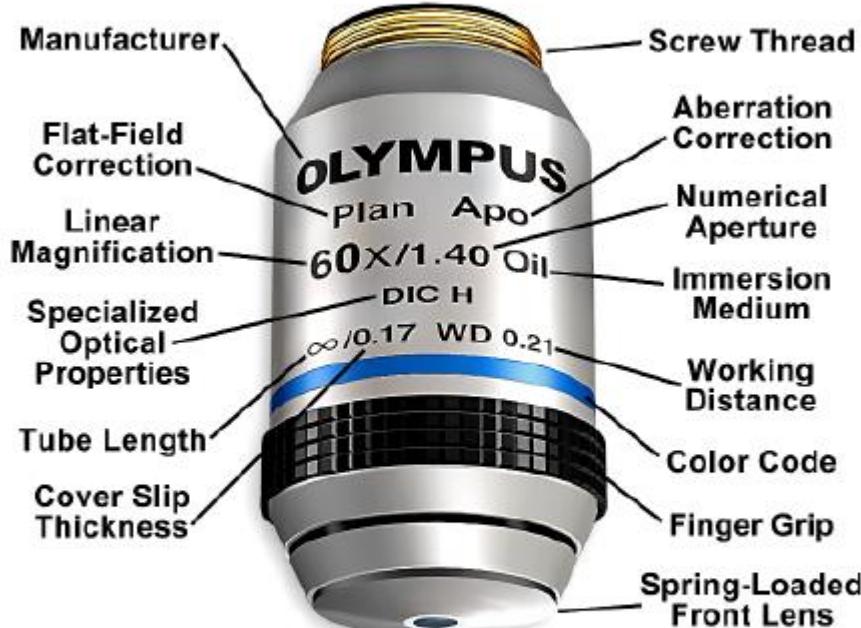
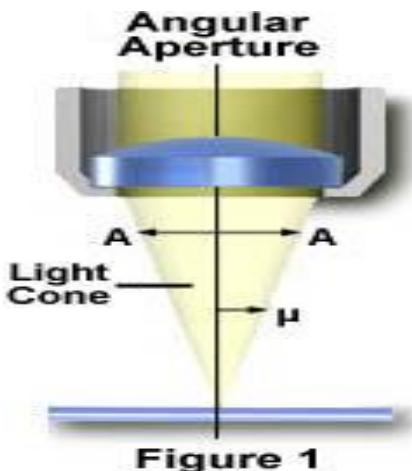
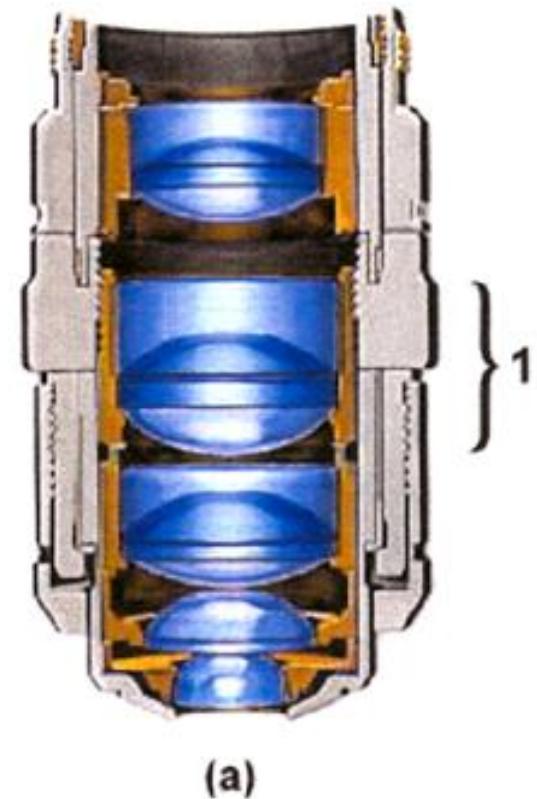


Figure 9. Specifications engraved on the barrel of a typical microscope objective. These include the manufacturer, correction levels, magnification, numerical aperture, immersion requirements, tube length, working distance, and specialized optical properties.



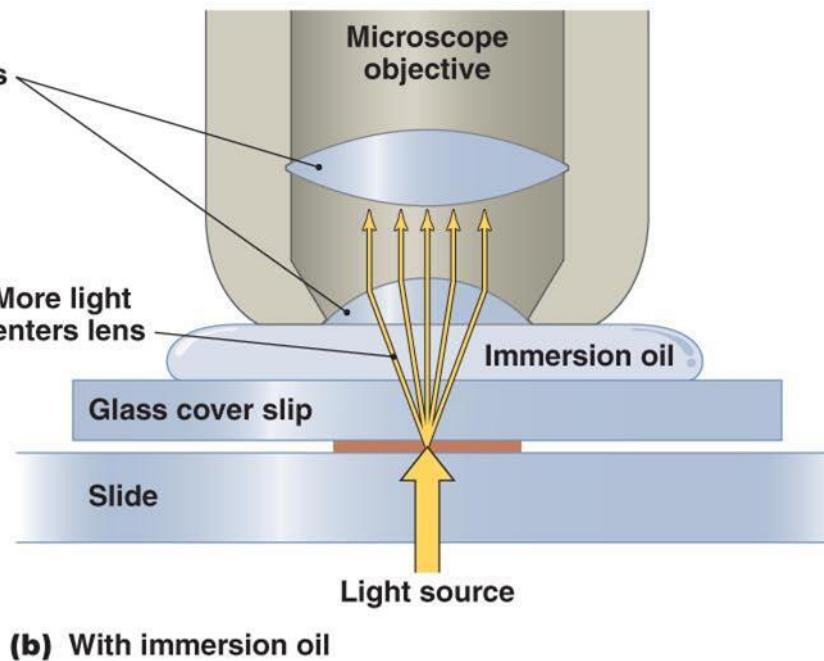
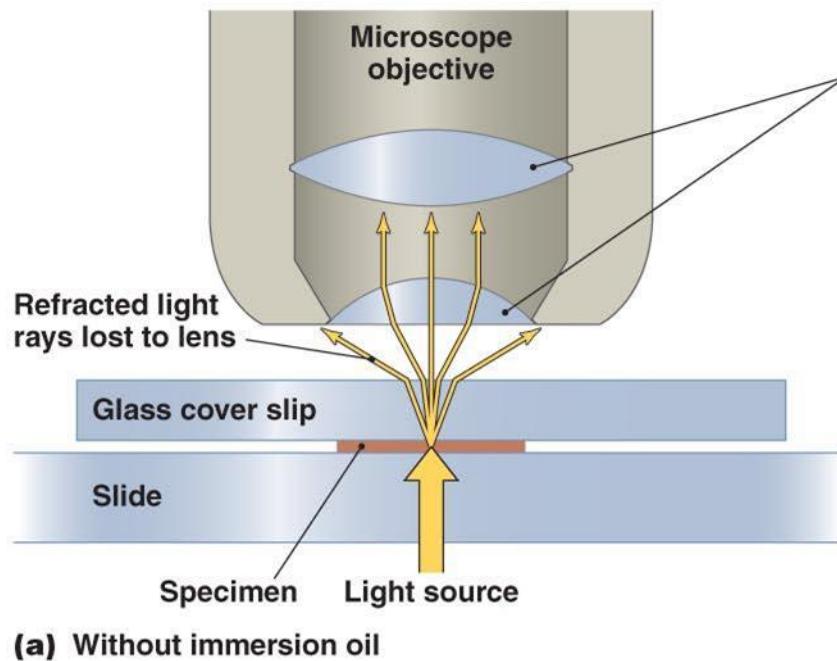
$$NA = n(\sin \alpha)$$



Object magnification ~ 1 to 150 x

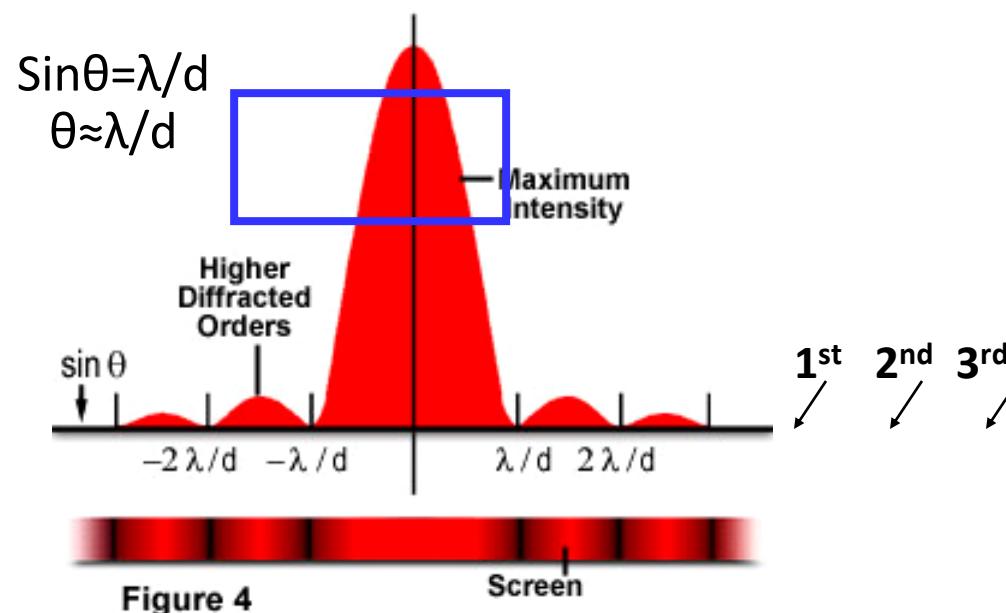
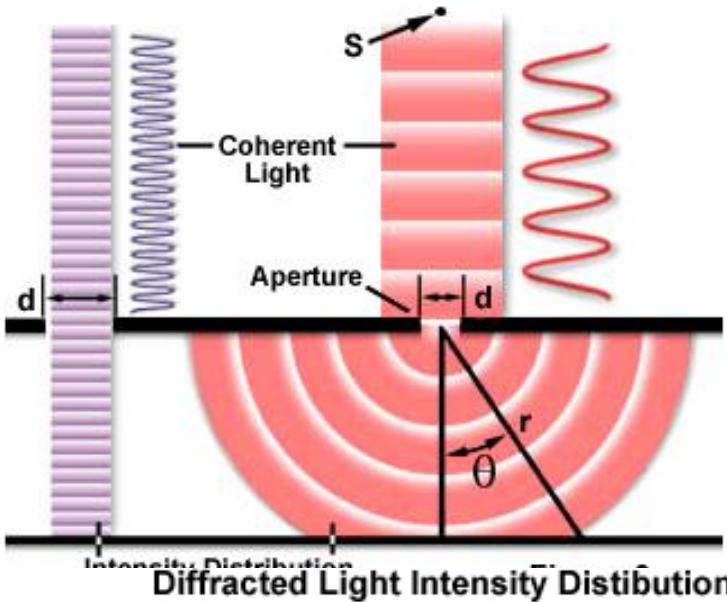
NA ~ 0.2 to 1.65

The effects of immersion oil on resolution



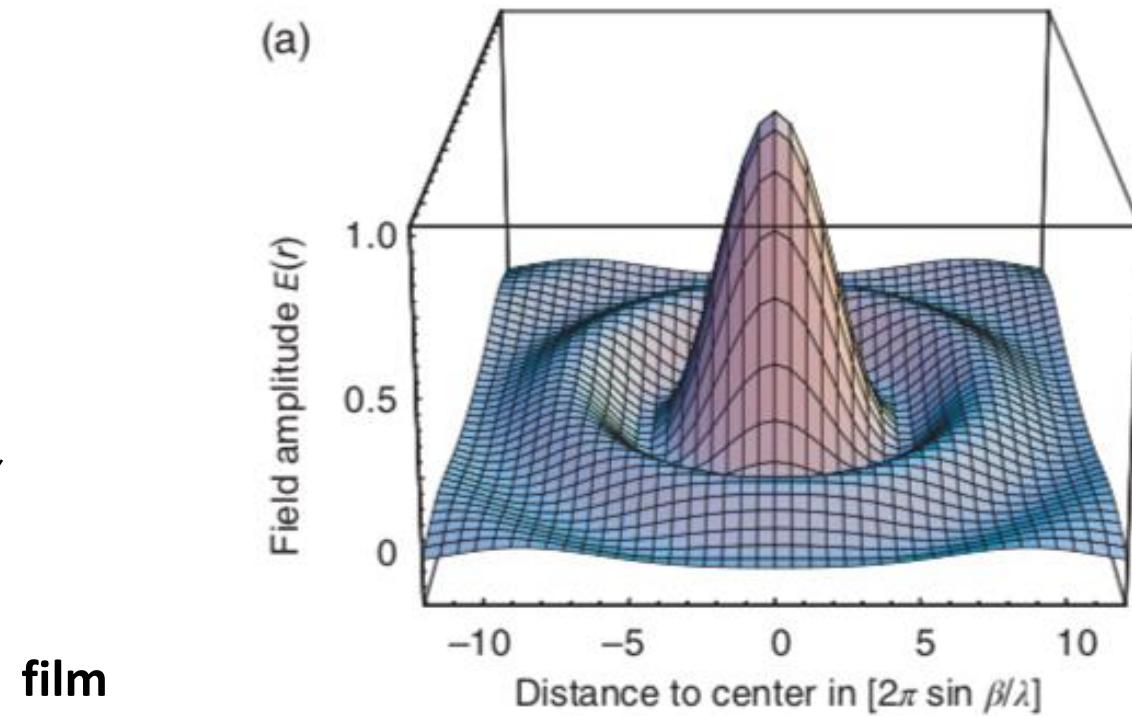
$$NA = n(\sin \alpha)$$

Diffraction of Coherent Laser Light



Diffraction of Light

Diffraction of light occurs when a light wave passes by a **corner** (or a **barrier**) or through an opening (or a slit) that is physically the **approximate size** of, or even smaller than that light's wavelength.



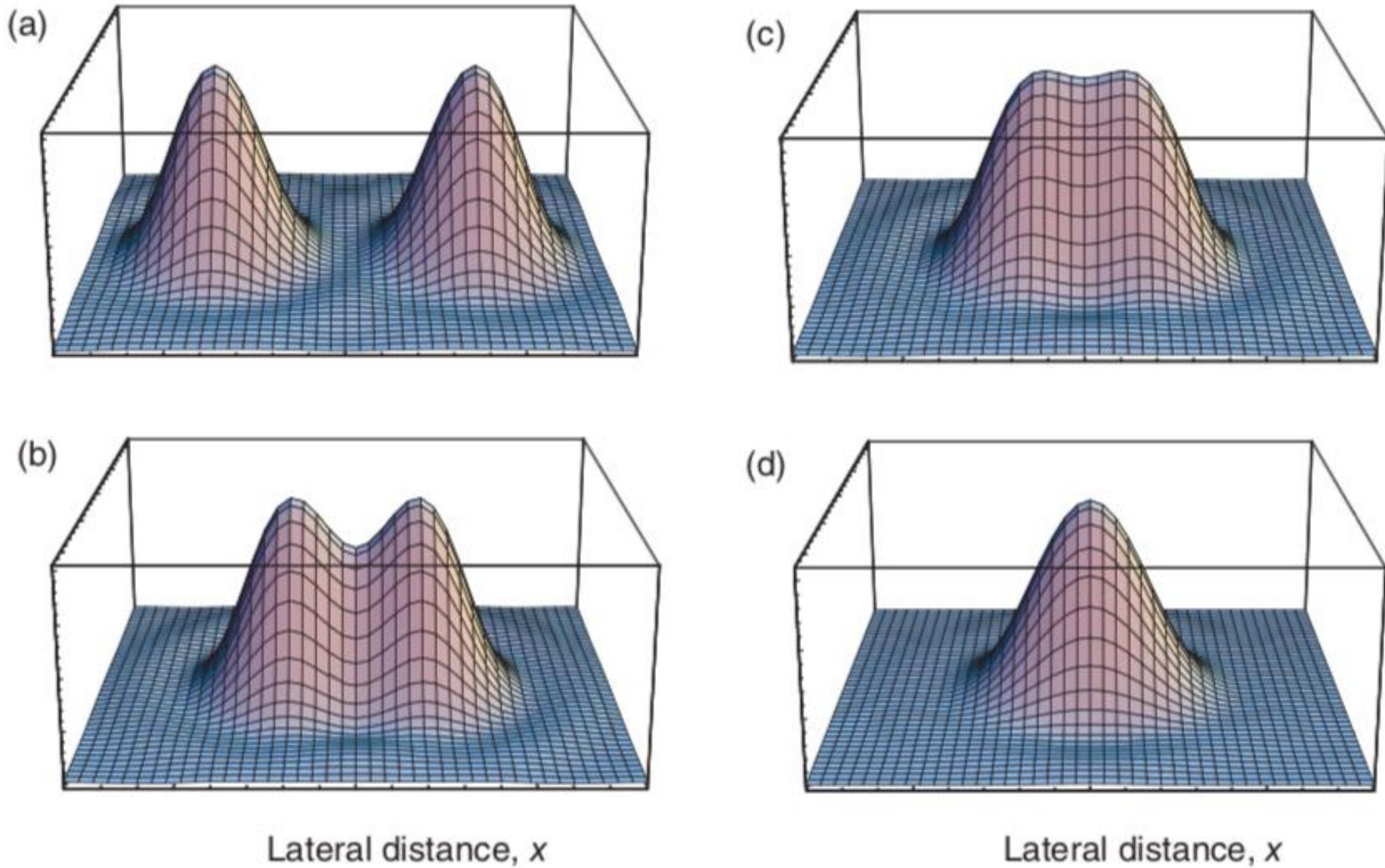
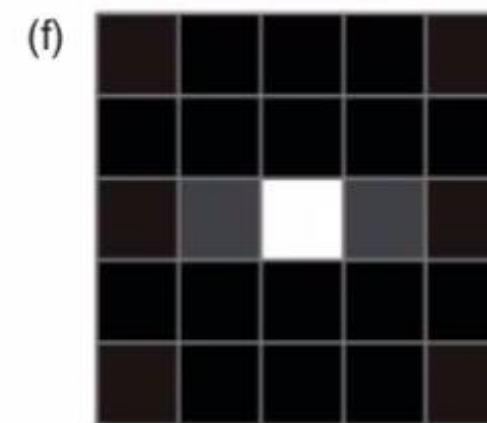
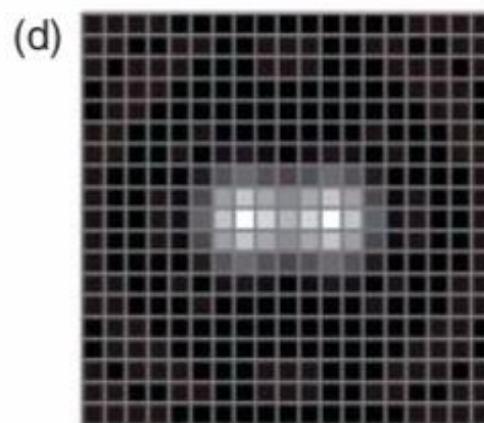
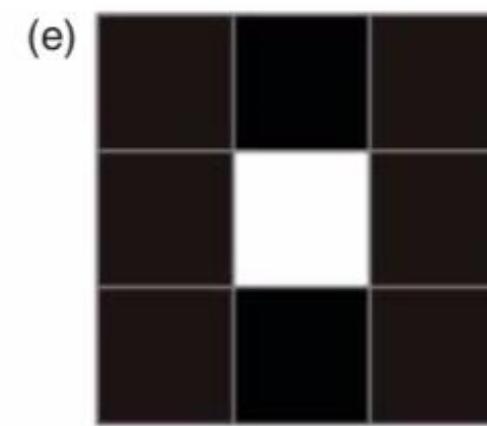
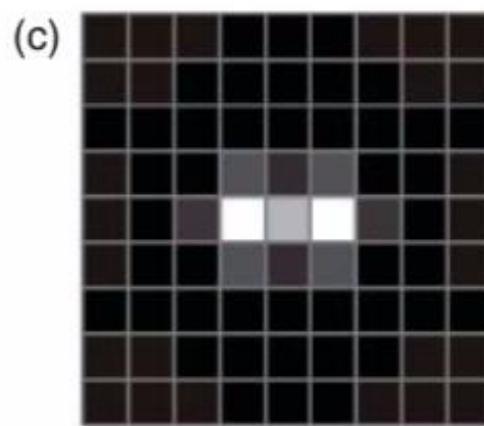
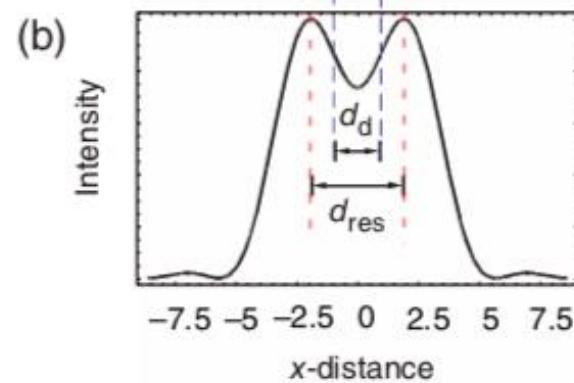
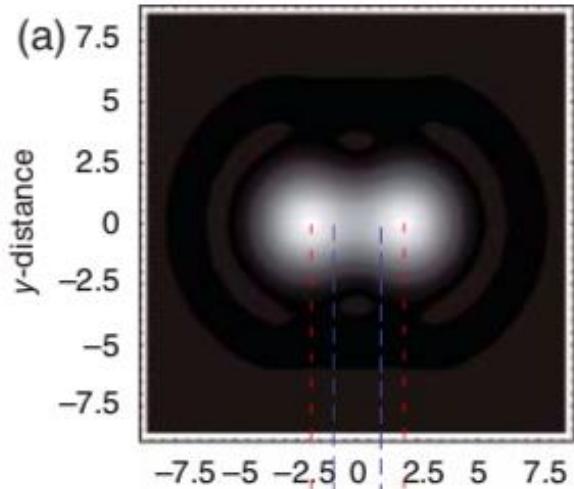


Figure 2.10. Surface plot of the intensity distribution in the $x-z$ plane of two incohorts.

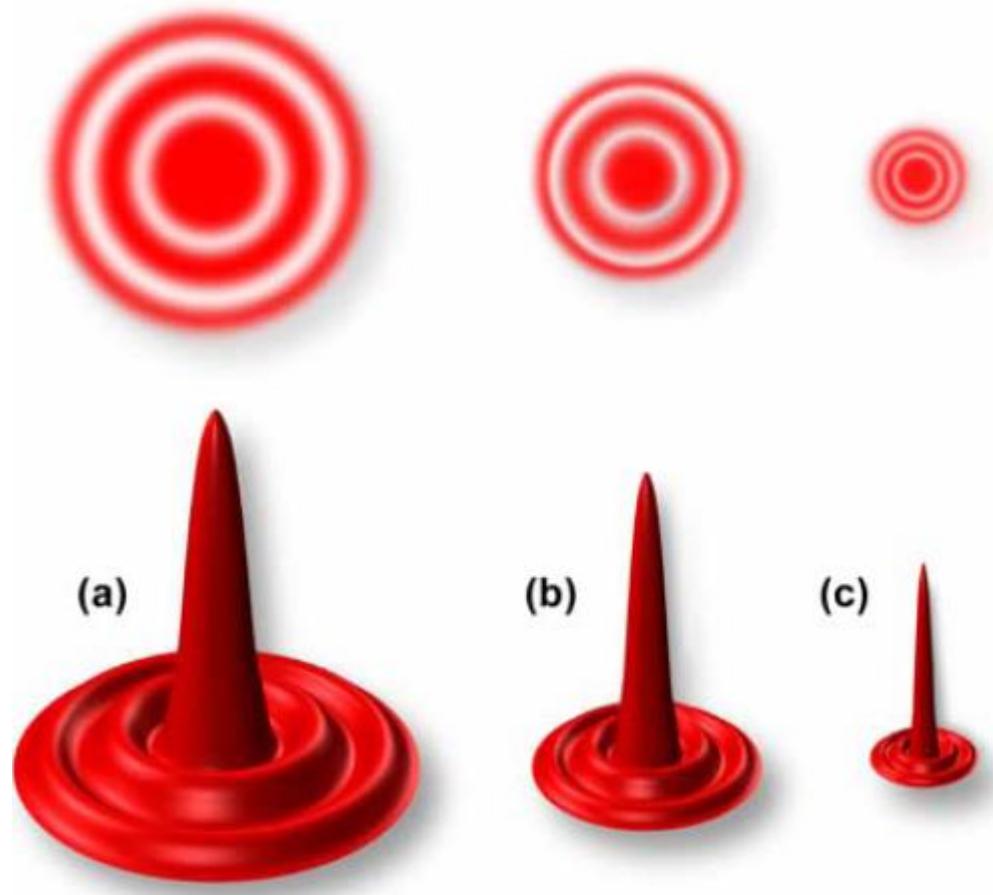
Role of CCD detector in resolution



Resolution of a Microscope (lateral)

The smallest distance between two specimen points that can still be distinguished as two separate entities

$$d_{\min} = 0.61\lambda/NA \quad NA=nsin(\alpha)$$



λ – illumination wavelength (light)

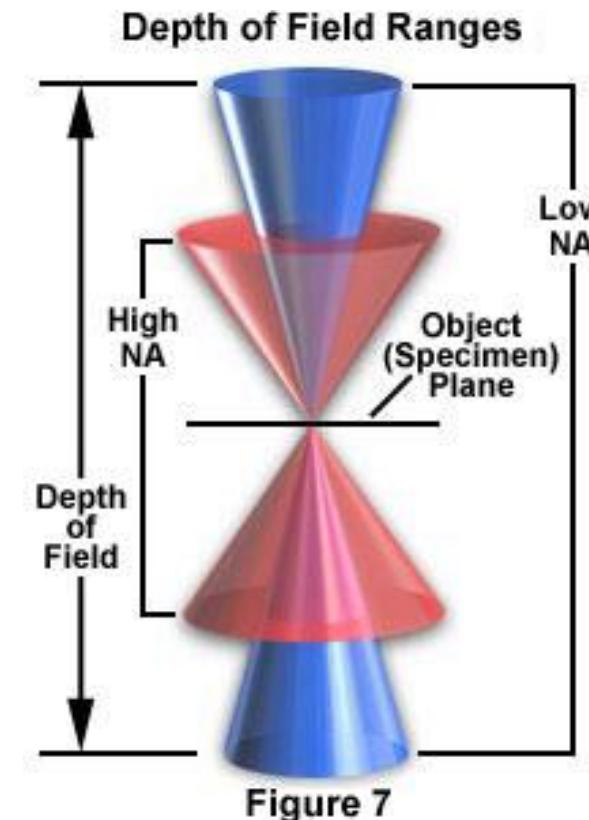
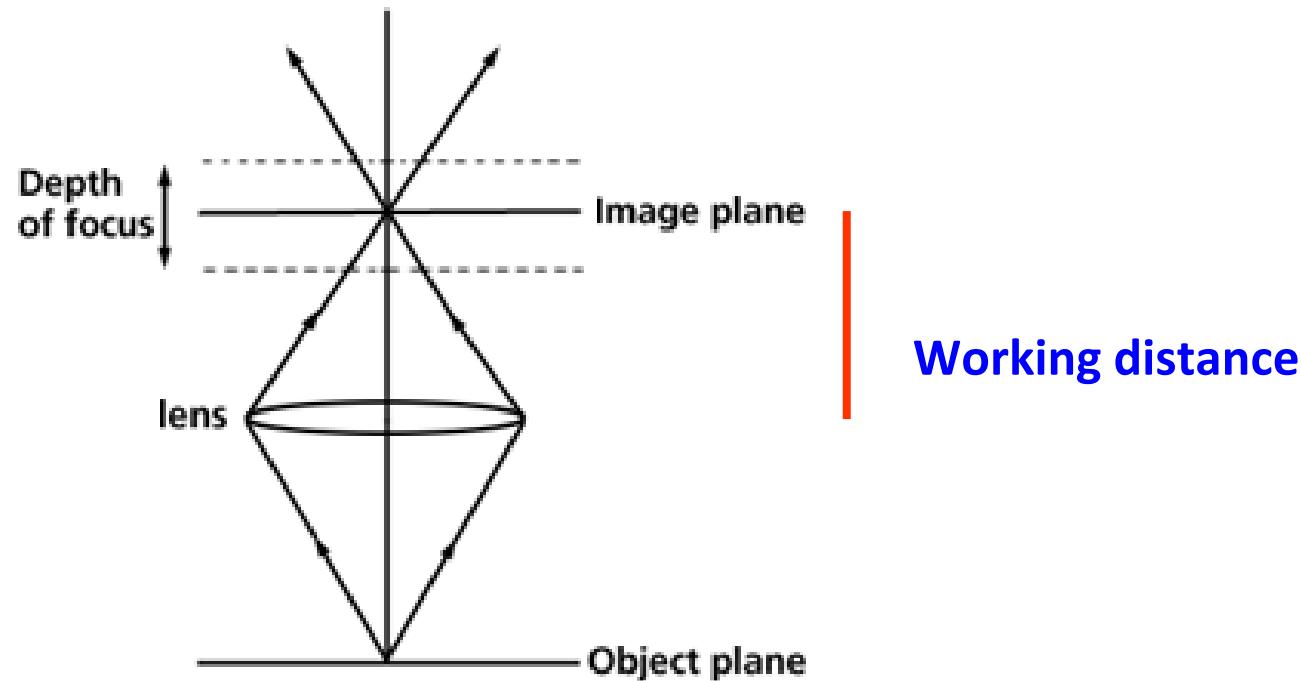
NA – numerical aperture

α -one half of the objective angular aperture

n-imaging medium refractive index

$d_{\min} \sim 0.3\mu\text{m}$ for a midspectrum λ of $0.55\mu\text{m}$

Axial resolution – Depth of Field



Depth of field =>

The axial range through which an **object** can be focused without any appreciable change in image sharpness

F is determined by NA.

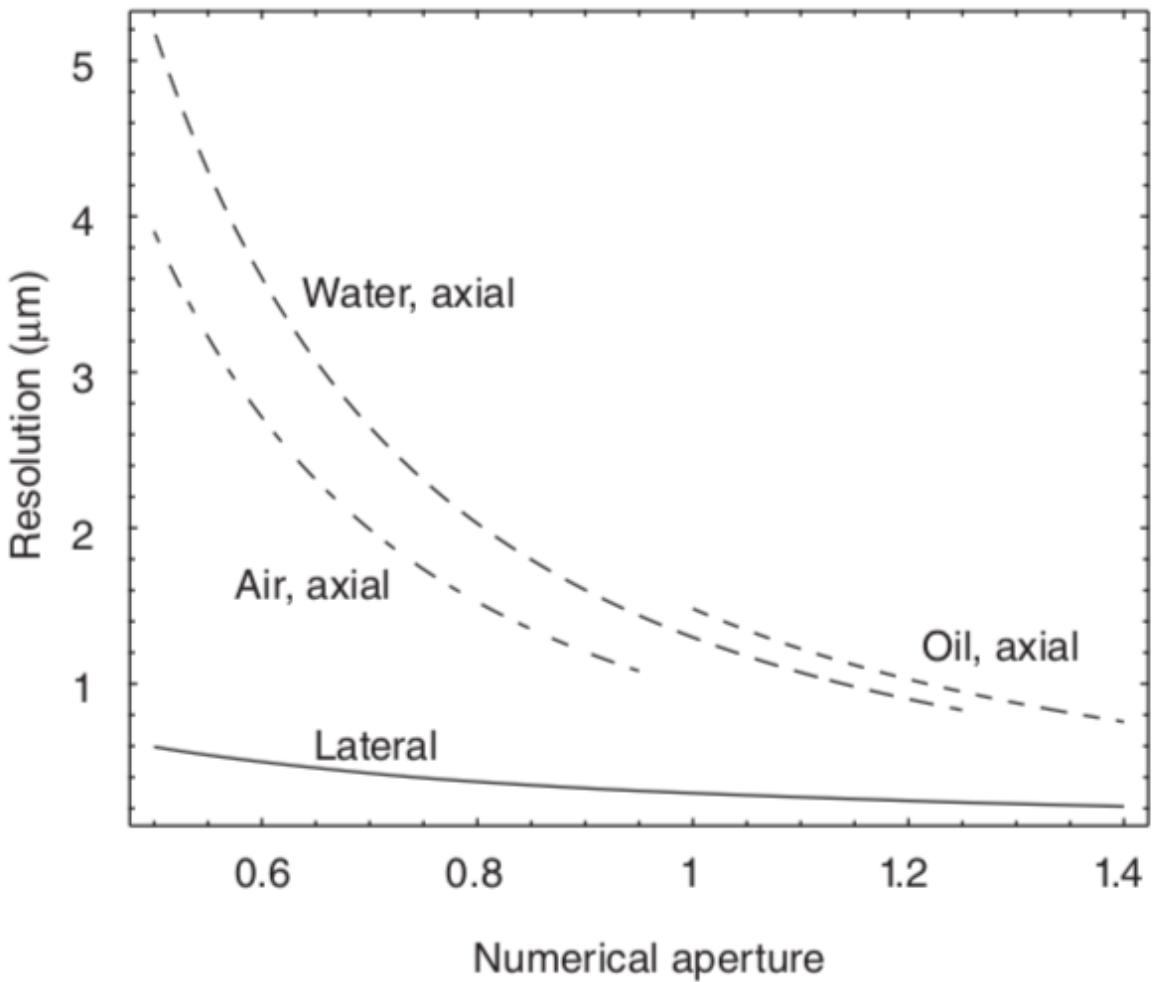
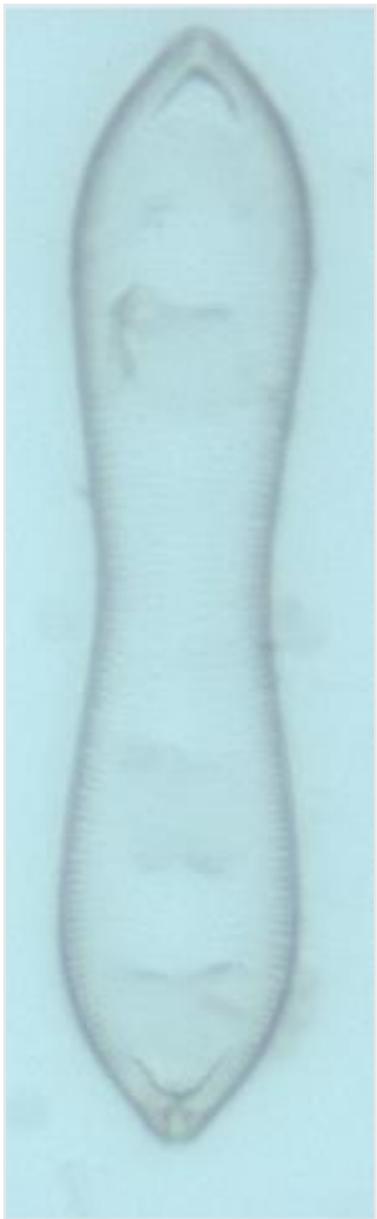
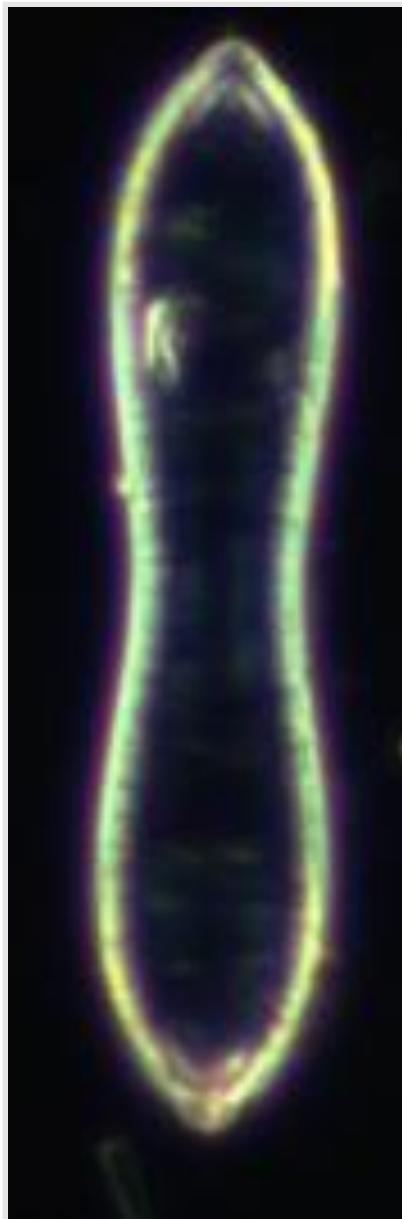


Figure 2.13 Axial and lateral resolution as a function of numerical aperture for air-, water-, and oil-immersion objectives. The curves were calculated according to Eqs (2.30) and (2.34) for indices of refraction of 1 for air, 1.33 for water, and 1.515 for oil, and for $\lambda = 488 \text{ nm}$.

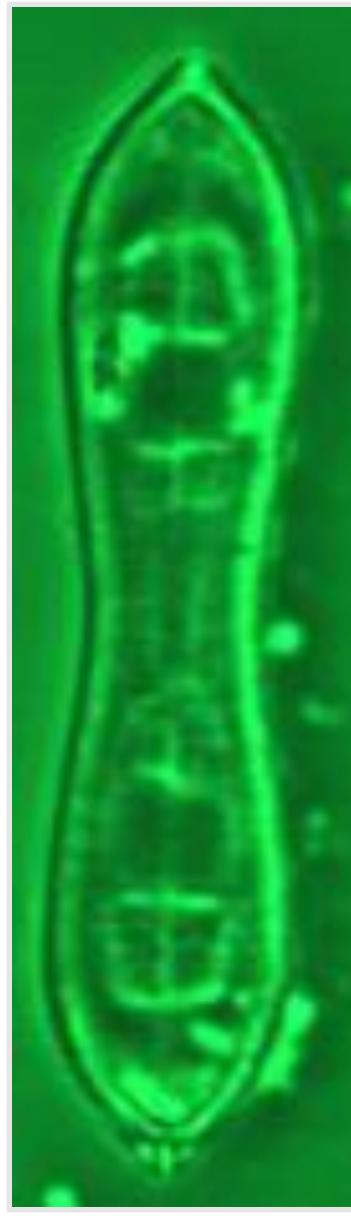
12-08-2022



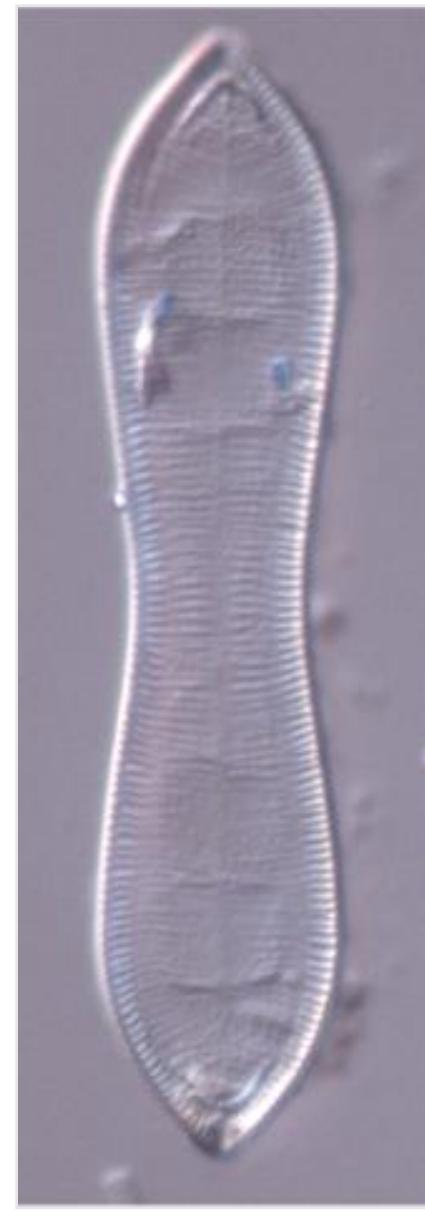
Bright Field



Dark Field



Phase Contrast



Differential
Interference Contrast

Bright Field Microscopy

Utilizes white light to visualize an object (“Naked Microscopy”)

Advantages:

- Simplicity of setup (thus lower cost)
- No manipulation of sample

Disadvantages:

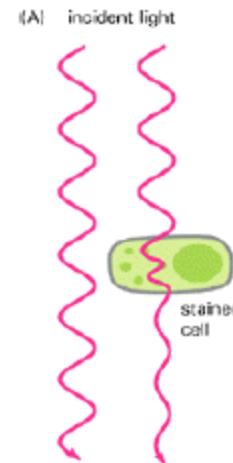
- Stains/dyes often needed to provide contrast (cells are colorless and translucent)
- Limited in magnification and resolution



Bright field image of a live zebrafish embryo at 24 hours post-fertilization.

Bright Field Microscopy: Use of Stains

Artificially amplifying minute differences in refractive indices



Staining Cells

Advantages:

- Provides contrast to distinguish features
(cells are colorless and translucent)

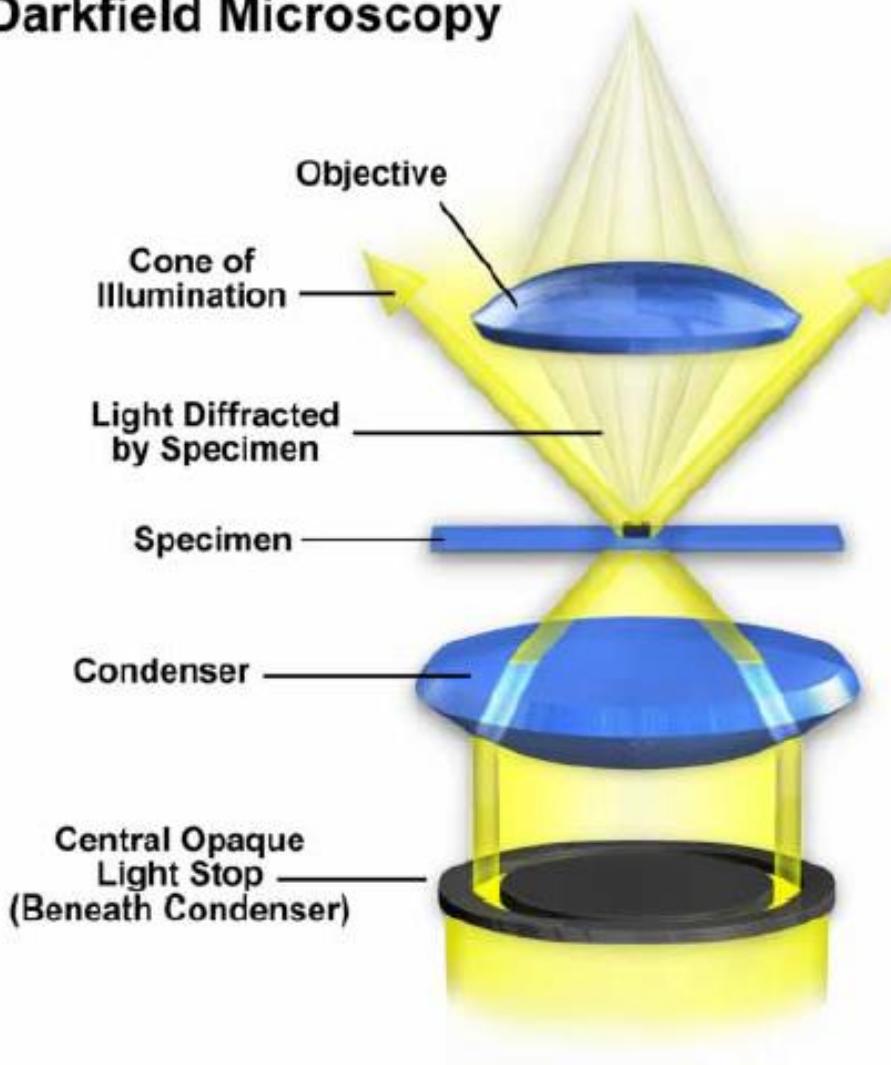
Disadvantages:

- You must fix and kill the sample
- Limited in magnification and resolution

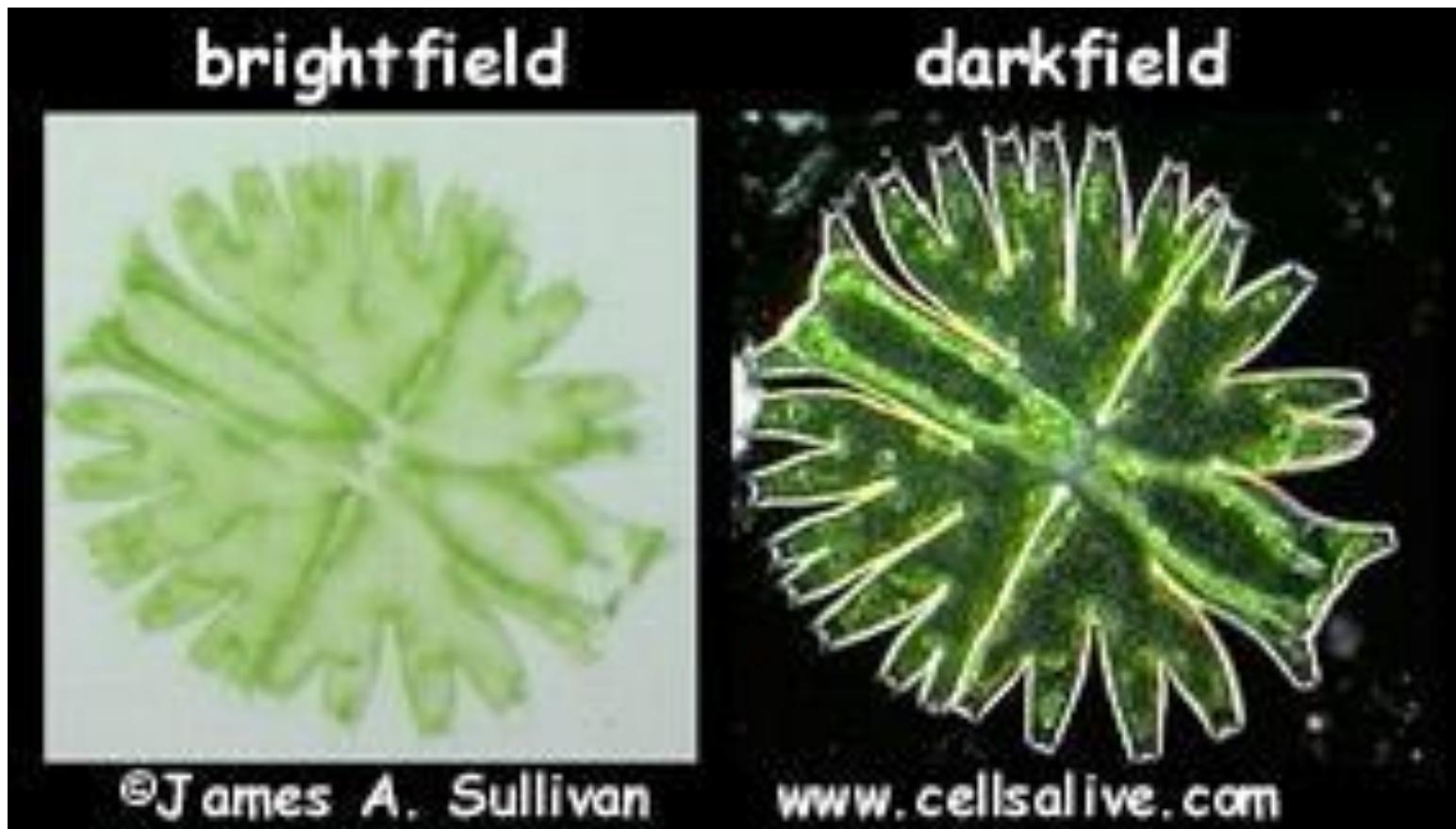
Darkfield Microscopy

Contrast:

$$C_I = \frac{I_{\text{obj}} - I_{\text{sur}}}{I_{\text{obj}} + I_{\text{sur}}}$$



Contrast Enhancement



OM images of the green alga *Micrasterias*

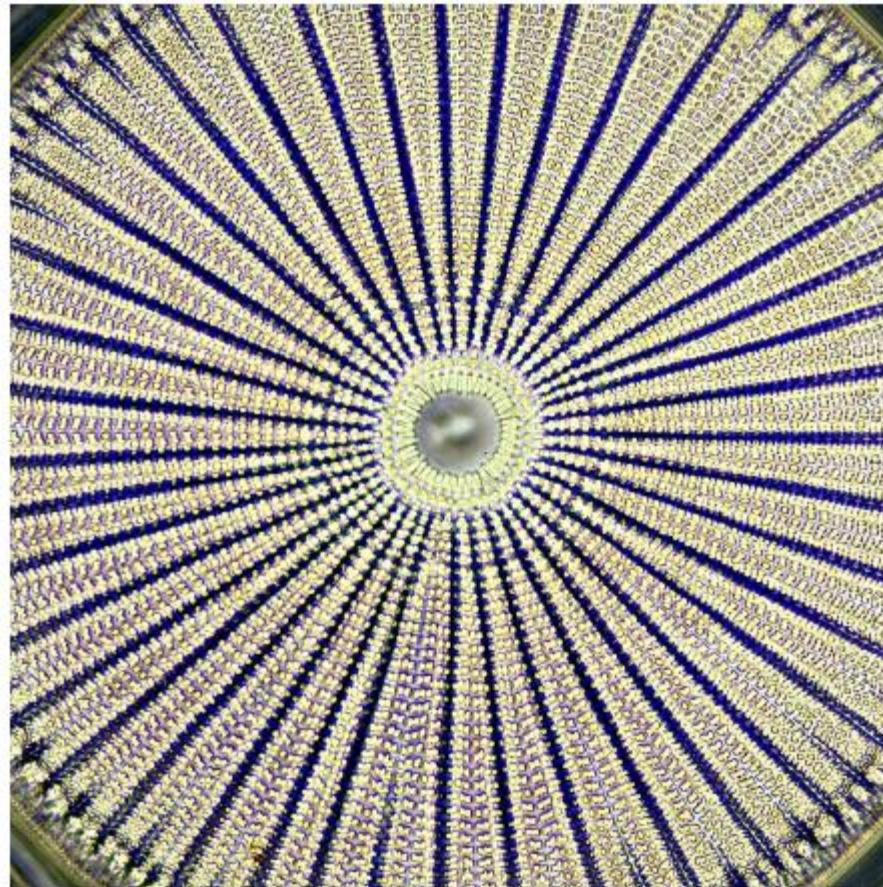


Figure 15. Darkfield photomicrograph of the diatom *Arachnoidiscus ehrenbergi* taken at high magnification using oil immersion optics and a 100x objective.

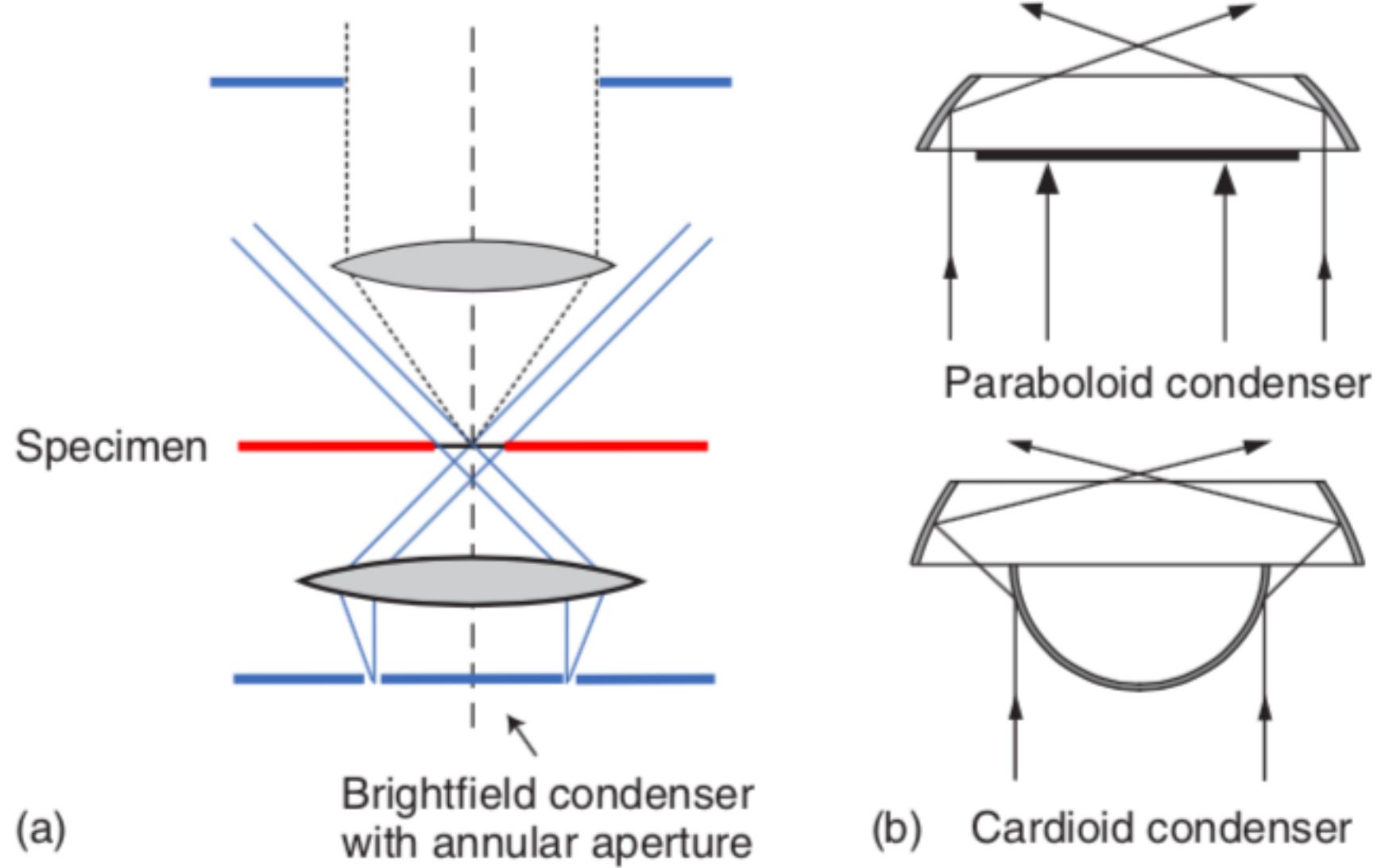
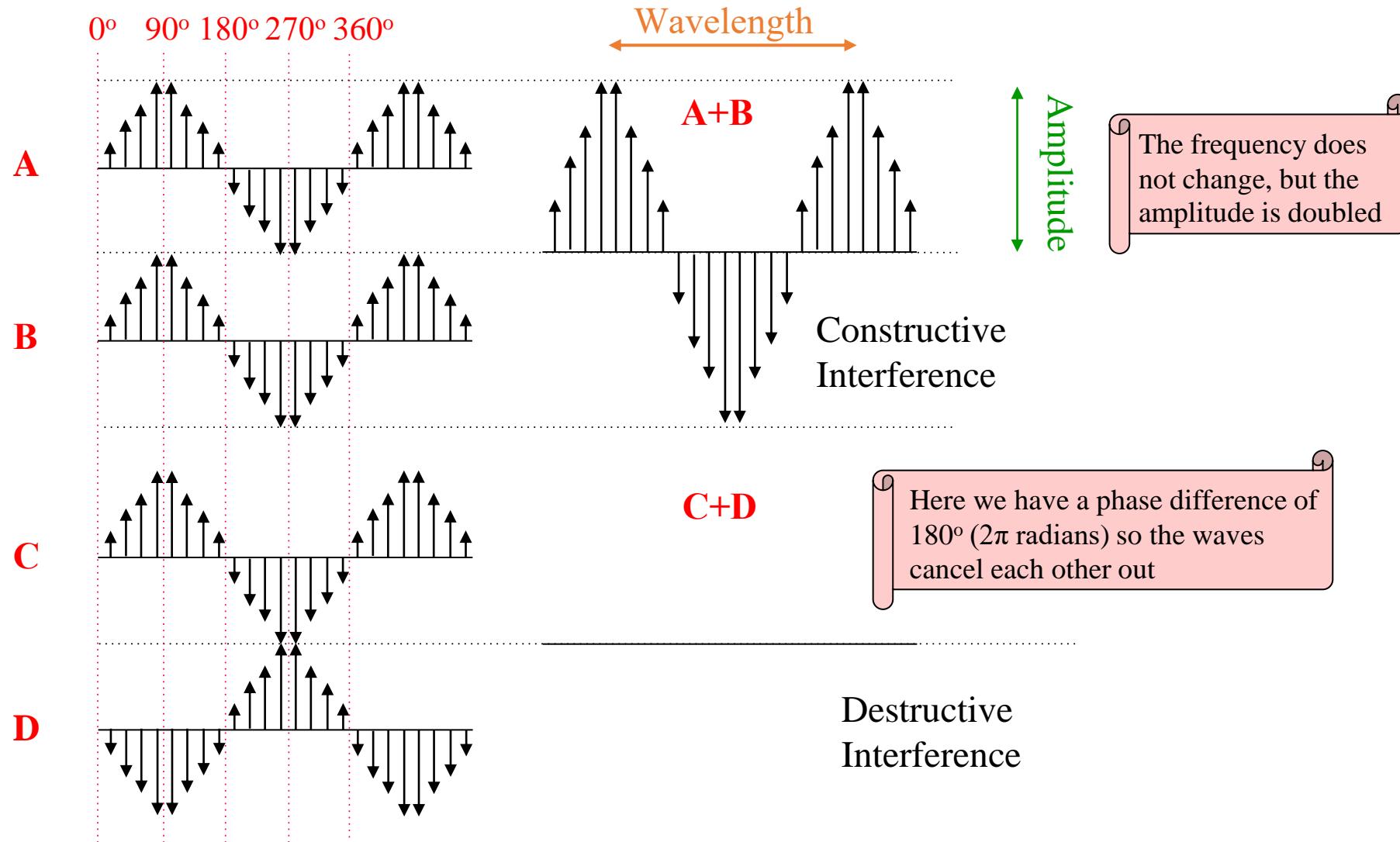


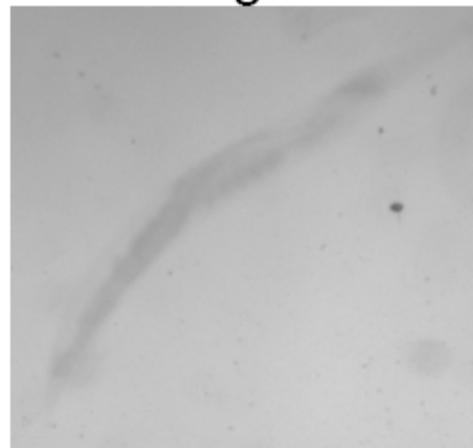
Figure 2.23 Dark-field contrast. (a) Principle of dark-field illumination and (b) dark-field condensers.

Interference

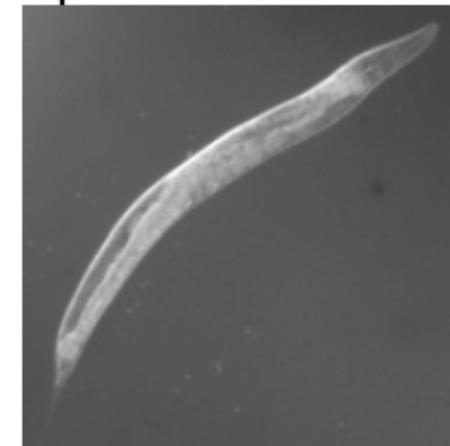


Phase Contrast Microscopy

- Amplification of minute differences in refractive indices within different regions of the cell.
- Contrast-enhancing optical technique to produce high-contrast images of transparent specimens.

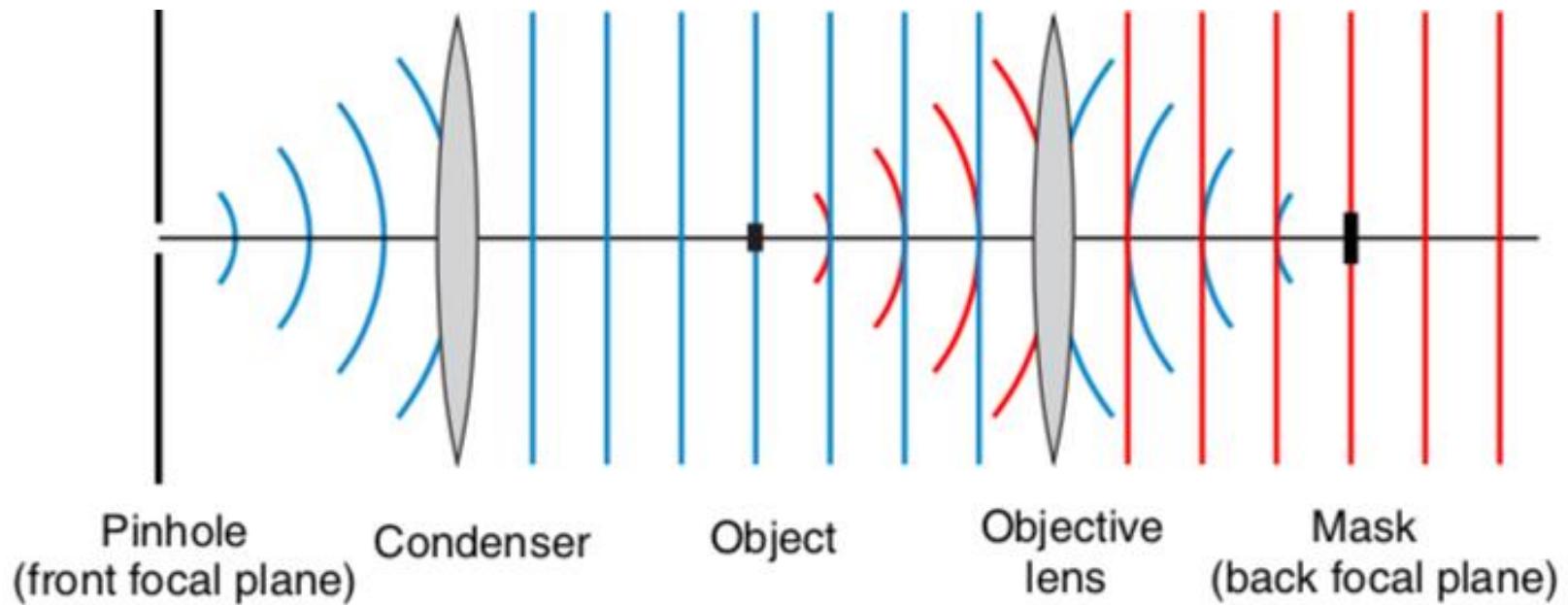
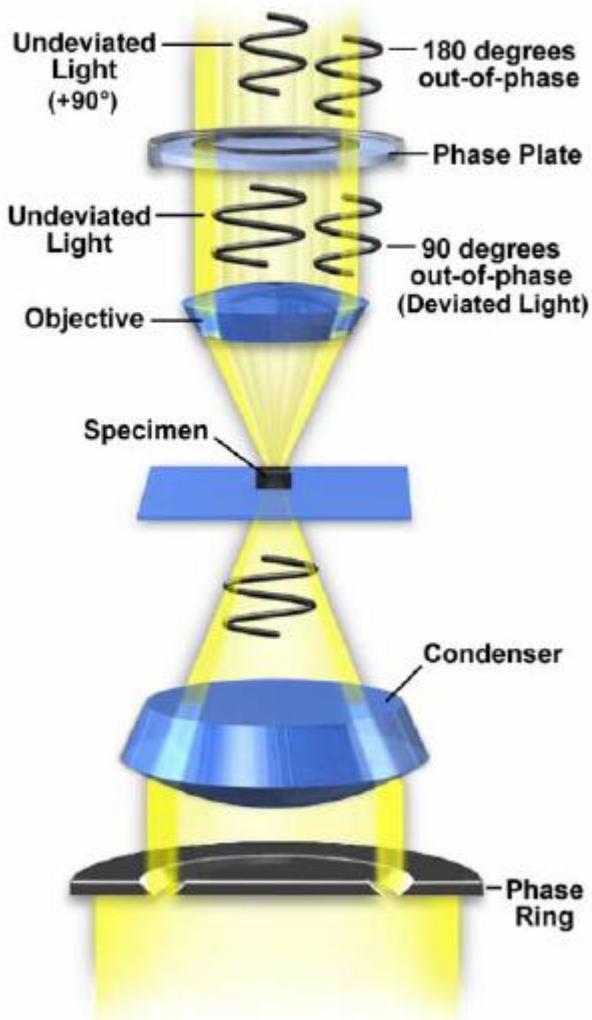


Bright Field



Phase Contrast

Phase Contrast Microscopy



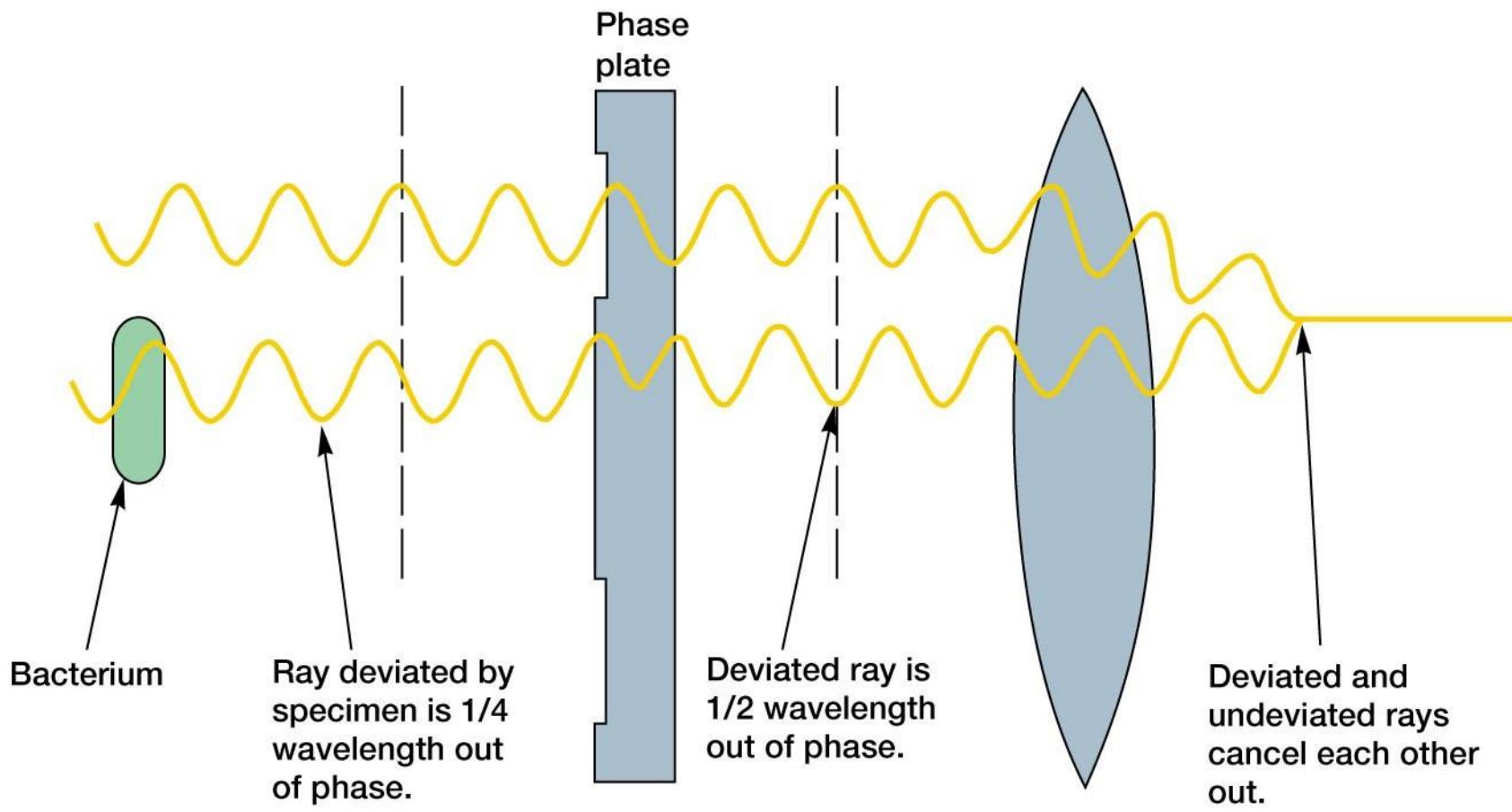


Figure 2.10

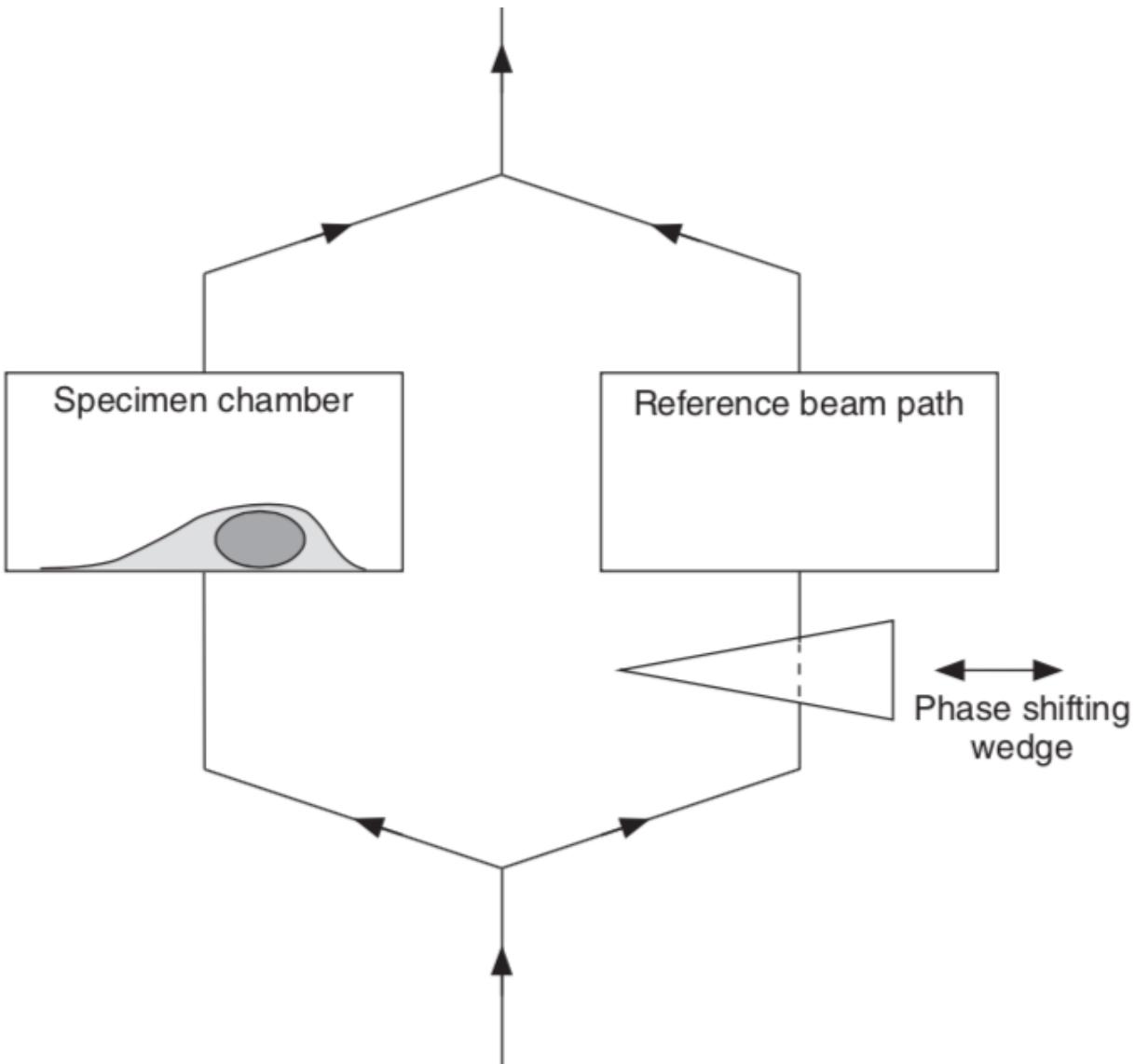


Figure 2.26 Principle of interference contrast.

Phase Contrast Microscopy

Advantages:

- Increase contrast without destroying sample
**Specimen can remain alive!
- Moderate cost

Disadvantages:

- Limited in magnification and resolution
- Only single cell or thin layer of cells is observable
- Halo effect

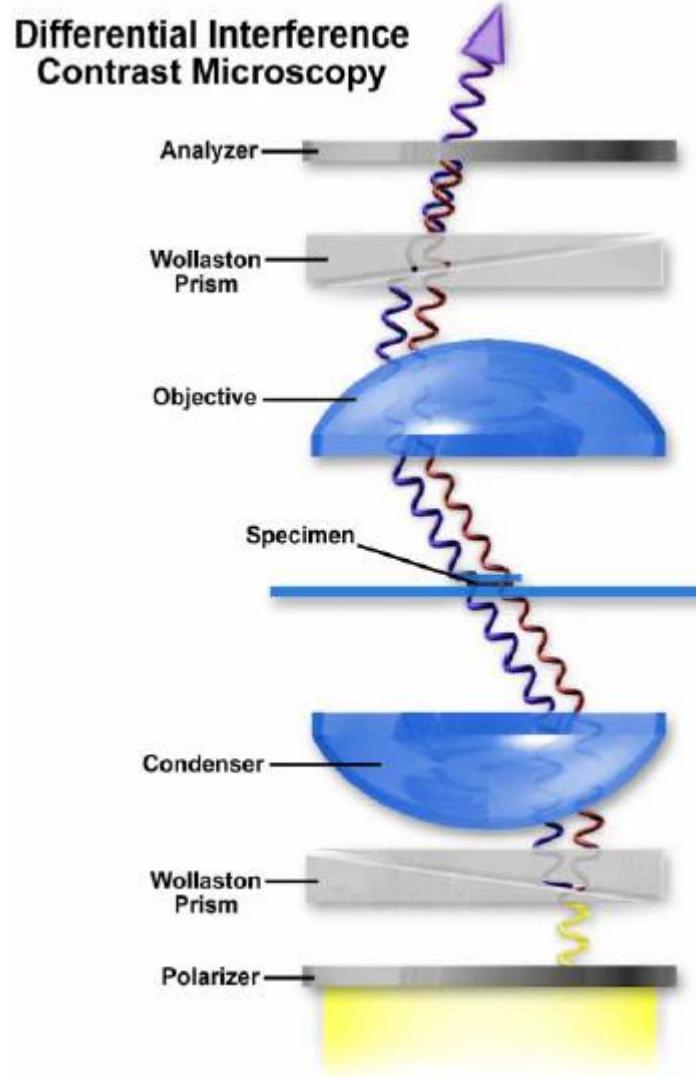
DIC Microscopy

Advantages:

- Increase contrast without destroying sample
- Superior resolution to phase contrast
- No halo effect
- Superior depth discrimination to phase contrast

Disadvantages:

- Limited in magnification and resolution
- Only single cell or thin layer of cells is observable
- High cost



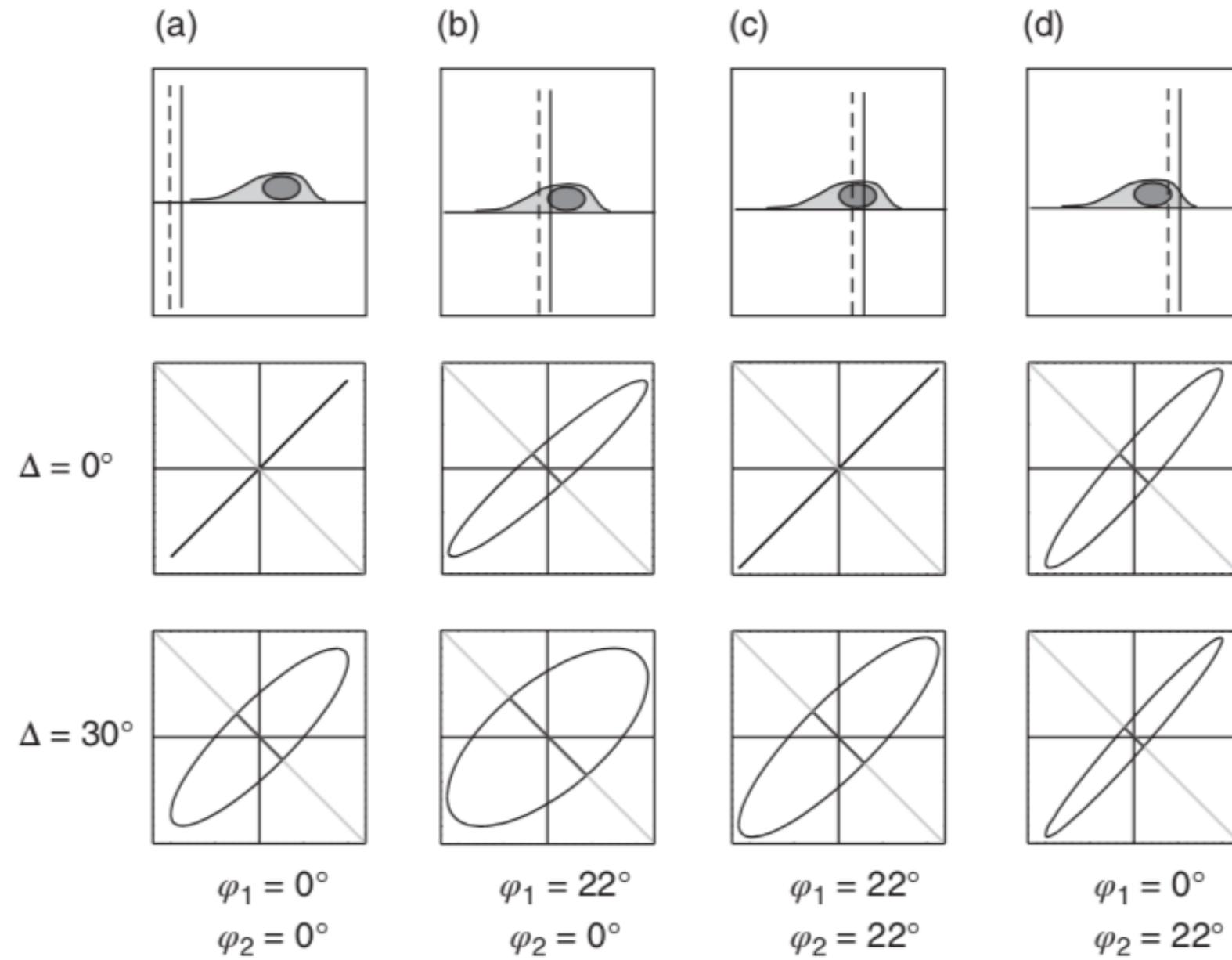
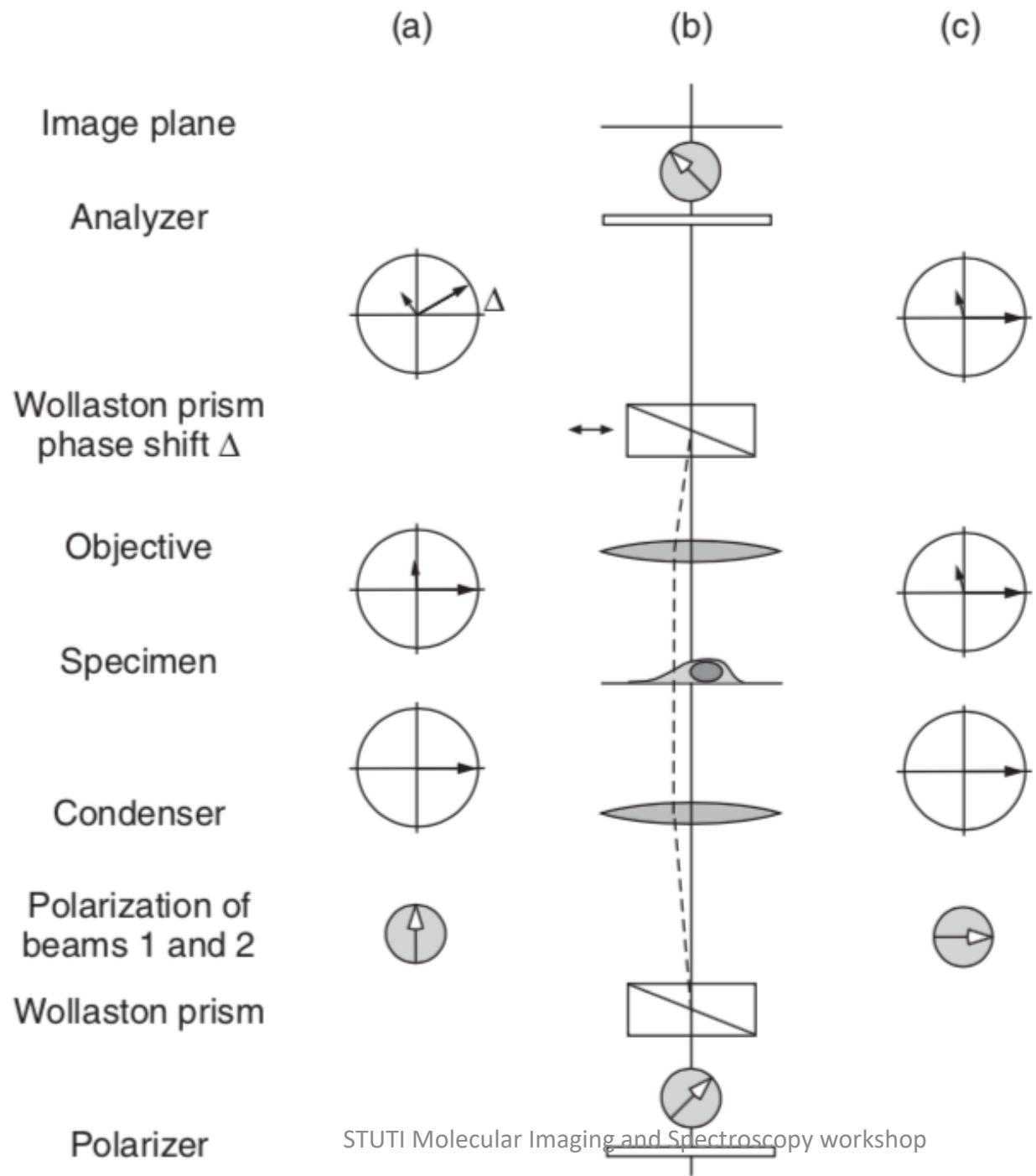
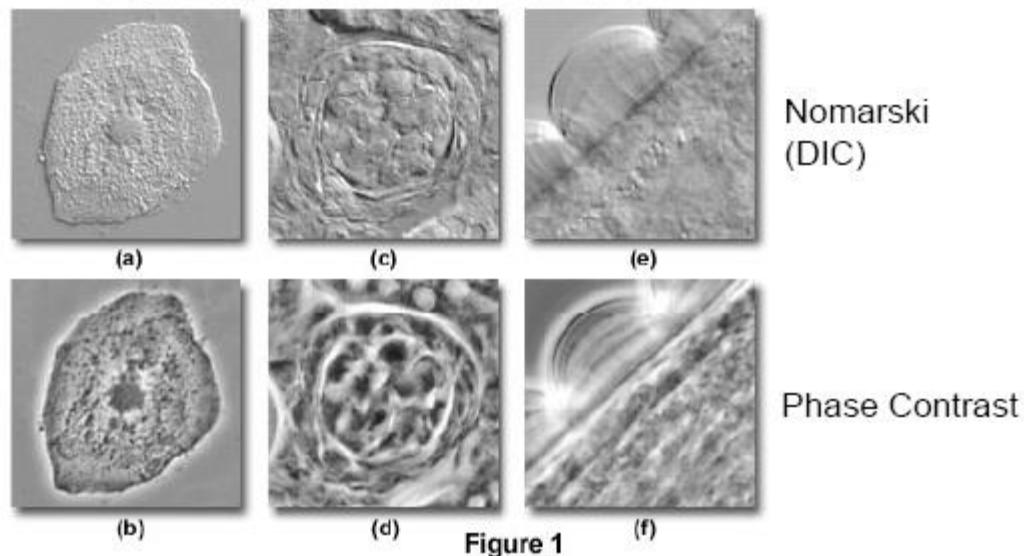


Figure 2.29 (a–d) Contrast generation, polarization, and phase relationships in DIC microscopy. For details, see the text.

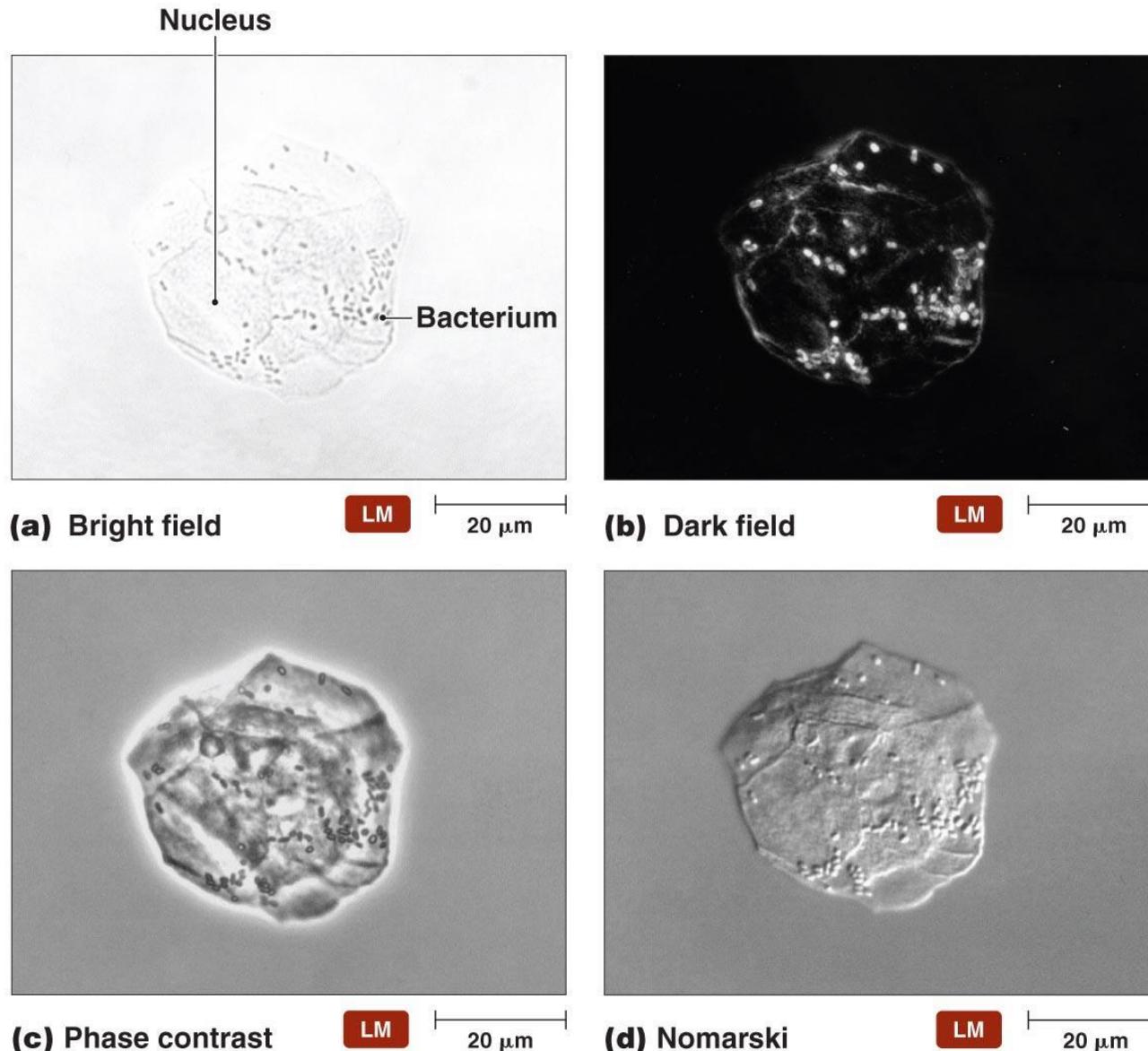


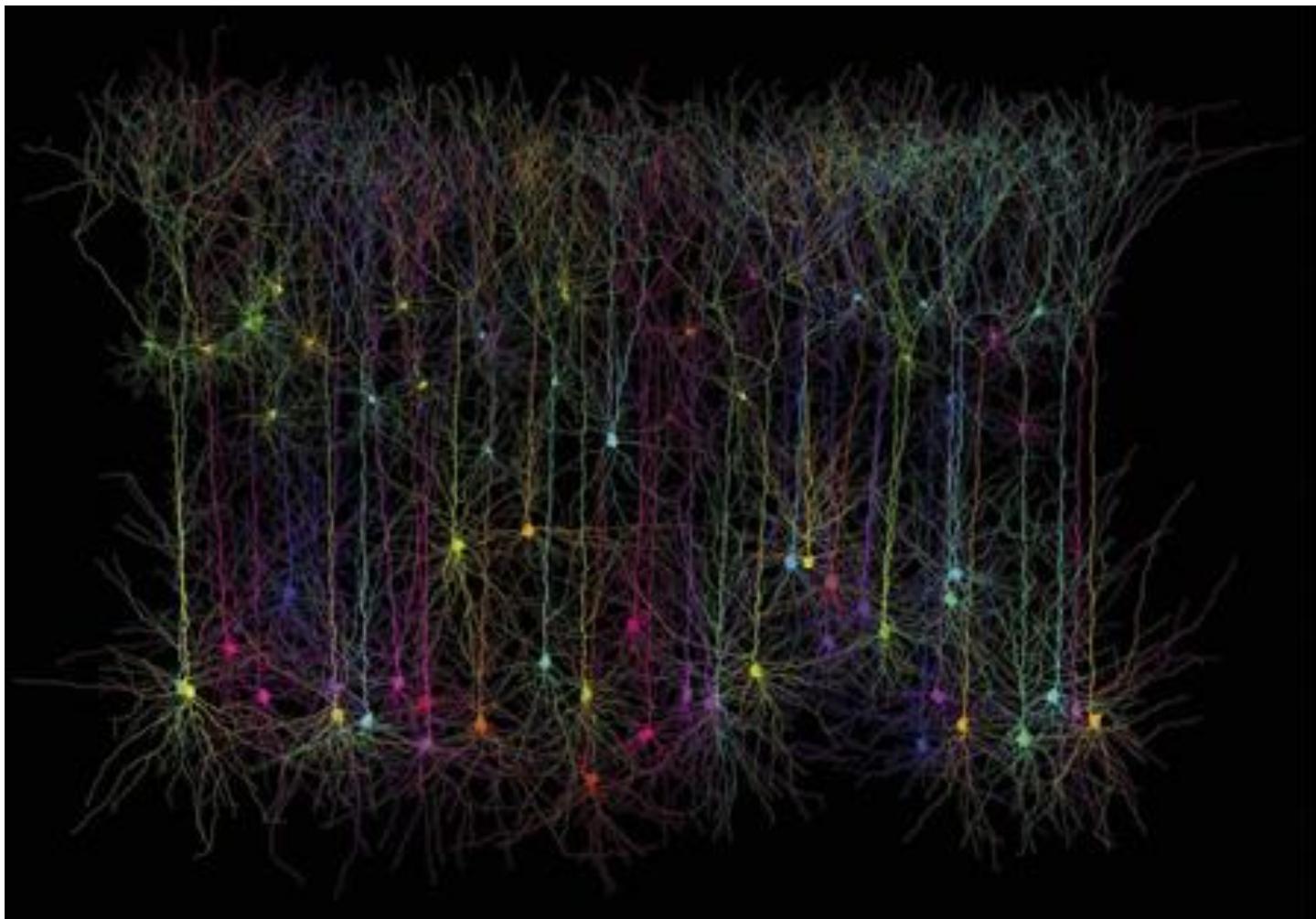
Phase Contrast vs. Nomarski (DIC)

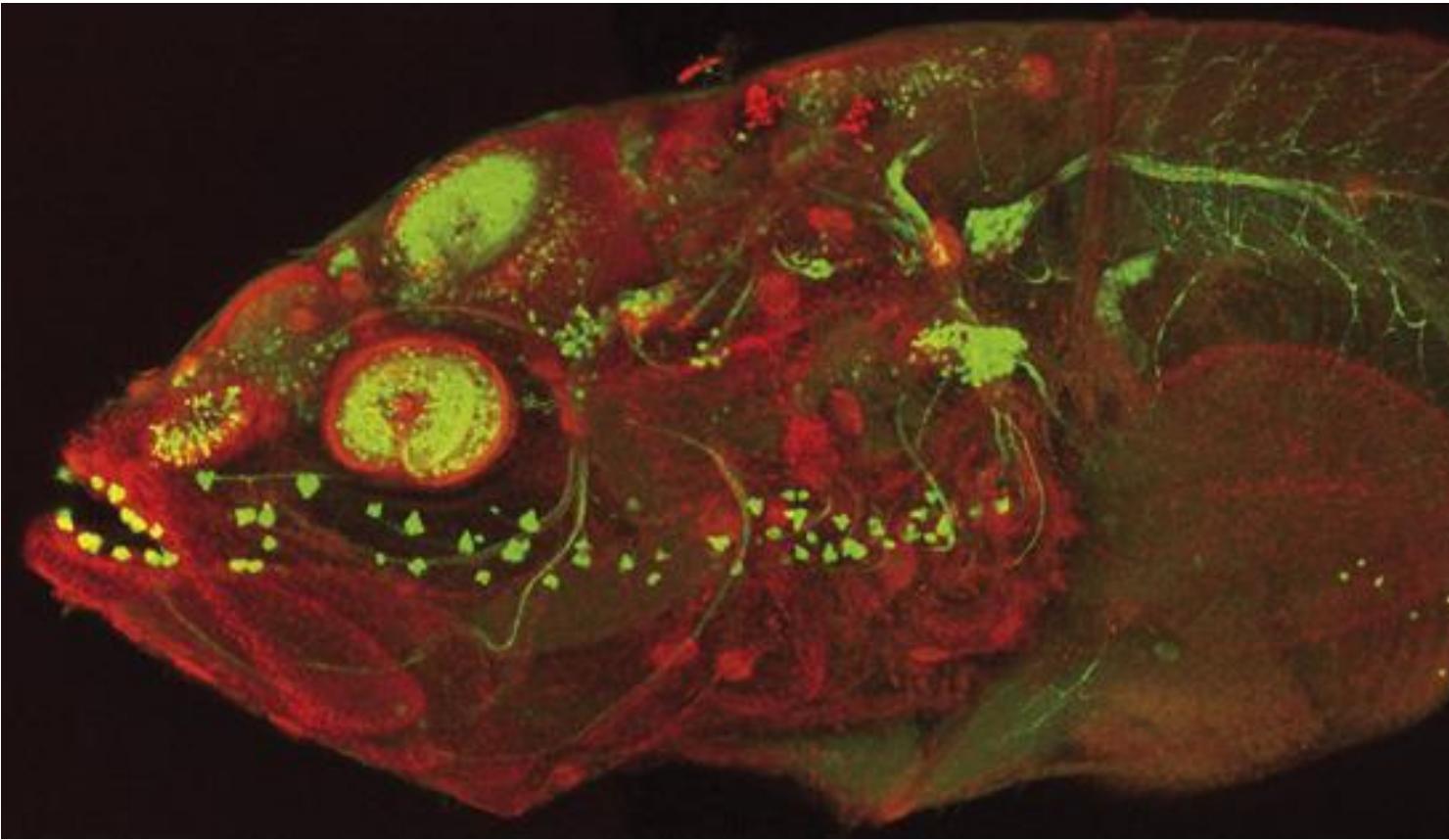
Transparent Specimens in Phase Contrast and DIC



Four kinds of light microscopy

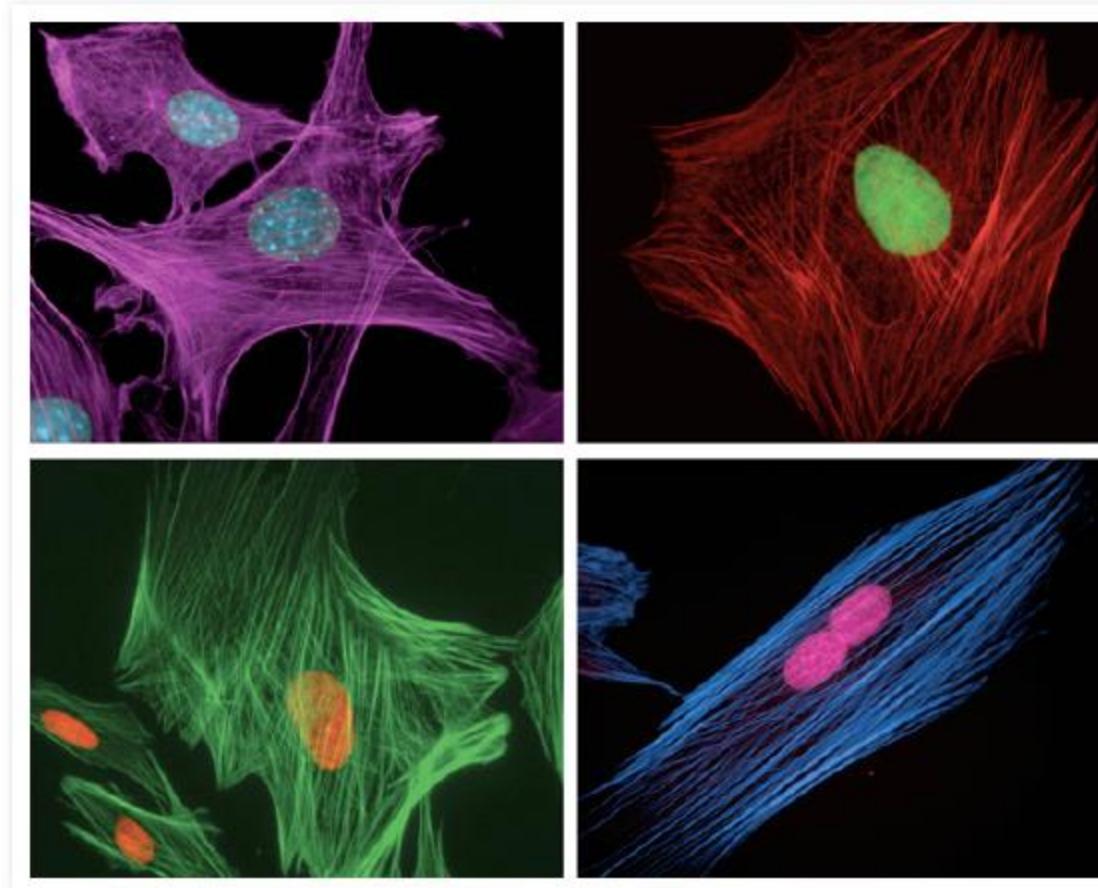




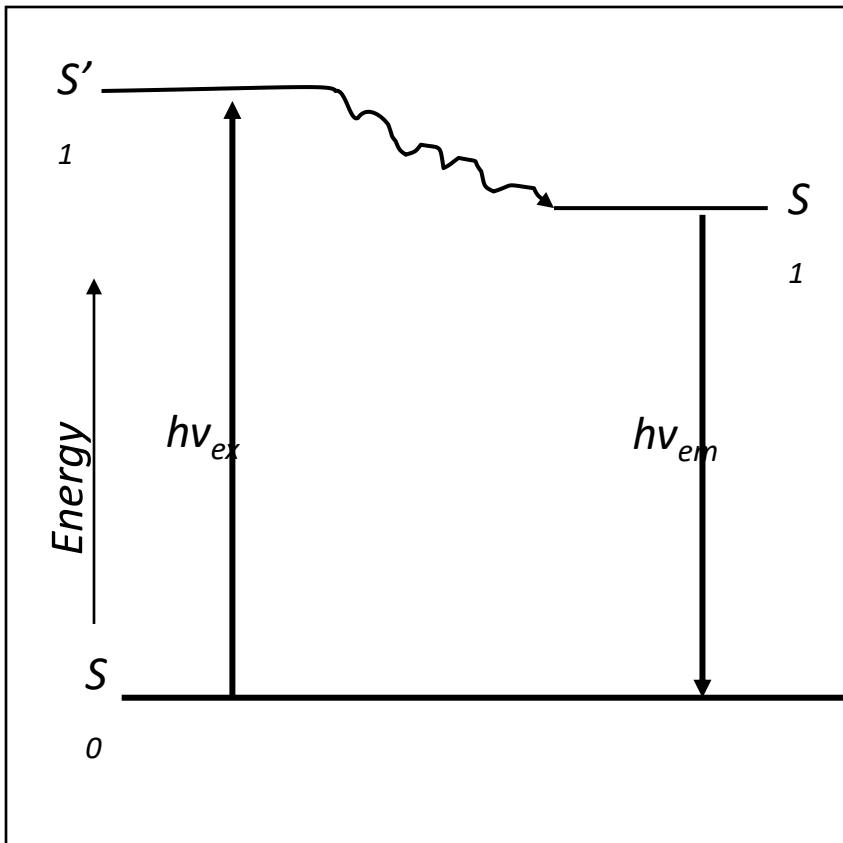


Confocal micrograph of a cavefish embryo at around five days post-fertilisation. The embryo has been stained with an antibody against a calcium-binding protein (in green) to show different neuronal types and their processes in the nervous system. This staining also reveals taste buds, which are located around the mouth and along the body of the cavefish.

Fluorescence Microscopy



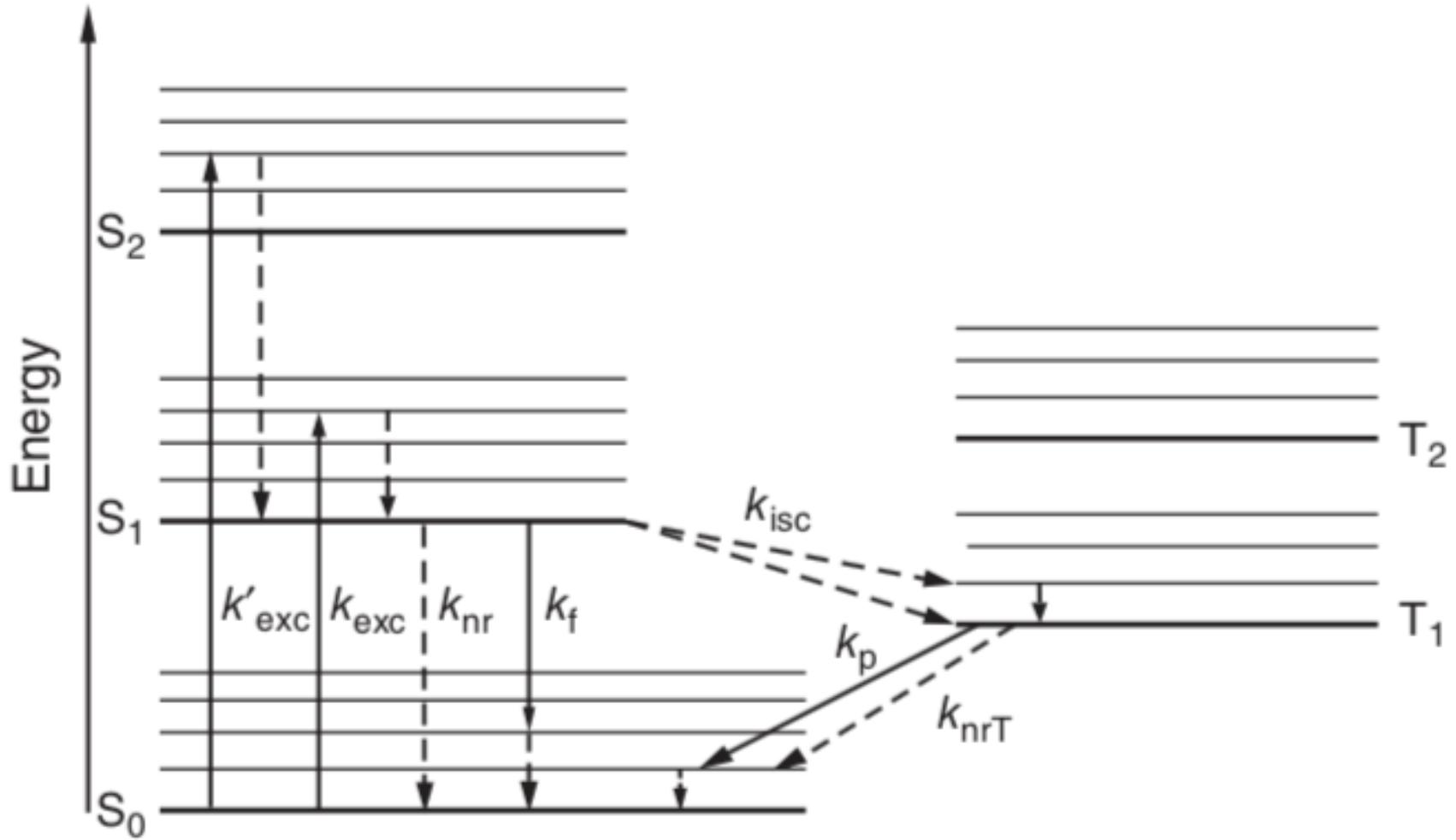
Simplified Jablonski Diagram for Fluorescence



A key point is that the electrons change their spatial distribution almost instantaneously – within femtoseconds – whereas the much heavier atomic nuclei move more slowly and take significantly longer – picoseconds – to relax to the new charge distribution of the electrons.

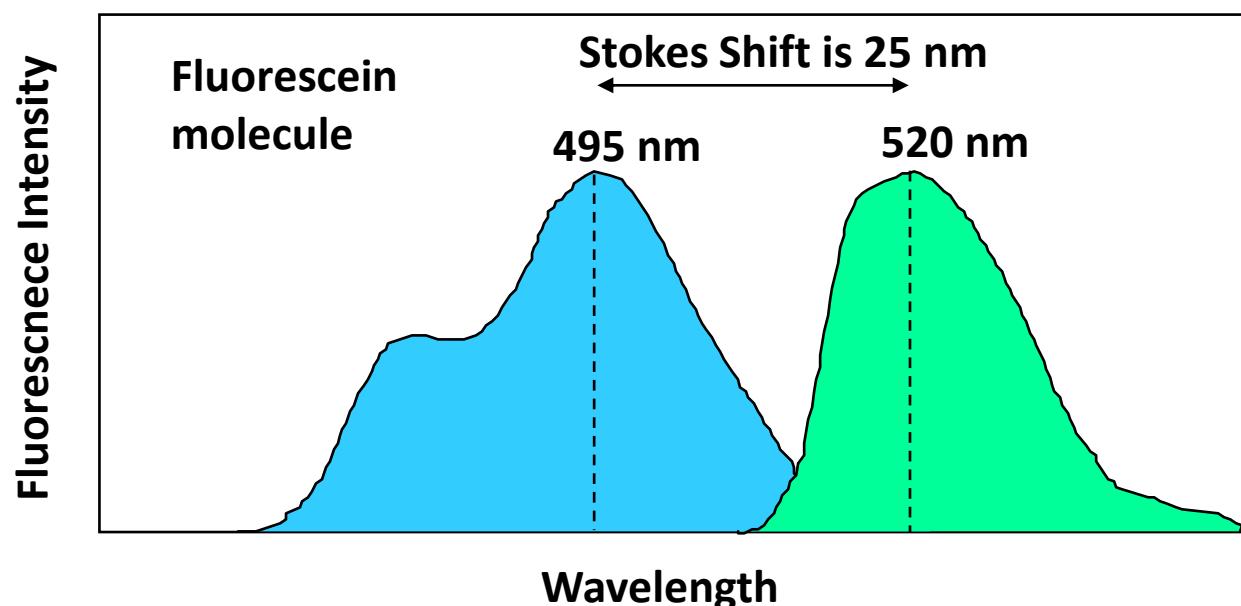
The physical and molecular basis for the theory of fluorescence emission was formulated by a Polish physicist Aleksander Jabłon'ski [4]

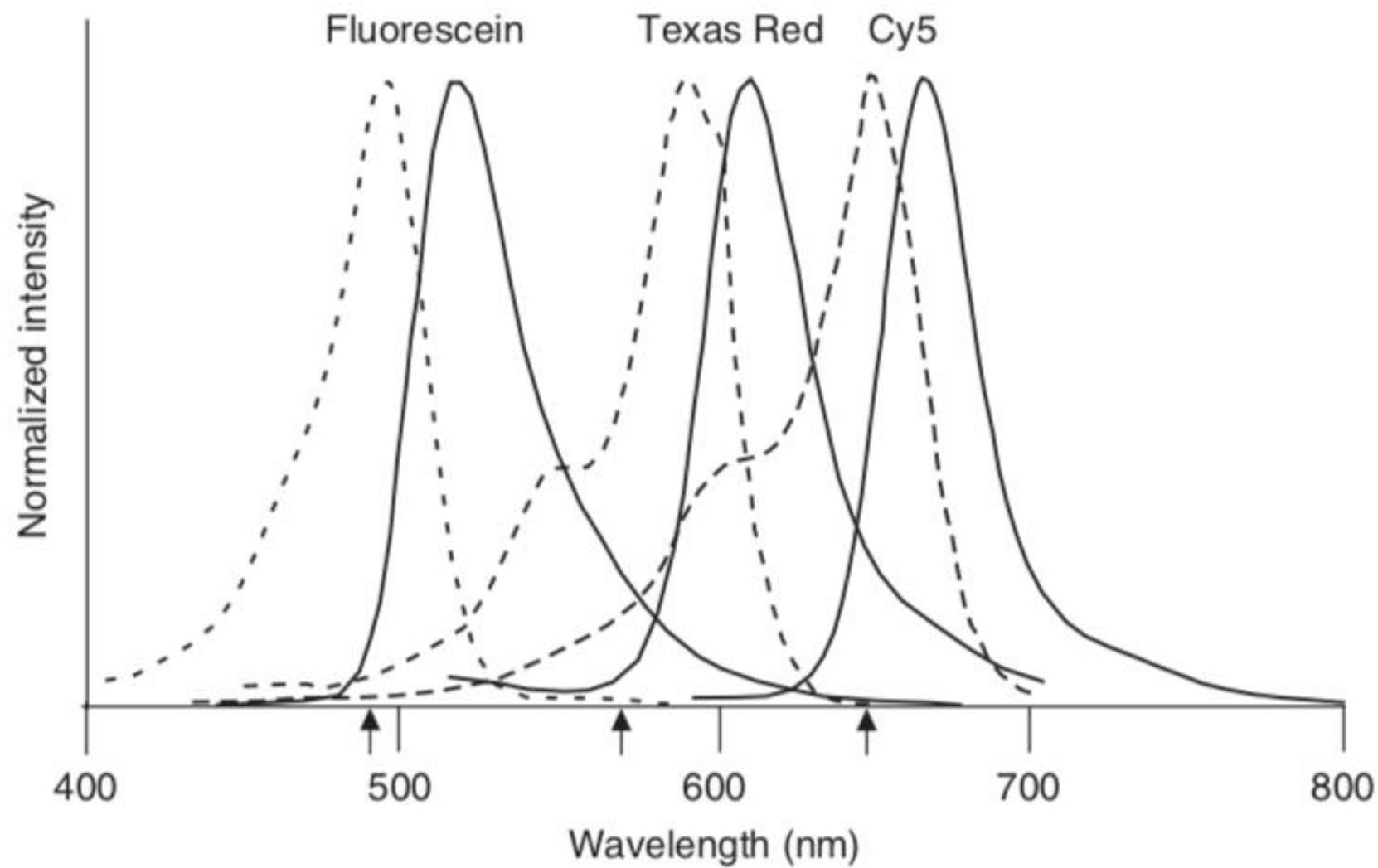
Different energy transfer process



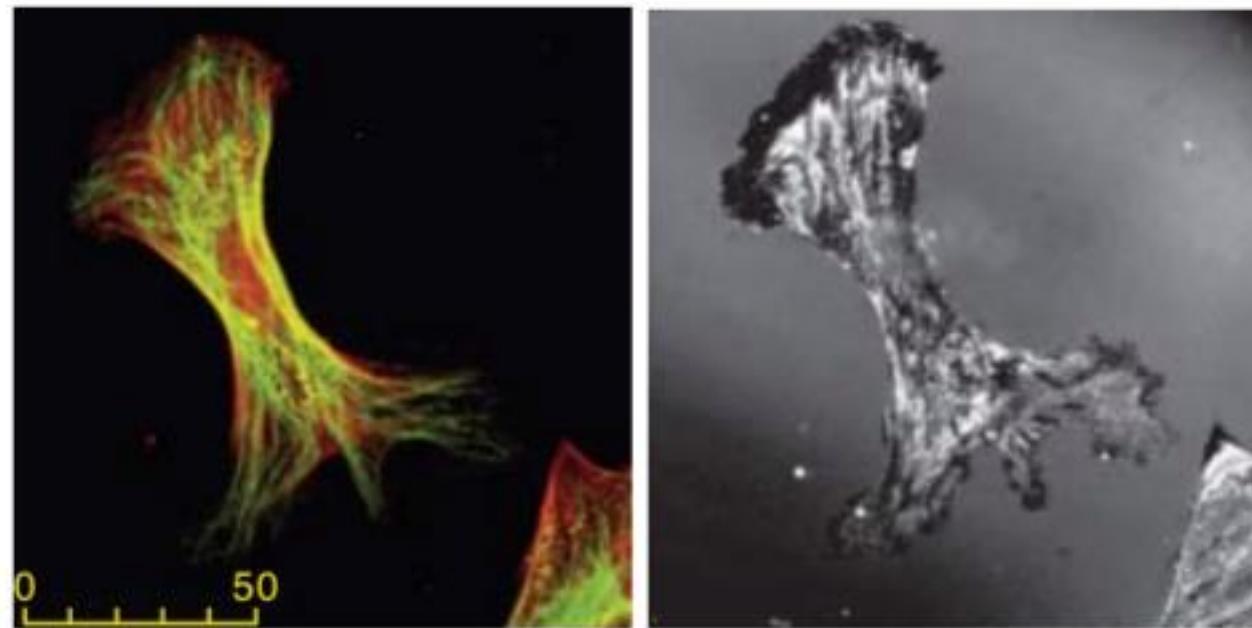
Stokes Shift

Due to broad energy levels, the absorption and emission are in spectral bands: $\lambda_{em} > \lambda_{ab}$



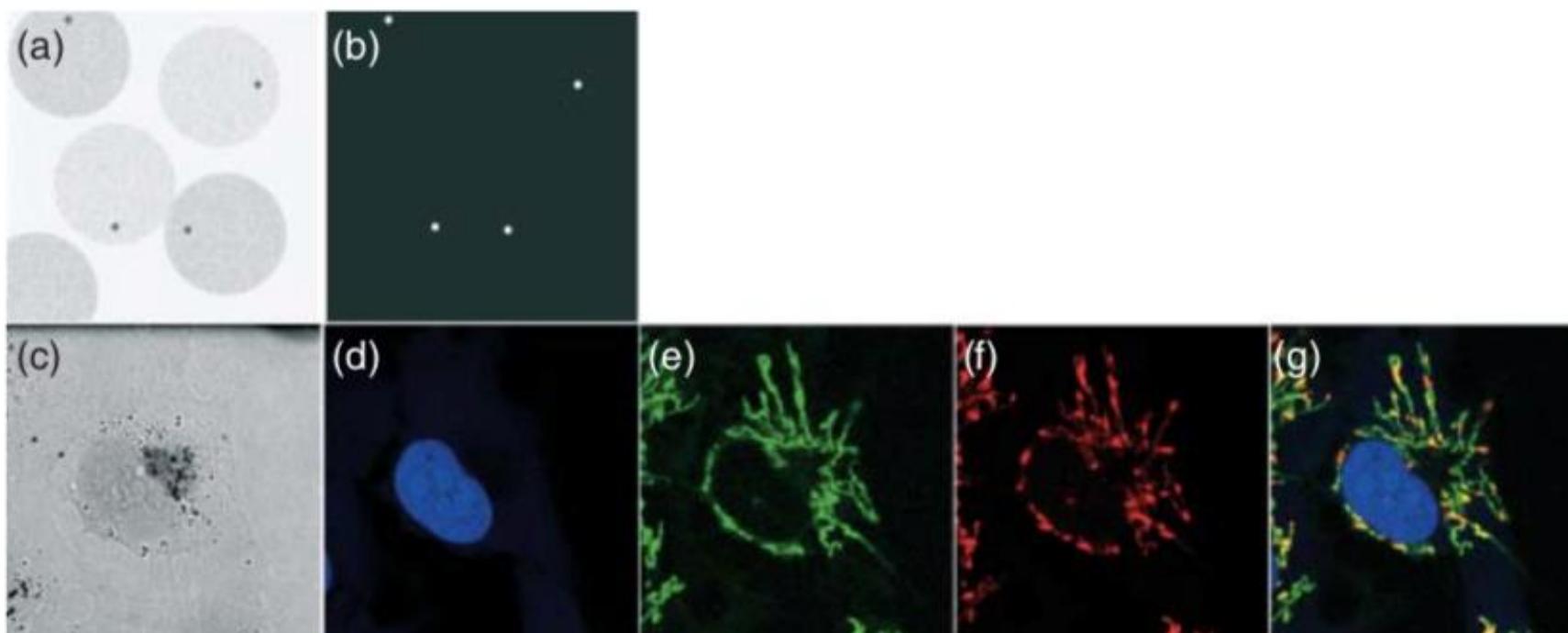


Contrast of bright light emission in the dark background



Molecular specificity:

1. DAPI
2. Immuno-label
3. Genetic engineering



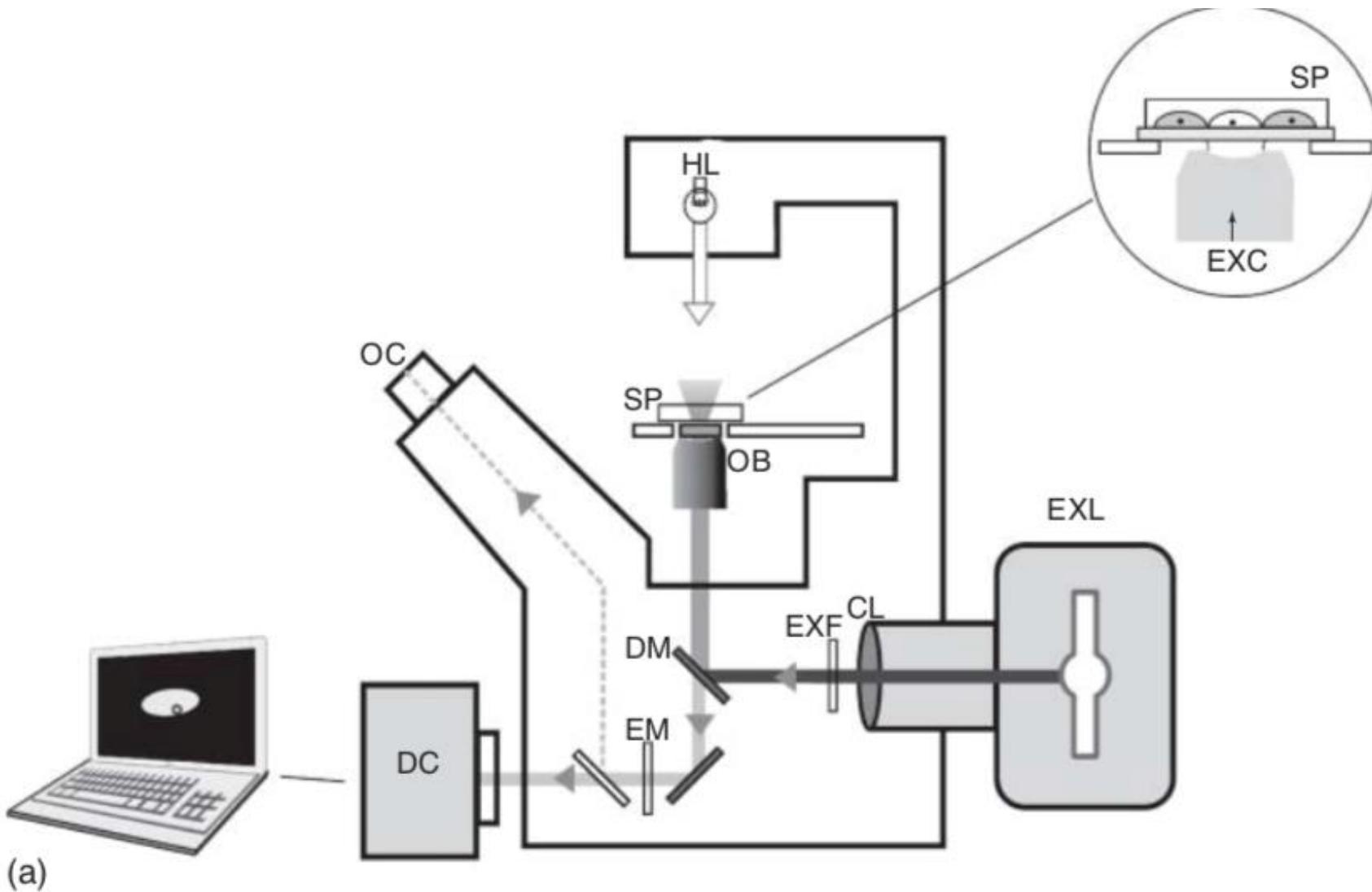
Fluorescence Microscopy

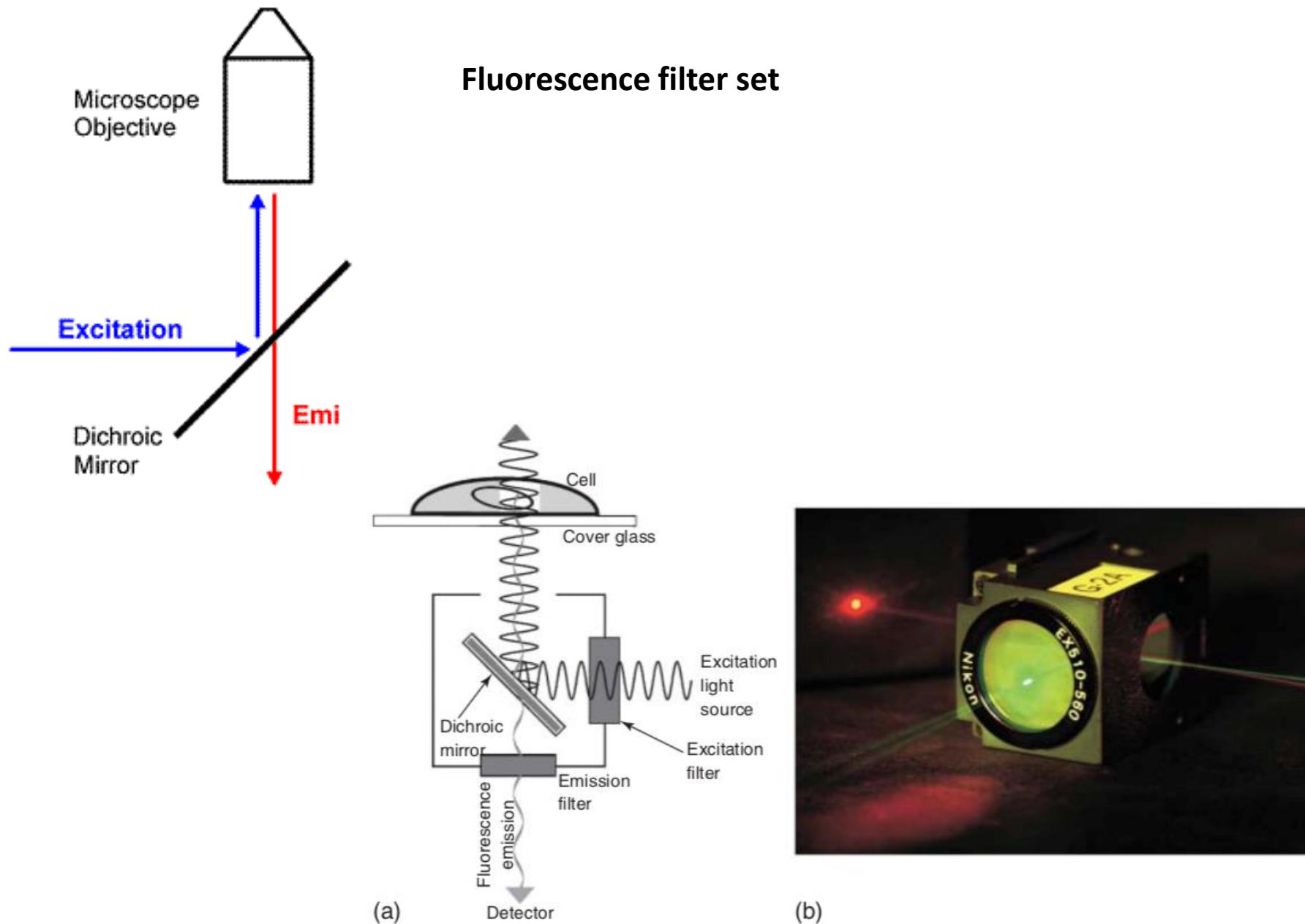
Advantages:

- Very sensitive
- Very specific (time/space of expression)
- Multiple labeling
- Sample can remain alive

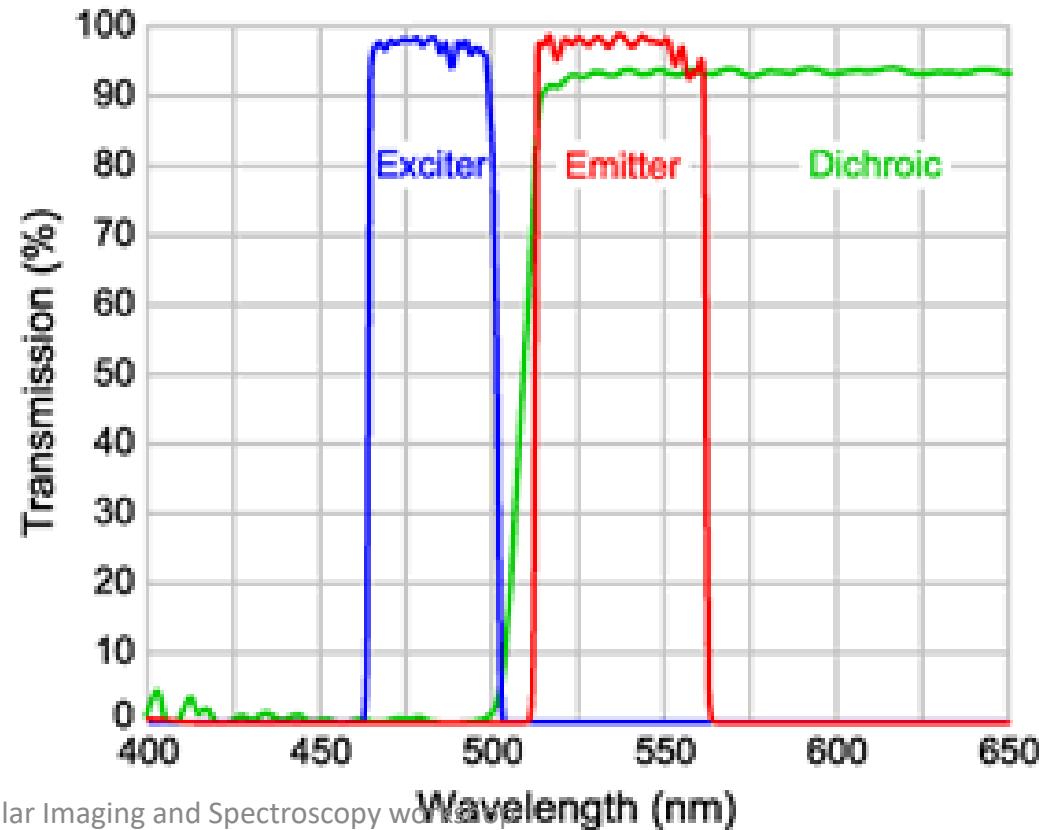
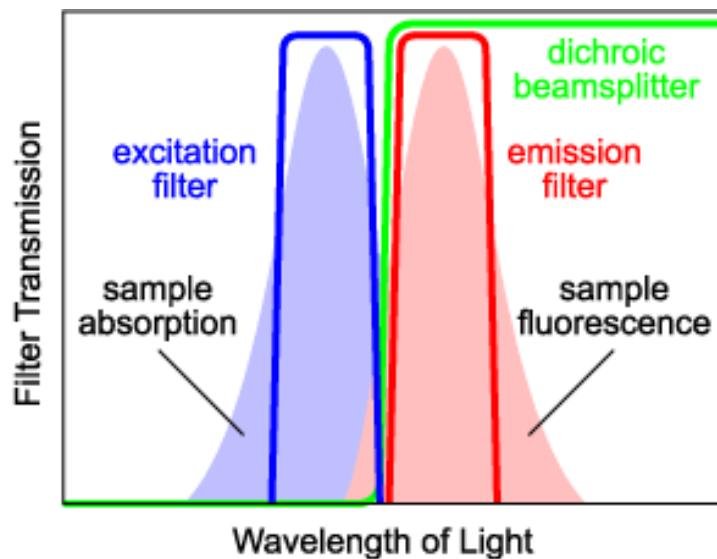
Disadvantages:

- Photo bleaching
- Bleed through from other layers in tissues or whole specimens

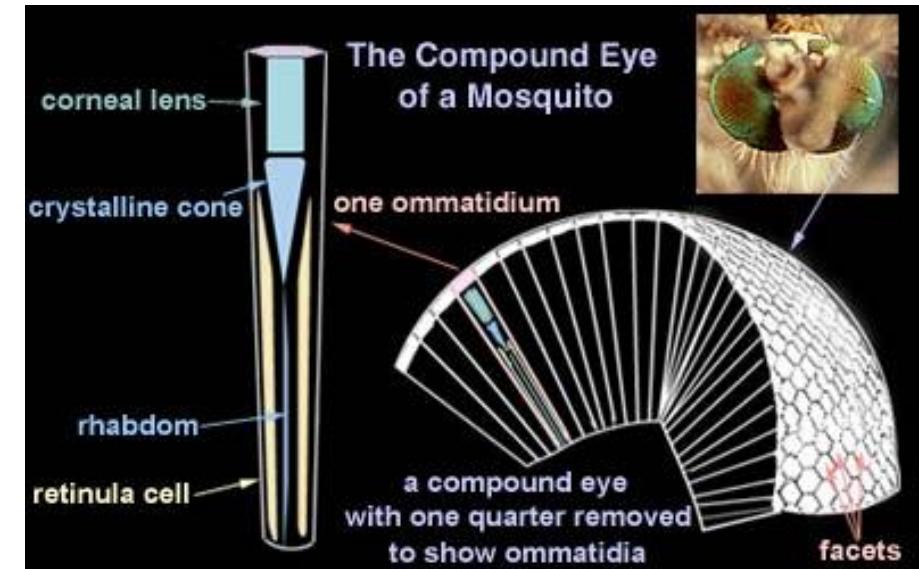
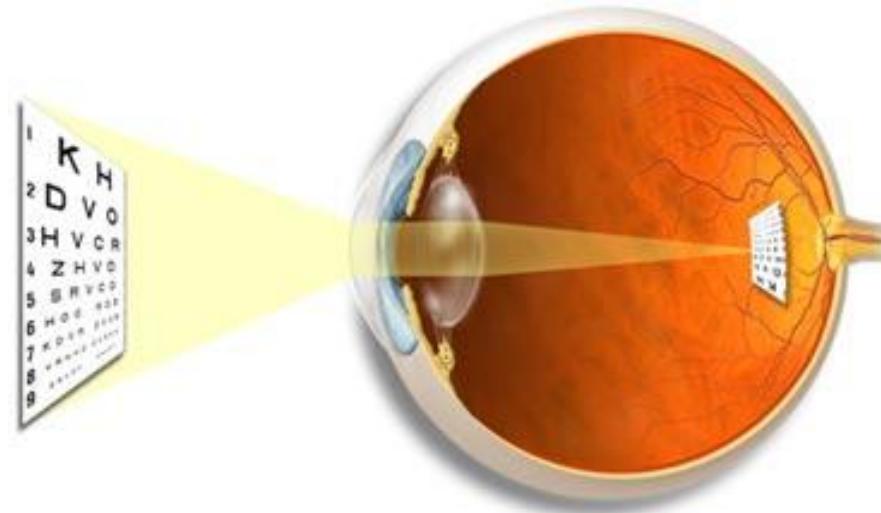




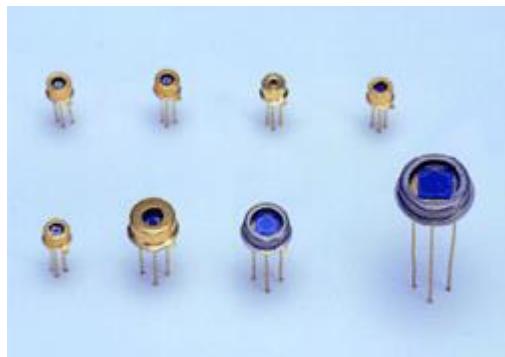
Bandpass Filters



Detectors



APD



Full-Frame CCD Architecture

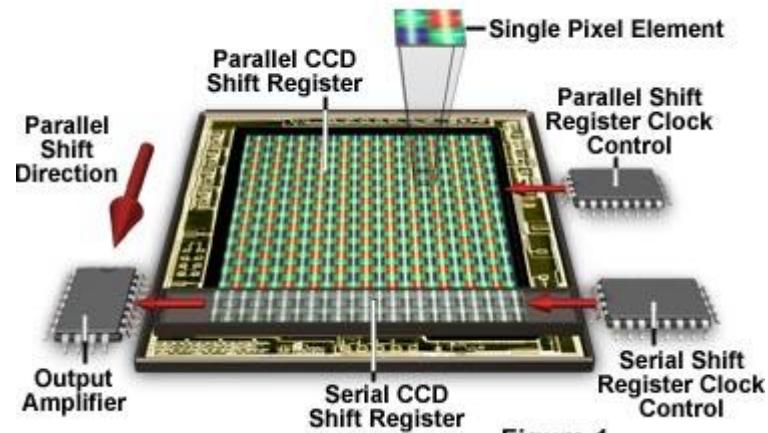


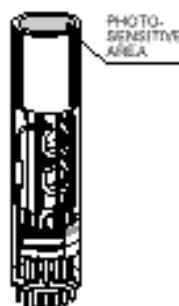
Figure 1

PMT Types

a) Side-On Type



b) Head-On Type



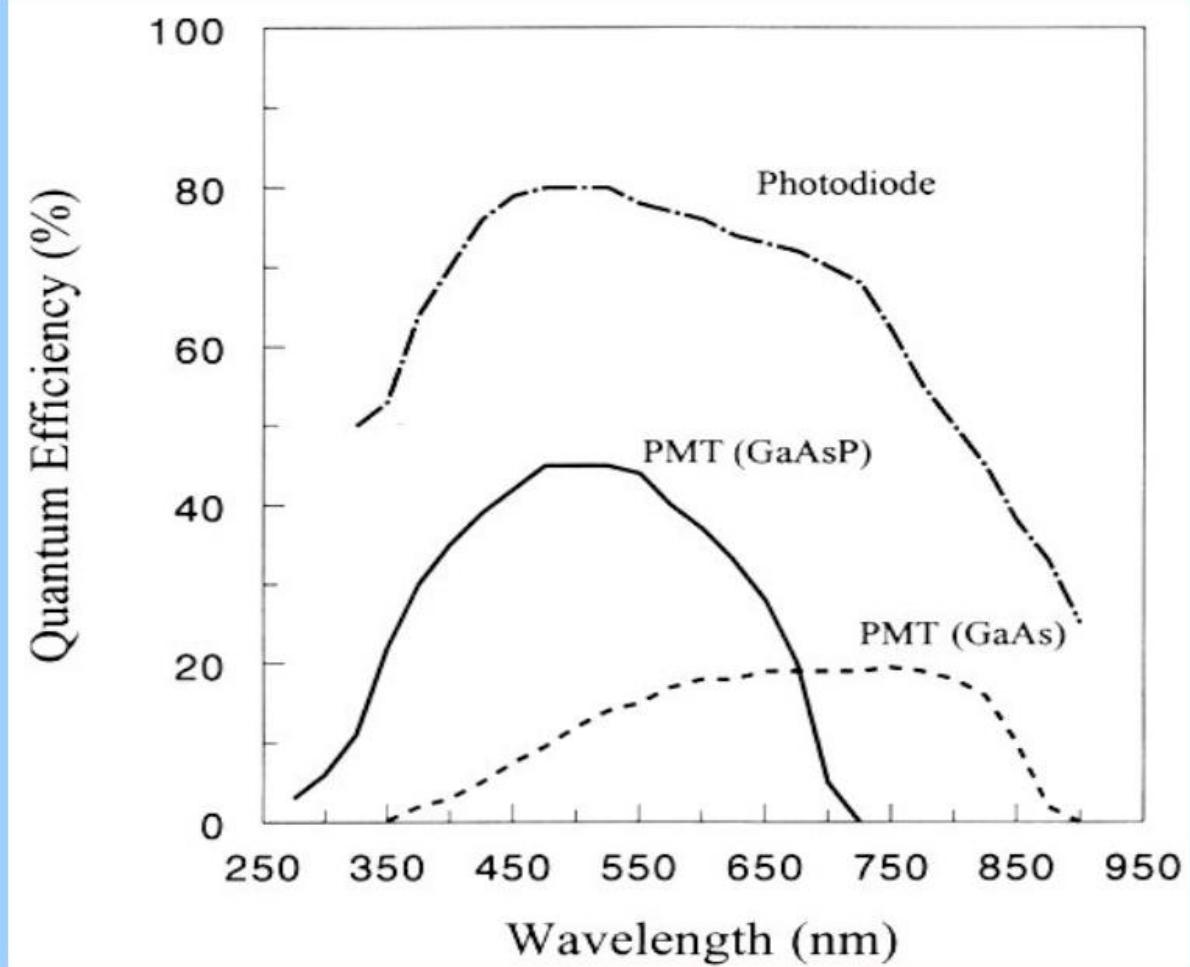
Single pixel

PMT
APD

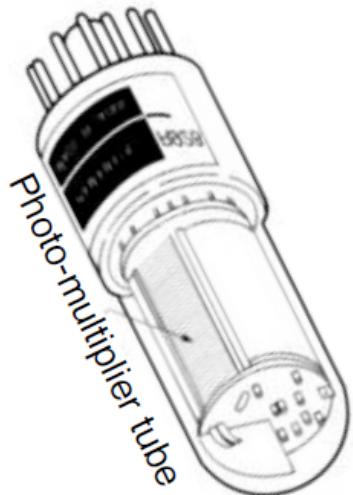
Multi pixel

- CCS
- CMOS
- ICCD
- EMCCD

Spectral sensitivity of confocal detectors

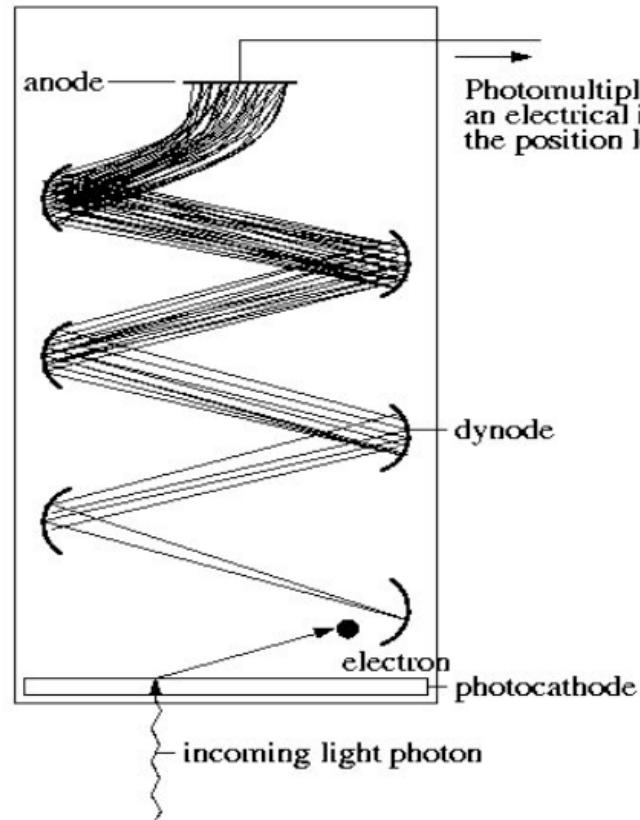


How PMT's work ...



Intensity measurements
without spectral information
(high sensitivity, pseudo colors)

Sequential single point measurements
-> coordinates get defined by position in the scan sequence

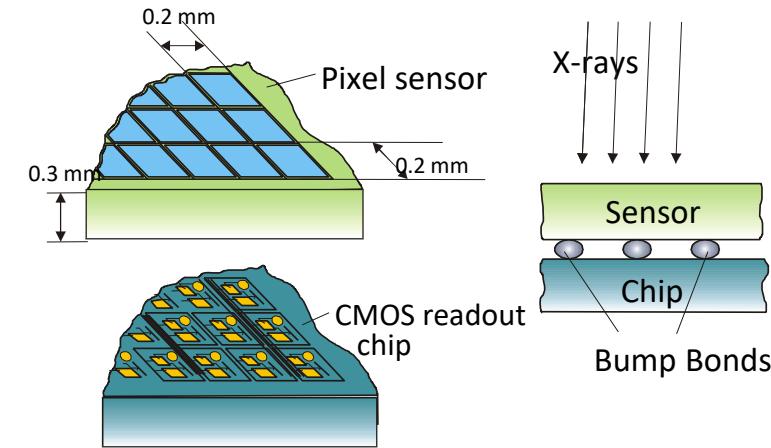


Principle of signal amplification

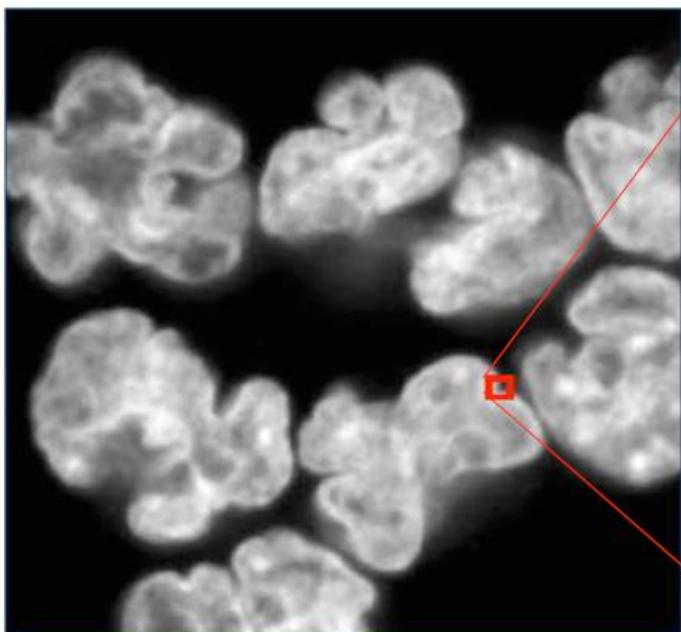
- 1) Conversion of photons into electrons
- 2) Multiplying electrons
- 3) Signal readout

Primary Features of CCD

- **Spatial resolution of the CCD array**
 - Number of Pixels in X and Y
 - Center to Center Distance of Pixels in microns
- **Full Well Capacity**
 - Related to Physical size and electronic design
 - Determines Maximum Signal level possible
- **Quantum Efficiency/Spectral Range**
 - Determines the usefulness of the camera
 - Major influence on exposure time
- **Camera Noise**
 - The limiting feature in low light applications
 - Influenced by Readout Speed / Readout Noise
 - Influenced by Dark Current / Time
- **CCD Chip Design**
 - Influences Total Frame Rate
 - Exposure time plus Readout time
 - **Total Photon Efficiency**
 - Quantum Efficiency and Exposure Cycle



Electronic grayscale image

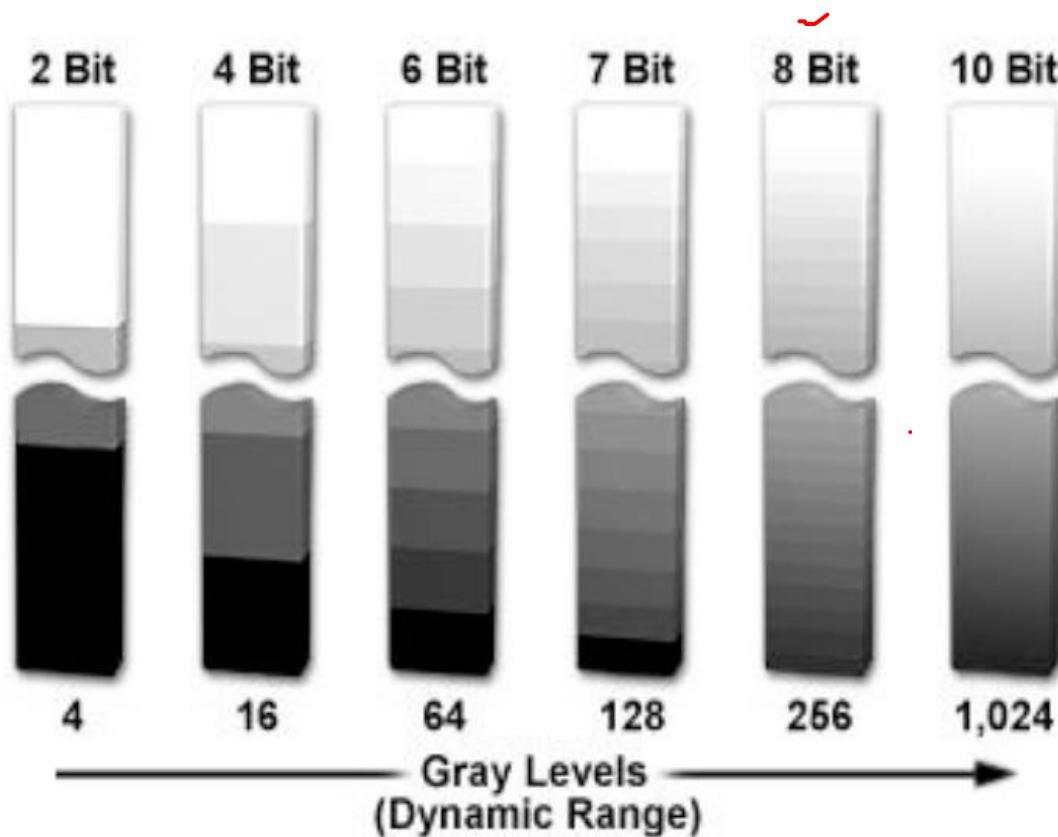


196	190	163	151	126	105	70	52	41
185	189	177	151	128	80	68	46	37
180	187	169	148	119	97	76	51	44
190	198	178	156	113	84	70	59	51
197	186	176	143	118	95	79	77	66
190	174	172	140	123	110	94	88	85
181	164	169	158	160	141	114	110	122
186	174	176	182	176	168	164	157	142
173	183	182	181	184	191	180	169	143

Each pixel (picture_element) has its coordinates and intensity values.

Dynamic range

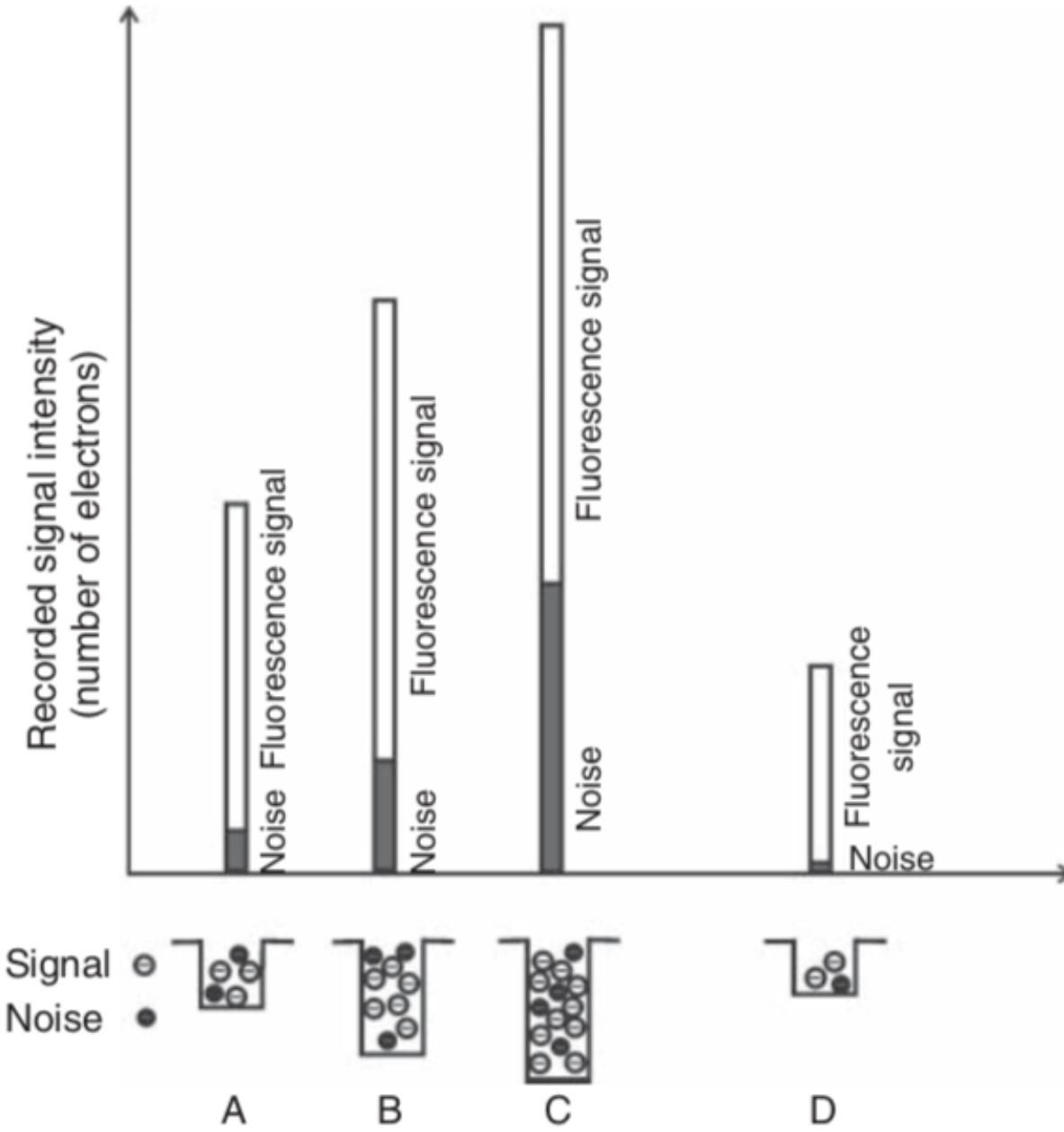
information depth - number of grey levels in an image, resolution of intensity



A higher dynamic range allows quantifications, image analysis.

The computer monitor displays 256 grey levels. The human eye can discriminate about 60 gray levels (6 bit).

More Bits need more storage space in the computer.



Fluorescent excitation sources

Mercury Arc Lamp



Figure 1

50 watts to 200 watts

The mercury burners do not provide even intensity across the spectrum from ultraviolet to infrared

Laser light source



- Highly coherent
- High power
- Single wavelength
- Pulsed sources



LED

Partially coherent
Low cost
Enough power

Z galvo stage provides fast z stacking

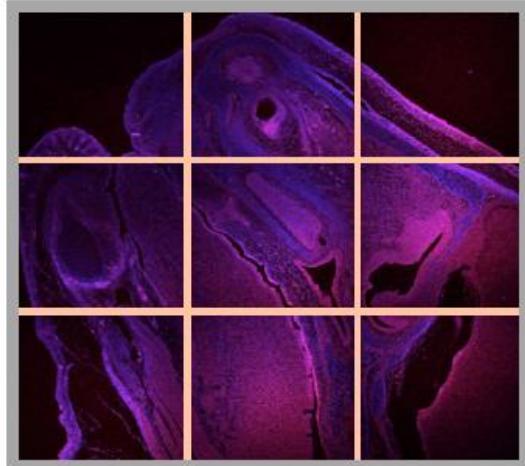


- ♣ Pivot-mounted arm with galvo motor
- ♣ $166 \mu\text{m}$ -z-range on SP2
 1.5 mm -z-range on SP5
- ♣ fast motor allows live xz-imaging
- ♣ Reproduceability 40nm
- ♣ Different inserts possible

Tile Scan

High Resolution Overview

Integration of Motorized xy-stage allows stitching of neighbouring data sets -> hight magnification in a larger field of view



Photobleaching

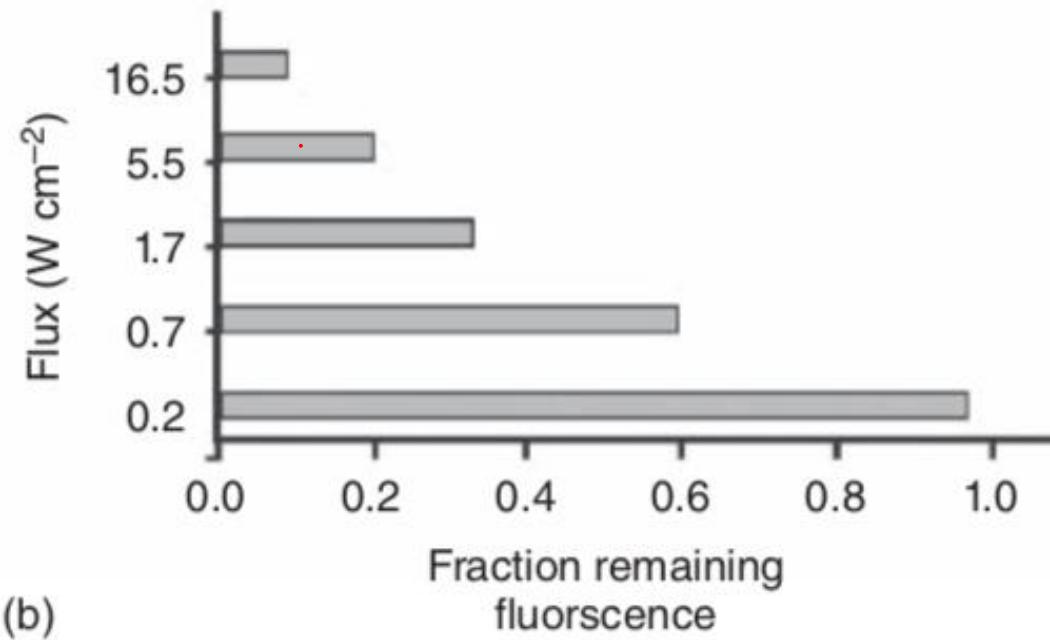
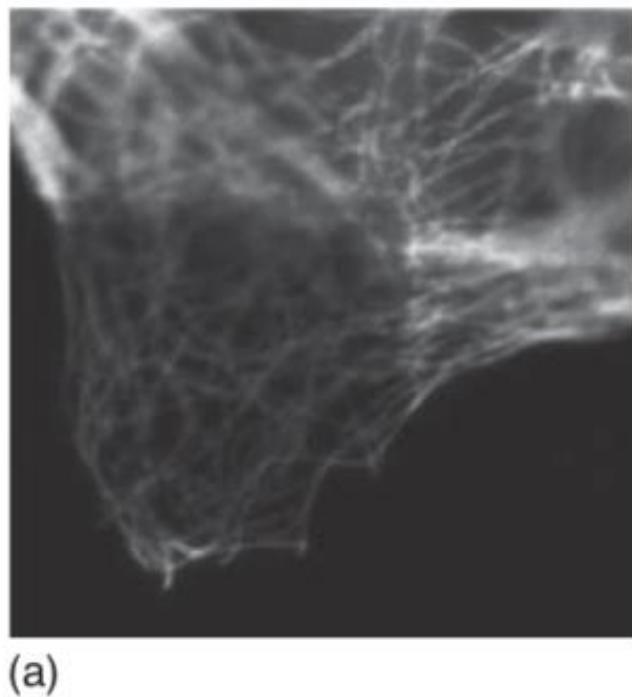
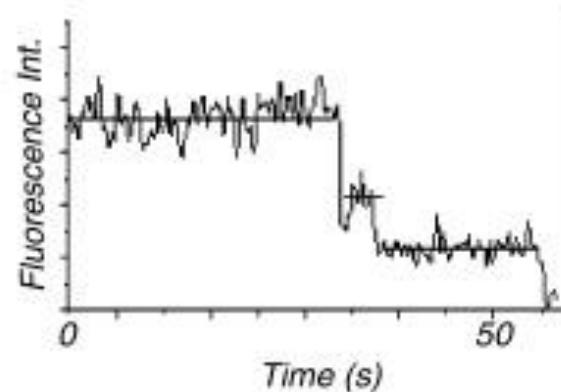
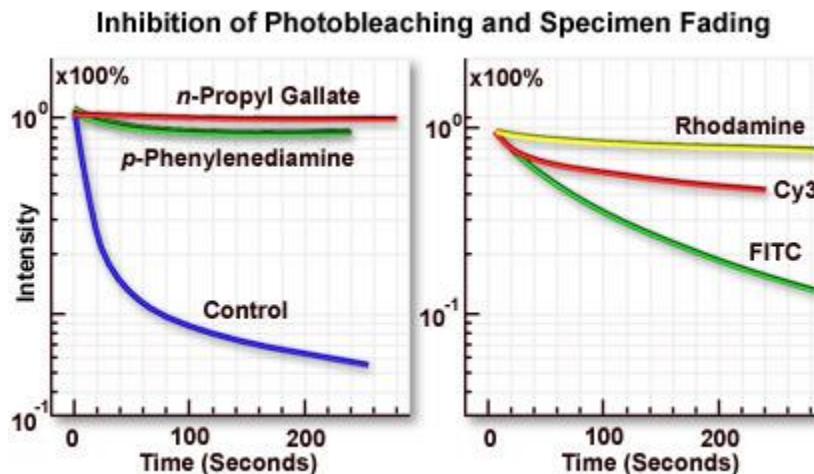


Figure 3.28 Photobleaching. (a) A square area of the fluorescent specimen was exposed to

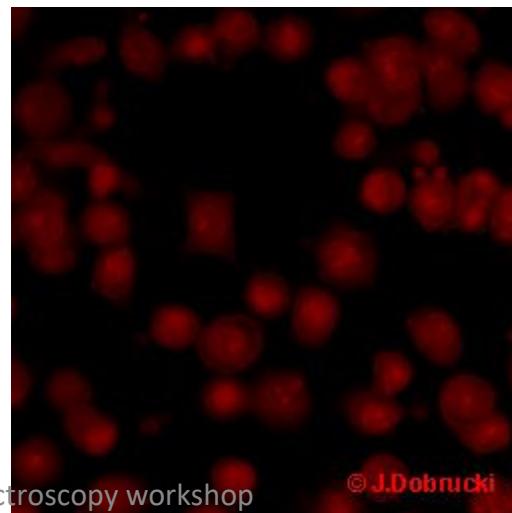
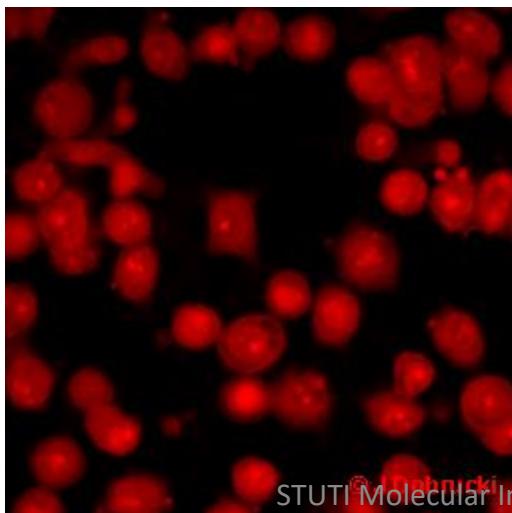
Photobleaching

- Defined as the [irreversible destruction](#) of an excited fluorophore.
- Methods for countering photobleaching
 - Scan for shorter times
 - Use high magnification, high NA objective
 - Use wide emission filters
 - Reduce excitation intensity
 - Use “[antifade](#)” reagents (not compatible with viable cells)



Antifade Agents

- Many quenchers act by **reducing oxygen concentration** to prevent formation of singlet oxygen.
- Satisfactory for fixed samples but not live cells!
- **Antioxidants** such as propyl gallate, hydroquinone, p-phenylenediamine are used.
- Reduce O_2 concentration or use singlet oxygen quenchers such as carotenoids (50 mM crocetin or etretinate in cell cultures); ascorbate, imidazole, histidine, cysteamine, reduced glutathione, uric acid, trolox (vitamin E analogue)



Type of fluorescent probes!

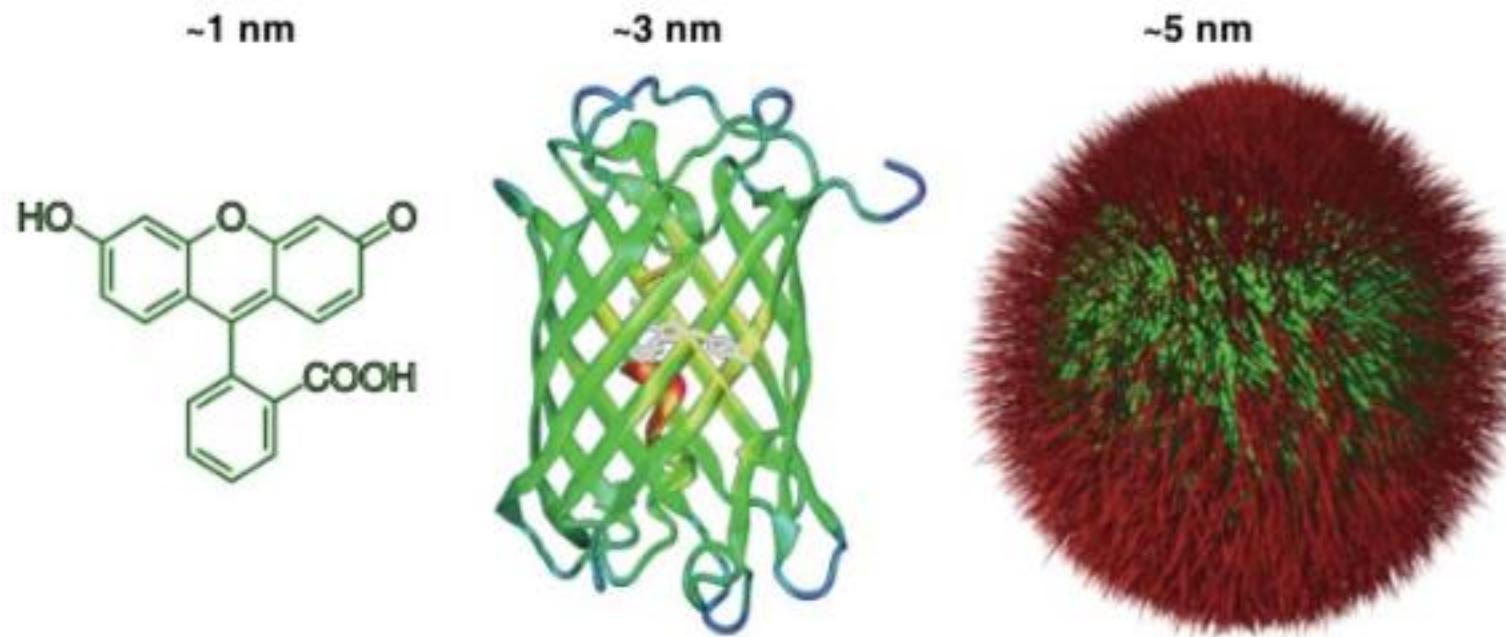


Figure 4.1 Fluorescent labels and their relative sizes. (from left to right) Organic dye, GFP-like fluorescent protein, and nanocrystal.

Organic dyes and QDs are intrinsically hydrophobic and have to be derivatized with functional groups such as carboxylates or sulfonic acids to render them soluble in aqueous solutions.

In traditional diffraction-limited fluorescence microscopy, the size of the fluorescence marker and its way of attachment to the structure of interest introduce negligible positional errors.

Probes for Proteins

<i>Probe</i>		<i>Excitation</i>	<i>Emission</i>
FITC		488	525
PE	575	498	
APC		630	650
PerCP™		488	680
Cascade Blue		360	450
Coumerin-phalloidin		350	450
Texas Red™		610	630
Tetramethylrhodamine-amines		550	575
CY3 (indotrimethinecyanines)		540	575
CY5 (indopentamethinecyanines)	640	670	

Key Parameters

- Extinction Coefficient (ϵ) : The fraction of light lost to scattering and absorbtion per unit distance in a *participating medium* (fraction per meter)
 - ϵ (epsilon) at a single wavelength (usually the **absorption maximum**)
- Quantum Yield
 - ϕ (phi) is a measure of the ratio of photon emission over absorbtion.

$$\phi = \frac{\text{Number of emitted photons}}{\text{Number of absorbed photons}}$$

Table 4.2 Key properties of fluorescent labels.

Property	Definition/Impact
<i>Optical properties</i>	
Excitation spectrum	Wavelength dependence of the ability of light to induce fluorescence emission of a chromophore
Extinction coefficient ϵ_λ ($M^{-1} cm^{-1}$)	Quantity characterizing a fluorophore's ability to absorb photons at a particular wavelength λ
Emission spectrum	Wavelength dependence of the emitted fluorescence
Stokes shift	Wavelength shift between excitation and emission bands
Fluorescence quantum yield (QY)	Ratio between the number of photons emitted and the number of photons absorbed
Molecular brightness	Product $QY \times \epsilon$ quantifies the relative rate of photon emission from different fluorophores under identical excitation conditions
Photostability	Resistance to photobleaching, quantified by the quantum yield of photobleaching, that is, ratio of the number of photobleached molecules to the total number of photons absorbed in a sample during the same time interval
<i>Physicochemical properties</i>	
Size	Most often, the smaller the better
Material	May affect chemical stability, toxicity, options to functionalize the probe
Solubility	Most biological applications require aqueous solvents
Cytotoxicity	Effect on sample viability
Phototoxicity	Effect on sample viability
Conjugation chemistry	Determines labeling target
Localization of target	Affects the choice of conjugation
Metabolism of target	Determines temporal stability of labeled construct in live cells

Table 4.1 Fluorescent markers – comparison of key properties.

Class	Examples	Brightness ^{a)}	Photostability ^{a)}	Biocompatibility ^{a)}	Environmental sensitivity ^{a)}
AlexaFluor	AlexaFluor 488	XXXX	XXXX	XXXX	XX
Atto	Atto 488	XXXX	XXXXX	XXXX	XX
BODIPY	BODIPY TMR	XXX	XX	XXXX	XXX
Coumarin	Coumarin 6	X	X	XXX	XXXX
Cyanines	Cy5, Cy7	XXX	XXXX	XXX	XX
Fluorescein	FITC	XXX	X	XXX	XXXX
Rhodamines	Rhodamine 6G	XXX	XXXX	XX	XXX
Quantum dots	—	XXXXX	XXXXX	X	X
GFP-like proteins	EGFP, EosFP	XX	XX	XXXXX	XX
Phycobiliproteins	R-phycoerythrin	XXXX	XX	XX	XX

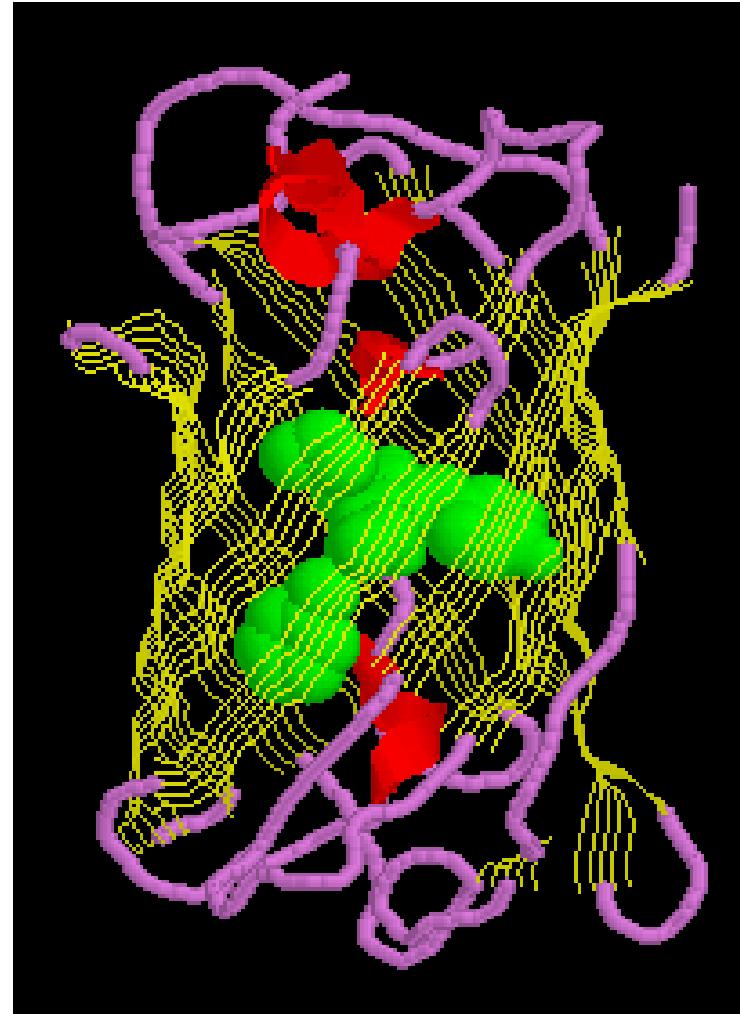
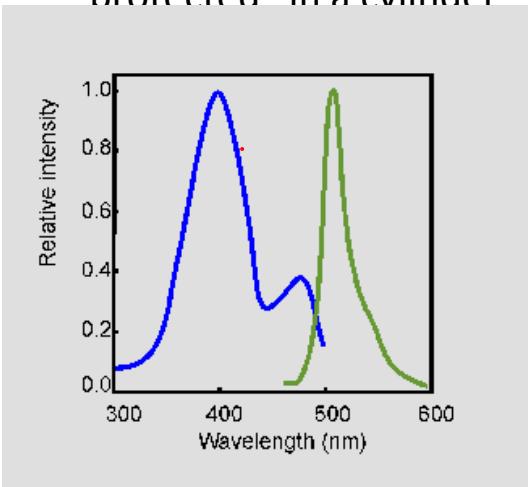
a) The number of 'x' symbols encodes a coarse scale for the shown quantity.

CdSe semiconductor QDs are toxic to live specimens because of their chemical composition. However, if the toxic nanocrystalline core is tightly encapsulated in a chemically stable shell

Beyond pure chemical toxicity, one also has to consider phototoxicity, which derives from the electronic properties of the photoexcited fluorophore. Most importantly, on excitation the fluorophore might react with molecular oxygen to yield a variety of reactive oxygen

GFP

- Discovered 1962 as companion to aequorin
- Cloned 1992, expression 1994
- 238 Aminoacids
- 27-30 kDa
- Fluorophore made by 3 aminoacids (65-67)
"protected" in a cylinder



A Japanese scientist, Osamu Shimomura, and two American scientists, Martin Chalfie and Robert Y. Tsien, were awarded the Nobel Prize for Chemistry in 2008 for "the discovery and development of the green fluorescent protein, 'GFP'".

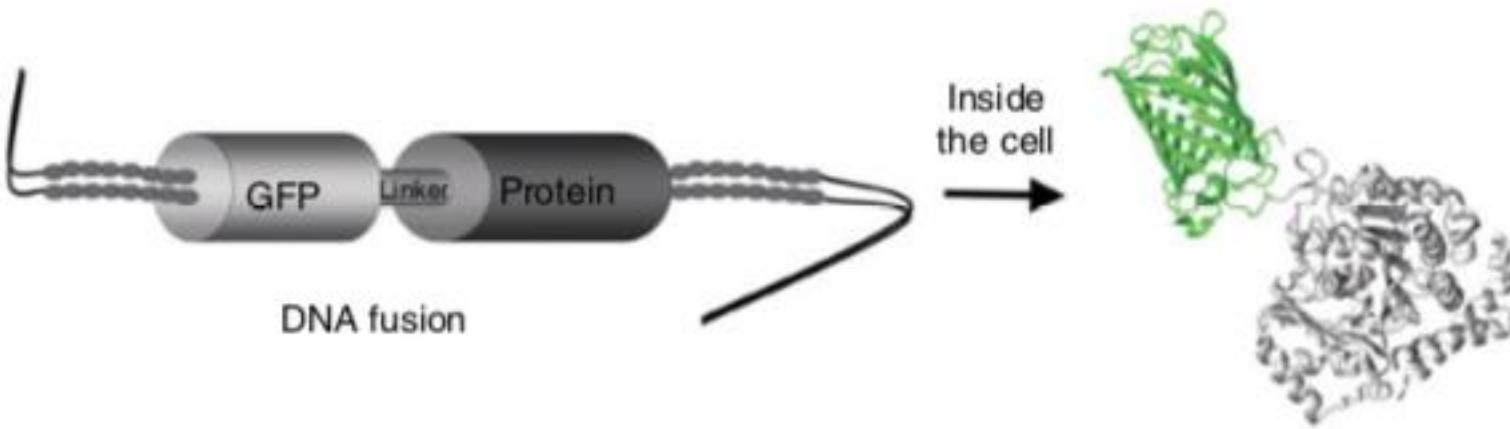


Figure 4.11 The gene coding for GFP is fused to the gene of the protein of interest on the DNA level. The fusion construct is subsequently expressed by the cell, eliminating the need for any additional conjugation chemistry.

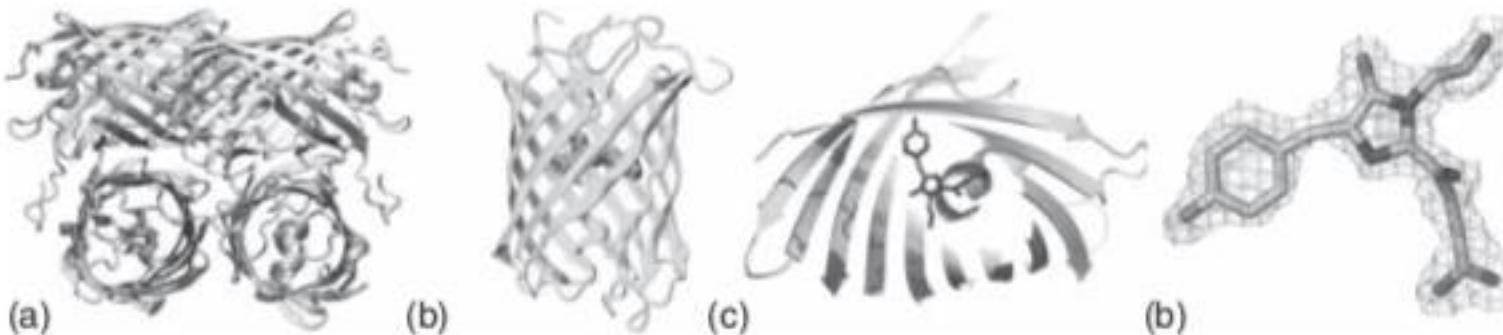


Figure 4.9 Molecular structure of GFP-like proteins: (a) quaternary arrangement, (b) monomer, (c) view into a monomer, and (d) the chromophore itself.

Table 4.3 Fluorescent proteins.

Name	Excitation (nm)	Emission (nm)	Brightness ^{a)}	Photostability ^{a)}	Oligomerization state ^{a)}
<i>Fluorescent proteins</i>					
GFP	395/475	508/503	×	×	D
EBFP	383	447	xx	x	D
mECFP	433/452	475/505	xx	xxx	M
mTFP1	462	492	xxxx	xxxx	M
EGFP	488	507	xxx	xxxx	D
EYFP	514	527	xxxx	xx	D
Venus	515	528	xxxx	x	D
DsRed	558	583	xxxx	xxxx	T
mRFP1	584	607	xx	x	M
tdTomato	554	581	xxxx	xxx	tD
mOrange	548	562	xxxx	x	M
mKO	548	559	xxx	xxx	M
mRuby	558	605	xxx	xxx	M
eqFP611	559	611	xxxx	xxx	T
mStrawberry	574	596	xxx	x	M
mCherry	587	610	xx	xxx	M
mKate	588	635	xxx	xxxx	M
mPlum	590	649	x	xx	M
mGarnet	598	670	x	xx	M
mCardinal	604	659	x	xx	M
eqFP670	605	670	x	xx	D

Fluorescent Probes

<i>Probe</i>	<i>Site</i>	<i>Excitation</i>	<i>Emission</i>
BODIPY	Golgi	505	
NBD	Golgi	488	
DPH	Lipid	350	
TMA-DPH	Lipid	350	
Rhodamine 123	Mitochondria	488	
DiO	Lipid	488	
dil-Cn-(5)	Lipid	550	
diO-Cn-(3)	Lipid	488	

BODIPY - borate-dipyrromethene complexes
DPH – diphenylhexatriene

NBD - nitrobenzoxadiazole
TMA - trimethylammonium

Immunofluorescent Staining

Immunofluorescent staining makes use of antibodies to locate and identify patterns of protein expression in cells.

Primary antibody binds to antigen.

Antibody-antigen complex is bound by a secondary antibody conjugated to a fluorochrome.

Upon absorption of high energy light, the fluorochrome emits light at its own characteristic wavelength (fluorescence) and thus allows detection of antigen-antibody complexes.

Suitable for:

- 1. frozen, non-fixed tissues and ethanol fixed tissues**
- 2. paraformaldehyde-fixed or methanol/acetone-fixed cells**

Basic Staining Technique

Cell Preparation

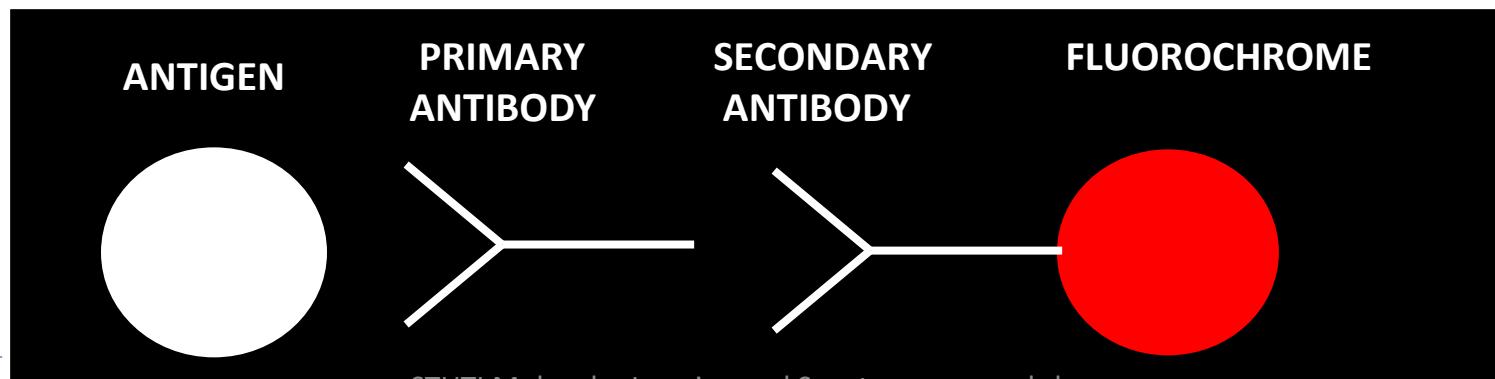
1. Culture cells on a glass coverslip in a 24-well plate. Cells may be transfected directly on the coverslip.
2. Fix cells using paraformaldehyde or methanol/acetone and then wash them 3 times in PBS

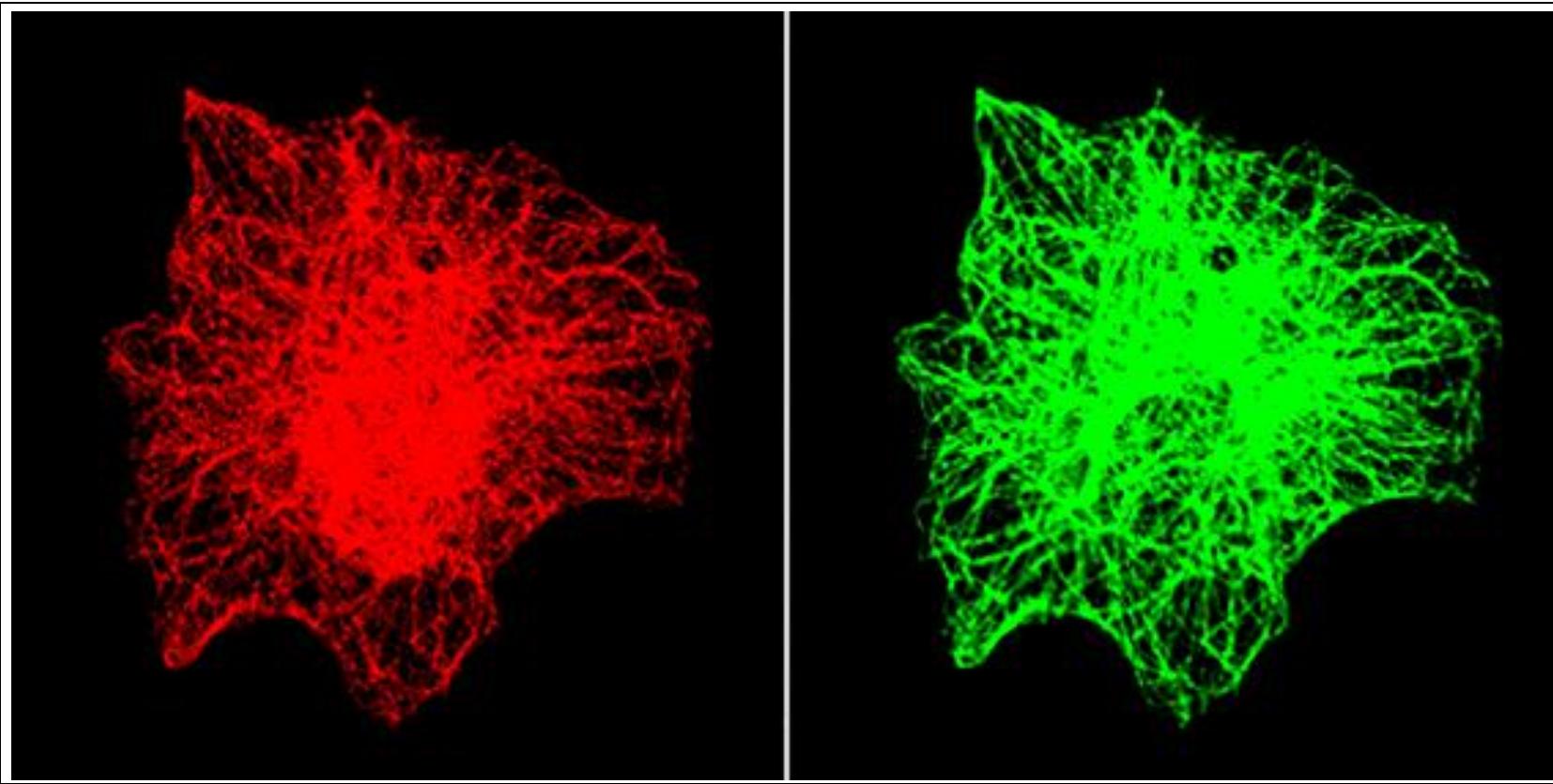
Cell Permeabilization

1. Incubate fixed cells in 1% Triton X-100 in PBS+0.02%BSA for 2 minutes at room temperature.
2. Wash the cells 3 times with PBS.

Immunofluorescent Cell Staining

1. Incubate cells with a blocking solution to minimize non-specific staining
2. Incubate cells with a polyclonal or monoclonal antibody specific for the protein of interest.
3. Incubate cells with a secondary antibody directed against the primary antibody.
The secondary antibody must be conjugated to a fluorochrome





PRIMARY ANTIBODY

sheep anti-p53 polyclonal

SECONDARY ANTIBODY

Texas Red conjugated anti-sheep

PRIMARY ANTIBODY

mouse anti-a tubulin monoclonal

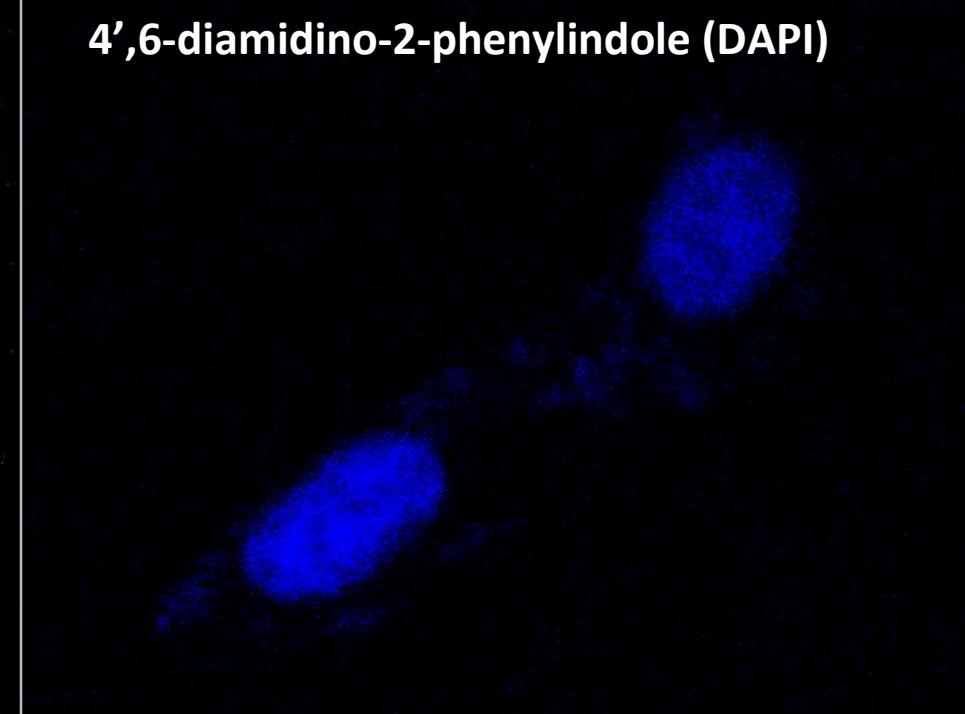
SECONDARY ANTIBODY

FITC conjugated anti-mouse

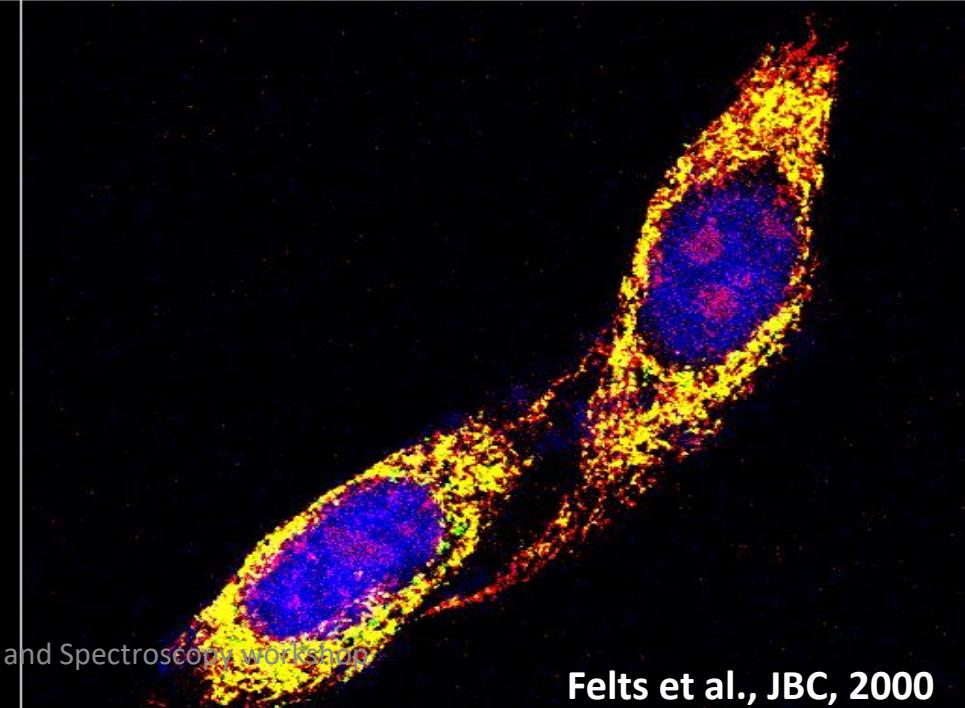
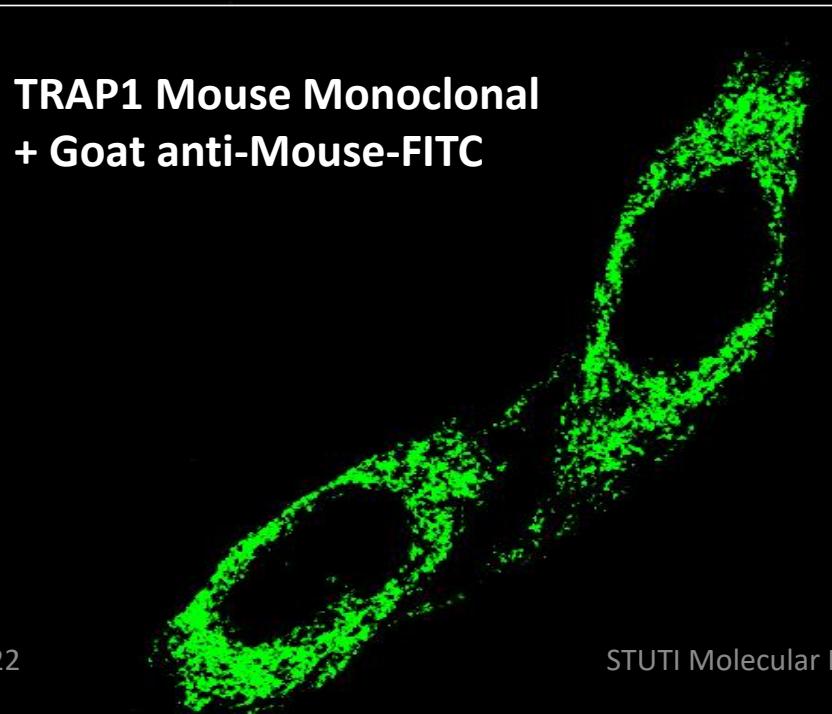
MitoTracker-Orange CMTMros



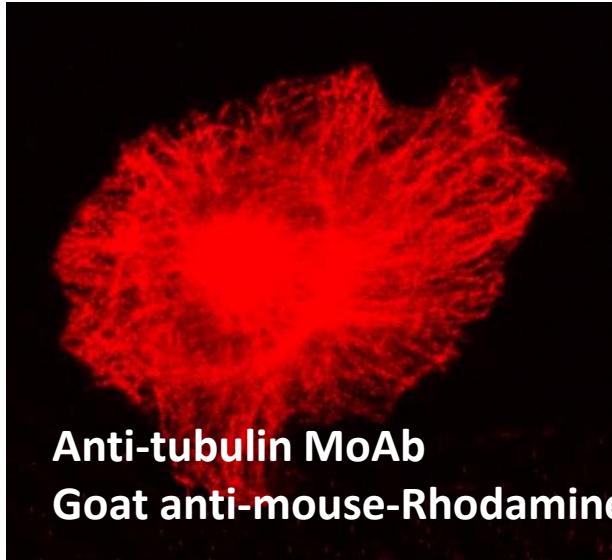
4',6-diamidino-2-phenylindole (DAPI)



TRAP1 Mouse Monoclonal
+ Goat anti-Mouse-FITC

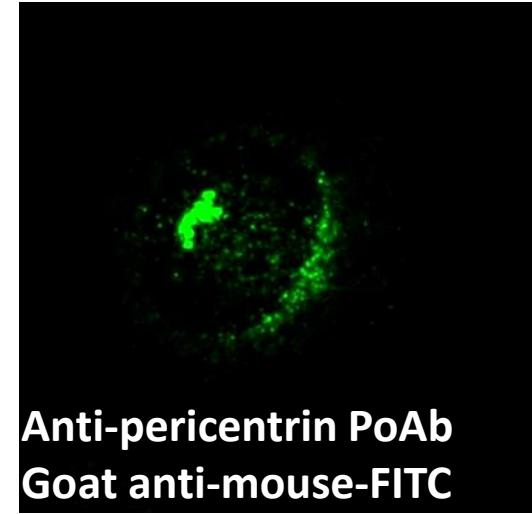


microtubules

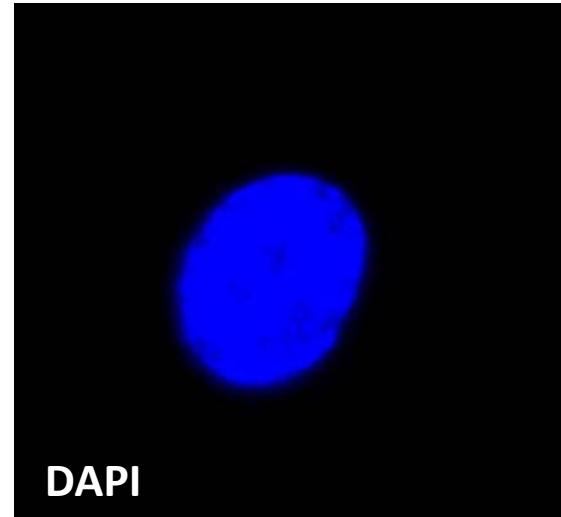


**Anti-tubulin MoAb
Goat anti-mouse-Rhodamine**

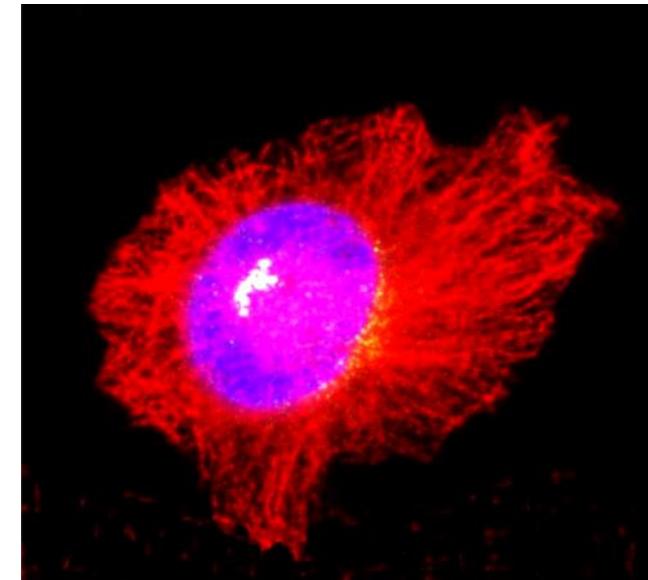
centrosomes



**Anti-pericentrin PoAb
Goat anti-mouse-FITC**



DAPI



nucleus

MERGE