MIC Microscopy Workshop 2022
Mixed Zoom / In-person Seminars

- **In Person**

Please wear a mask, your neighbor may have a medical condition!
35 sqft = 6 ft away from anybody else
Please save questions for Q. breaks
Questions from in-person audience repeated *by* speaker for Zoom

- **Zoom**

Mute your microphone
Do not talk in your microphone, in-person audience cannot hear you
Questions in chat will be relayed *to* the speaker

Presentation PDFs from MIC staff available on demand
Session is being recorded

Microscopy Workshop, Spring 2022

Vincent Schram, Ph.D.

- **LIGHT MICROSCOPY 1: TRANSMISSION AND FLUORESCENCE**

  Monday May 9, B35 / GG607, 11 am - 1:30 pm

- **LIGHT MICROSCOPY 2: CONFOCAL, 2P AND LIGHT SHEET**

  Tuesday May 10, B35 / GG607, 11 am - 1:30 pm

- **LIGHT MICROSCOPY 3: SUPER-RESOLUTION IMAGING**

  Wednesday May 11, B35 / GG607, 11 am - 1:30 pm

- **IMAGE ANALYSIS WORKSHOP: IMAGEJ**

  Thursday May 12, B35 / GG607, 9:30 am - 12:30 pm / 1 pm - 4 pm

- **CONFOCAL MICROSCOPY HANDS-ON**

  Friday May 20, B35 / GD922, 9:30 am - 4 pm (4 groups)

LIGHT MICROSCOPY IN B.35

Light Imaging Facility (LIF)

- NINDS confocal facility
- Open to everyone in Porter building
- Carolyn Smith, smithca@nih.gov

Microscopy & Imaging Core (MIC)

- NICHD full-service microscopy facility
  - Light and confocal microscopy: Vincent Schram
  - Well-equipped histology lab: Dr. Ling Yi
  - Transmission electron microscope / EM wet lab: Chip Dye
- Priority to NICHD, B.35 NINDS, limited for other B.35 Institutes

No charge, but acknowledgement required

Microscopy Resources

- Zeiss: http://zeiss-campus.magnet.fsu.edu/

- Nikon: https://www.microscopyu.com/

- Olympus: https://www.olympus-lifescience.com/en/microscope-resource/

- Leica: https://www.leica-microsystems.com/science-lab/topics/basics-in-microscopy/

- MIC site: http://mic.nichd.nih.gov/

- FAES courses:

  BIOC 035 (microscopy, no schedule)
  BIOC 053 (super-resolution, March 23-26)
  BIOC 062 (ImageJ, June 29-30)

LIGHT MICROSCOPY Pt 1

TRANSMISSION AND FLUORESCENCE IMAGING

Target audience: life scientists unfamiliar with common light microscopy techniques

- **No biomedical research without microscopy (cells / tissues)**
- **Biologist not trained or proficient in microscopy methods**

→ ***Principles underpinning common light imaging techniques:***

Right experimental decisions
Optimize equipment choice and configuration
Discover and use available technologies

→ ***METHODS talks***

TRANSMISSION IMAGING

Light Fundamentals

Nature of light, Energy and Spectra, Refractive index, Polarization

Optics Fundamentals

Positive and Negative lenses, Aberration, Optical resolution

Light Microscope

Overview, Light sources, Kohler illumination

Contrasting Methods

Dark Field, Polarization, Phase Contrast, Differential Interference Contrast

LIGHT FUNDAMENTALS

Nature of light

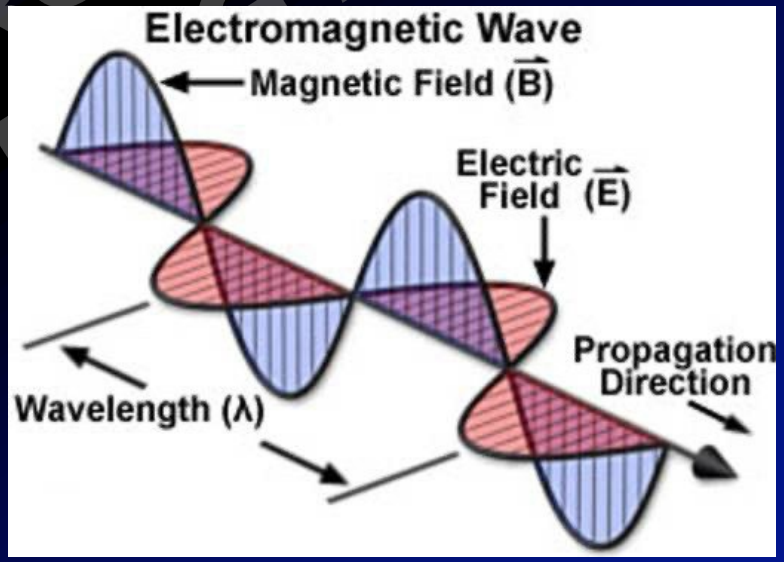
- **Light = electromagnetic radiation**

Energy emitted or absorbed by charged particles

- **Superposition of magnetic and electric field**

- **Dual wave and particle behavior:**

Photon has mass and energy (particle) and wavelength (wave)



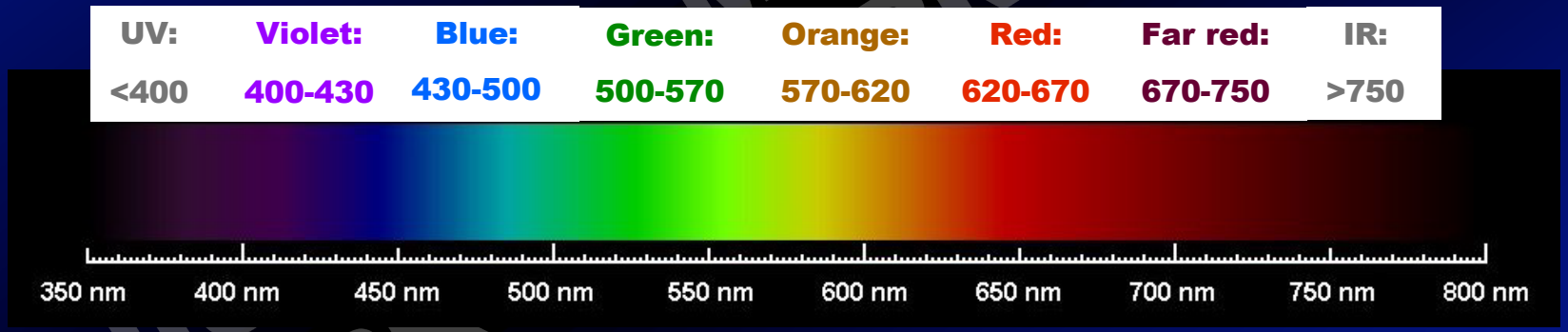
Energy and Spectra

- **Photon energy:** $E = h \cdot \nu = h \cdot C / \lambda$

  ν = frequency C = speed of light

  h = Planck's constant, $6.62\ 10^{-34}$ J.sec λ= wavelength (nm)

- **Energy inversely proportional to wavelength (nm)**



- **White light: continuous emission across visible spectra**

  Reddish = low, blueish = high color temperature

Refractive Index

- Speed of light varies with medium
- Index of refraction: $n = c / v$

  c = speed of light in vacuum

  v = speed of light in medium

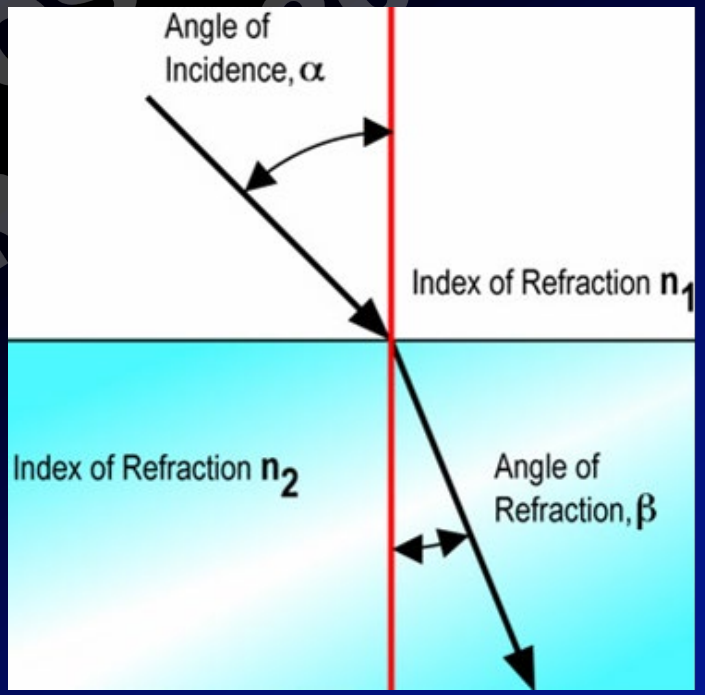
- Common refractive indexes

  Air = 1

  Water = 1.33

  Glycerol = 1.45

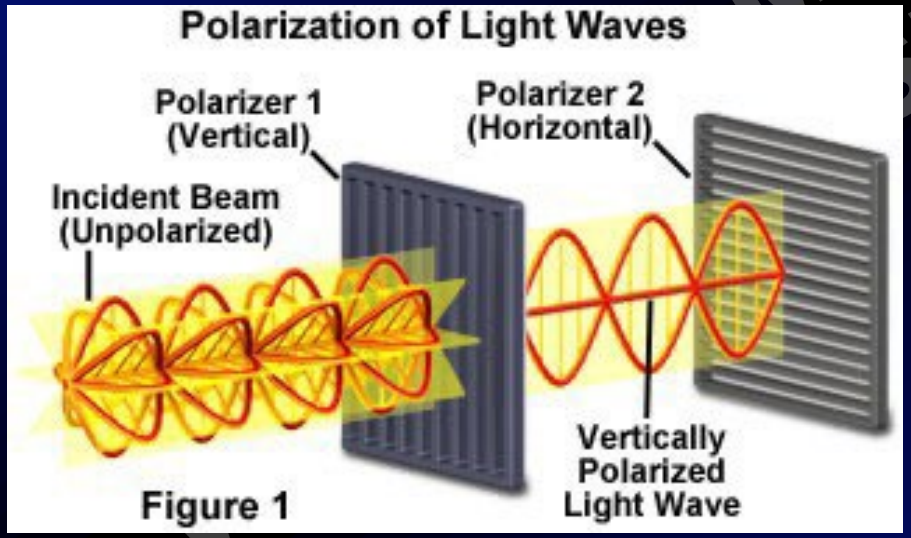
  Glass = 1.52

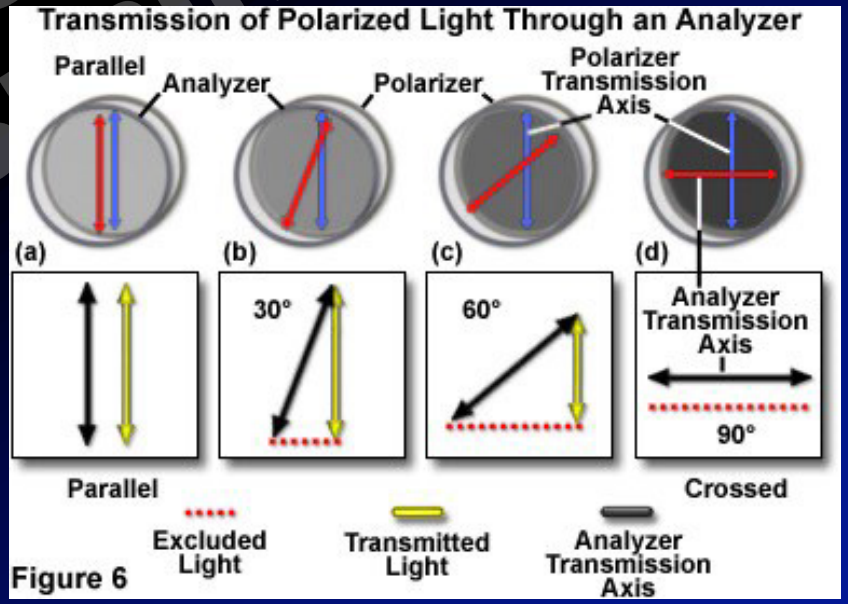


- Snell's law: $n1 . \sin \alpha = n2 . \sin \beta$

Polarization

- Polarization = orientation of light's electric field
- Human eye not sensitive to polarization

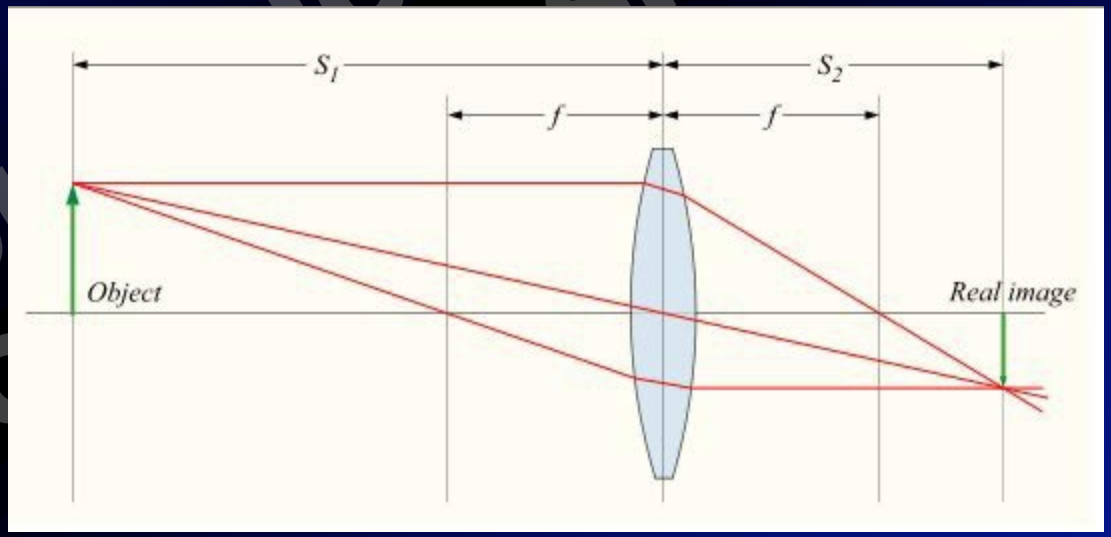




OPTICS FUNDAMENTALS

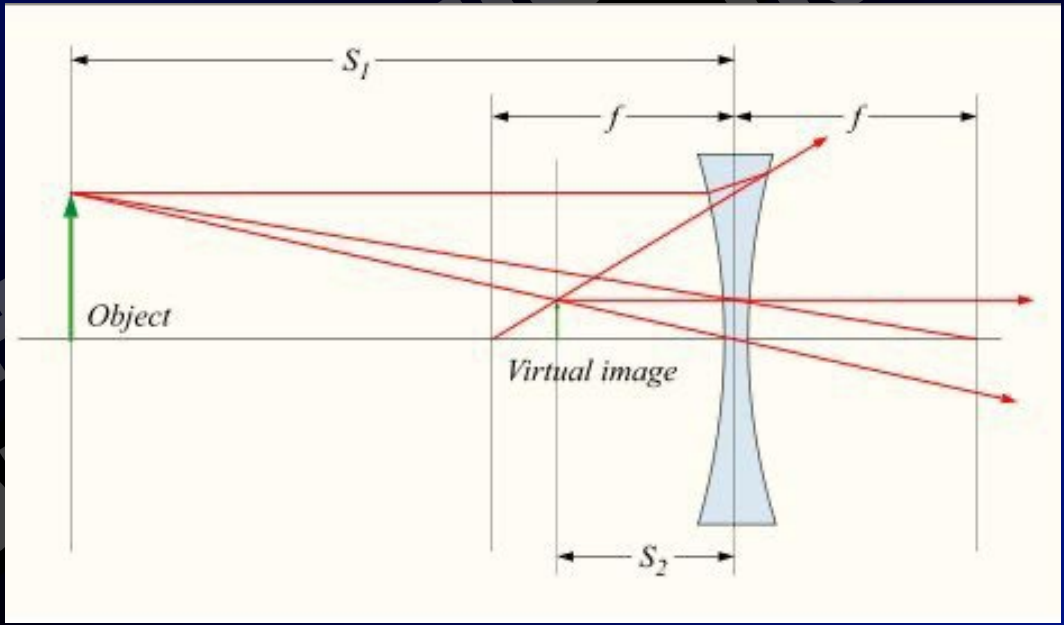
Positive Lens

- **Positive or converging lens: plano-convex or bi-convex**
  - **Converge collimated beam**
  - **Collimate point source (fiber)**
  - **Form real image (screen)**

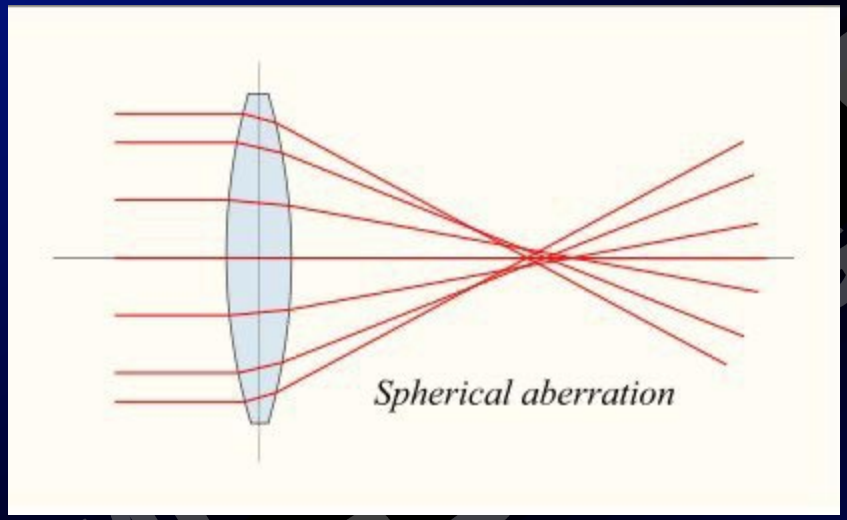


Negative Lens

- **Negative or diverging lens: plano-concave or biconcave**
  - **Diverge converging or collimated beams**
  - **Form virtual image (eye)**

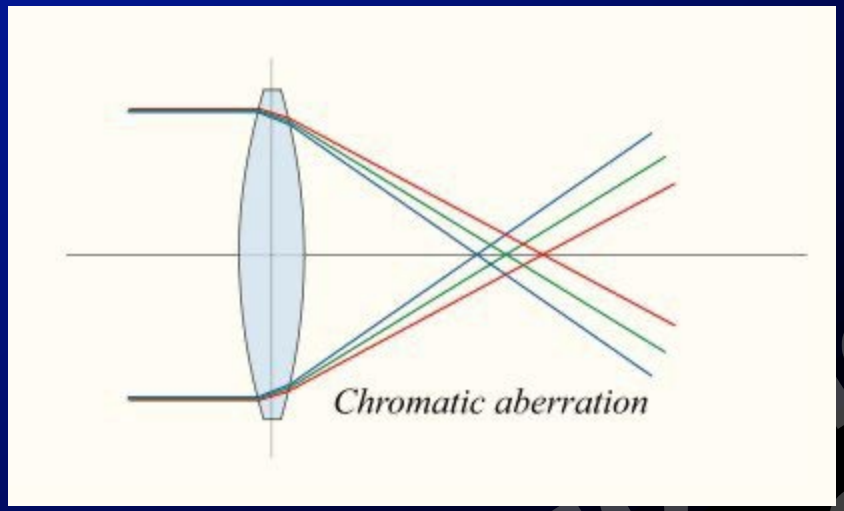


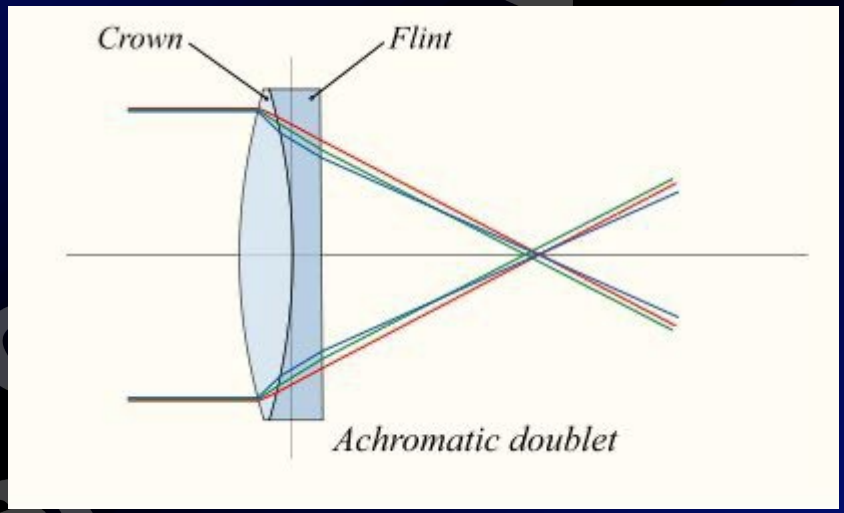
Spherical Aberration



- Surface of spherical lens deviates from theoretical shape
  → Rays at the edge of the lens refocus before focal point
- Manifests as image blur, non-flat depth of field
- Expensive, complex non-spherical lenses

Chromatic Aberration





- **Refractive index varies with λ → Red light refocus beyond blue light**
- **Manifests as color fringes**
- **Corrected with complex doublet lens**

→ ***Spherical, chromatic, and other aberrations corrected with additional lenses***

Optical resolution

- **Lateral resolution = 1.22 . λ/ (2 . NA)**
  - **→ Approx. 0.2 μm with visible light**
- **Numerical aperture: NA = n . (sin μ)**
  - **n = refractive index**
  - **μ = Angular aperture (A) / 2**

→ Max. theoretical NA at μ = 90 deg

Air = 1, Water = 1.33, Oil = 1.52

→ Immersion media with high refractive index increases light collection efficiency

→ High NA at the expense of working (focal) distance, unless front lens diameter increases

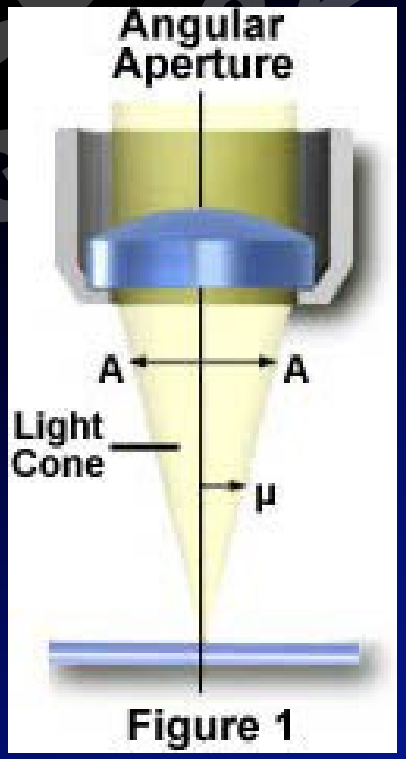
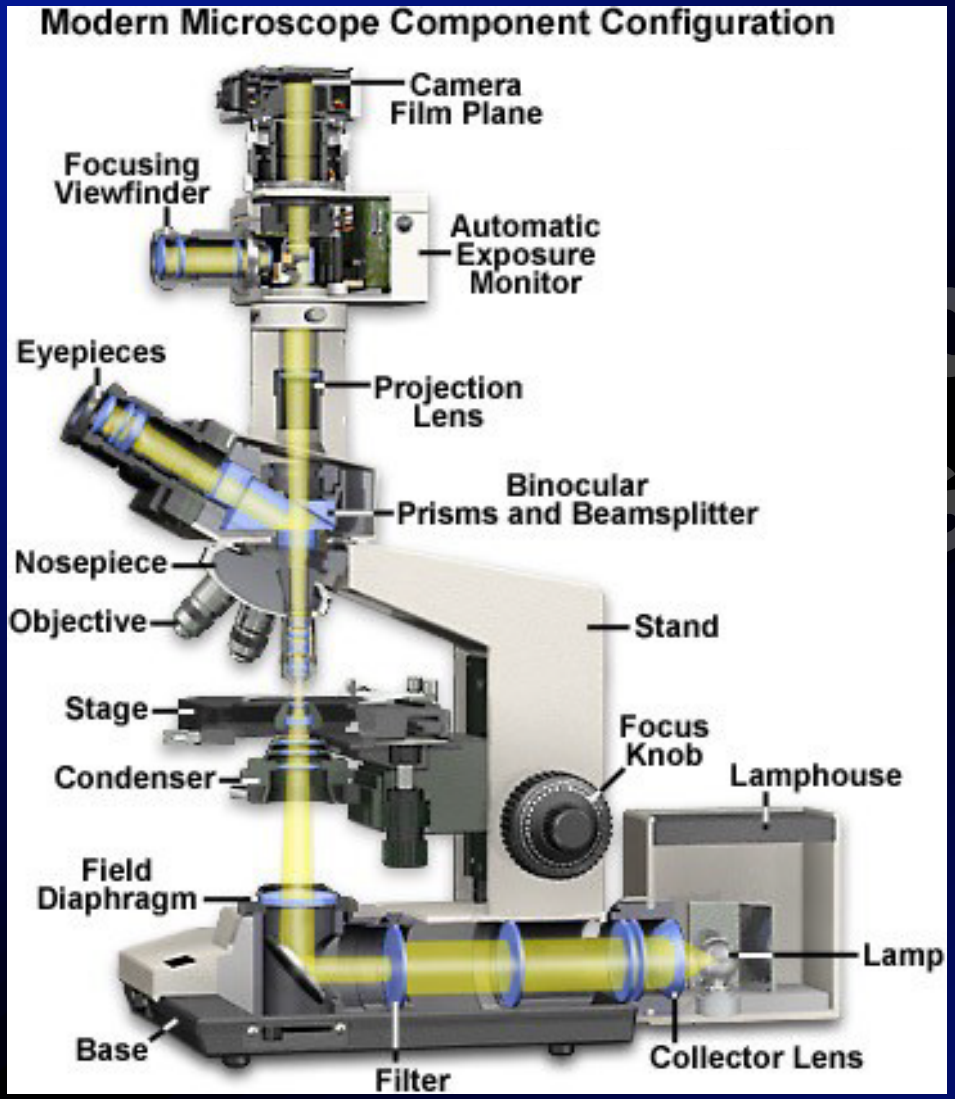


Figure 1

TRANSMISSION MICROSCOPE



- **Infinity corrected system:**

Light emerging from the objective is collimated

Microscope tube lens re-forms the image

- **Condenser diaphragms:**

Aperture stop: attenuation

Field stop: area in FOV

- **Total magnification:**

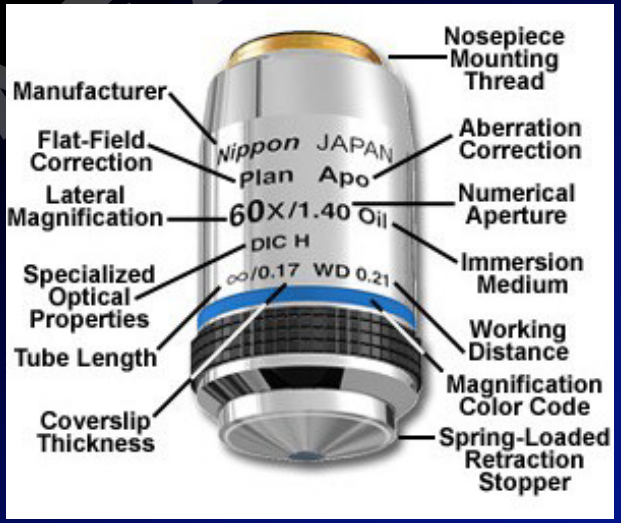
Eye: objective x eyepiece

Camera: objective x scaling factor

Microscope Objective

➔ ***Most important component of the microscope…!***

- **Achro, Achromat, Apo: chromatic aberrations**
- **Plan, Pl, Plano: spherical aberrations**
- **Achroplan, Plan Apo: chromatic + spherical**
- **Corr, W/Corr, CR: correction collar (coverslip, temperature, immersion…)**
- **I, Iris, W/Iris: iris diaphragm (NA)**
- **Oil, Oel, Water, WI, Wasser, Gly: immersion media**
- **DIC, NIC: Nomarski**
- **Ph 1, 2, 3: phase condenser annulus 1, 2, 3**

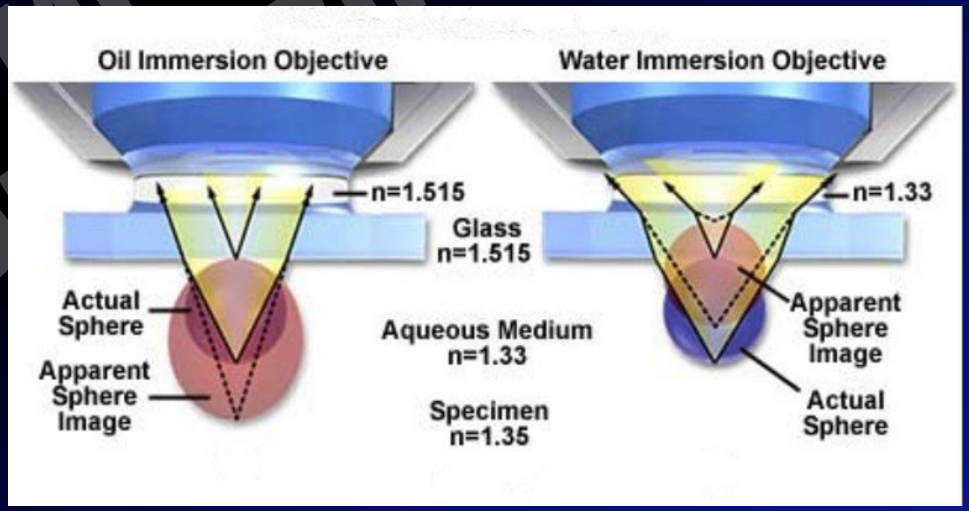


Objective Characteristics

- **Working distance:**

5x/0.13: 17 mm, 10x/0.45: 2 mm, 20x/0.8 air: 0.55 mm, 40x/1.3 oil: 0.21 mm, 63x/1.4 oil: 0.19 mm
WD value are past the coverslip
→ 10x or less for multi-well plate (1 mm plastic bottom)
→ FOV translation with up/down focus = reached WD

- **Always match refractive indexes of immersion and mounting media**
→Lower apparent NA, especially when going deep



- **Coverslip correction: carefully check water-immersion lenses…!**

Coverslip

- **Coverslip thickness & quality critical for high-resolution imaging:**
  - **#0 coverslip: 0.09 – 0.13 mm**
  - **#1 coverslip: 0.13 – 0.16 mm**
  - **#1.5 coverslip: 0.16 – 0.19 mm**
  - **#2 coverslip: 0.19 – 0.23 mm**
  - **…**
- **Microscope objectives built for #1.5 coverglass assuming no extra layer of mounting medium**
  - **→ #1 coverlips for mounted tissue sections**
  - **→ #1.5 coverslips for plated cells, especially for high-resolution**
- **Glass quality matters for high-resolution: "German" glass**
- **Excessive coating: may be a problem for TIRF or super-resolution**
- **Avoid using 10mm coverslips (leak mounting media)**

Condenser: Kohler Illumination

- **Perfectly defocused image of filament**
  - → **Most even illumination (not perfect)**

- **Condenser adjustment procedure:**

Focus objective on specimen

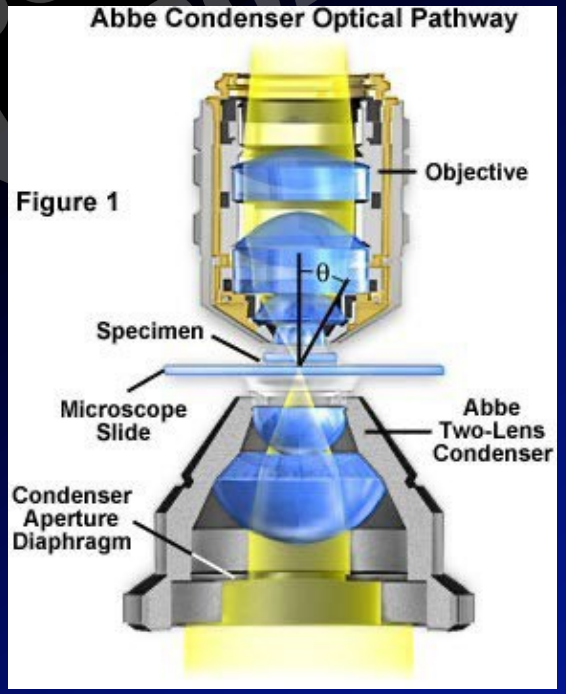
Close field stop

Move condenser up or down for sharp edges

Center condenser

Open field stop until barely beyond edges

→ ***Critical for high-quality transmission image***



Transmission Imaging

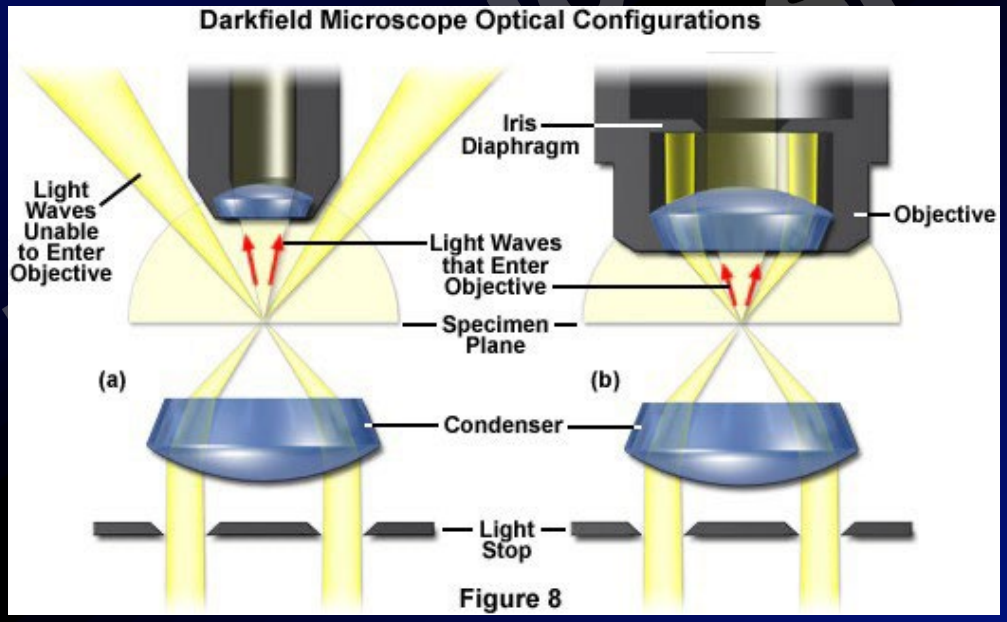
- **Light source**
  - Halogen lamp: cheap but inconsistent color temperature
  - LED: stable color temperature and long life
  - Uneven illumination → shading correction
- **Detector**
  - High-resolution CMOS color camera (white balance)
- **Very sensitive to dust – dirt: dust cover, clean lab, air filters (proximity to specimen or camera)**
  - → Shading correction…!
- **Biological specimen = low contrast**
  - → Chemical dye (crystal violet, eosin, AEC, DAB…)
  - → Contrasting technique to enhance contrast

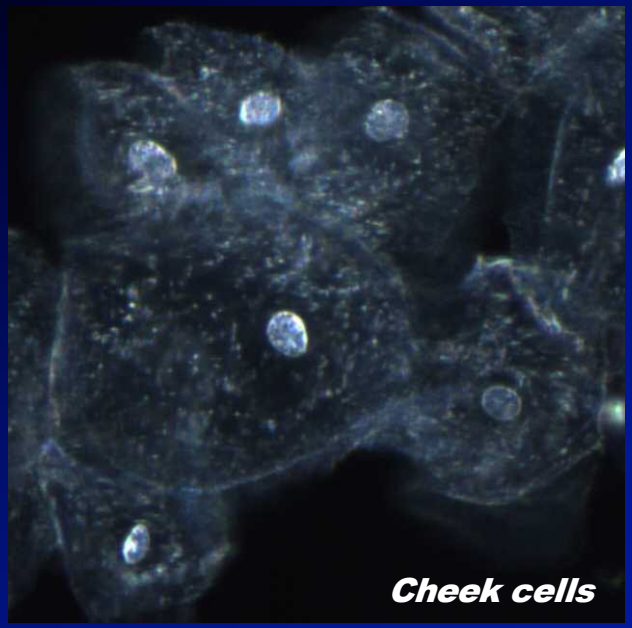
CONTRASTING METHODS

Dark Field

Diffraction / refraction of light by specimen

- ➔ Hollow cone of illumination does not reach detector
- ➔ Bright image on dark background
- ➔ Requires special high-NA condenser
- ➔ Super-resolution possible ("Ultra" dark field)

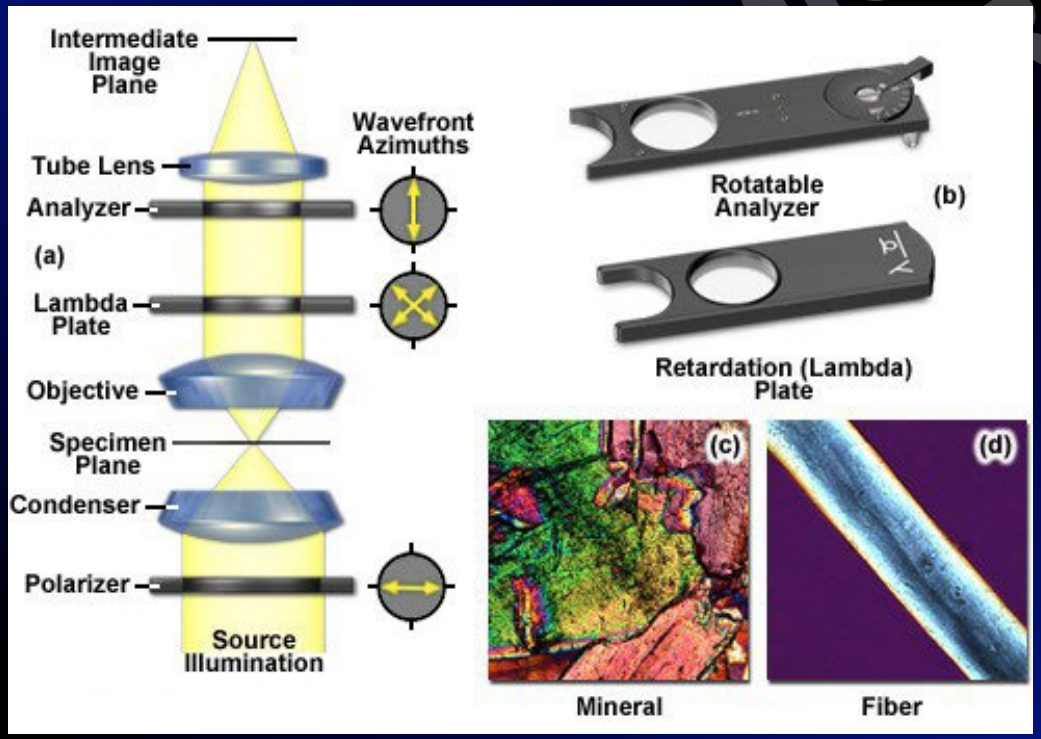


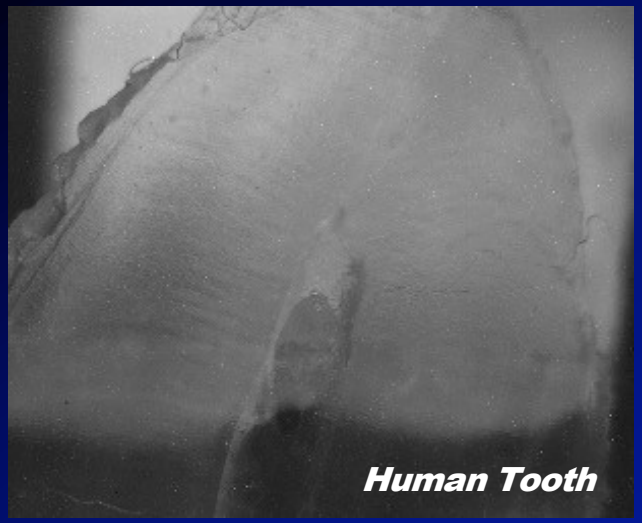


Polarization

Change in light polarization

- ➔ **Bi-refringent (anisotropic) specimen only (material / earth sciences)**
- ➔ **Image created by constructive or destructive interferences through analyzer**
- ➔ **Affected by plastics and strain on lenses ➔ special optics**





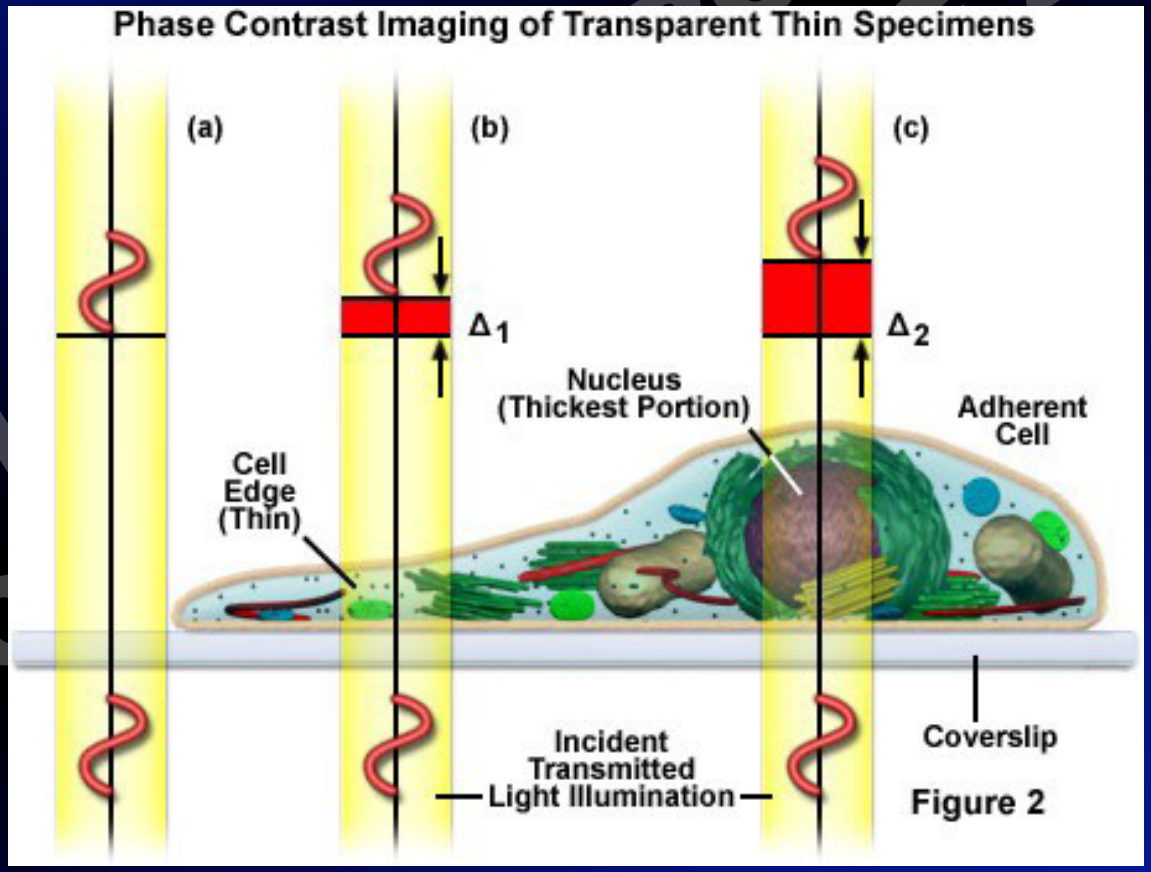
Phase Contrast (1)

Change in refractive index

Slight differences between specimen and media:

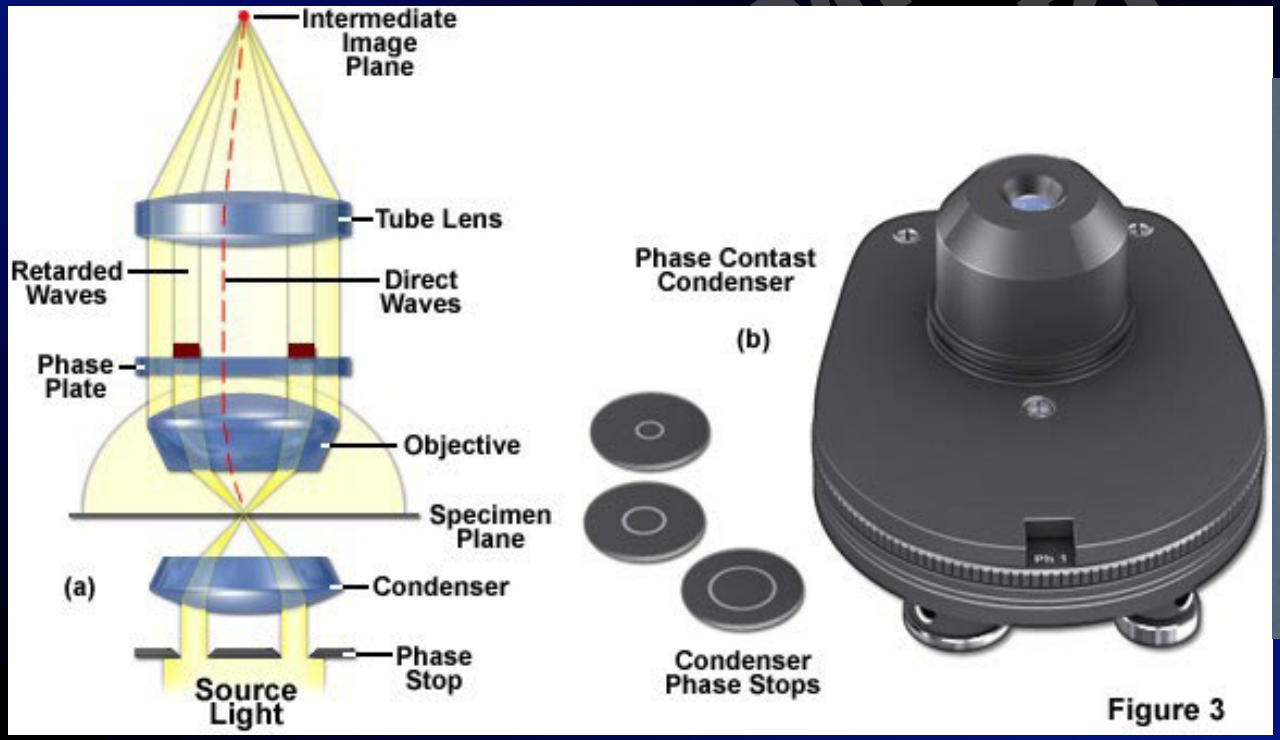
→ Retardation (change of phase) of light

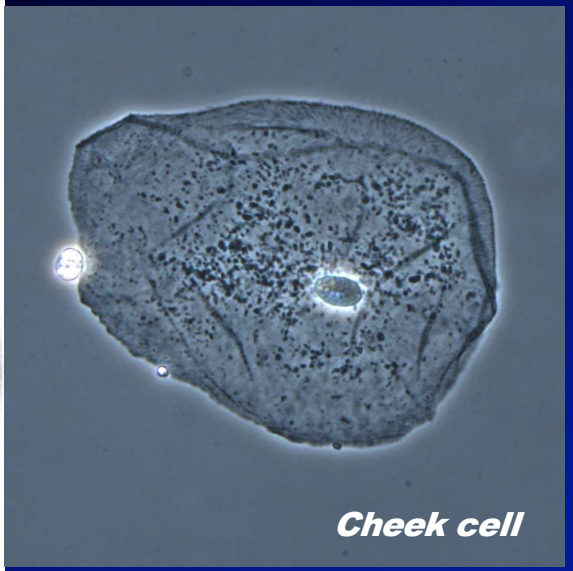
Eye not sensitive to phase change → requires special setup



Phase Contrast (2)

- Phase stop creates hollow cone of light
- Transmitted light shifted by phase ring
- Scattered light not influenced by phase ring (phase objective)
- Image built from constructive or destructive interferences
  - ➔ Widespread, well-suited for tissue culture (plastic vessels)
  - ➔ Phase objective and condenser ring matched, aligned



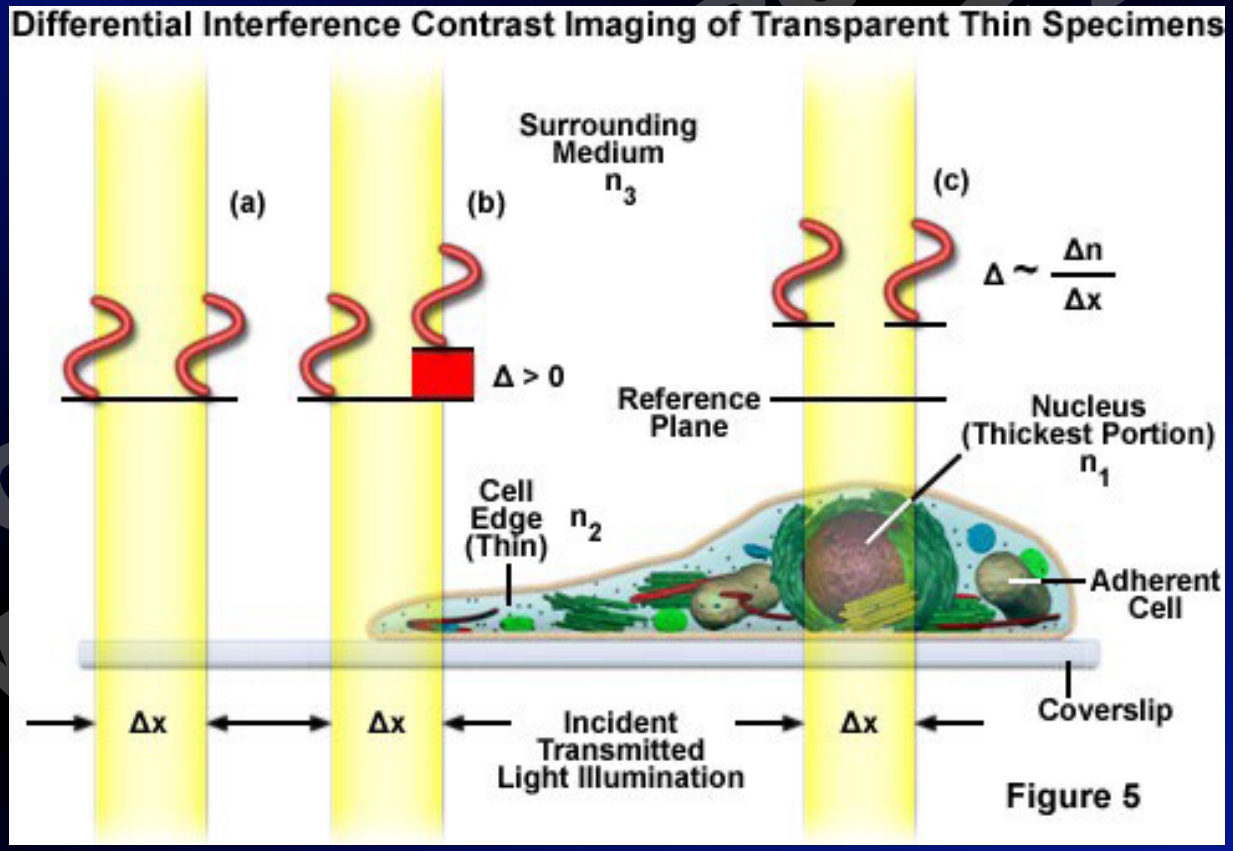


Differential Interference Contrast (1)

Change in refractive index GRADIENT

Wollaston prism splits polarized light onto orthogonal components:

→ **Spatially shifted, but not resolved by objective**



Differential Interference Contrast (2)

Interference image re-built with secondary Wollaston prism and analyzer:

- ➔ **Better performance and resolution than phase contrast**
- ➔ **Pseudo-3D aspect of plasma membranes / organelles**
- ➔ **Unsuitable for plastic containers (no TC)**
- ➔ **DIC = Phase with orthogonal components and higher NA**

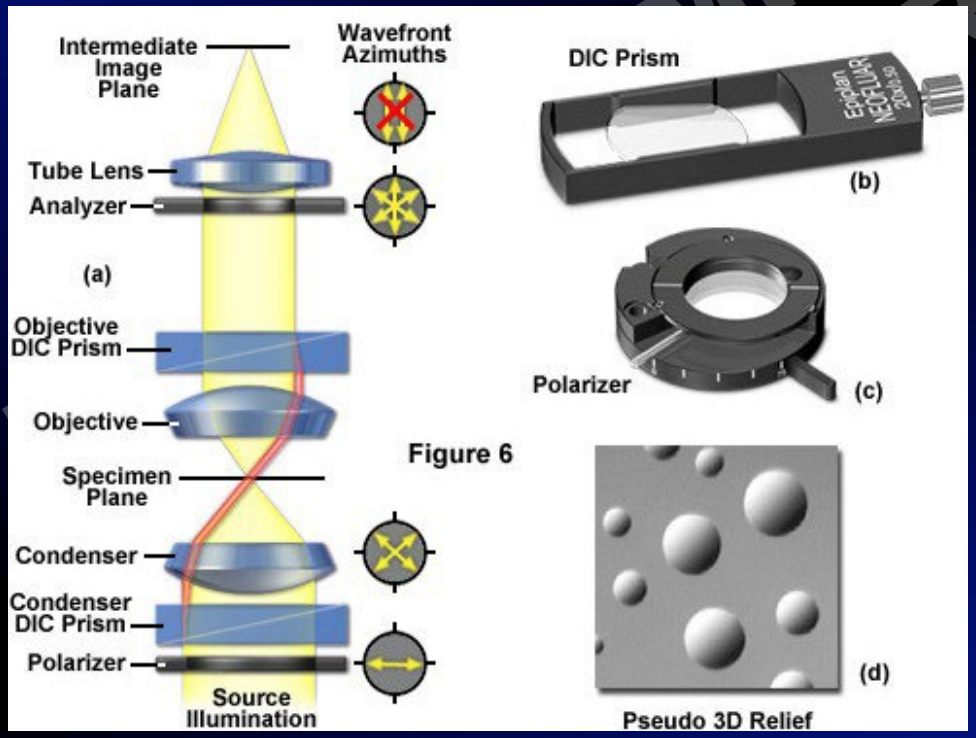
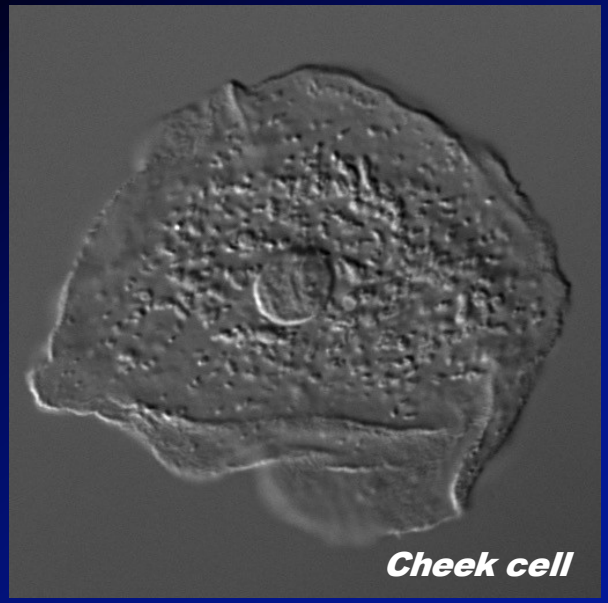


Figure 6



TRANSMISSION IMAGING TAKE-HOME MESSAGE

- **Pros:**
  - Easy and cheap
  - High resolution (especially point scanner w/ tPMT)
  - No staining required
  - Works well with live cells
  - Already built into most microscopes

- **Cons:**
  - Sensitive to dirt and dust
  - Precise alignment
  - Low tolerance on specimen (DIC)
  - Organelle morphology not enough for identification

→ ***Transmission imaging often under-estimated***

QUESTIONS

FLUORESCENCE MICROSCOPY

Fluorescence Fundamentals

Nature of fluorescence, Stokes shift, Spectra, Quantitation

Fluorescence Microscope

Overview, Light sources, Filters, Detectors

Fluorescent Dyes

Chemicals, Endogenous markers: GFP, Mutants, Photo-switchers

Techniques

Immunostain, Transfection, Photobleaching, FRET

Experimental Considerations

Dye combinations & Crosstalk

FLUORESCENCE FUNDAMENTALS

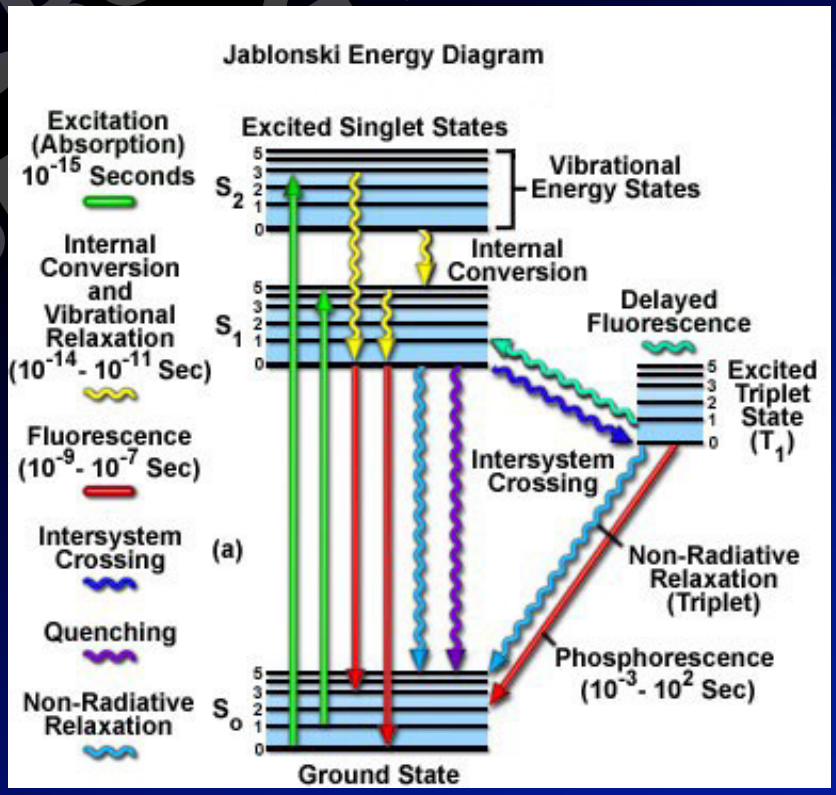
Nature of Fluorescence

The Most important technique in Life Sciences microscopy

- **Part of Luminescence family,**
  →Atoms, molecules and nanoparticles (Qdots)

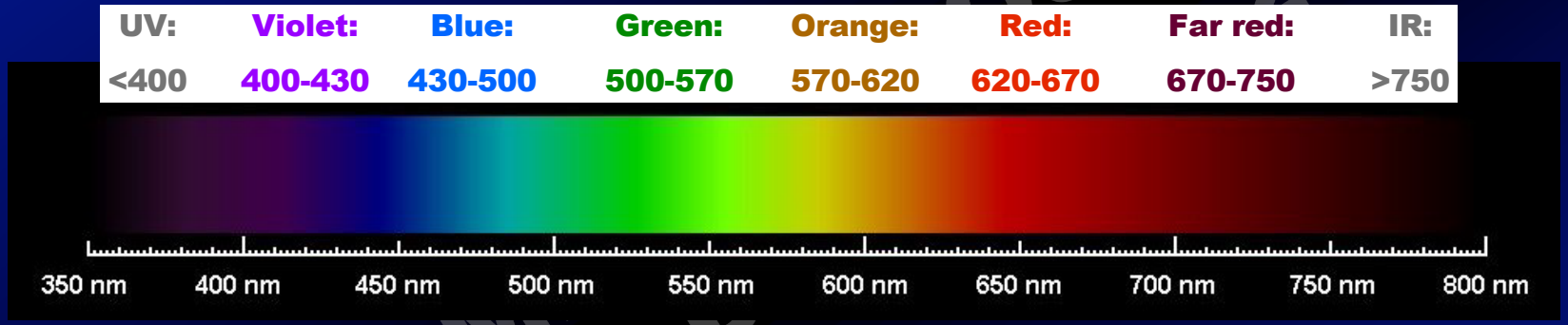
- **Relaxation of an excited orbital electron by emitting a photon**

- **Non-radiative internal conversion**
  → Emitted photon of lower energy



Stokes Shift

Lower energy of emitted photon → *Emission shifted toward the red*



+Energy

Purple laser = 405 Ex → Blue fluorescence

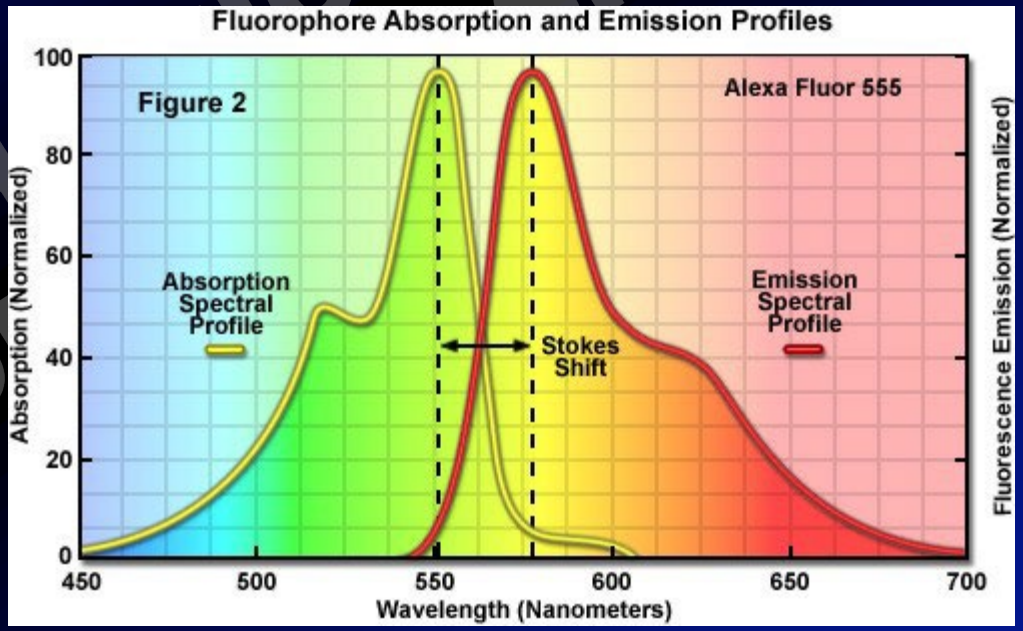
Blue laser = 488 Ex → Green fluorescence

Green laser = 568 Ex → Red fluorescence

Red laser = 633 Ex → Far-red fluorescence

Excitation and Emission Spectra

- **Excitation spectra:**
  Measure intensity at maximum emission wavelength, scan excitation
- **Emission spectra:**
  Excite at maximum excitation wavelength, scan emission
- **Stockes shift = difference absorption / emission maxima**
  ➔ Energy losses in non-radiative processes



Quantitation

- **Quantum yield:** ϕ = Photons emitted / Absorbed

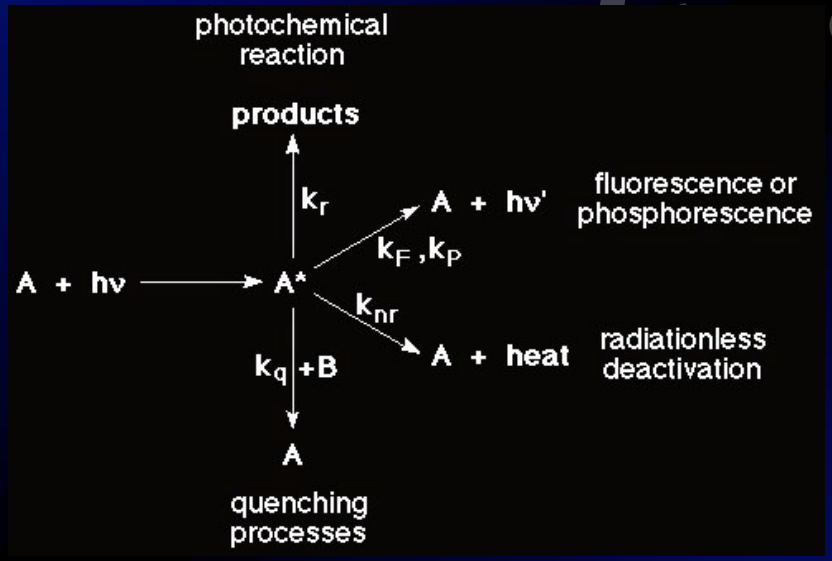
- **Fluorescence Lifetime:** $I(t) = I(t=0) \cdot \exp -(t / \tau)$

  First-order exponential, decays in 0.5 – 20 ns

  Depends on molecular dynamics and environment

  More polar environments induce red-shift

  → ***Best way to measure FRET***



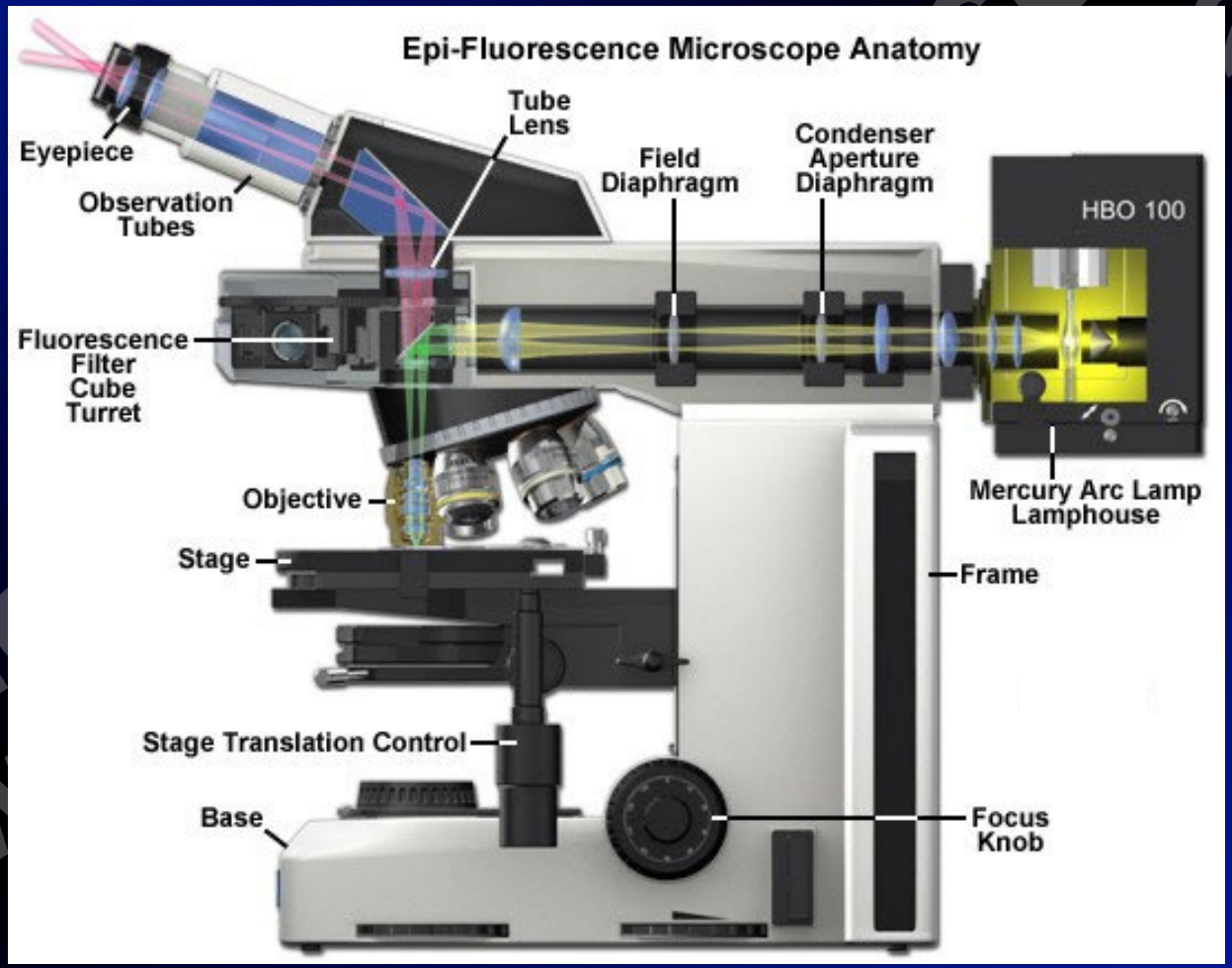
- **Competing de-excitation processes**

  →Lifetime = kinetic of radiative de-excitation, steady-state intensity

→Fluorescence ntensity is NOT quantitative...!!!!

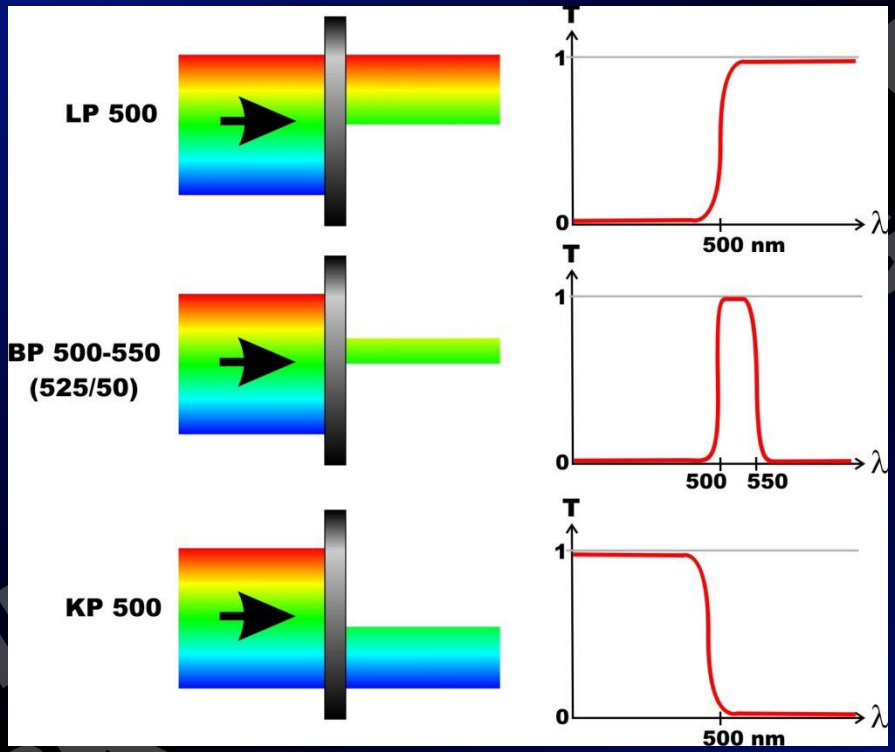
FLUORESCENCE MICROSCOPE

Overview



Fluorescence Filters

- **Long-pass, band-pass or short-pass filters:**



- **Manufactured by thin-film coating technology:**
  - → **Sensitive to incidence angle (90, 45 deg or low-angle)**
  - → **High damage threshold**
  - → **Multiple bandwidths possible (505-550 / 560-620 = green / red)**

Filter Selection

- **Depends on fluorophore:**

➔ **Excitation filter at or near maximum Ex**

➔ **Dichroic splits excitation and fluorescent light**

➔ **Emission filter near max Em, as wide as possible**

➔ **Sequential excitation for multiple colors…!**

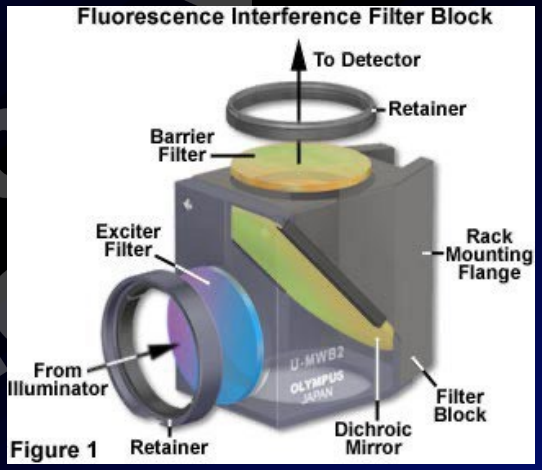
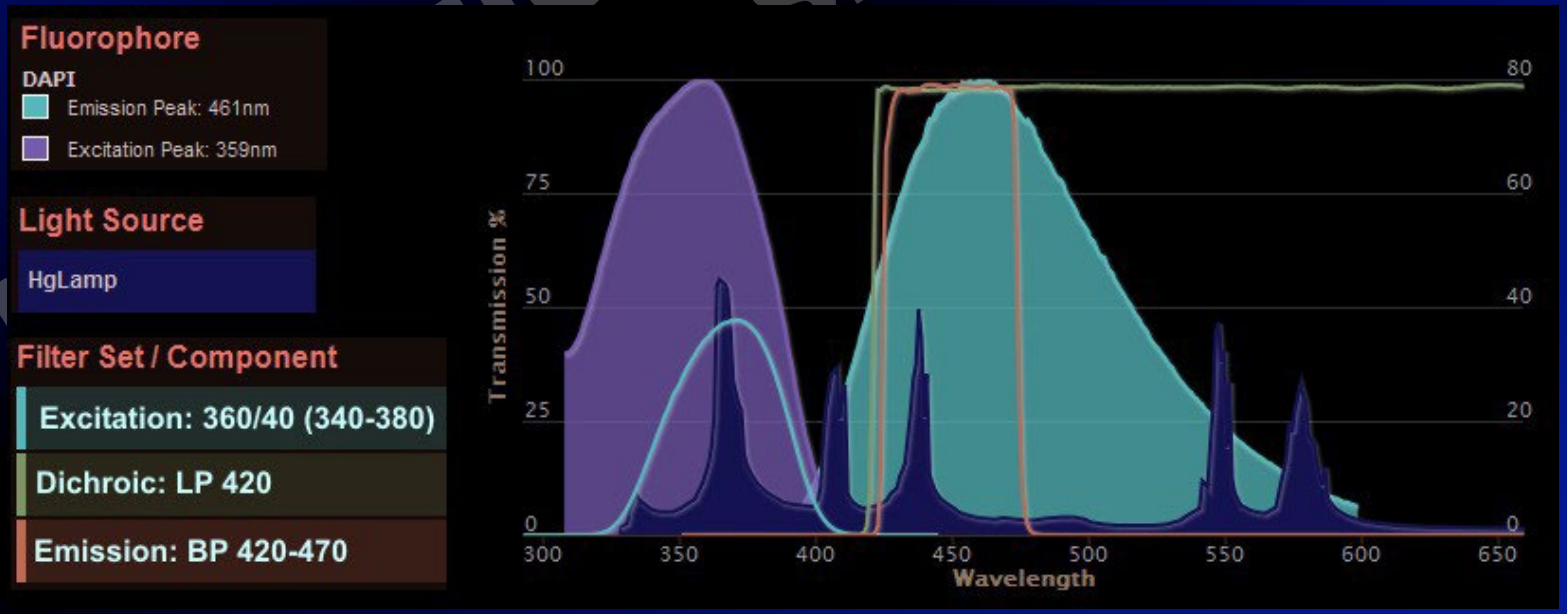


Figure 1



Light Sources: Broadband

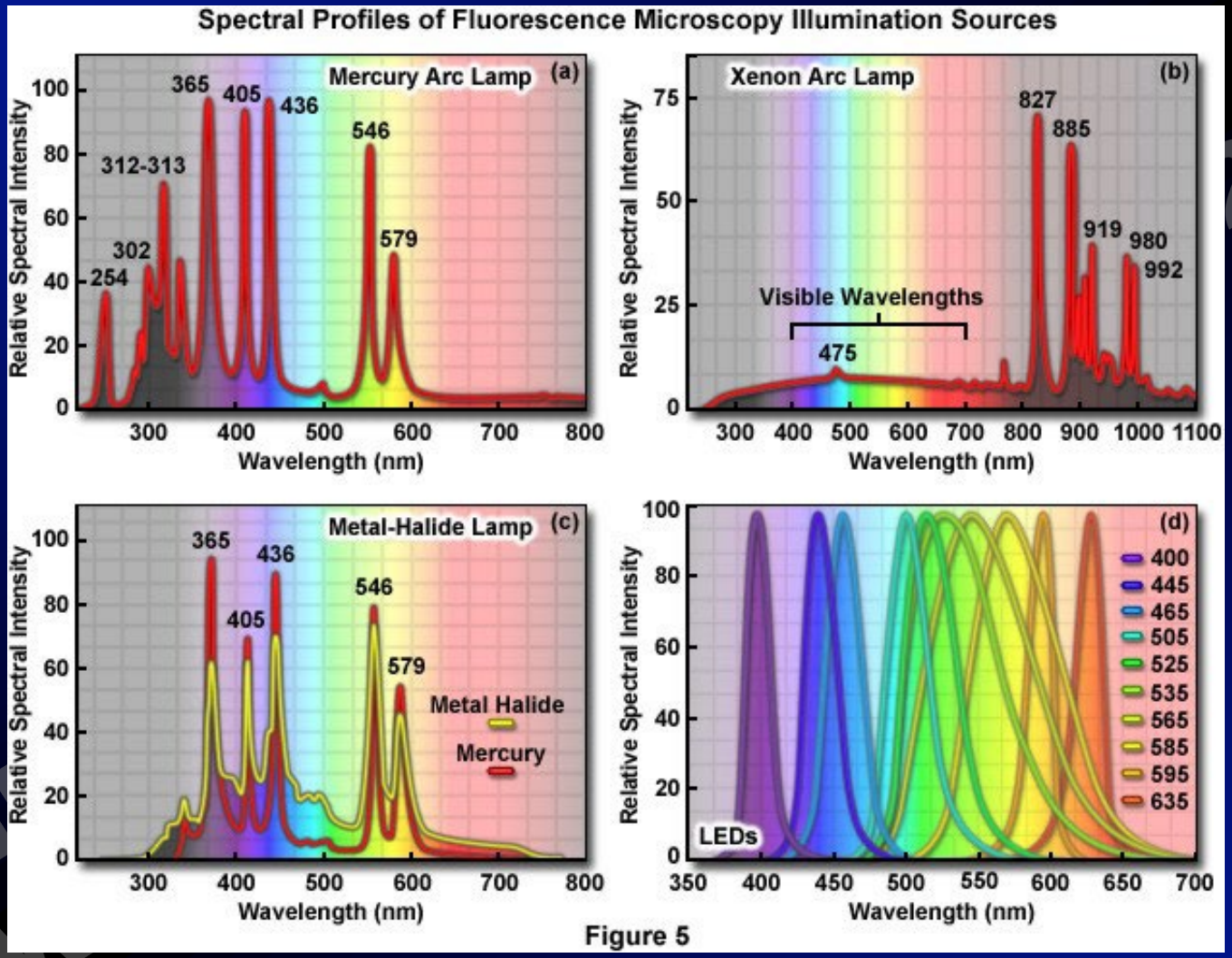


Figure 5

- Mercury lamp: 200 - 500 hours, cheap
- Metal Halide: 2,000 - 3,000 hours, pricey
- LED: > 10,000 hours

Light Sources: Lasers

- **Laser: stimulated emission of inverted population in resonant cavity**

  →Monochromatic coherent light, perfectly collimated

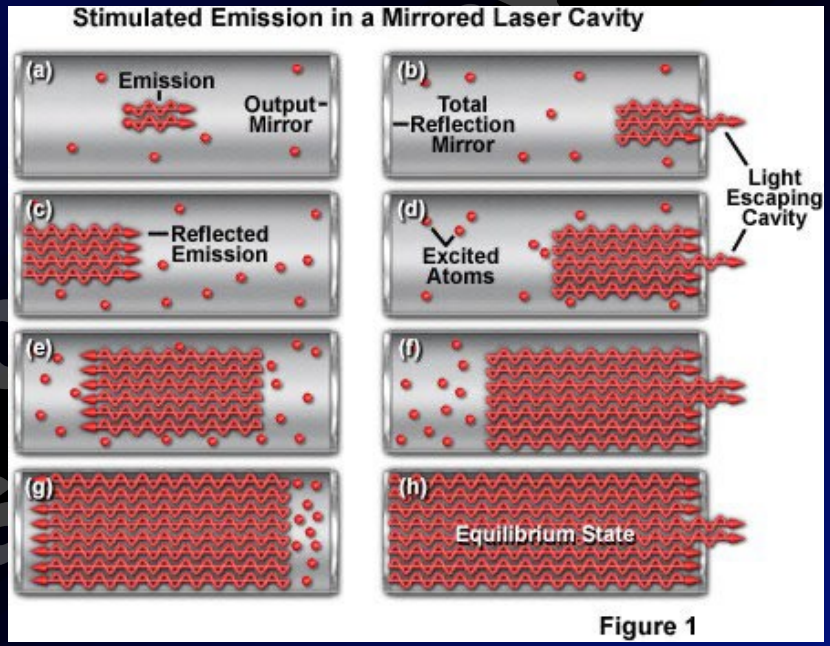


Figure 1

- **Gas lasers:**

  Poor efficiency, costly, not reliable

  Ar: 458 / 488 / 514 nm

  Ar / Kr (omnichrome): 488 / 568 / 633 nm

  HeNe: 543, 633 nm

- **Solid-state lasers:**

  Glass with rare earth dopant in resonant cavity

  Cheap, very reliable and power-efficient

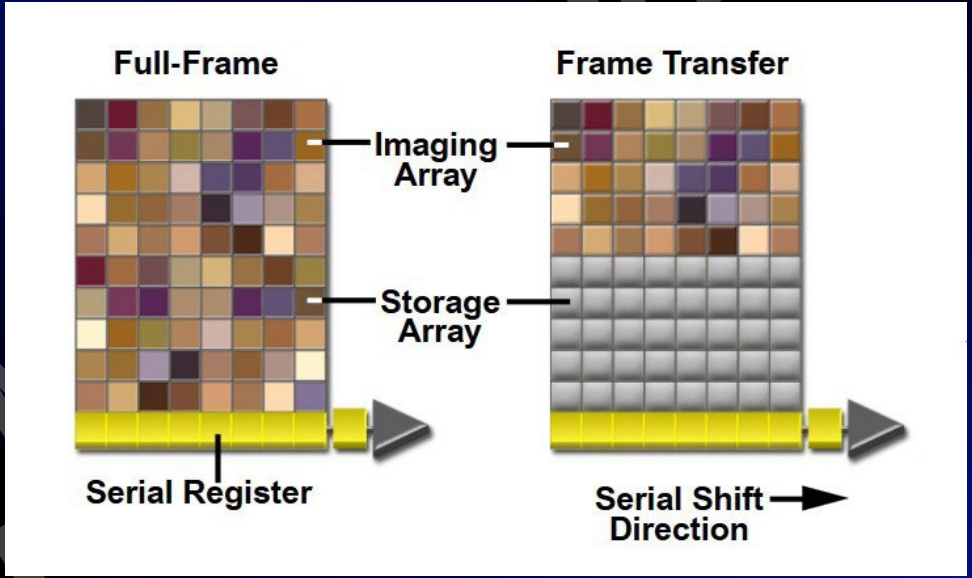
  Available in many colors and powers

- **Pulsed IR and white-light lasers: costly, specialized applications**

Detector - CCD camera

- **Charge Coupled Device: Array of elements, acquire charge when exposed to light (exposure)**

- **Charge to voltage conversion: array content moved sequentially to readout register**



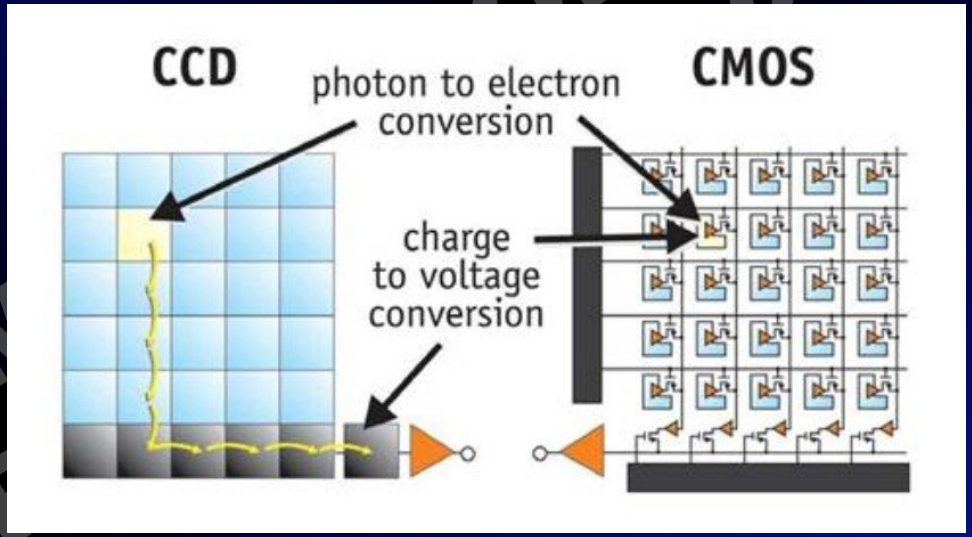
- **Electron multiplied (EM): extra register with electron-multiplying capability**

- **Older technology, high sensitivity, costly, slow, large pixels**

- **Crop from bottom for speed**

Detector - CMOS camera

- Complementary Metal Oxide Semi-conductor

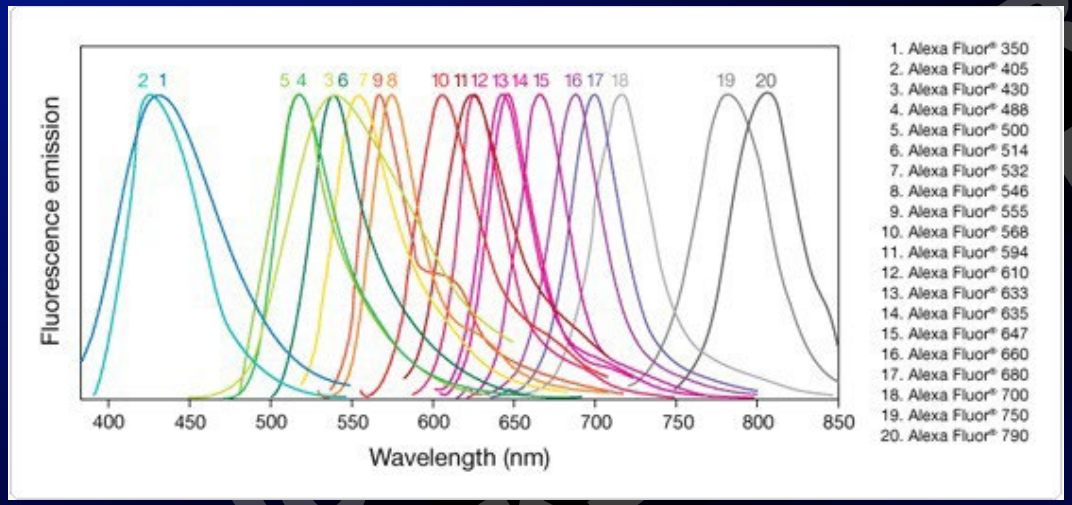


- Acquire charge on exposure
- Charge converted to voltage on each pixel
- Readout with conventional wires
- No gain control, crop from center of the field for speed
- Modern technology: low cost, high speed, small pixels, sensitive

FLUORESCENT DYES

Chemical dyes

- DAPI (DNA), FITC, Rhodamins, Cyanin dyes, etc...



- Alexa

  Widely used in immunostains

  Stable, bright (esp. reds)

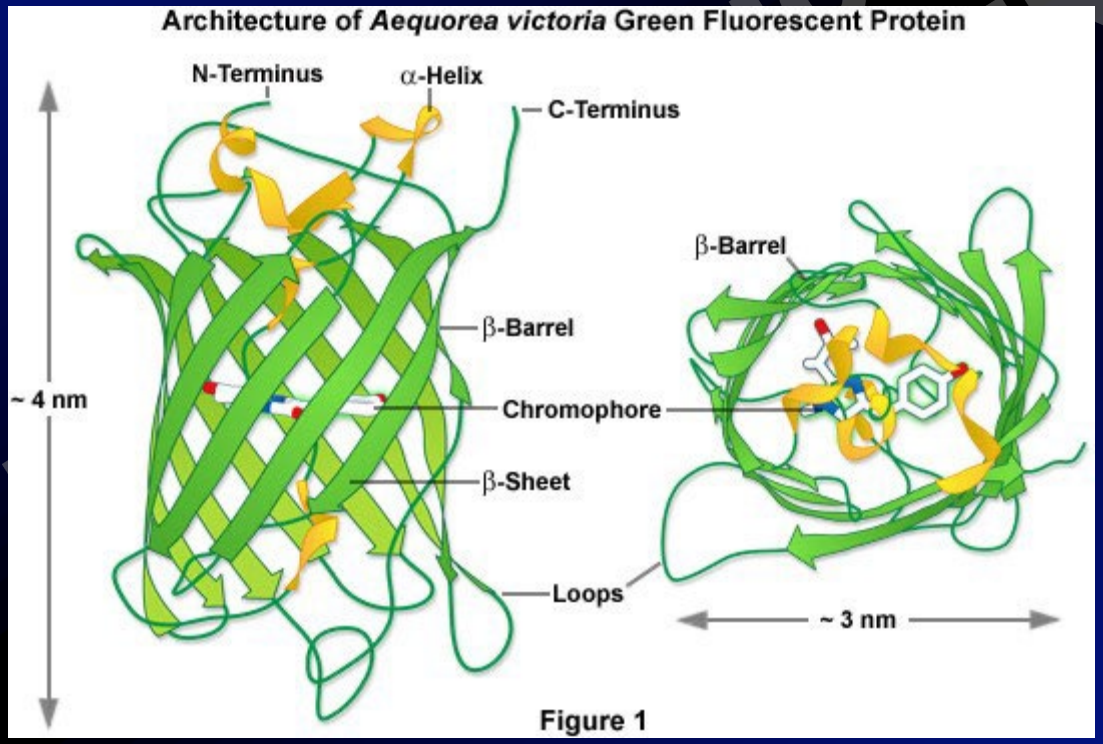
- Dylight

- Etc...

- In vivo dyes:
  - → Calcium dyes: fluo-4, indo, fura...
  - → Organelles, functional, viability, proliferation dyes...
  - → Typically loaded by mixing in DMSO / buffer

Endogenous Markers: GFP

- **GFP: green fluorescent *PROTEIN***
  - → **Native fluorescent marker**
  - → **238 AA β-barrel**
  - → **Cyclization of inner Ser65–Tyr66–Gly67 = chromophore**



→Revolutionized live microscopy

Fluorescent Proteins

Directed mutagenesis:

Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*In vivo* Structure	Relative Brightness (% of EGFP)
GFP (wt)	395/475	509	21,000	0.77	Monomer*	48
Cyan Fluorescent Proteins						
ECFP	439	476	32,500	0.40	Monomer*	39
Cerulean	433	475	43,000	0.62	Monomer*	79
Green Fluorescent Proteins						
EGFP	484	507	56,000	0.60	Monomer*	100
Yellow Fluorescent Proteins						
EYFP	514	527	83,400	0.61	Monomer*	151
Venus	515	528	92,200	0.57	Monomer*	156
Orange Fluorescent Proteins						
dTomato	554	581	69,000	0.69	Dimer	142
dTomato-Tandem	554	581	138,000	0.69	Monomer	283
DsRed	558	583	75,000	0.79	Tetramer	176
DsRed2	563	582	43,800	0.55	Tetramer	72
DsRed-Express (T1)	555	584	38,000	0.51	Tetramer	58
DsRed-Monomer	556	586	35,000	0.10	Monomer	10
Red Fluorescent Proteins						
mRFP1	584	607	50,000	0.25	Monomer	37
mCherry	587	610	72,000	0.22	Monomer	47

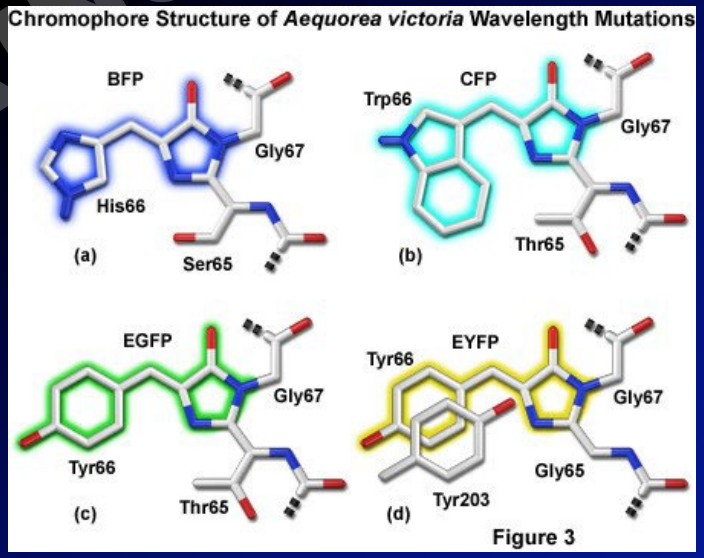
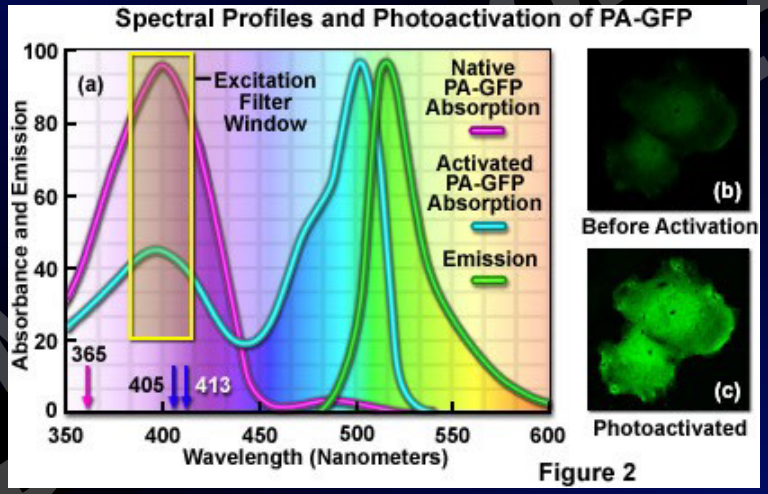


Figure 3

Photo-Switchable Proteins

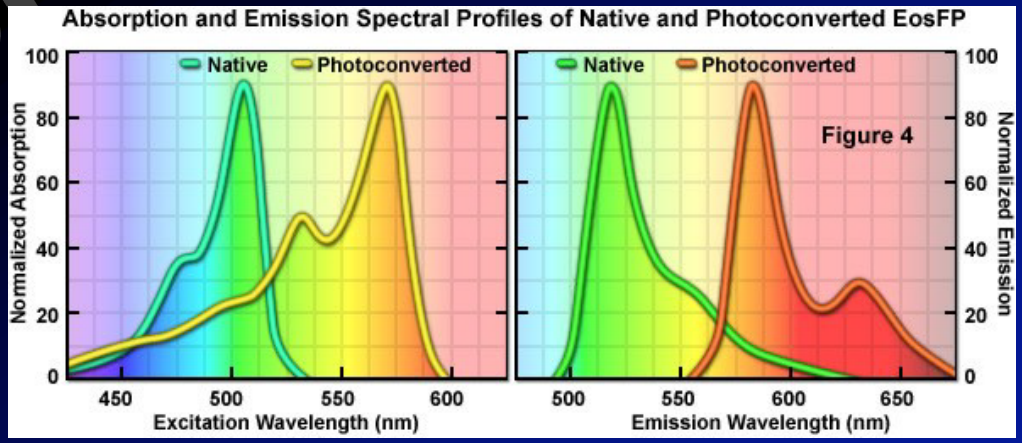
- **Develop color or change spectral characteristics after irradiation with UV light**

- **Dark to bright:**
  PA-GFP, PA-mCherry



- **Green to red: mEOS2, dendra2**

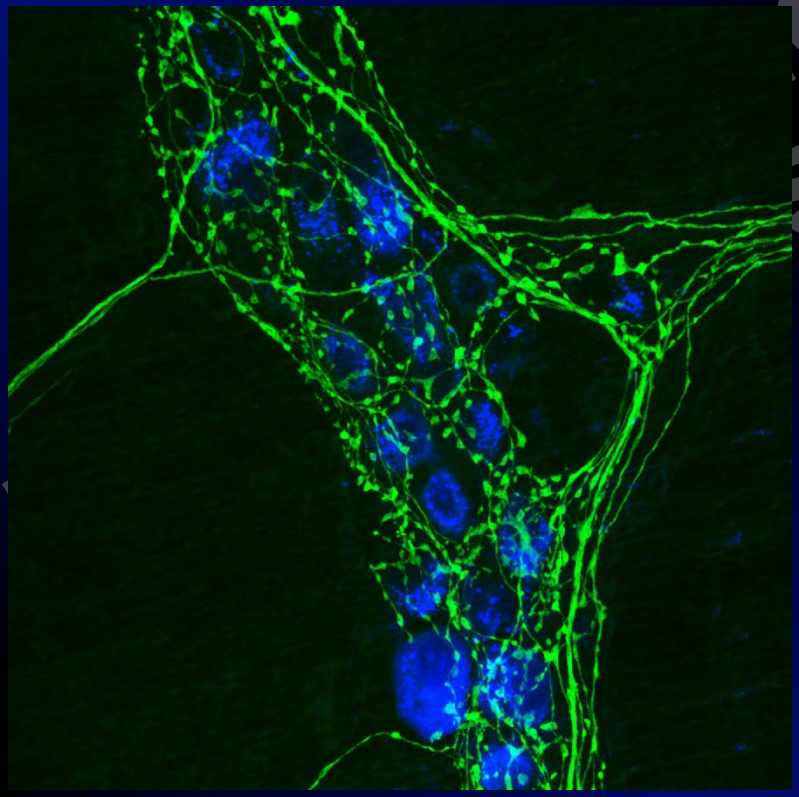
- **Reversible switchers: DRONPA**

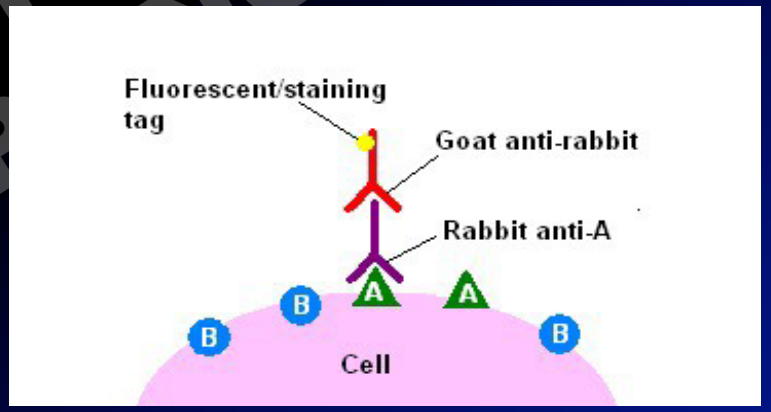


TECHNIQUES

Immuno-Fluorescence

- Primary antibody detects specific epitope
- Fluorescent-tagged secondary targets primary



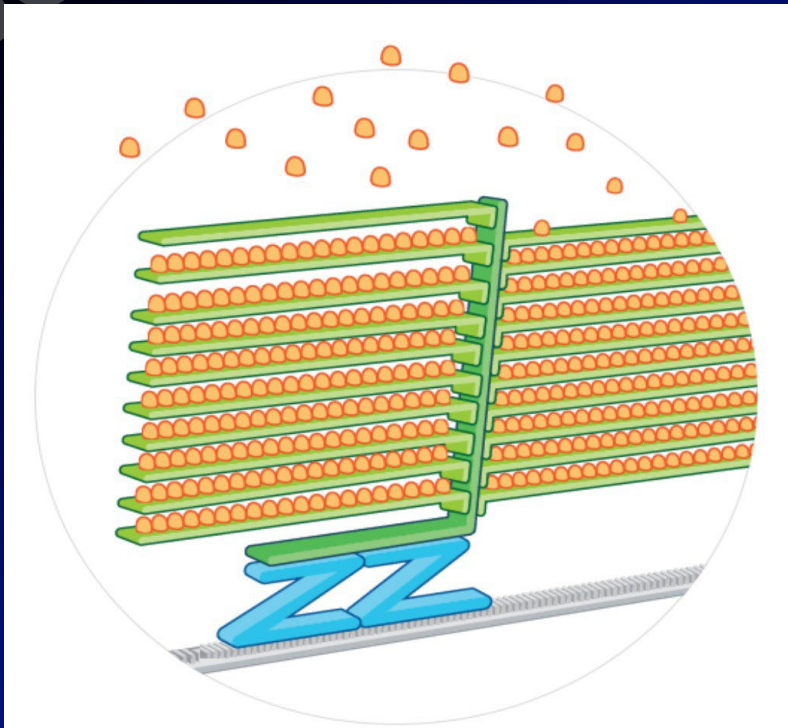


→Highly sensitive, specific (AB permitting), and versatile

RNAscope

- **Proprietary mRNA in situ hybridization developped by Advanced Cell Diagnostics (ACD)**
- **Highly specific ZZ-pair amplification → detects single mRNAs**
- **Paraffin embedded → chromogenic stains (2 markers max.)**
- **Fresh frozen / perfusion fixed: Fluorescence**
  - **→ 5 makers max. (Multiplex)**
  - **→ 15 markers max. (HiPlex, 3 rounds)**
- **Requires specialized oven and pricey chemicals from ACD**

→ Single-cell expression patterns



Transfection

- **Construct:**

Protein of interest fused to FP

- **Vector: expression system**

- **Transfection methods:**

  Lipofectamin

  Electro-poration

  Virus

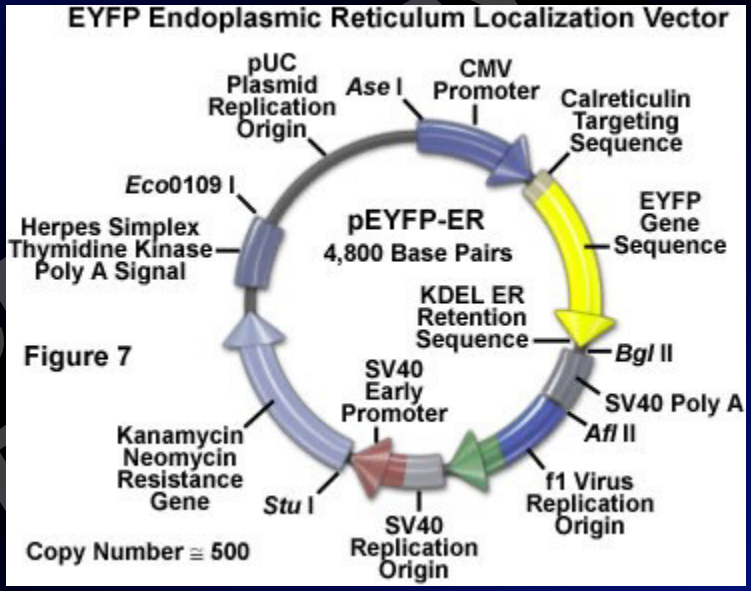


Figure 7

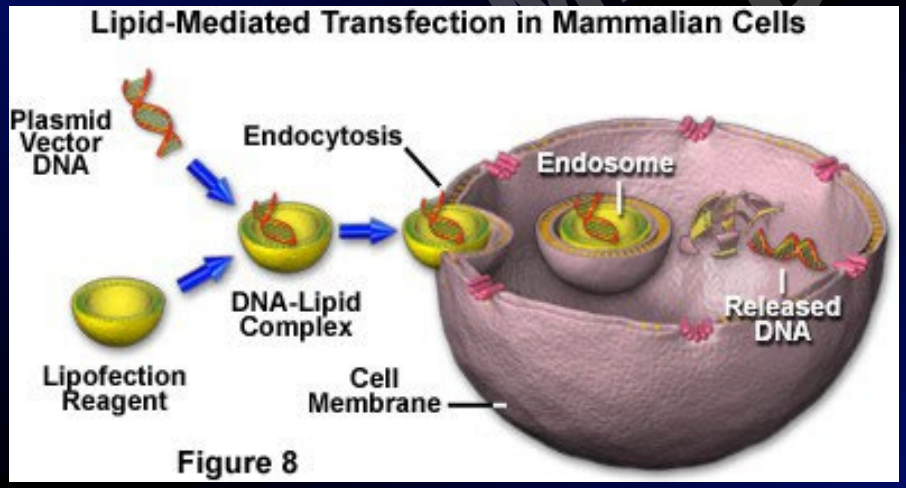


Figure 8

- **Experimental considerations:**

Expression level

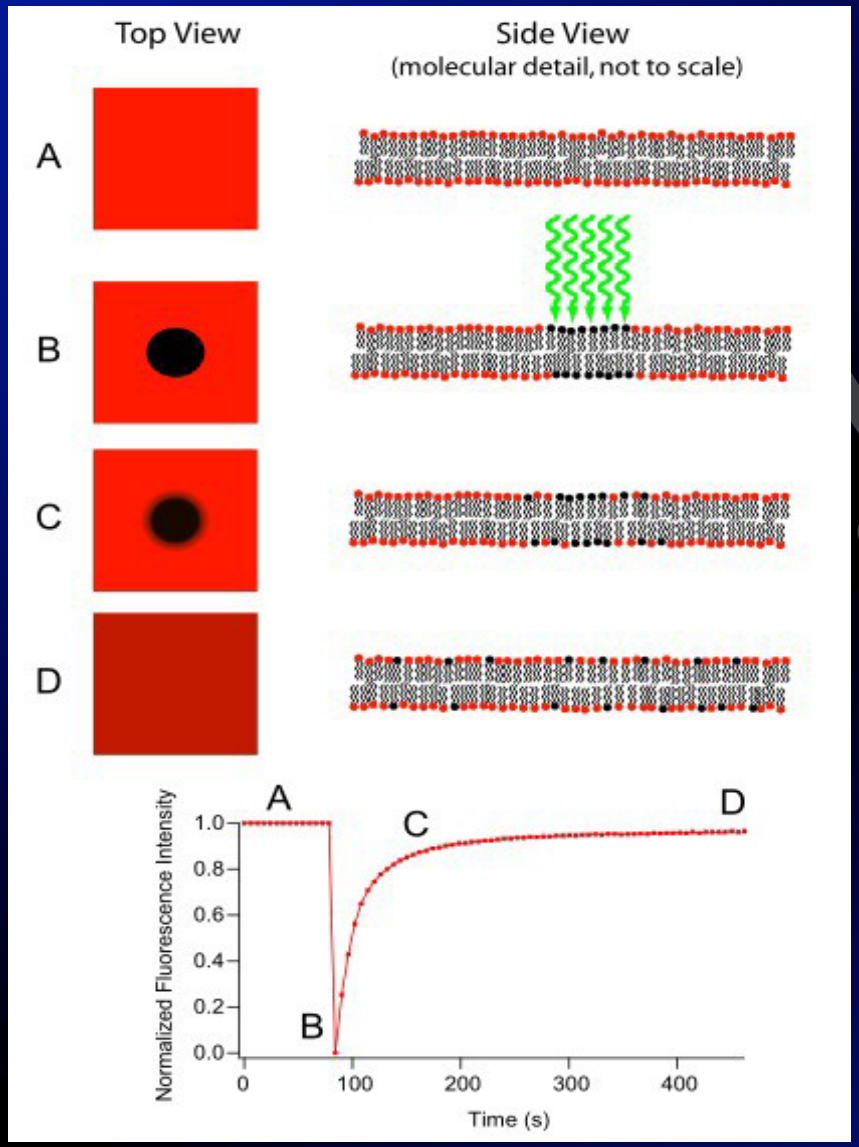
Cell physiology

Self-aggregation of the FP

Tagged protein property change

etc…

Photobleaching / Photoactivation



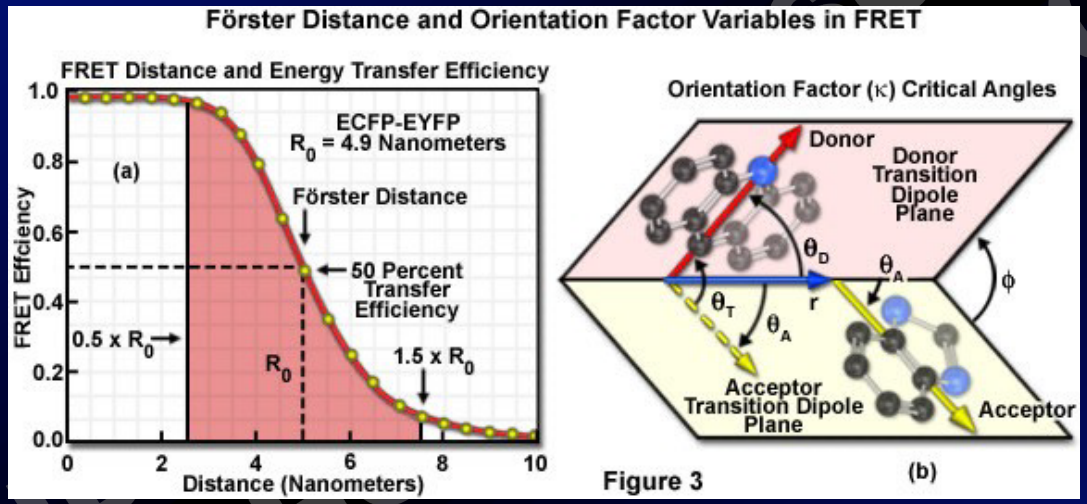
- **Bleaching:**
  Localized, intense illumination bleaches fluorochromes
- **Recovery:**
  Unbleached molecules re-enter bleached region
  →Diffusion, active transport characteristics
- **Works with all FPs**
- **Difficult to quantify in 3D**
- **Photoactivation = FRAP on photoswitchable proteins**

FRET

- Foster Resonance Energy Transfer

→ Non-radiative energy transfer between donor andacceptor

→ Overlapping emission (donor) and excitation (acceptor) spectrum



- Short range, 2 to 8 nm $(Distance)^{-6}$

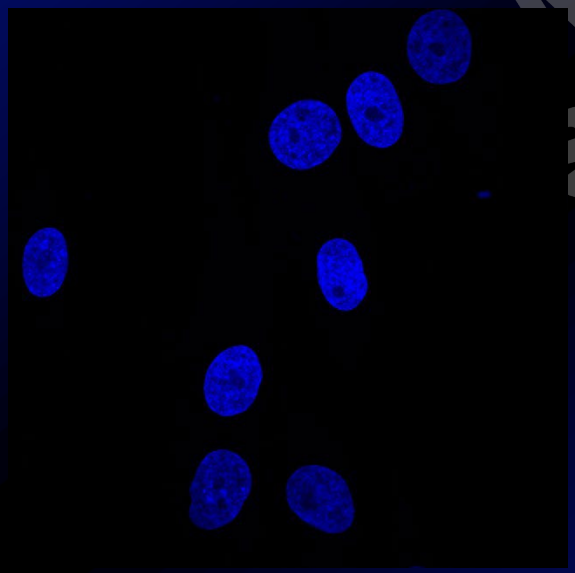
- Intermolecular FRET = 2 to 5% change, not advisable on point scanning confocal

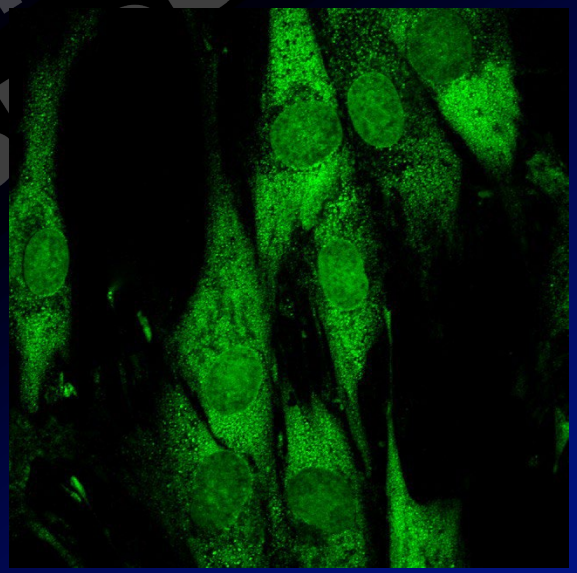
- Sensitive to relative orientation, dynamics and environment

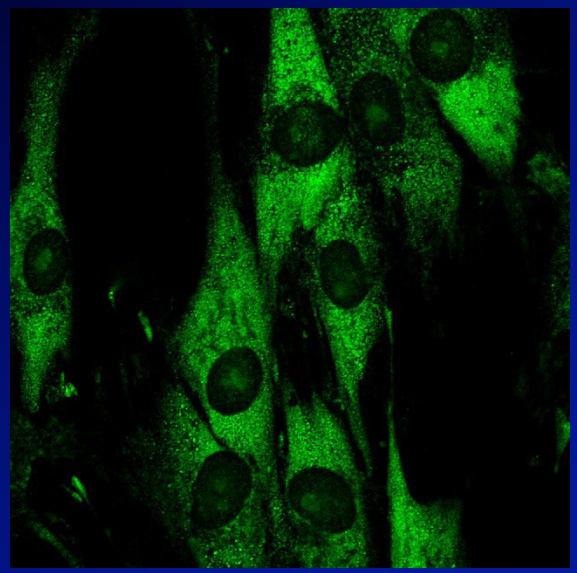
→ ***FRET best measured with fluorescence lifetime***

EXPERIMENTAL CONSIDERATIONS

- Check equipment first: excitation, filters, detectors…
- Chemical dyes brighter and more photo-resistant than FPs
- Limit light exposure for live cells, especially below 410 nm
- Crosstalk: simultaneous vs. sequential illumination:







FLUORESCENCE MICROSCOPY TAKE-HOME MESSAGE

Fluorescence widely used in life science imaging

- **Pros:**
  - **Large selection of dyes**
  - **Large choice of techniques**
  - **Extreme sensitivity**
  - **In-vivo chemicals or endogenous markers**
  - **Very versatile: localization, dynamics, proximity, etc…**
- **Cons:**
  - **Proper use requires basic understanding of fluorescence**
  - **Not suited for long-term (clinical) archival**

QUESTIONS