MICRO-561

Biomicroscopy I

Syllabus (tentative)

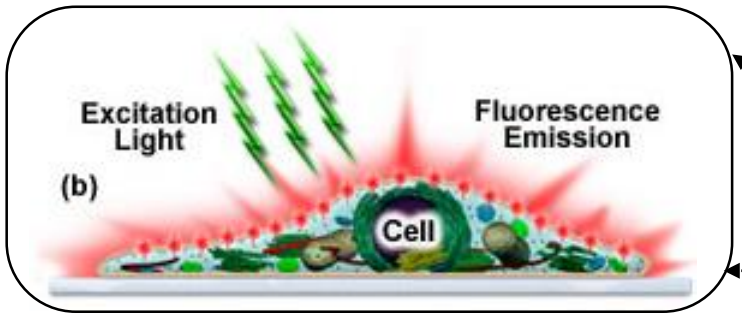
Lecture 1	Introduction & Ray Optics-1
Lecture 2	Ray Optics-2 & Matrix Optics-1
Lecture 3	Matrix Optics-2
Lecture 4	Matrix Optics-3 & Microscopy Design-1
Lecture 5	Microscopy Design-2
Lecture 6	Microscopy Design-3 & Resolution -1
Lecture 7	Resolution-2
Lecture 8	Resolution-3
Lecture 9	Resolution-4, Contrast-1
Lecture 10	Contrast-2, Fluorescence-1
Lecture 11	Fluorescence-2, Sources
Lecture 12	Filters
Lecture 13	Detectors
Lecture 14	Bio-application Examples

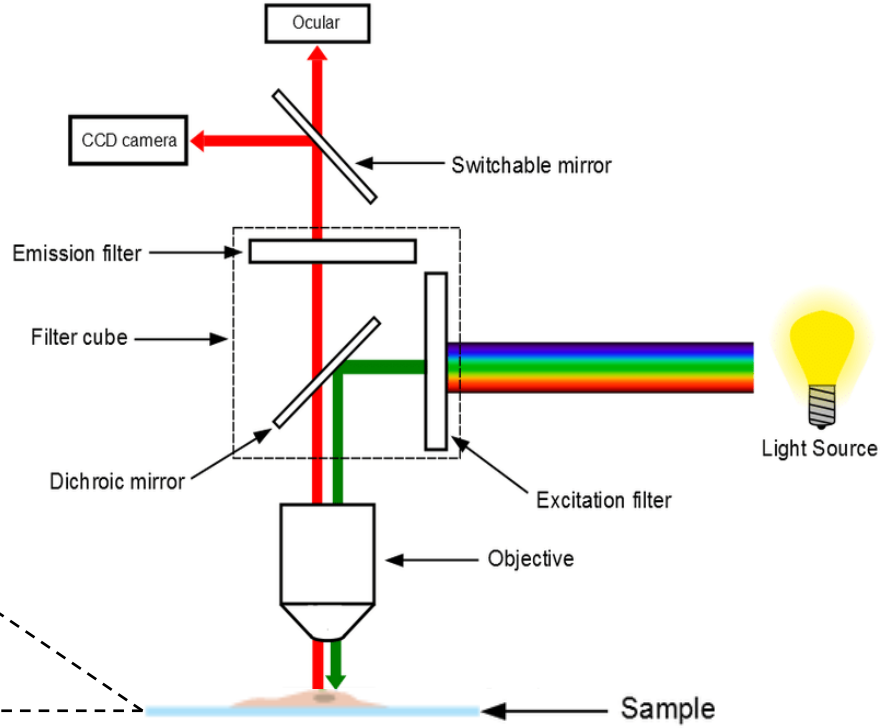
Exam: 28 January 2025 (Tuesday)
9am – noon
CE13

Outline

- To understand fluorescence microscopy we need to be familiar with:
  - **Basic principles of fluorescence**
  - **Properties of fluorescent dyes**
  - **Different kinds of fluorescence markers**
  - The important optical components
    - Filters and filter sets
    - Excitation Sources
    - Detectors
    - Also, their proper positioning in the optical train of the microscope

Fluorescence labelled cell:

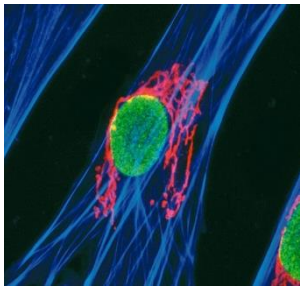




Reminder: There are different types of fluorescence probes

Organic fluorophores:

1. Synthetic dyes

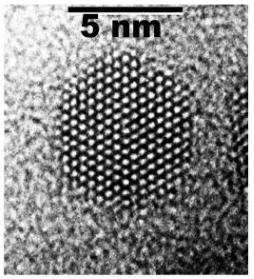


2. Fluorescent proteins

Inorganic fluorophores:

1. Lanthanides

2. Quantum dots



Probes, markers, labels, tags, dyes ..

Reminder: Synthetic Dyes as Fluorescent Markers

- These dyes are typically small molecules with molecular weight < 1000 Da [Dalton]

- Small molecule dyes penetrate easily through cell membranes.

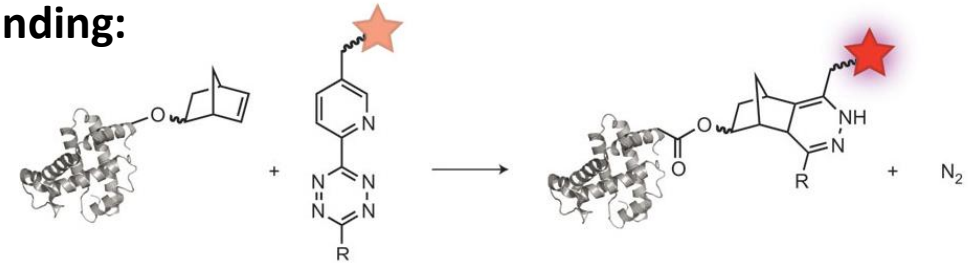
- Small molecule dyes minimally disturb the molecule they attach to.
  - **Example:** They are widely used in DNA staining where small DNA molecule folding or hybridization can be disturbed by larger marker molecules.

Question:
How can we introduce synthetic fluorescence labels on the sample?

There are multiple methods for introducing fluorescence labels

Three commonly used approaches to couple a fluorescence label to a **specific** biomolecule:

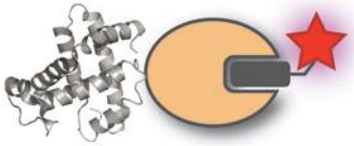
1) **Crosslinking with covalent bonding:**



Some of the most used functional targets and reactive groups in crosslinking:

- Primary amines (–NH2)
- Carboxyls (–COOH)
- Sulfhydryls (–SH)
- Carbonyls (–CHO)

2) **Dye molecule is linked to a specific antigen that binds to a target biomolecule**



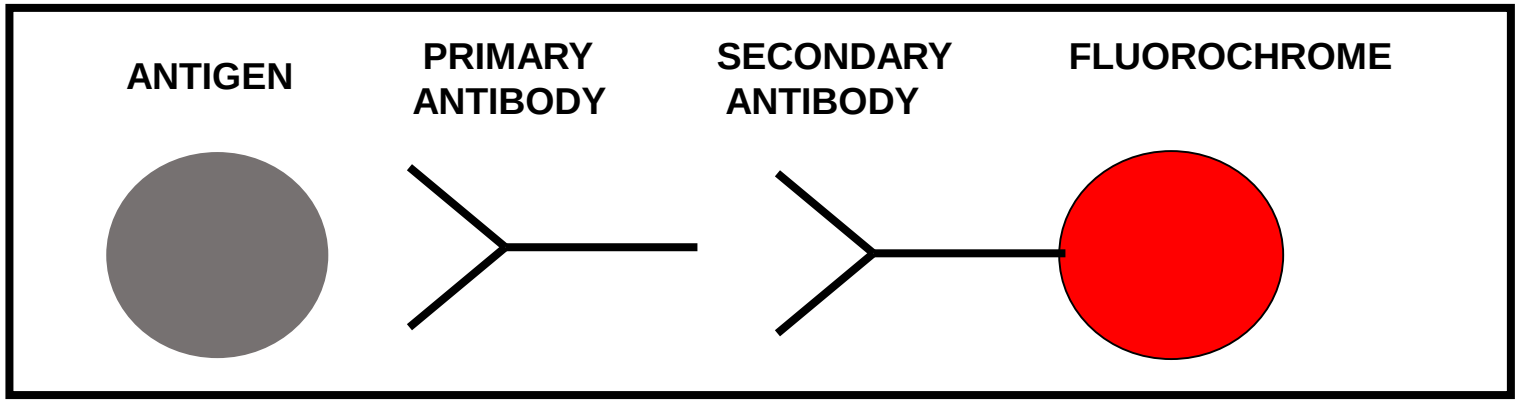
There are multiple methods for introducing fluorescence labels

3) Immuno-fluorescent staining.
This method uses antibodies to locate and identify the patterns of protein expression in cells:

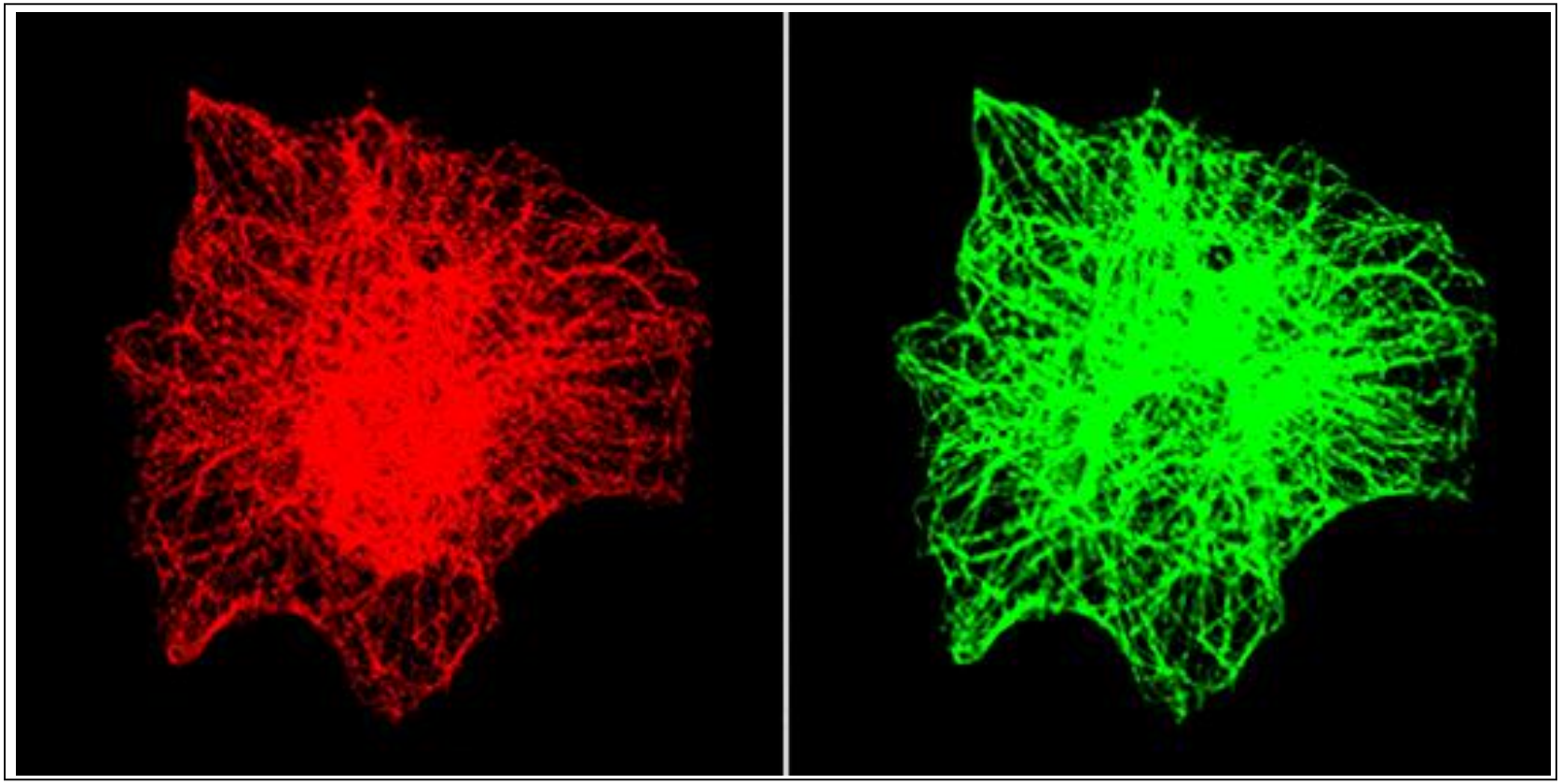
i) Primary antibody binds to antigen.

ii) Antibody-antigen complex bounds to a secondary antibody that is conjugated to a fluorochrome.

iii) Upon absorption of proper light, the fluorophore emits light at its own characteristic wavelength, thus allowing the detection of the antigen-antibody complexes.



Example for immuno-fluorescent staining



PRIMARY ANTIBODY
sheep anti-p53 polyconal

SECONDARY ANTIBODY
Texas Red conjugated anti-sheep

PRIMARY ANTIBODY
mouse anti-α tubulin monoclonal

SECONDARY ANTIBODY
FITC conjugated anti-mouse

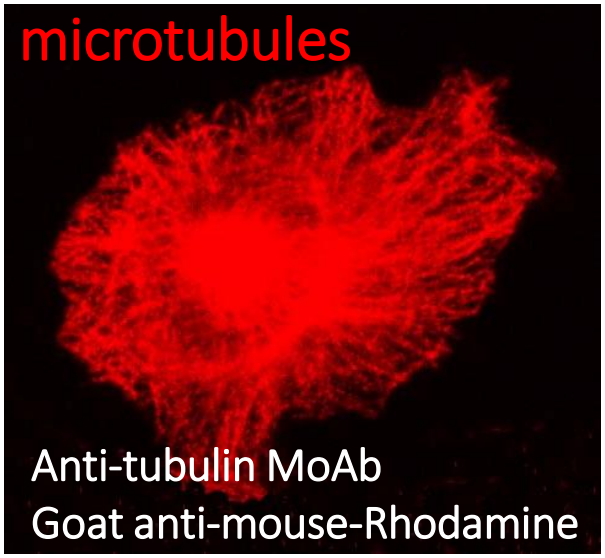
Giannakakou et al., Nature Cell Biology,2000

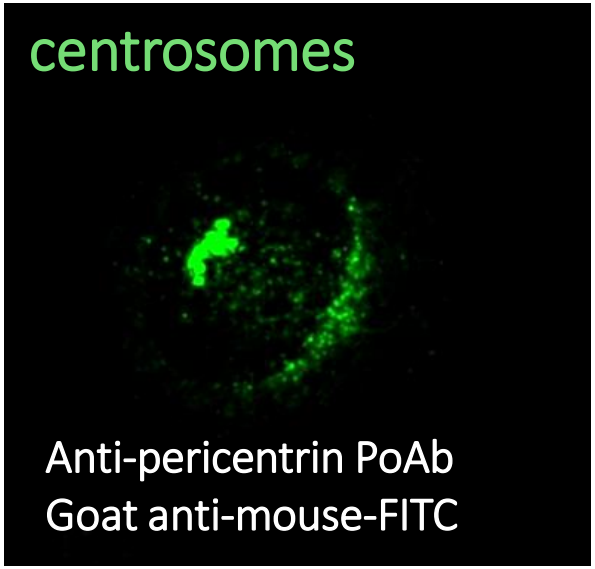
Fluorescence labels can offer target (biomolecular) specificity: direct staining of specific cell structures

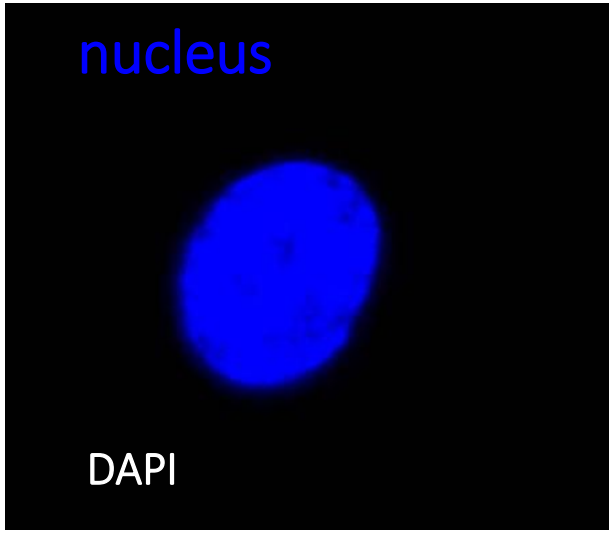
Target	Fluorescent Probe	Mechanism
Mitochondria	MitoTracker	mitochondrial membrane potential
Lysosomes	LysoTracker	hydrolytic activity of enzymes
ER and Golgi	Lectin conjugates	lipid composition
Stress fibers	Phalloidin-conjugaes	bind F-actin
Nuclei	DAPI	binds to minor groove of ds-DNA

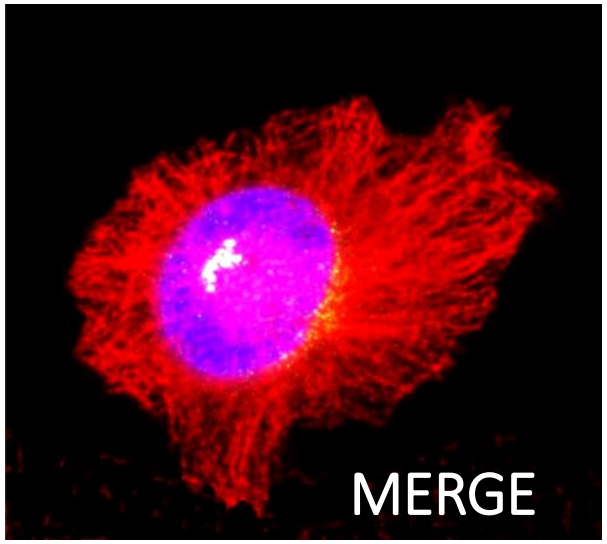
Multi-Color Imaging & Specificity

Differently colored dyes can be addressed to the different parts of the cell:

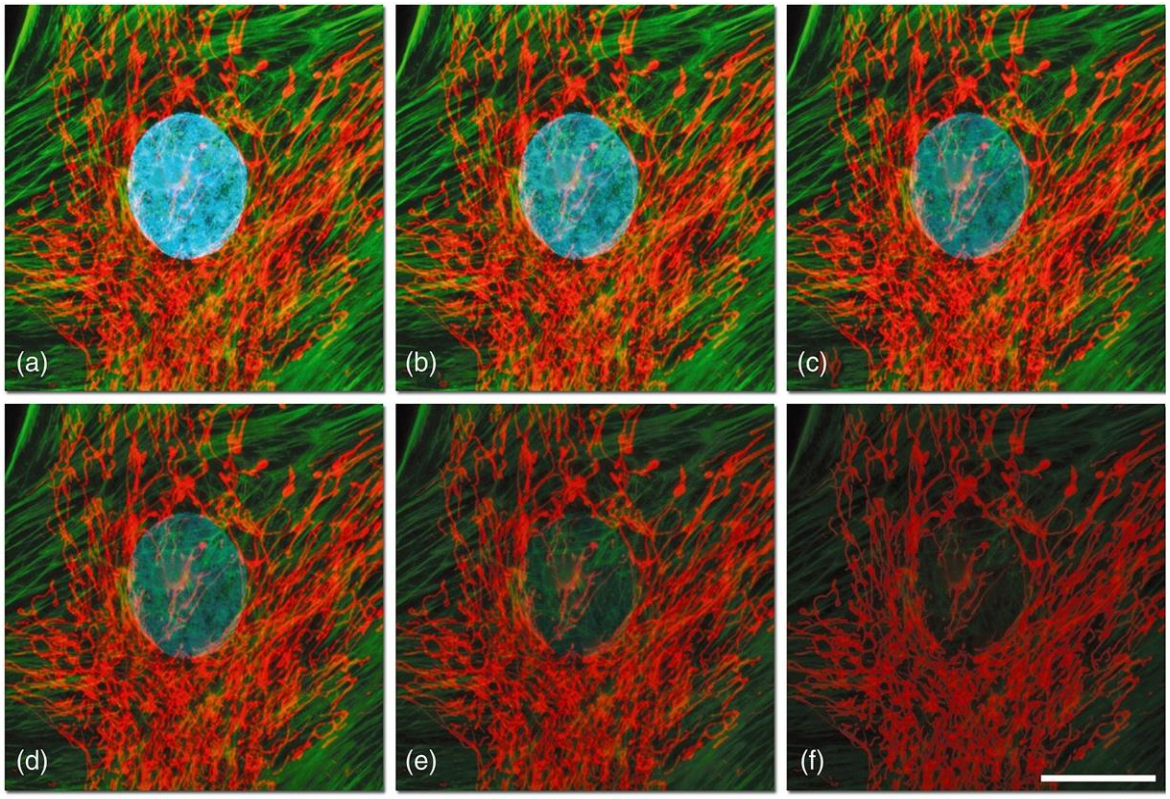








Concern in multi-color fluorescence: photobleaching



- Photobleaching observed in a series of bioimages captured at different time points for a stained culture of Indian Muntjac deerskin fibroblast cells.
  - The nuclei were stained with DAPI (blue fluorescence)
  - The mitochondria were stained with MitoTracker Red (red fluorescence)
  - The actin cytoskeleton were stained with Alexa Fluor 488 (green fluorescence).
- Time points were taken in 2-minute intervals by exciting the three fluorophores simultaneously while also recording the combined emission signals.
- Note that all three fluorophores have a relatively high intensity in panel a, but the DAPI (blue) intensity starts to drop rapidly at 2 minutes and is almost completely gone at 8 minutes.
  The mitochondrial and actin stains are more resistant to photobleaching, but the intensity of both drops over the course of the timed sequence (10 minutes).

(a) *t* = 0. (b) *t* = 2 m. (c) *t* = 4 m. (d) *t* = 6 m. (e) *t* = 8 m. (f) *t* = 10 m. Bar = 20 *μ* m.

Environmental and external effects can change fluorescence properties

Effect of coupling:

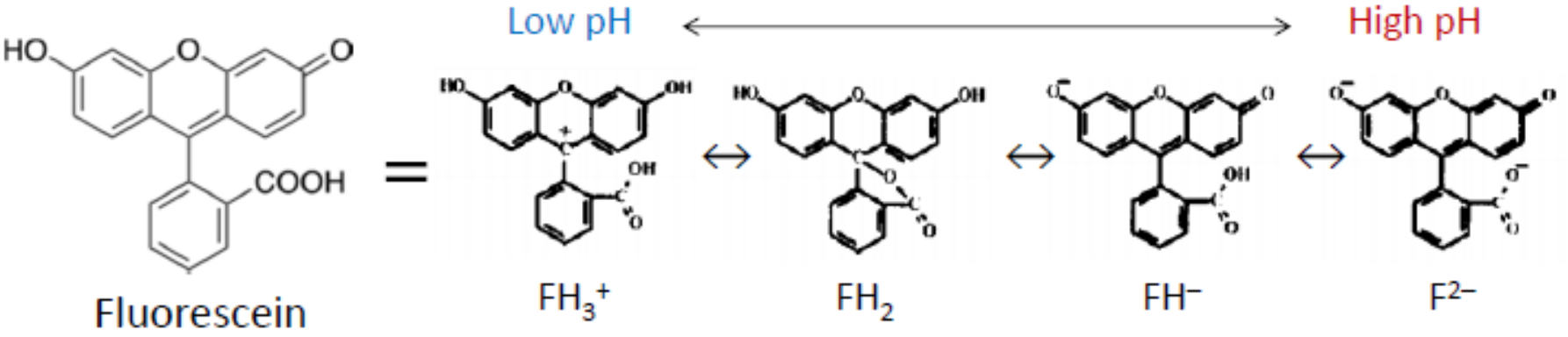
- Emission spectra may shift by coupling a dye to a protein or bacteria.

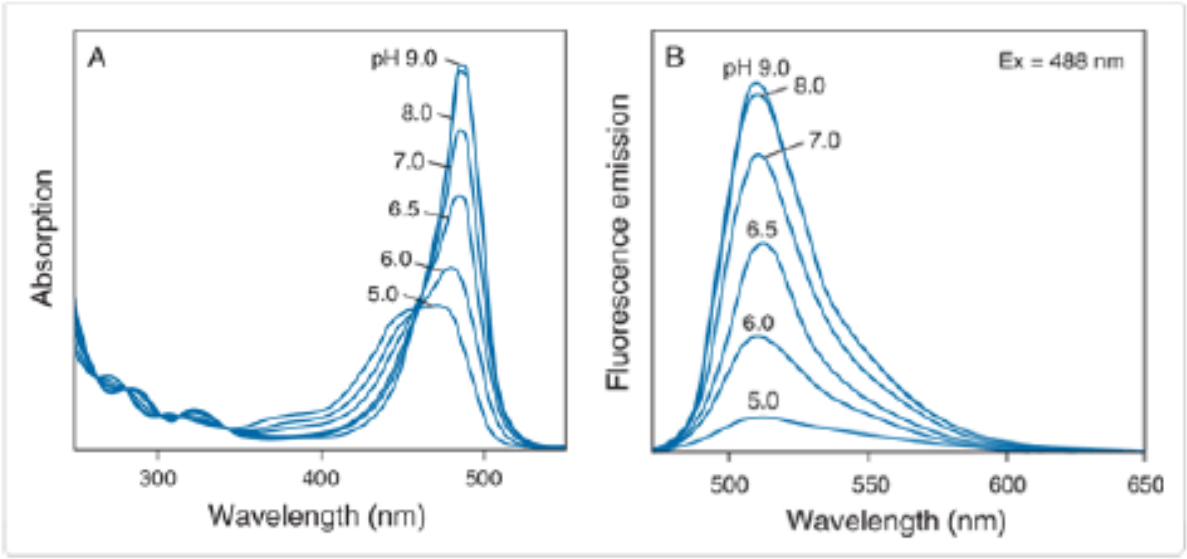
- Quantum efficiency may change by coupling a dye to a protein.
  - **For example:** coupling Fluorescein to a protein may reduce the dye quantum yield Q by 60% and ε by 10 %.

Other environmental and external factors:

- Solvent pH, solvent type, solvent polarity, and other quenching species can change fluorescence properties.

Example: The influence of pH (environment) on dye brightness





Invitrogen

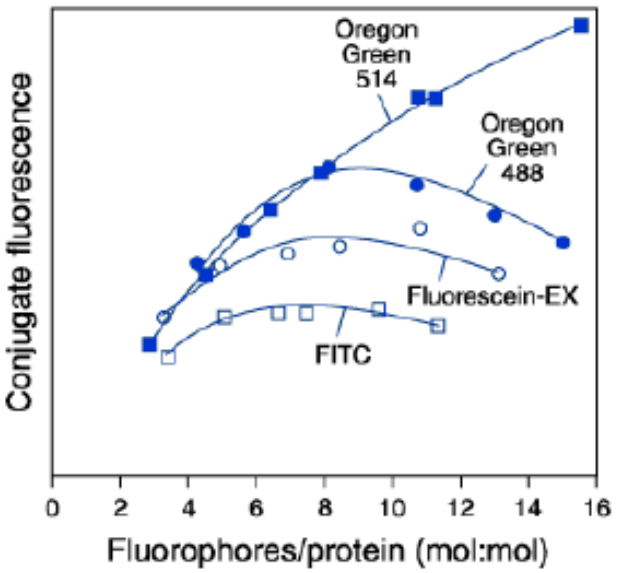
Examples: The influence of dye concentration & excitation light intensity

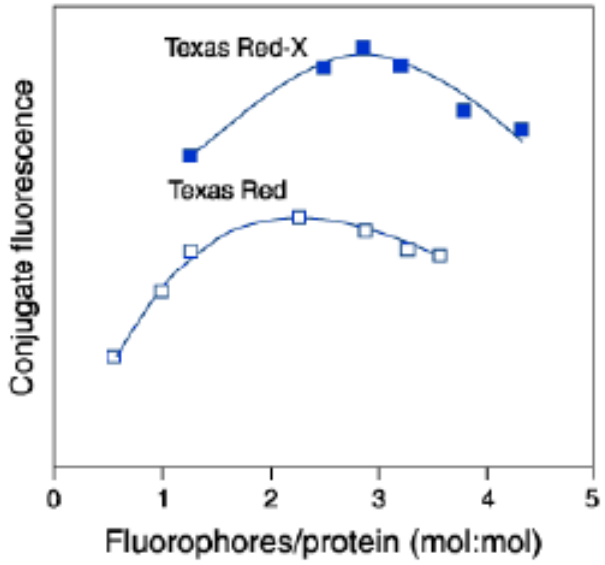
- **At high concentration of dye:**

Quantum efficiency (ϕ_F) thus emission intensity may decrease due to:

- Increased dye-dye interaction.
- Self-quenching: self-absorption will happen at overlap regions between excitation and emission spectra.

Increasing dye concentration: increasing # of dyes per protein





- **Under high intensity illumination:**

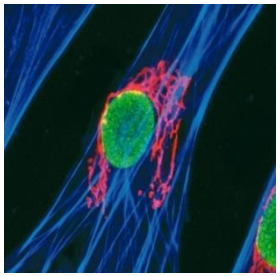
Irreversible destruction or photo-bleaching of the excited dye limits emitted intensity.

Outline

- **There are different types of fluorescence probes.**
- **Let's cover next fluorescence proteins**

Organic fluorophores:

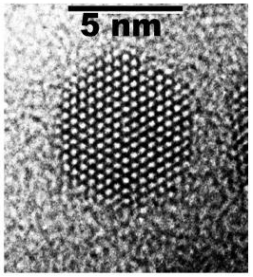
1. Synthetic dyes



2. Fluorescent proteins

Inorganic fluorophores:

1. Quantum dots
2. Lanthanides

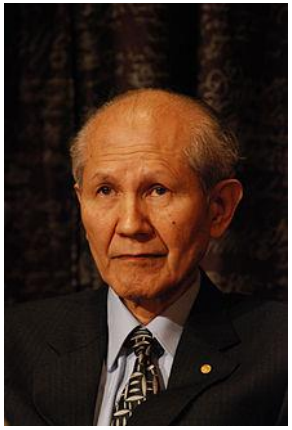


Fluorescence Proteins

- Fluorescent proteins are discovered first in the form of "**Green Fluorescent Protein**, GFP" in the early 1960s.
- Dr. Shimomura worked in the Department of Biology at Princeton to study bioluminescent jellyfish Aequorea Victoria that he collected at the Friday Harbor Laboratories of the University of Washington. In 1962, he isolated GFP from Aequorea Victoria.

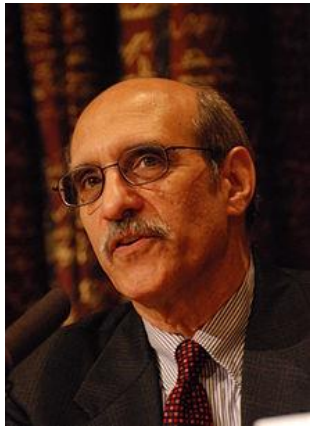
- Fluorescent proteins opened up **a new era in cell biology**:
  - They enabled to apply molecular cloning methods, fusing the fluorophore moiety to a wide variety of protein and enzyme targets, in order to **monitor cellular processes in living systems using optical microscopy.**

2008 Nobel Prize for chemistry is awarded for the discovery & development of GFP



Osamu Shimomura
Organic chemist & marine biologist

Roger Tsien
biochemist

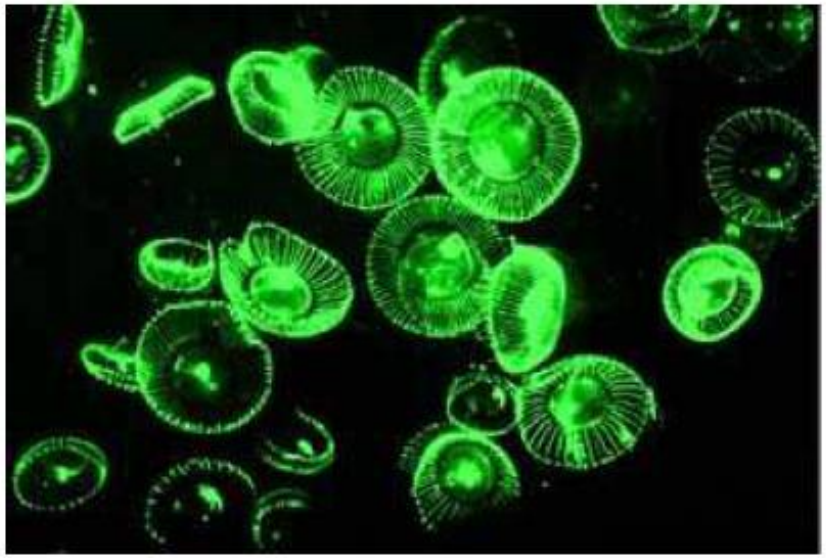


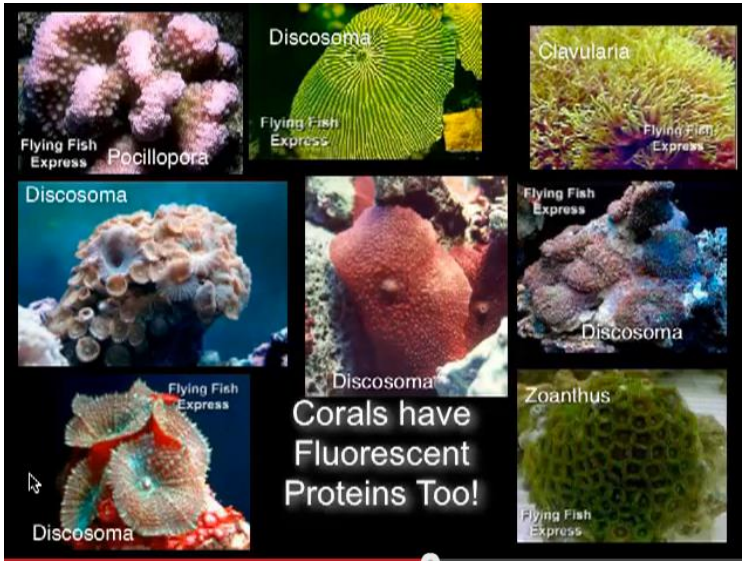
Martin Chalfie
neurobiologist

Green Fluorescent Protein - GFP

- GFP is a protein composed of 238 amino acid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range.

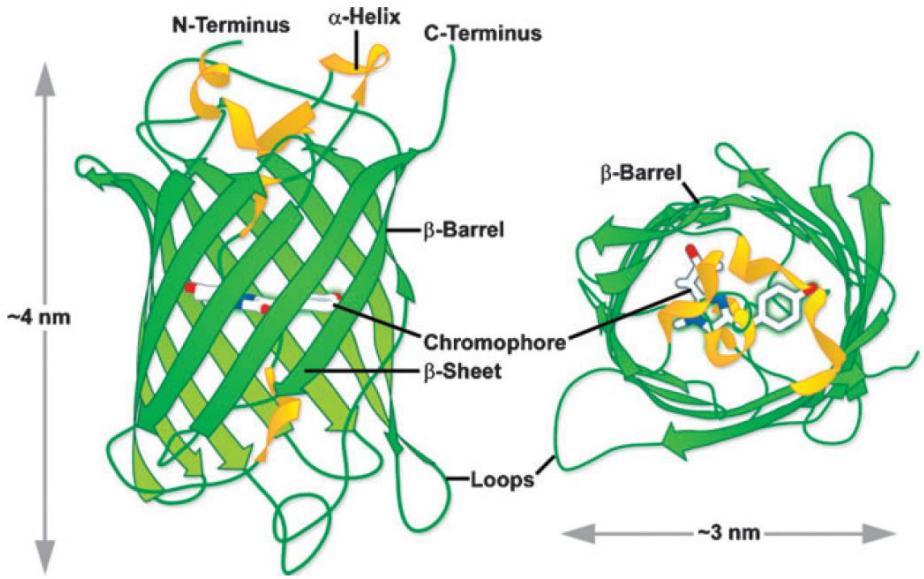
- Although the protein is first isolated from the **jellyfish** Aequorea Victoria, many other marine organisms, like coral reefs have similar fluorescent proteins.

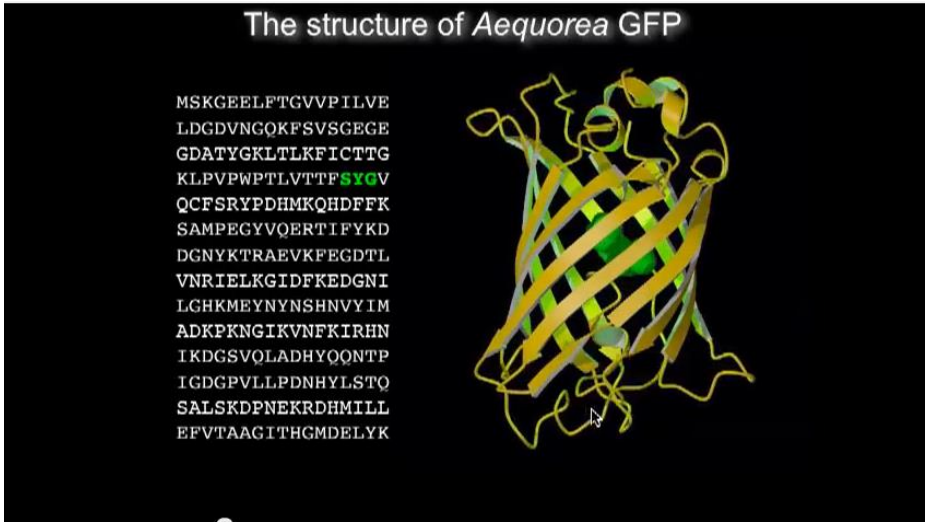




Green Fluorescent Protein - GFP

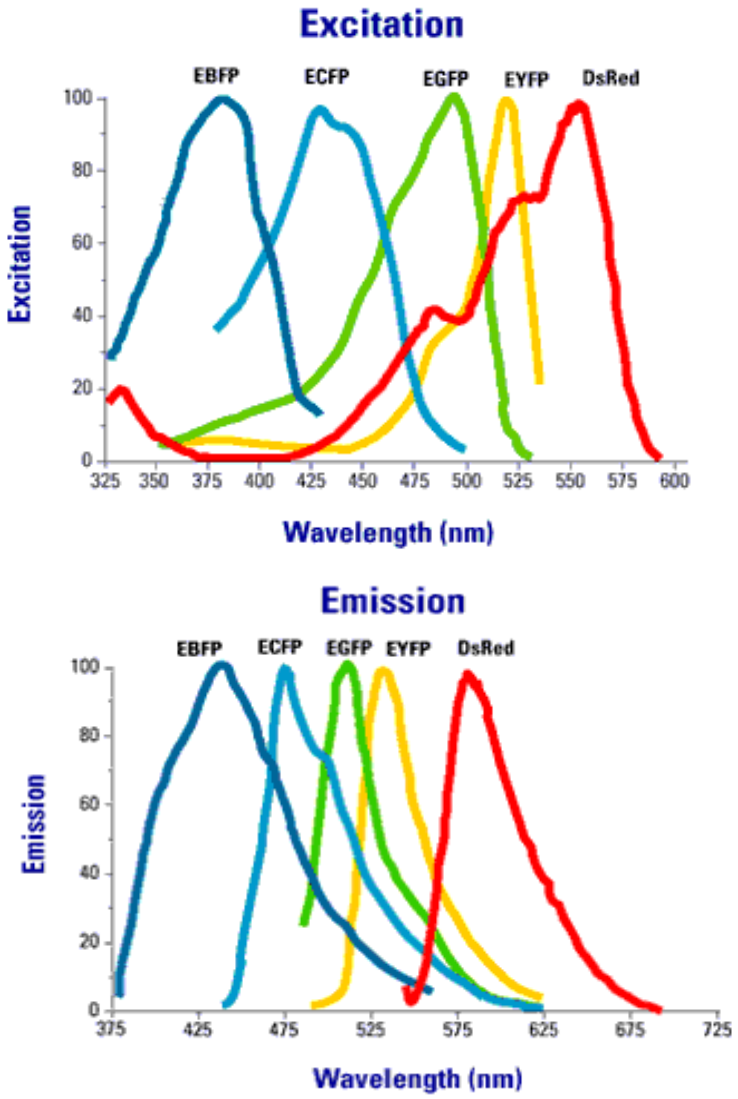
- The fluorophore of GFP sits in the center of the β-barrel
- The chromophore is formed (from Ser-65, Tyr-66, Gly-67) upon folding of the polypeptide chain, without the need for enzymatic synthesis.

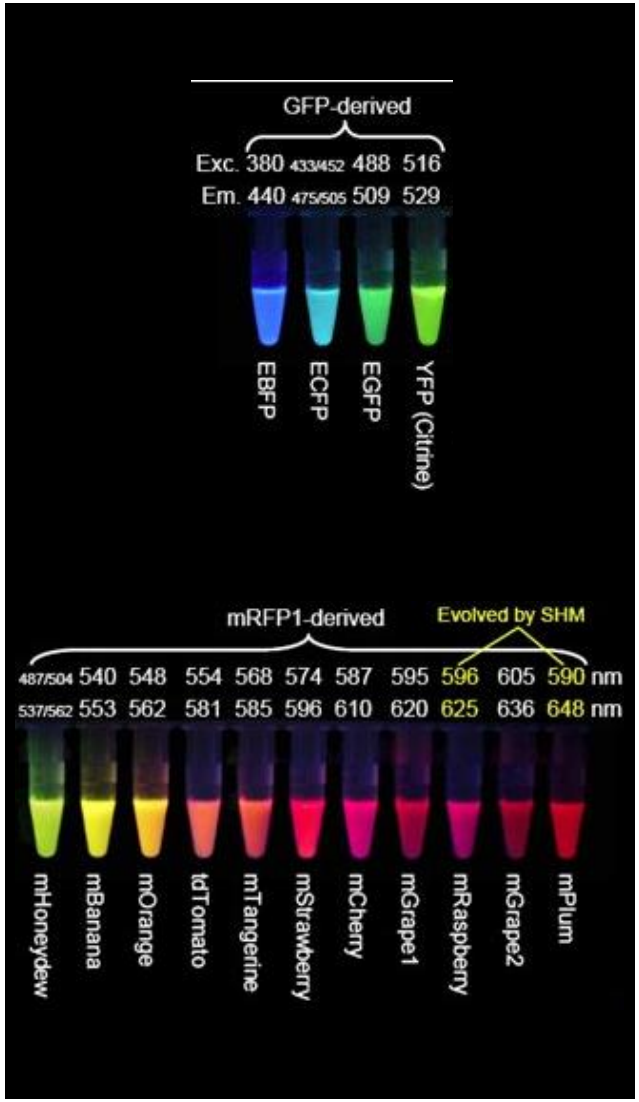




The fluorescent protein palette: tools for cellular imaging.
Richard N. Day and Michael W. Davidson
Chem. Soc. Rev., 2009, 38, 2887–2921.

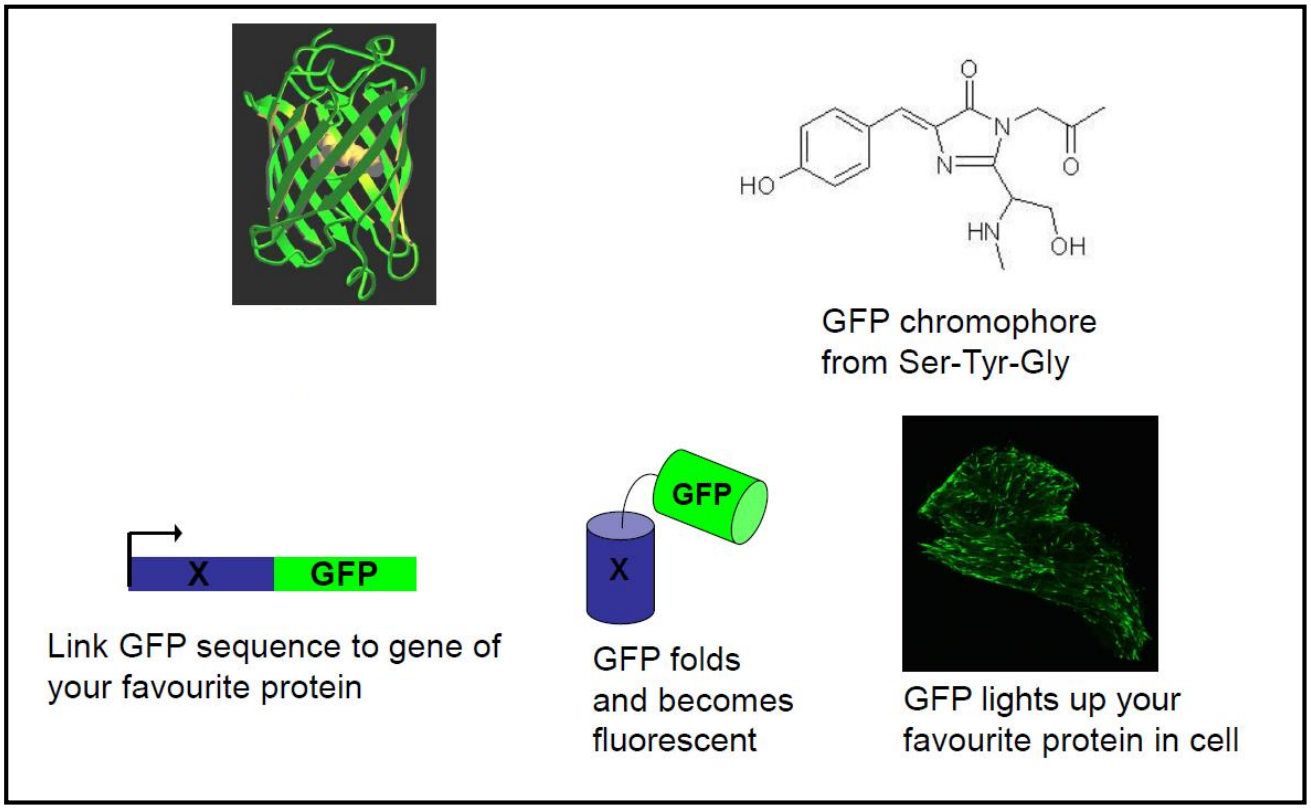
Fluorescent proteins can have different colors





Advantageous of Fluorescent Proteins-1

- If the DNA vectors are provided (purchasable), they can be used to construct fluorescent proteins that can be observed in cells **after transfection with the engineered vectors**.
- This technique avoids the problem of purifying, tagging, and introducing labelled proteins into cells or having to produce specific antibodies.
- In cell and molecular biology, the GFP gene is frequently used as a reporter of expression.
- **Thus, fluorescent proteins are suitable for studying protein dynamics in living cells.**



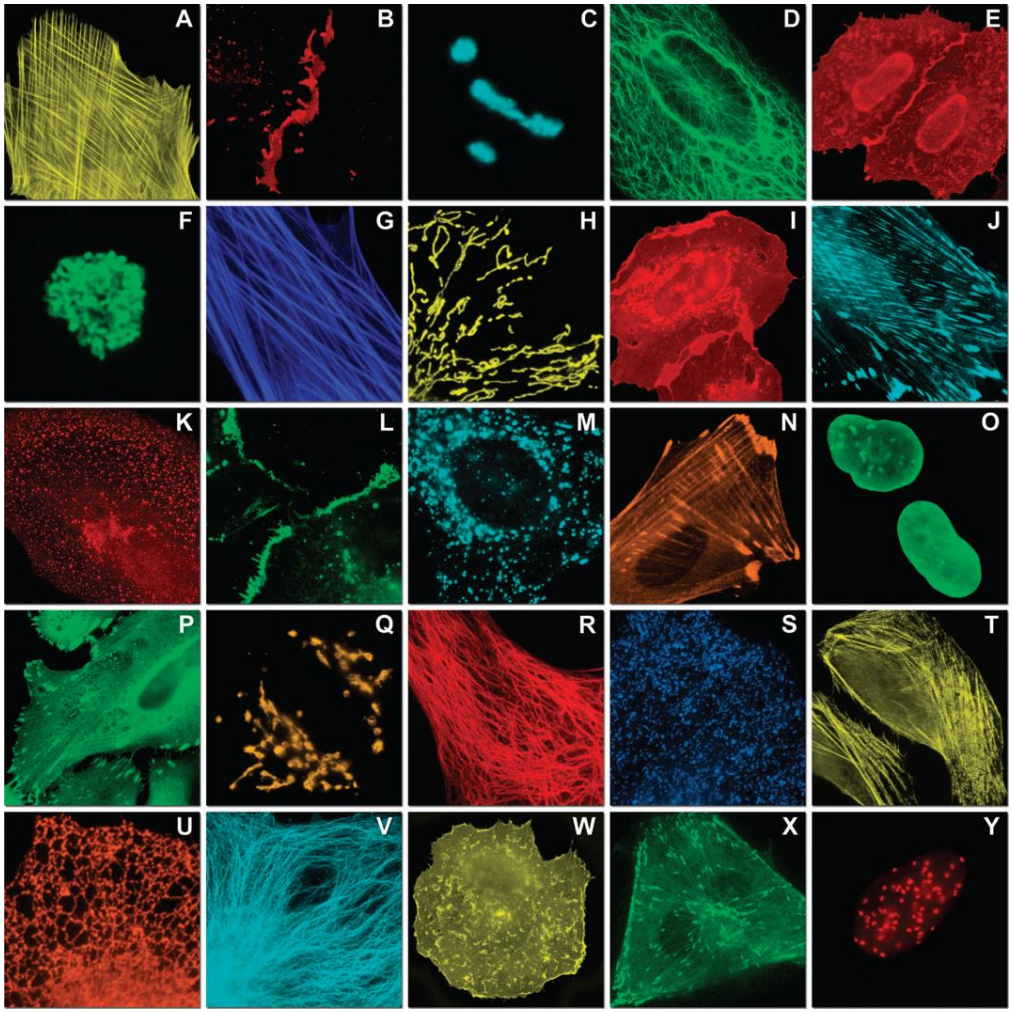
Advantageous of Fluorescent Proteins -2

GFP gene can also be inserted into living organisms and maintained in their genome through breeding…

Example: Applications of fluorescent proteins

The fluorescent protein palette: tools for cellular imaging.

Richard N. Day and Michael W. Davidson, *Chem. Soc. Rev.*, 2009, 38, 2887–2921.



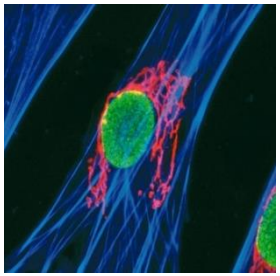
(A) mOrange2-b-actin-C-7.
(B) mApple-Cx43-N-7.
(C) mTFP1-fibrillarin-C-7.
(D) mWasabi-cytokeratin-N-17.
(E) mRuby-annexin (A4)-C-12.
(F) mEGFP-H2B-N-6.
(G) EBFP2-b-actin-C-7.
(H) mTagRFP-T-mitochondria-N-7.
(I) mCherry-C-Src-N-7.
(J) mCerulean-paxillin-N-22.
(K) mKate-clathrin (light chain)-C-15.
(L) mCitrine-VE-cadherin-N-10.
(M) TagCFPlysosomes-C-20.
(N) TagRFP-zyxin-N-7.
(O) superfolderGFP-lamin B1-C-10.
(P) EGFP-a-v-integrin-N-9.
(Q) tdTomato-Golgi-N-7.
(R) mStrawberry-vimentin-N-7.
(S) TagBFP-Rab-11a-C-7.
(T) mKO2-LC-myosin-N-7.
(U) DsRed2-endoplasmic reticulum-N-5.
(V) ECFP-atubulin-C-6.
(W) tdTurboRFP-farnesyl-C-5.
(X) mEmerald-EB3-N-7.
(Y) mPlum-CENP-B-N-22.

Outline

- **There are different types of fluorescence probes.**
- **Let's focus next on inorganic fluorophores.**

Organic fluorophores:

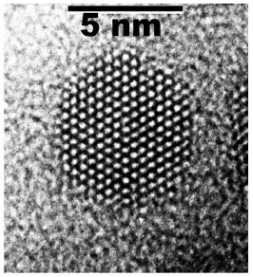
1. Synthetic dyes



2. Fluorescent proteins

Inorganic fluorophores:

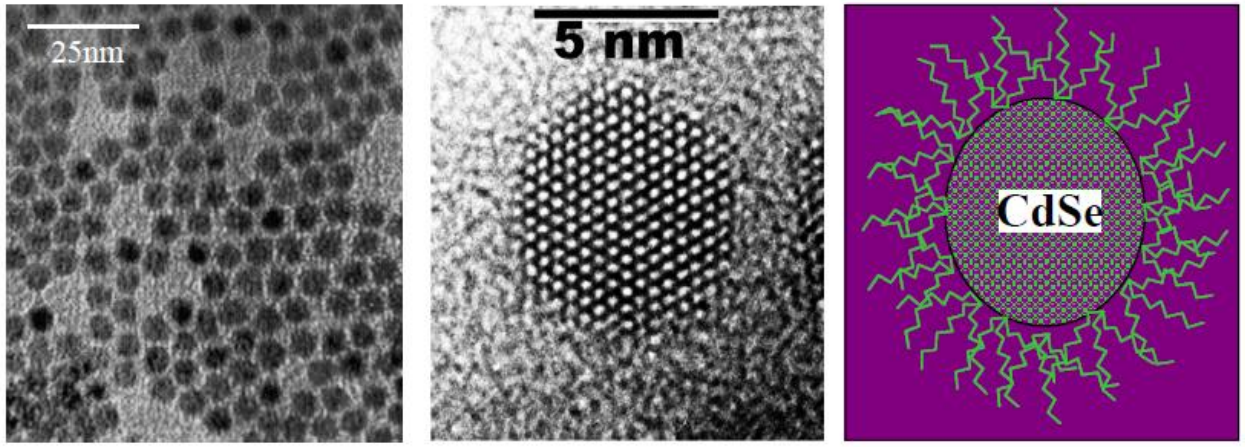
1. Quantum dots
2. Lanthanides

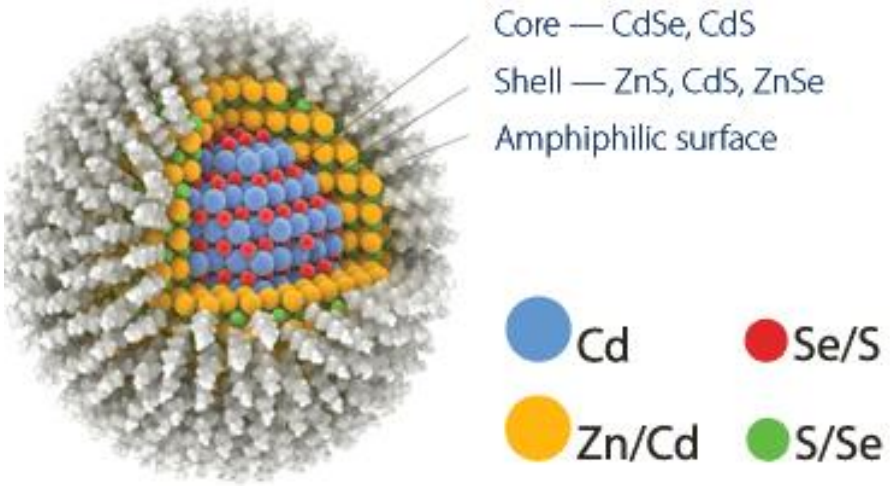


Quantum Dots (QD)

QD are man-made molecular-sized semiconductor nanocrystals that fluorescence

TEM images



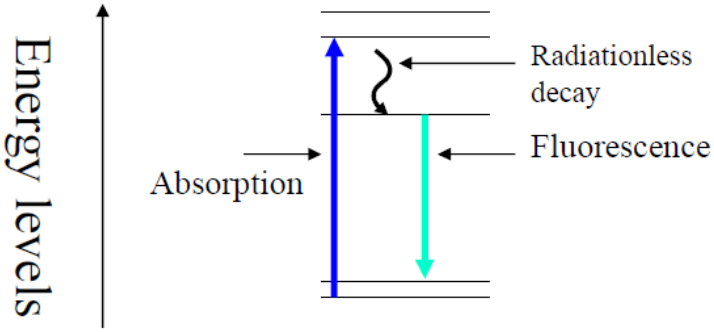


Size: Typically tuned from ~2-10 nm
Structure: highly crystalline

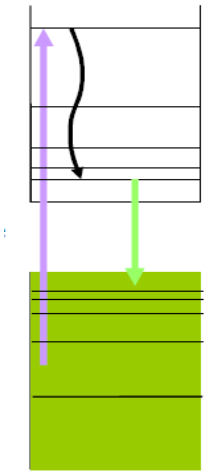
Properties:

- *Higher extinction coefficient than organic fluorophores*
- *High photostability and resistance against photobleaching.*

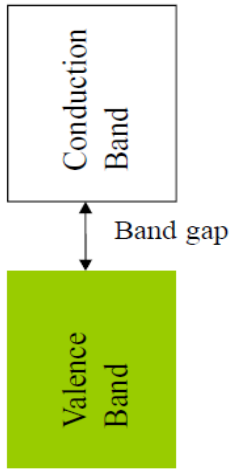
Quantum Dots: Fluorescent semiconductor nanoparticles



a) Atoms & Molecules:
Discrete energy levels

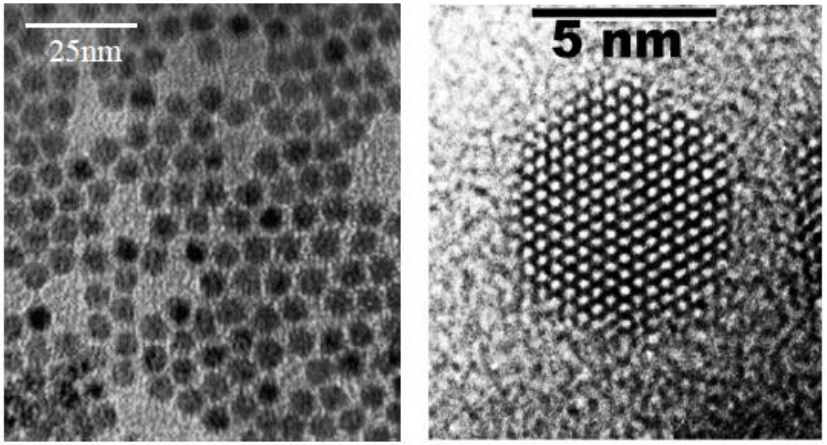


b) Q-dots:
Discrete energy levels (like molecules)



c) Semiconductors:
No energy discretization (i.e. quantization)
Energy levels are so close that they form "bands"

Semiconductor nanoparticles with diameters lower than the exciton Bohr radius, often called **"artificial atoms"**.



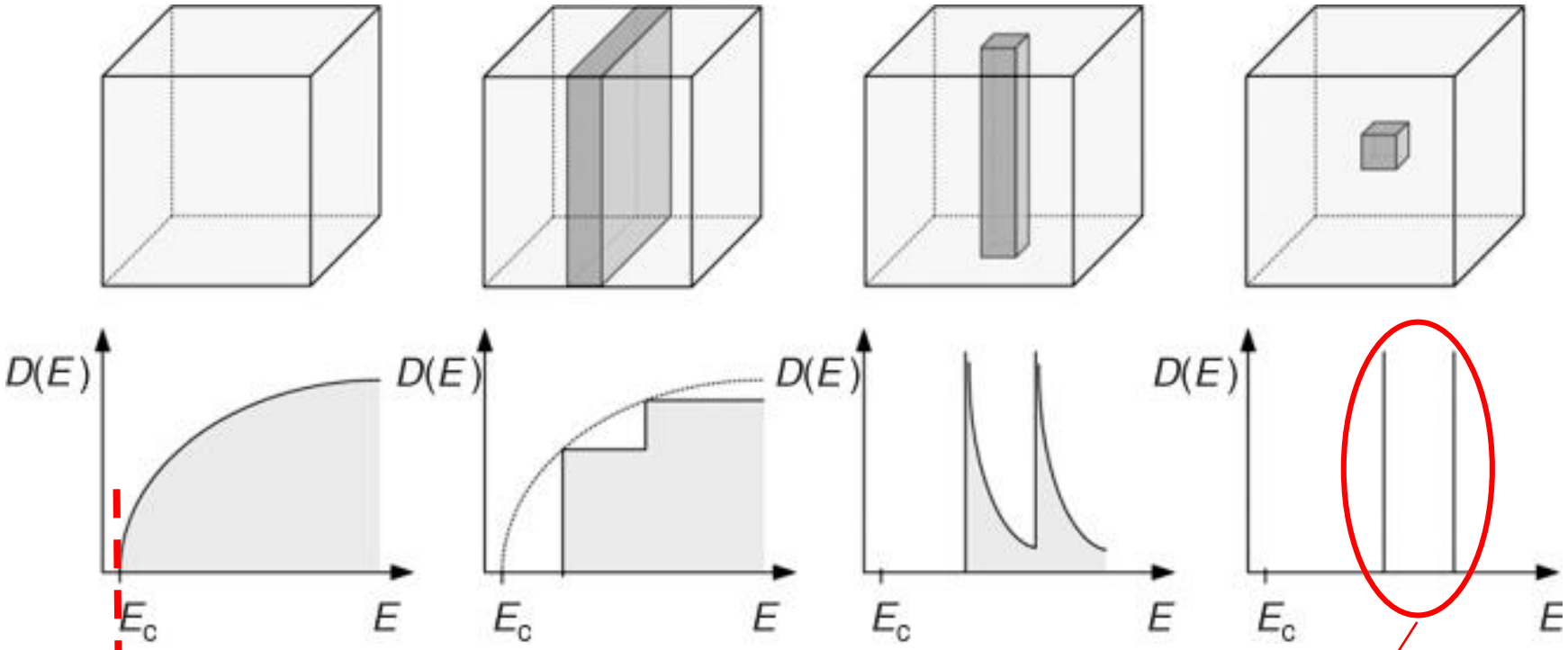
Quantum Confinement

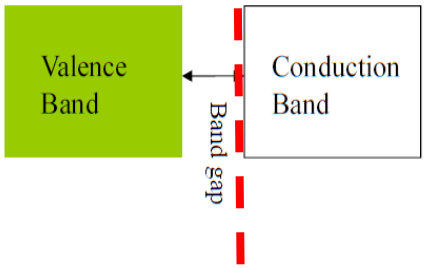
No Confinement
Bulk material (i.e. semiconductors)

1D Confinement
"Quantum well"

2D Confinement
"Quantum wire"

3D Confinement
"Quantum dot"

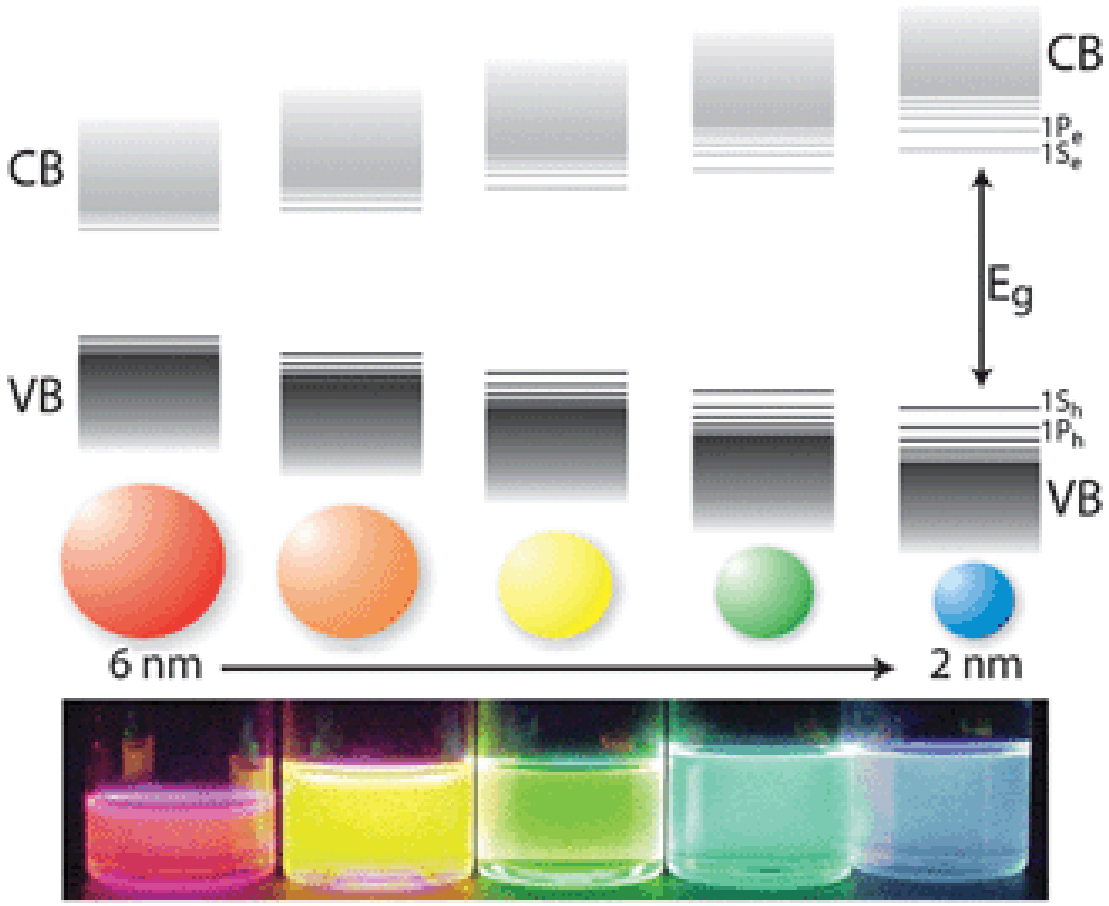




E_c is conduction band edge
D(E) is density of states as a function of frequency

3D confinement results in energy quantization as in "atoms"

Tuning of Quantum Dot Emission



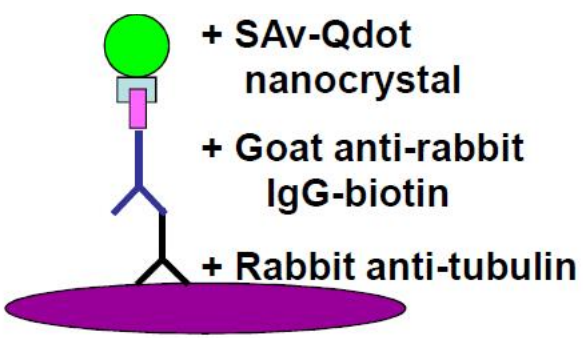
Optical properties of Q-dots:

- The energy levels depend on the particle size.
- With size reduction (which required forcing the electrons to confine better in a small space), the energy gap increases.

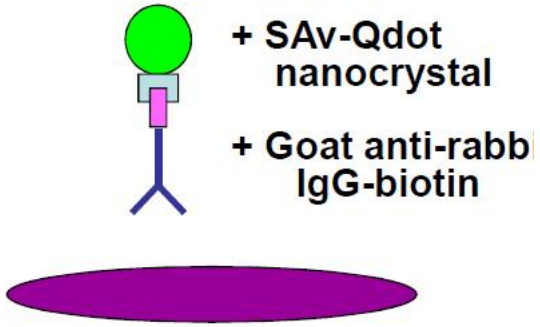
➔ **Smaller sizes result in "blue" tuning.**

Example: specific cellular staining by Immuno-fluorescence

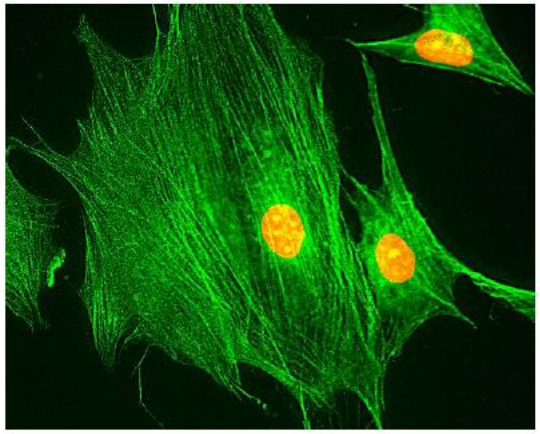
Tubin staining with Quantum Dot (Qdots)

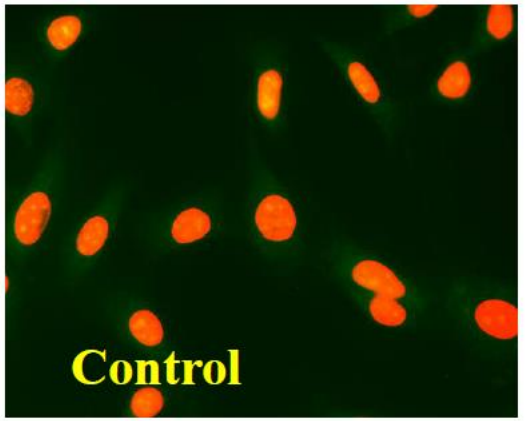


SPECIFIC BINDING



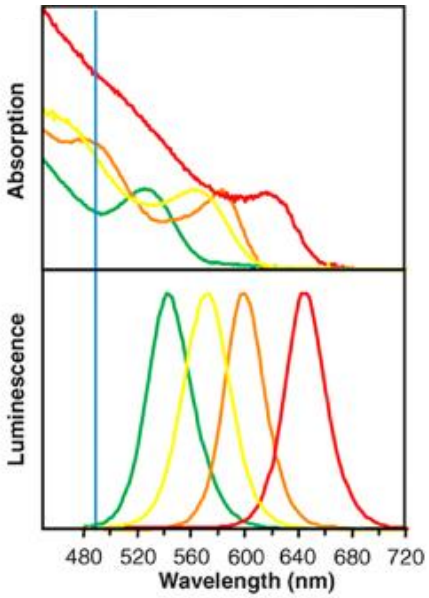
NEGATIVE CONTROL

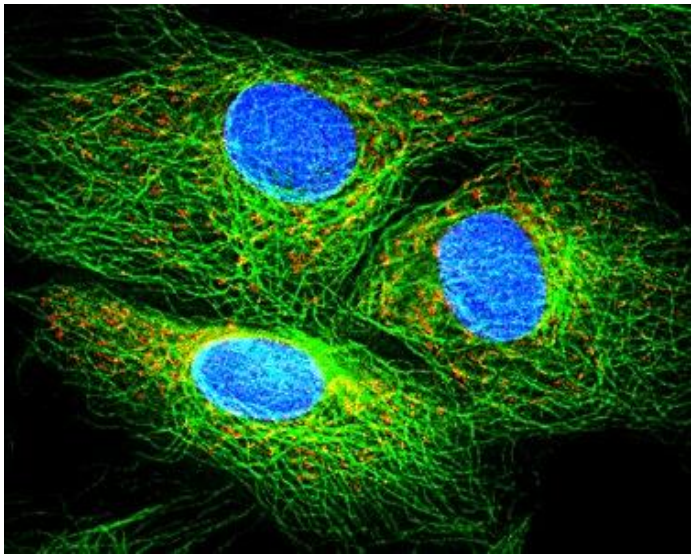




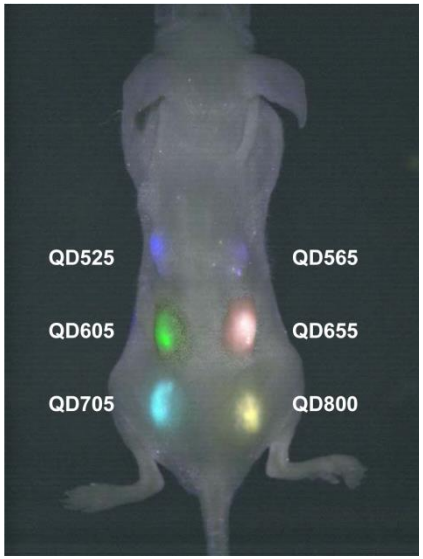
Streptavidin: is a 52.8 kDa protein purified from the bacterium *Streptomyces avidinii*. Streptavidin has an extraordinarily high affinity for biotin (aka vit. B7 or vit. H). With a dissociation constant (K_d) of ~10^{-14} mol/L, biotin-streptavidin binding is one of the strongest non-covalent interactions known in nature.

Bio-imaging with fluorescent quantum dots





X. Wu et al, Nat. Biotech. (2003)



- Excitation in the UV for all particle sizes allows easy color multiplexing.
- Long photostability allows the analysis of particle biodistribution
- **But, toxicity issues** require quantum dot synthesis using other semiconductors without Cd or Se, for instance. Capsulation of core (containing Cd) with shell & protein layers can be alternative.

Lanthanide Chelate Complexes

- The fluorescence of lanthanide complexes is very long-lived with lifetime between a few 100s to a few ms.
- They are typically excited by UV light and emit fluorescence in the visible region.
- Large stokes shift
- Their emission profile is very sharp, and the wavelength is specific to each metal.

  Example: Eu^{3+} complexes can emit at 615 nm & Tb^{3+} complexes can emit at 545 nm

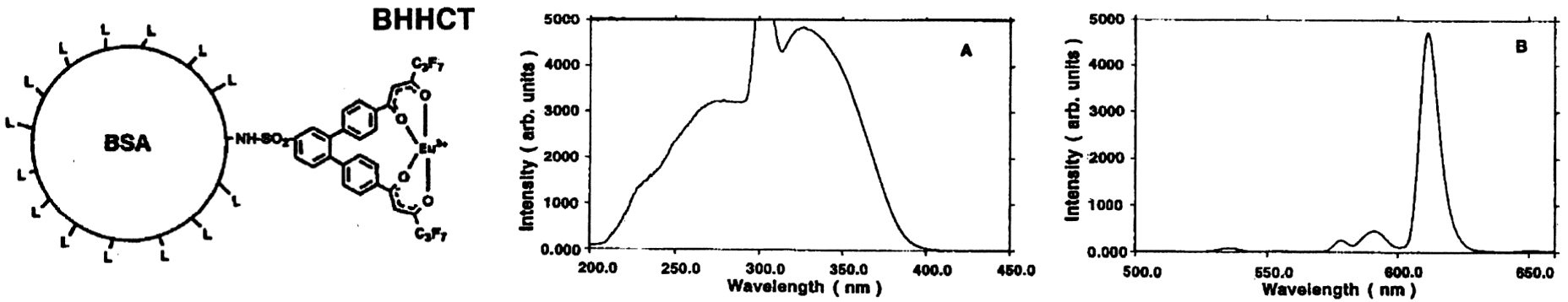
	lifetime	Stokes shift	λex,max	λem,max
	ns	nm	nm	nm
Fluorescein (FITC)	4.5	28	492	520
Rhodamine (RBITC)	2	35	550	585
Eu(β-NTA)$_3$[a)]	500,000	276	339	615

Matsumoto et al. Fluorescent Lanthanide Chelates for Biological Systems. Macromol. Symp. 186, 117-121 (2002)

Cho U. & Chen J.K. Lanthanide-based optical probes of biological systems (2020) Cell Chemical Biology

Lanthanide Chelate Complexes

Excitation (A) and emission (B) spectra of Bis-heptafluoro-hexanedion-chlorosulfo-o-terphenyl (BHHCT) labelled with BSA in the presence of Eu^{3+}.

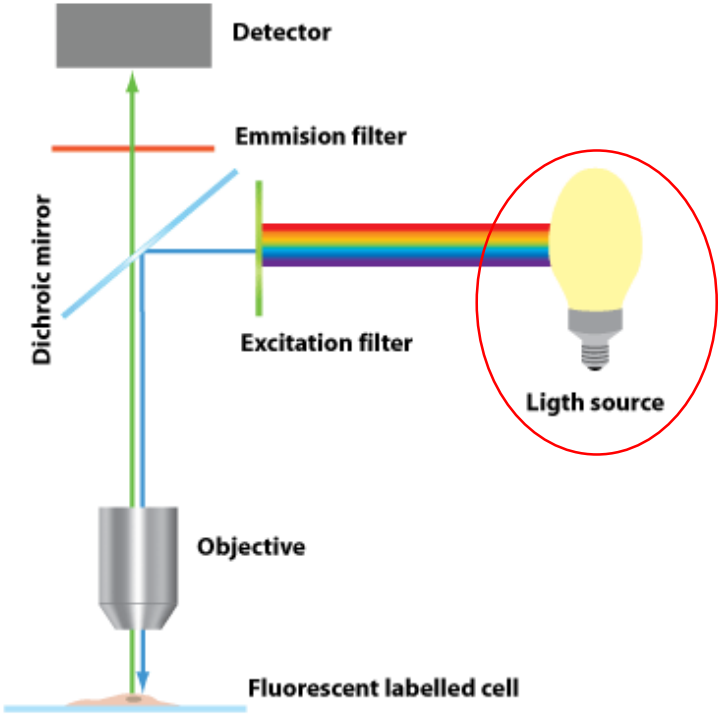


Example: BHHCT-Eu^{3+} is suitable for immunoassay & DNA hybridization

Matsumoto et al. Fluorescent Lanthanide Chelates for Biological Systems. Macromol. Symp. 186, 117-121 (2002)

Outline

- To understand fluorescence microscopy we need to be familiar with:
  - Basic principles of fluorescence
  - Properties of fluorescent dyes
  - Different kinds of fluorescence markers
  - The important optical components
    - Illumination sources
    - Filters and filter sets
    - Detectors
    - Their proper positioning in the optical train of the microscope



Illumination in Microscopy

- To obtain optimal imaging performance, the specimen must be properly illuminated.
- This requires:
  - proper selection of the wavelength and the intensity of the illumination source
  - correct alignment & focusing of the source (i.e. recall Kohler illumination)

Commonly used illuminators in optical microscopy (bright-field & fluorescence):

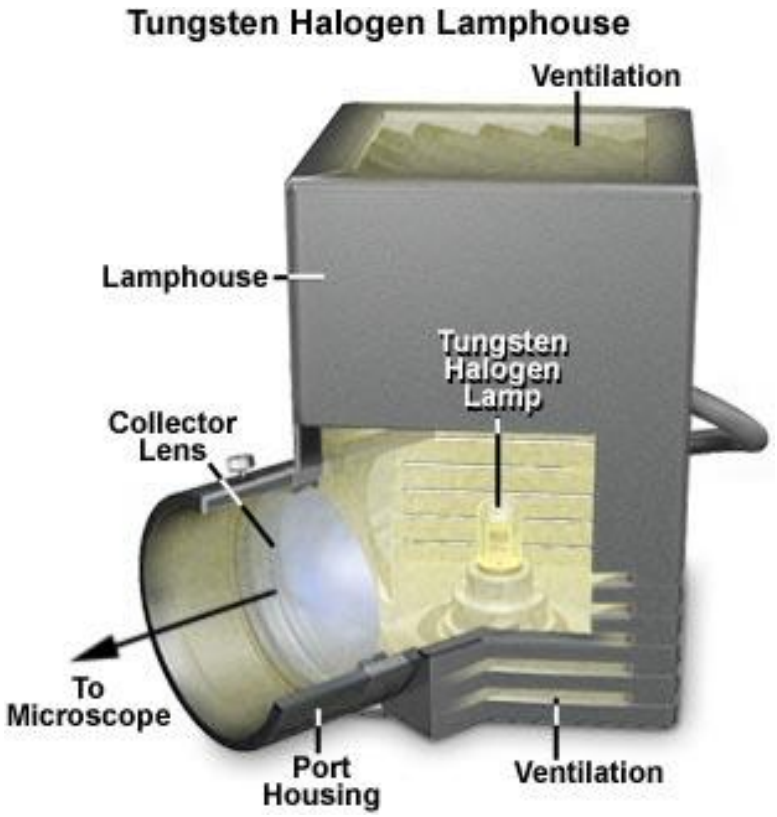
- Incandescent lamps
  Quartz tungsten-halogen
- Ion arc lamps
  Mercury, Xenon
- Metal halides
- Solid-state sources:
  LEDs & Lasers

Incandancent Lamps

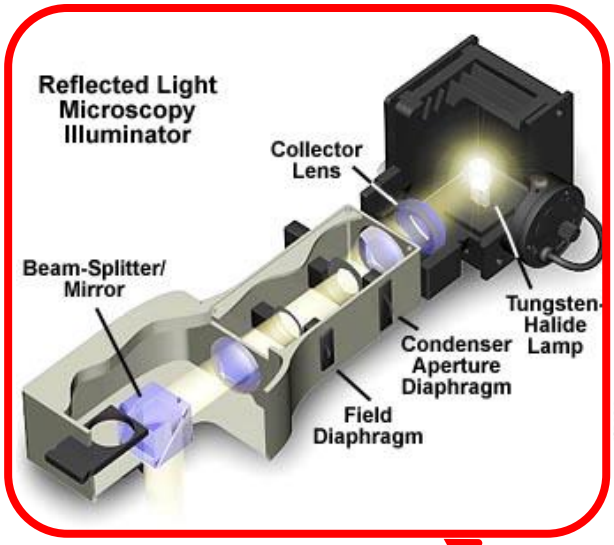
- **Incandescent lamps** with wire filaments and inert gases are frequently used in microscopy (especially in bright-field microscopy), because they are:
  - Cheap & easy to use
  - Provide sufficiently bright & even illumination when used properly (e.g. with a ground glass filter).

- Quartz tungsten-halogen is a commonly used incandescent lamp.
- Operation:

- It is based on a wire filament (such as **tungsten**) that is heated to a high temperature by passing an electric current through it until it glows with visible light (**incandescent)**.
- The bulb consist of an air-tight glass enclosure. The bulb is typically made of fused silica (quartz) since it is very strong, the gas pressure can be higher.
- The bulb is filled with an inert gas for reducing evaporation of the filament and preventing its oxidation.
- **Halogen** (such as iodine or bromine) increases the lifetime of the bulb and prevents its darkening.

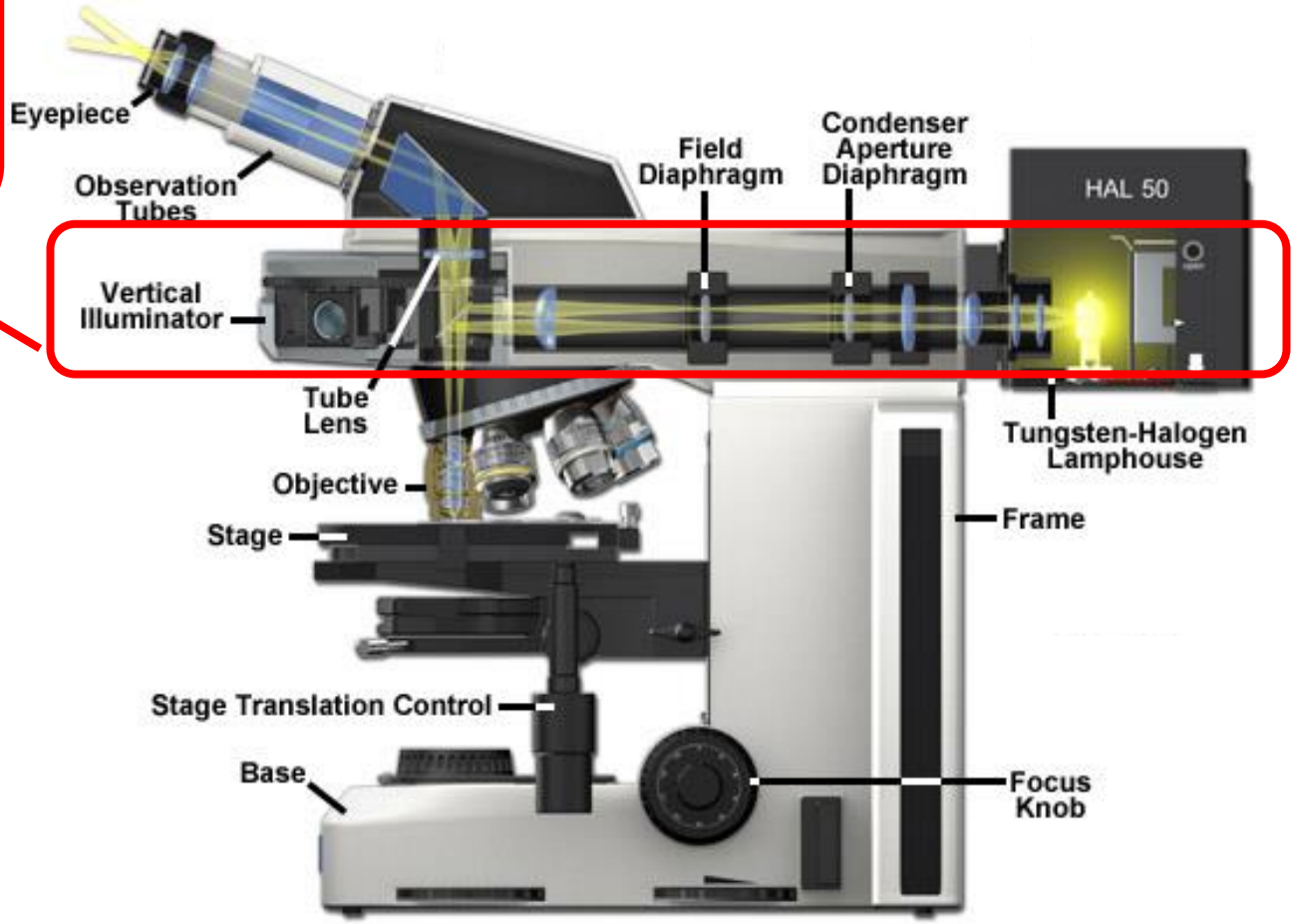


Example: Use of incandescent lamp

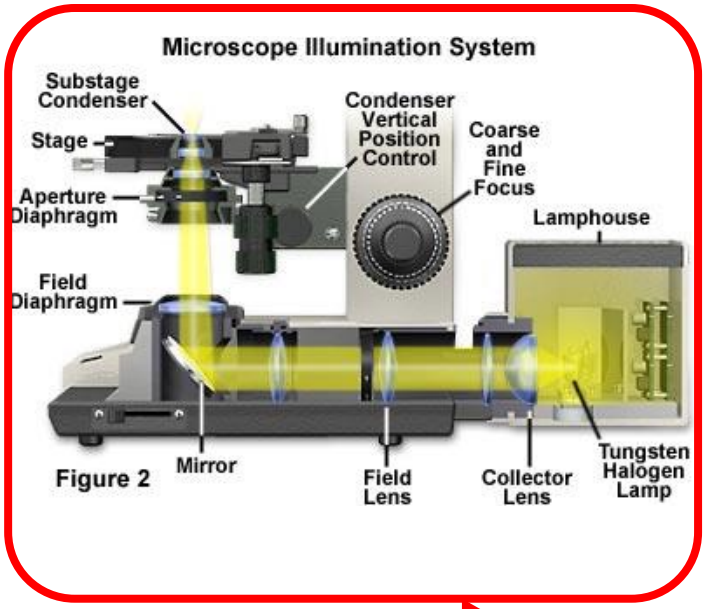


Anatomy of an Upright – **Reflected** Light Microscopy:

- Objective lens is on top of the sample stage.
- Light sources is delivered above the sample stage.

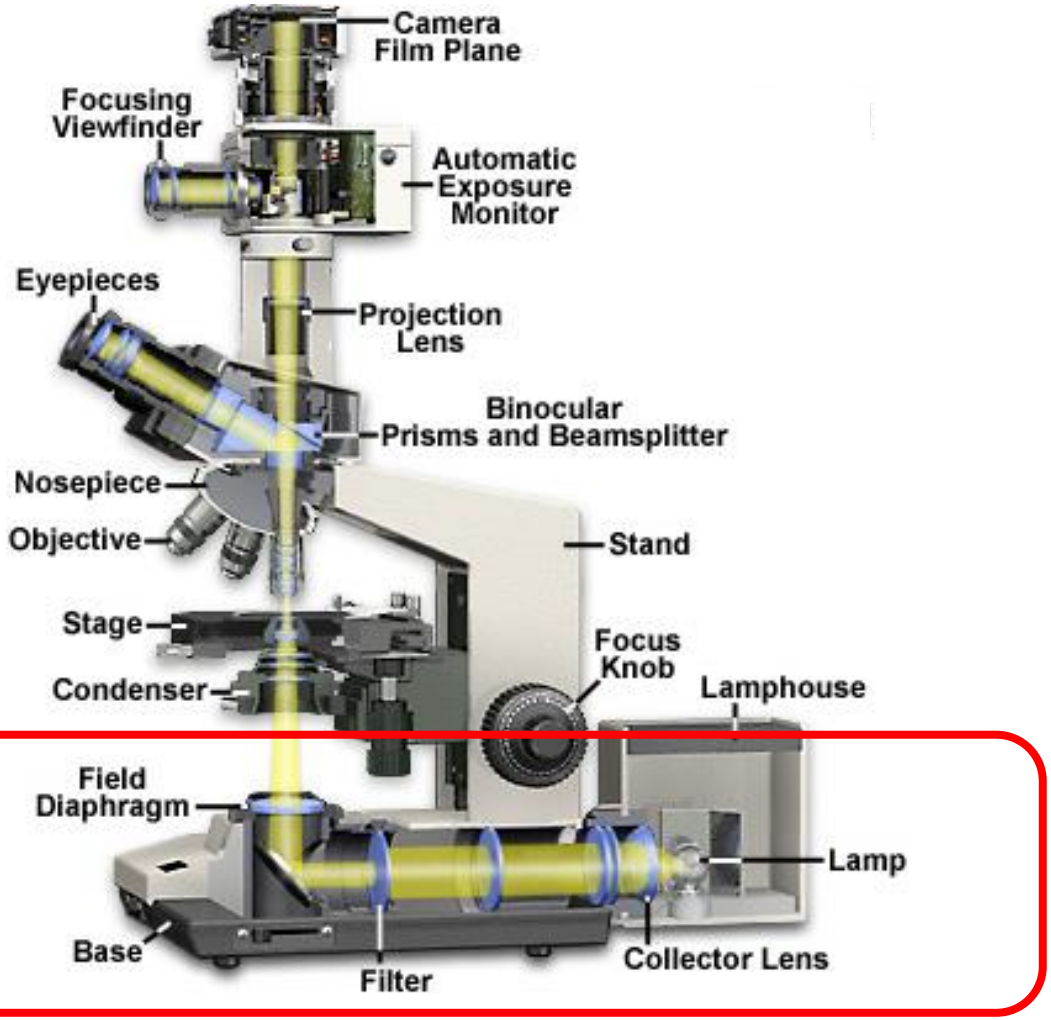


Example: Use of incandescent lamp



Anatomy of an Upright –**Transmitted** Light Microscopy:

- Objective lens is on top of the sample stage.
- Light sources is delivered below the sample stage.

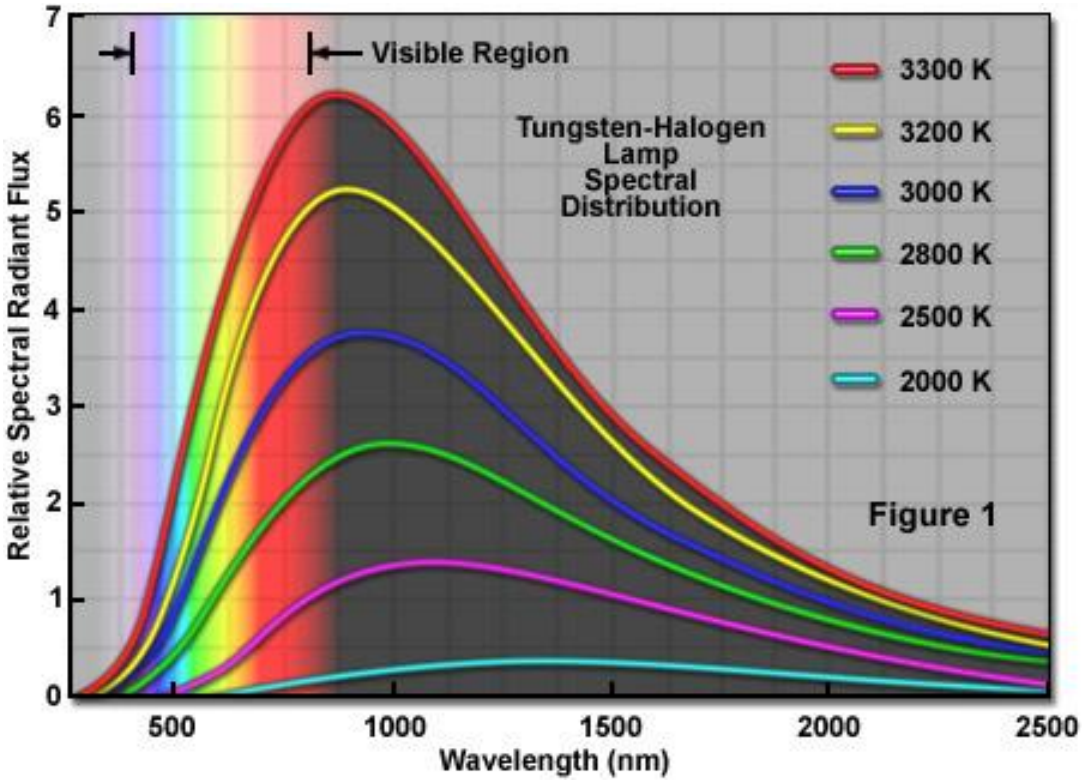


Spectrum of Incandescent lamps

- Quartz halogen tungsten filament lamp has black-body radiation spectrum:
  → it has "continuous spectrum" (and also broad spectrum)
- Figure shows lamp spectrum at different temperature → it has significant power in the infrared!

- The peak wavelength depends on the body temperature.
- With increasing voltage (thus temperature), brightness increases but also the spectrum shifts to shorter wavelengths.

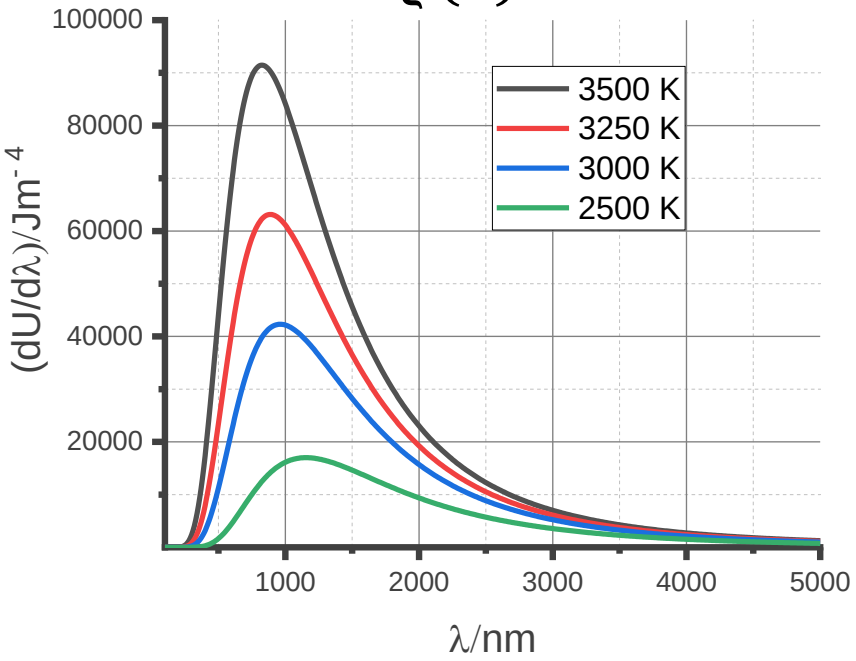
→ In microscopy application, it is better to fix the voltage and adjust the intensity with an absorbing filter.

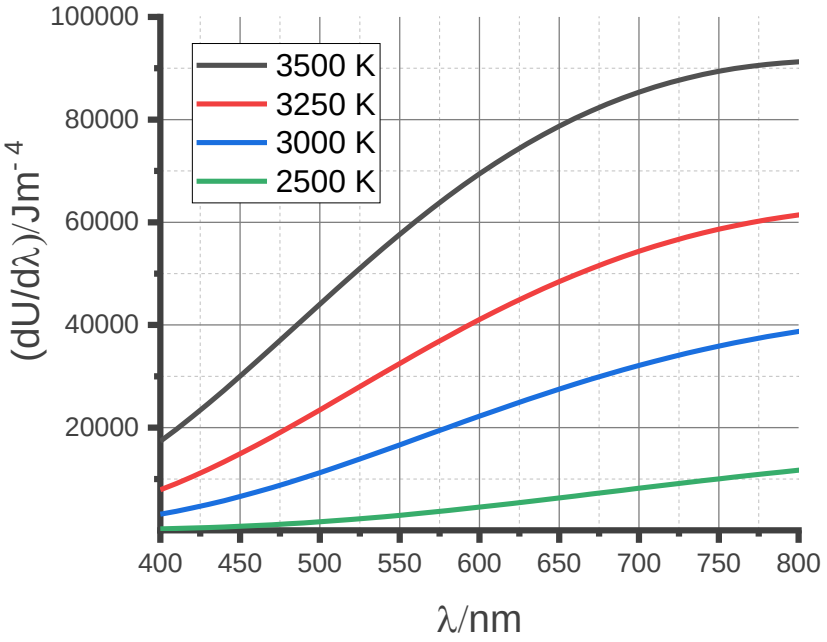


Spectrum of blackbody radiation

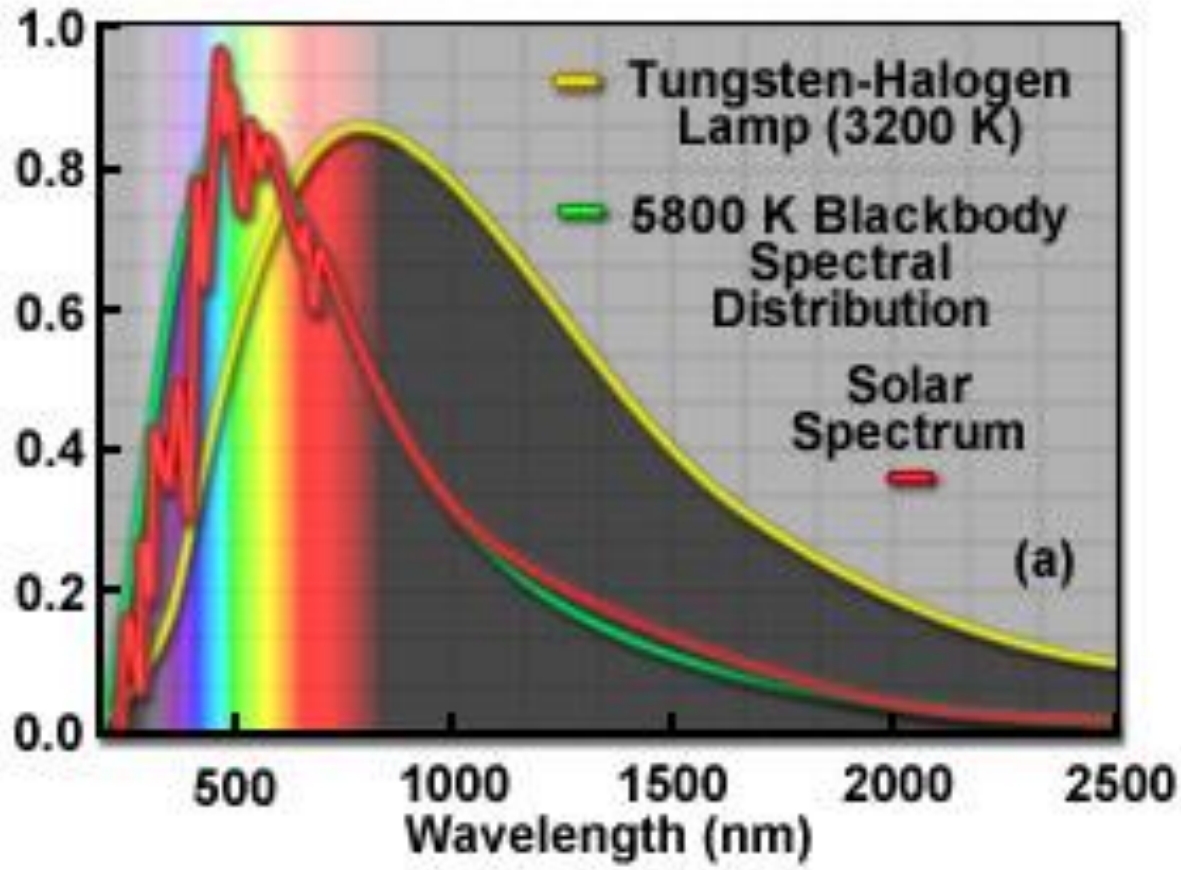
$$\varrho(\lambda) = \frac{8\,\pi\,h\,c}{\lambda^5}\left\{\frac{e^{-\frac{hc}{\lambda kT}}}{1-e^{-\frac{hc}{\lambda kT}}}\right\}$$

$$d\mathcal{U} = \varrho(\lambda)d\lambda$$





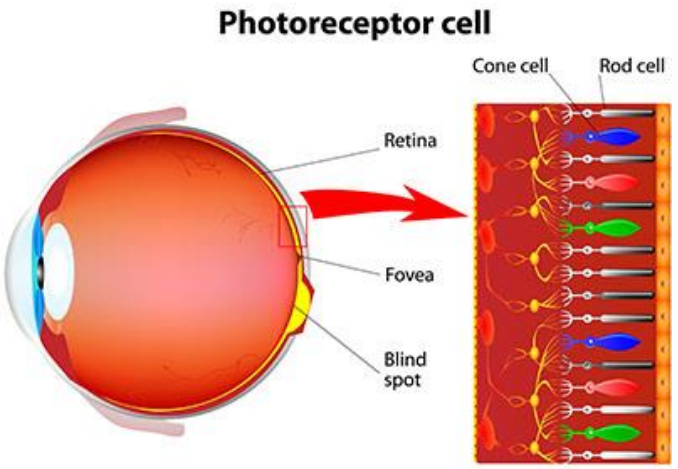
Blackbody radiation: Incandescent lamp & sun

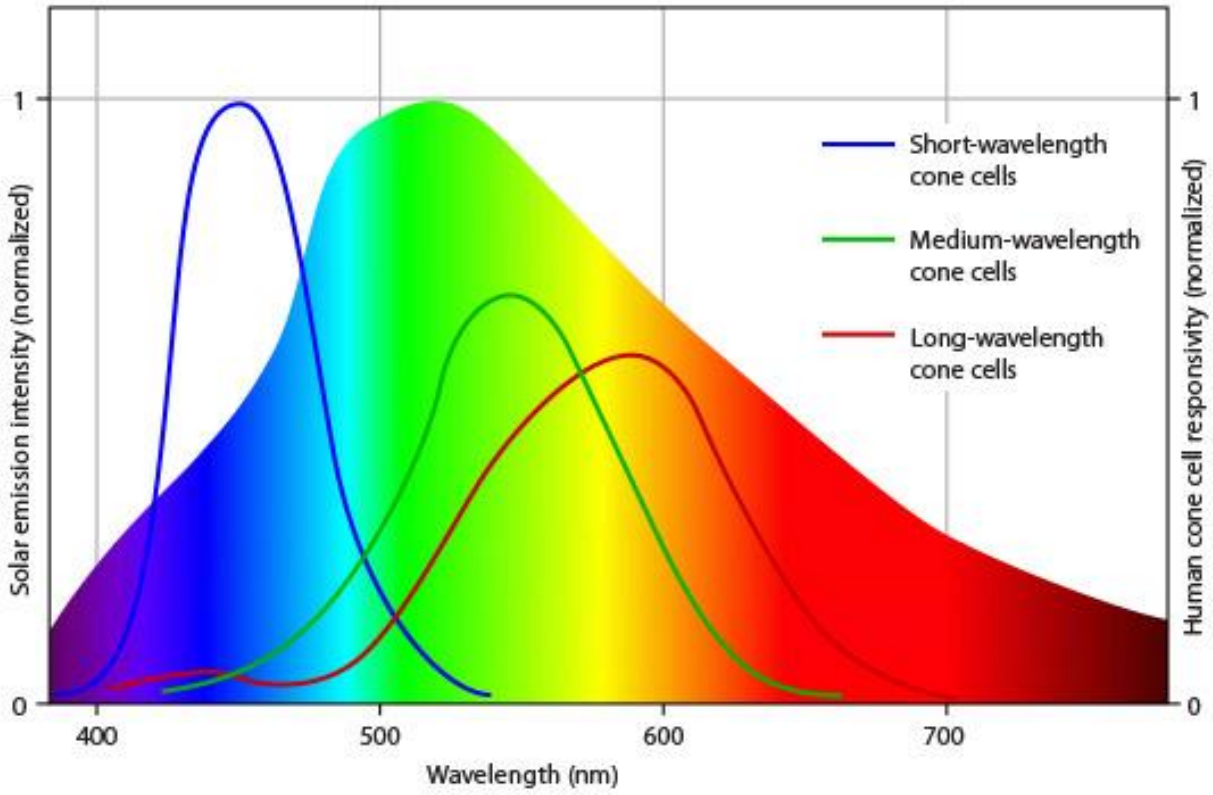


Example:

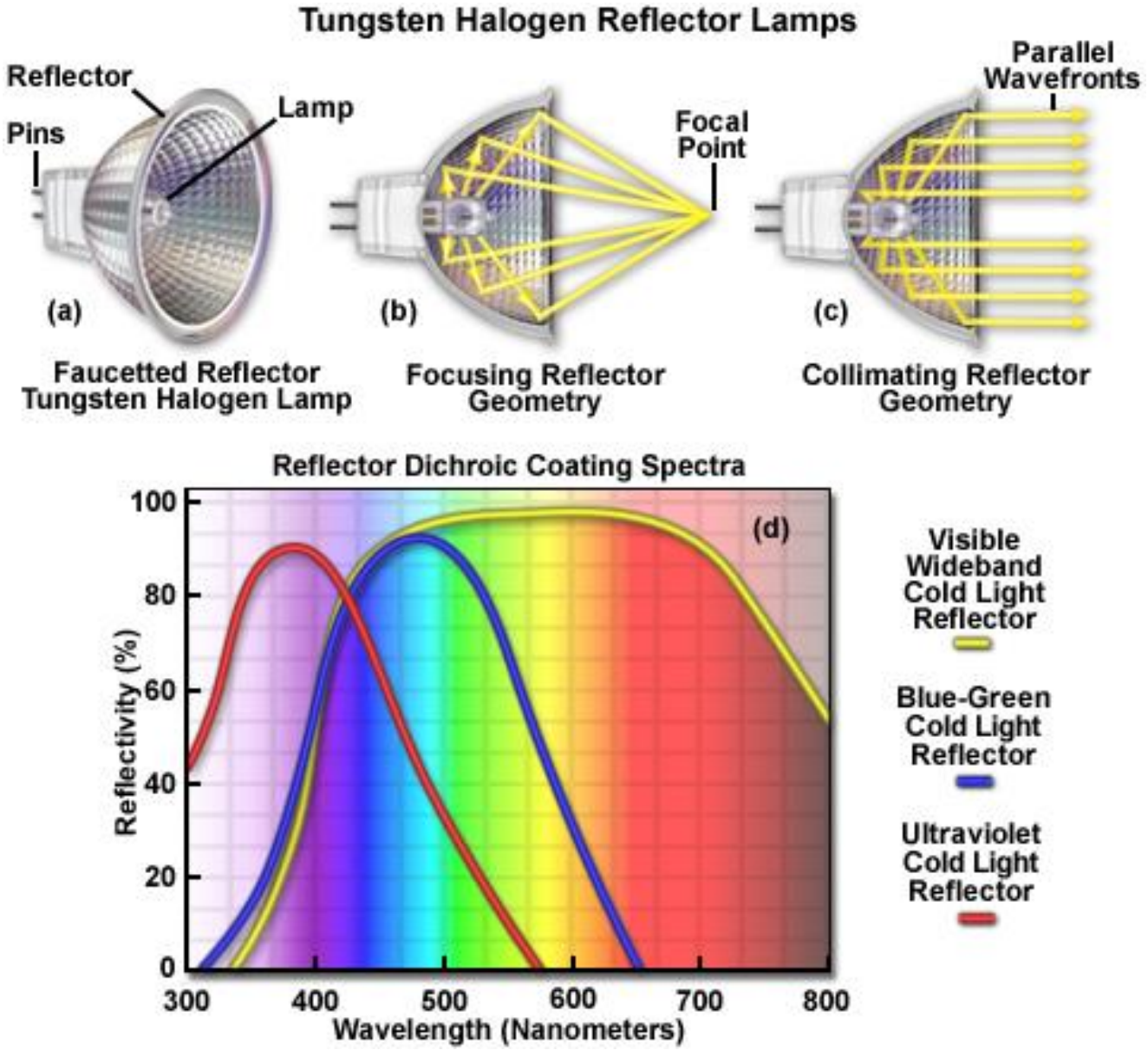
Sun, with an effective temperature of **~5800 K**, is an approximately black body with an emission spectrum peaked in the central, yellow-green part of the visible spectrum, but with significant power in the ultraviolet as well.

Adaptation of human eye to the sun

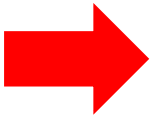




Reflector can be used to control the light from an incandescent lamp



Illumination in Microscopy



Commonly used illuminators:

- Incandescent lamps
  - Quartz tungsten-halogen
- Ion arc lamps
  - Mercury, Xenon
- Metal halides
- Solid-state sources:
  - LEDs & Lasers

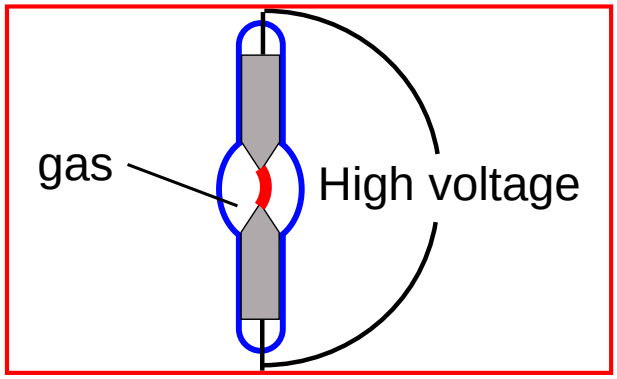
Ion Arc Lamps

Operation:

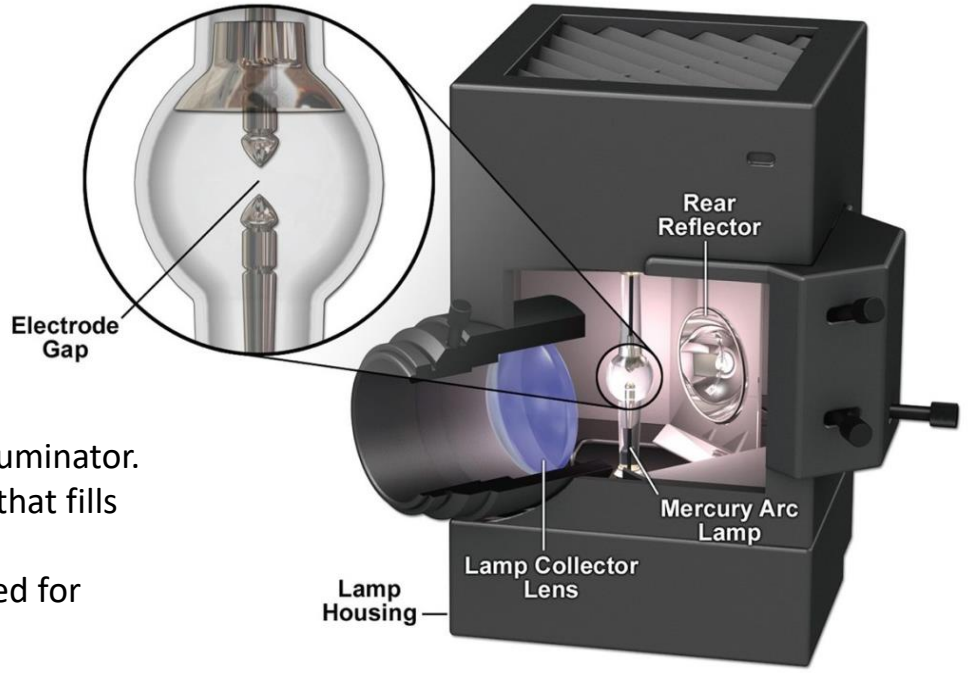
- The gap between the two electrodes contains a bright ionized plasma discharge.
- A high voltage is pulsed across the lamp to ignite the arc at the gap between the two electrodes. After ignitation, discharge can be maintained at a lower voltage.
- Arc lamp produces light by the electric arc, which is a plasma discharge that occurs when a gas is ionized.

Features:

- 10-100X brighter than incandescent lamps
- provide brilliant monochromatic illumination when combined with an filter
- But, they are expensive & have shorter lifetime



Commonly used ones are mercury arc lamp & xenon arc lamp:



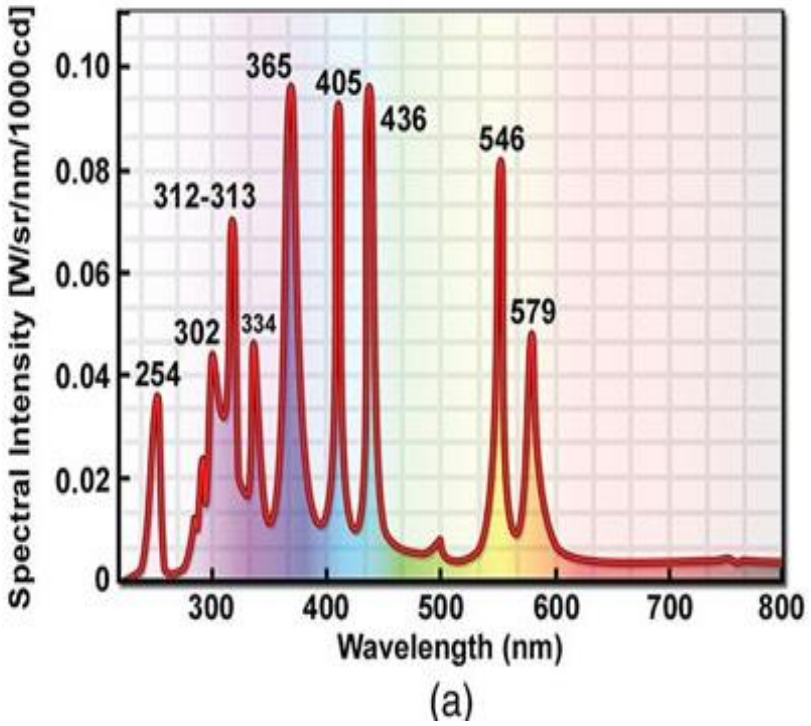
- A rear reflector to increase the output efficiency of the illuminator.
- The collector lens produces a magnified image of the arc that fills the rear aperture of the objective.
- Uniform coverage of the objective rear aperture is required for optimal resolution and illumination of the specimen.

Commonly used Ion Arc Lamps: Mercury & Xenon

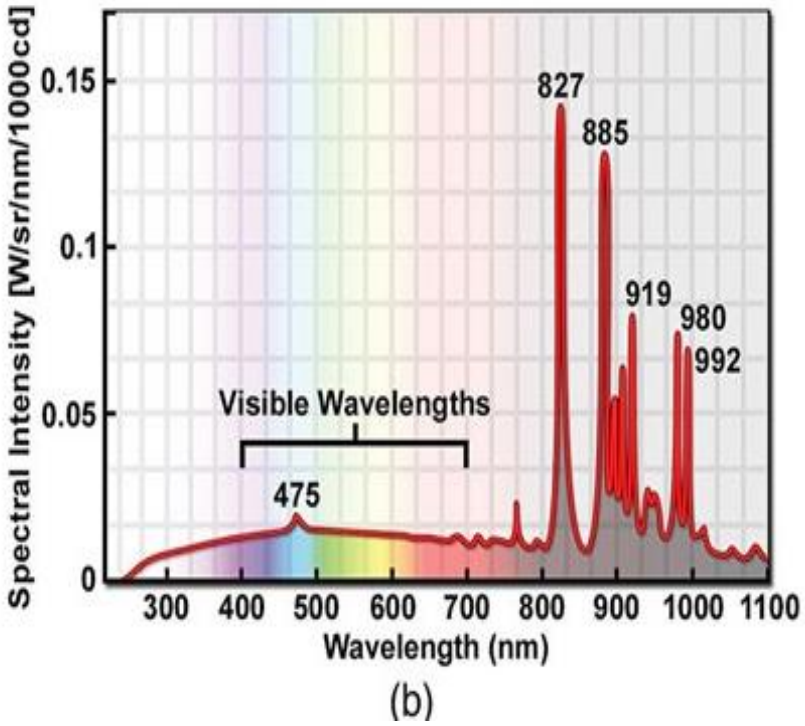
- Both produces “continuous spectra” across the entire visible range, extending into the UV and IR.
- Only ~1/5 of the output spectrum is in visible, remainder is in the UV & IR.

→ this demands to use special blocking filters when examining living cells, which are sensitive to UV & IR radiation

mercury arc lamp

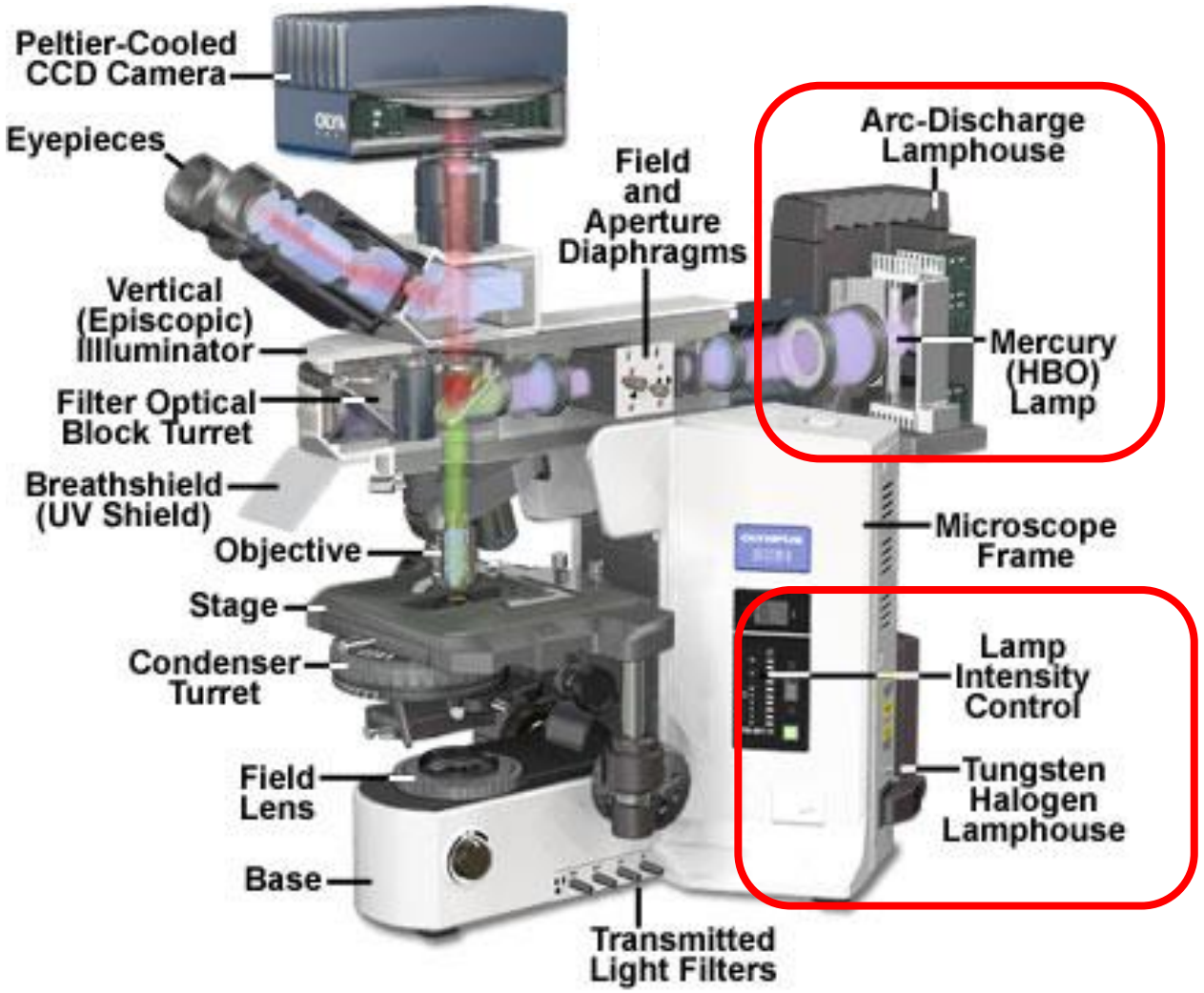


Xe arc lamp



Ion Arc Lamps in Microscopy

Anatomy of an <u>upright</u> microscope that can operate both in transmission & reflection mode
→ It is suitable for both bright-field & fluorescence microscopy



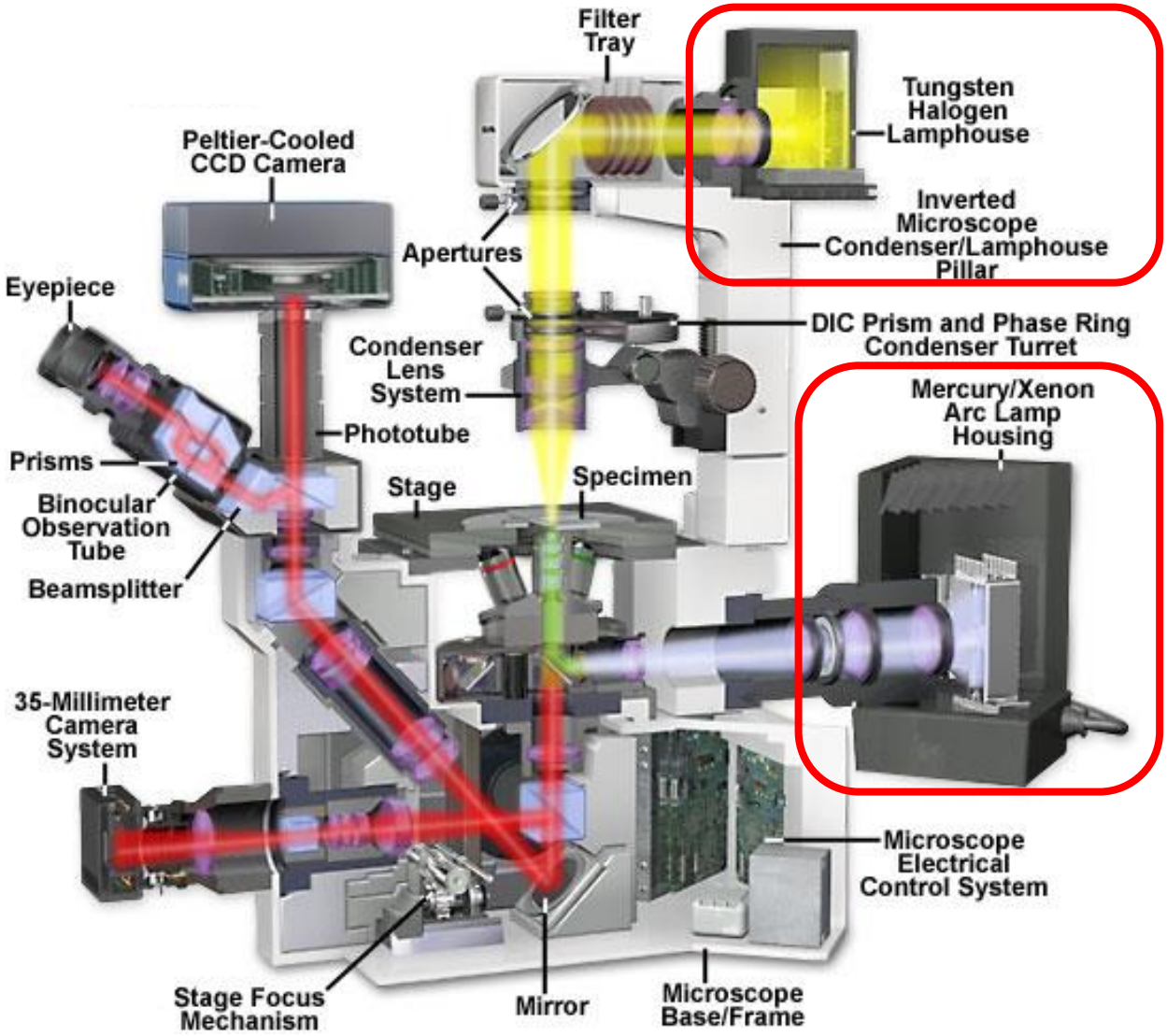
Reflection arm:
with Ion lamp

Transmission arm:
With basic lamp

Ion Arc Lamps in Microscopy

Anatomy of an <u>inverted</u> microscope that can operate both in transmission & reflection mode

→ It is suitable for both bright-field & fluorescence microscopy



Transmission arm:
With basic lamp

Reflection arm:
With Ion arc lamp

Commonly used light sources in microscopy

- Incandescent lamps
  Quartz tungsten-halogen
- Ion arc lamps
  Mercury, Xenon
- Metal halides
- Solid-state sources
  LEDs, lasers

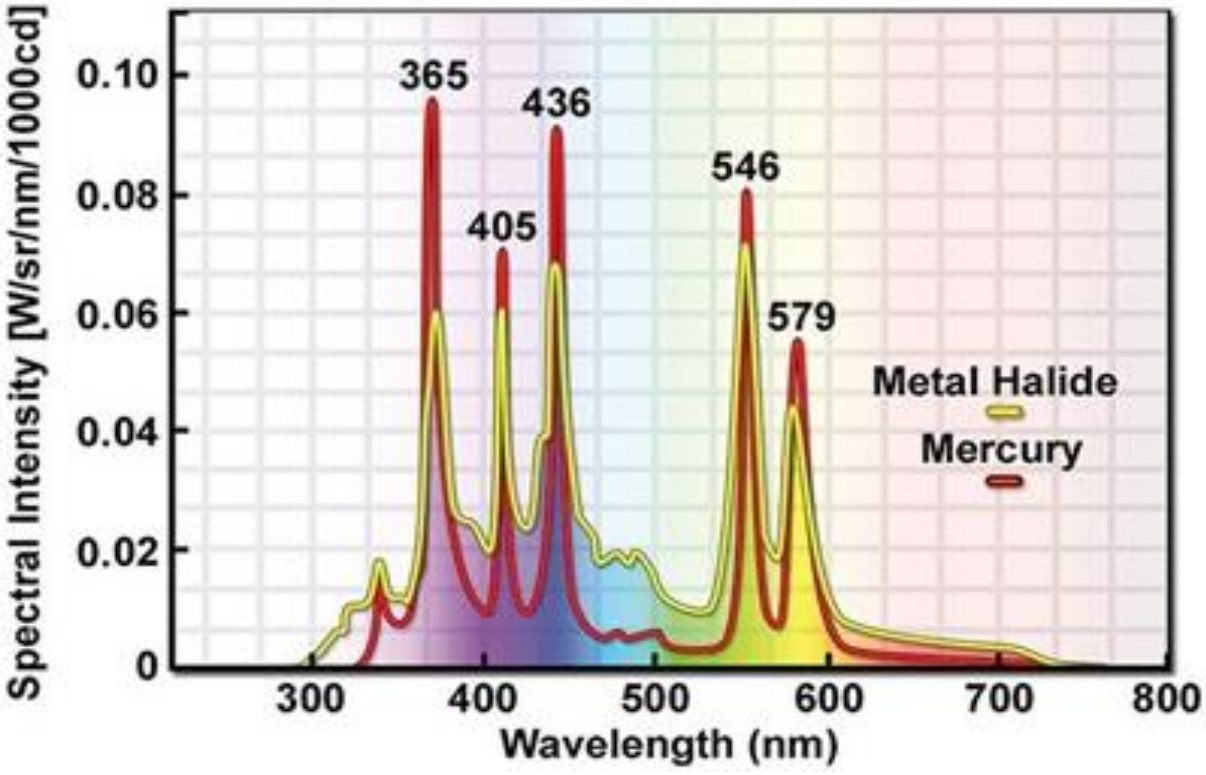
Metal Halide Lamps

Operation:

- A metal-halide lamp produces light by ionizing a mixture of gases in an electric arc.
- The arc tube contains a mixture of mercury, argon or xenon and a variety of metal halides, such as sodium iodide.

Features:

- They produce a spectrum with emission lines very similar to mercury.
- They are **bright,** have **long life (2000 hours)**, and give **homogenous illumination**
- They are more suitable than mercury arc lamp for exciting dyes such as fluorescein and GFP
- But, the bulb & power supply come at **high cost**.



Metal Halide Lamps

- Unlike ion arc lamps, they are **housed in an external unit** that is coupled to microscope using a liquid light guide and a collimating lens that spreads the illumination to fill the objective back aperture.