MICRO-561

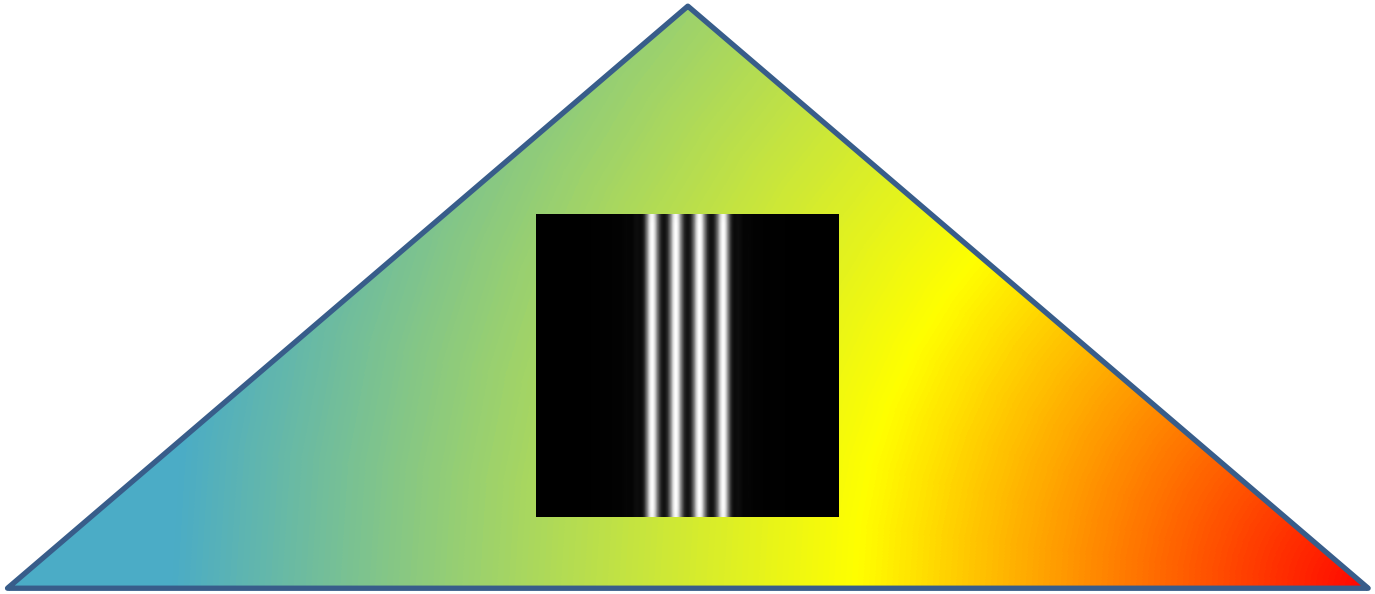
Fundamentals of Biomicroscopy

Syllabus (tentative)

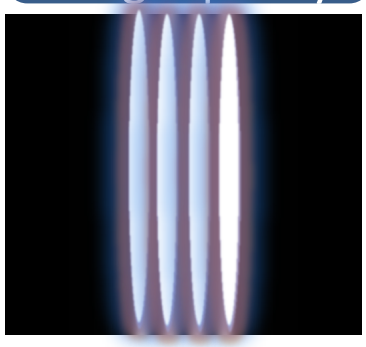
Lecture 1	Introduction & Ray Optics-1
Lecture 2	Ray Optics-2 & Matrix Optics-1
Lecture 3	Matrix Optics-2
Lecture 4	Matrix Optics-3 & Microscopy Design-1
Lecture 5	Microscopy Design-2
Lecture 6	Microscopy Design-3 & Resolution -1
Lecture 7	Resolution-2
Lecture 8	Resolution-3
Lecture 9	Contrast
Lecture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

Important aspects in microscopy

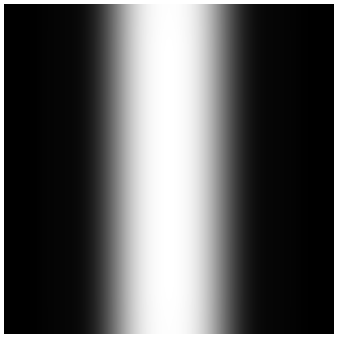
Magnification



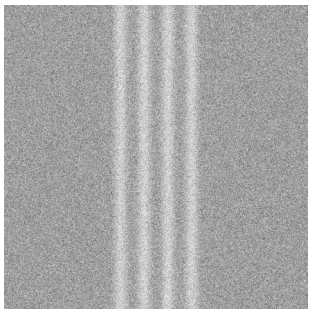
Aberrations – image quality



Resolution



Contrast



Important aspects in microscopy:

- Magnification
- Image quality – aberrations, alignment, illumination condition etc
- Resolution
- **Contrast**

- **Contrast** is necessary to detect/differentiate details from *background*.
- Contrast can be achieved when the captured light from an object has different in **intensity** or color (= wavelength) from the background light.

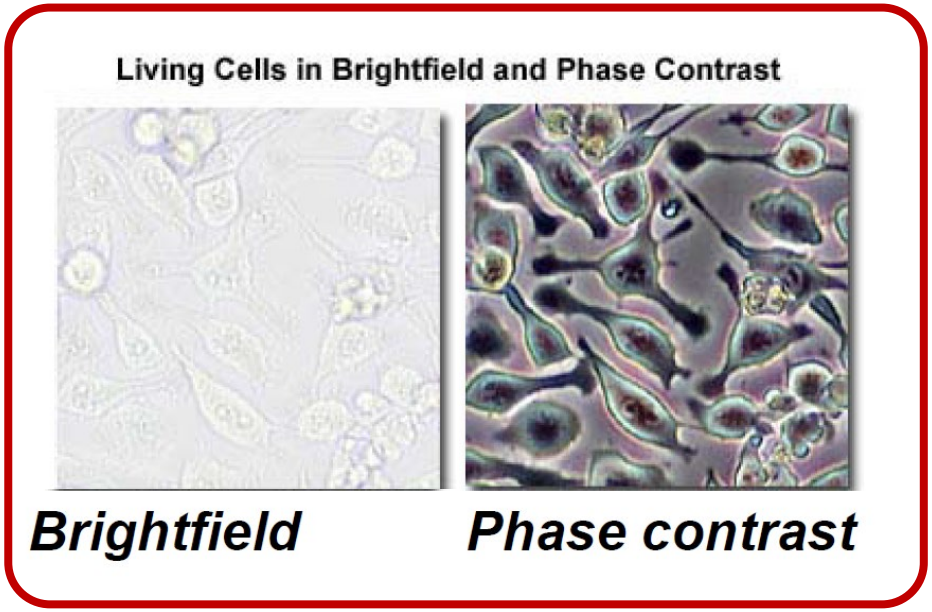
Contrast in Bright-Field Microscope

Why is the contrast low?

Most of the biological samples (e.g. cell, tissue etc..) are optically thin and transparent:

➔ They do not absorb, scatter etc.. ➔ **we get low contrast w.r.t. background**

➔ They are hard to see!!



Contrast depends on the difference between the sample brightness and background brightness

Background signal and contrast

$$\frac{Brightness\ of\ Specimen}{Brightness\ of\ Specimen + Brightness\ of\ Background} \times 100\%$$

Background: 100 Units
Specimen: 50 Units

50/(100+50) X 100% = 33%

Background: 50 Units
Specimen: 50 Units

50/(50+50) X 100% = 50%

Background: 0 Units
Specimen: 50 Units

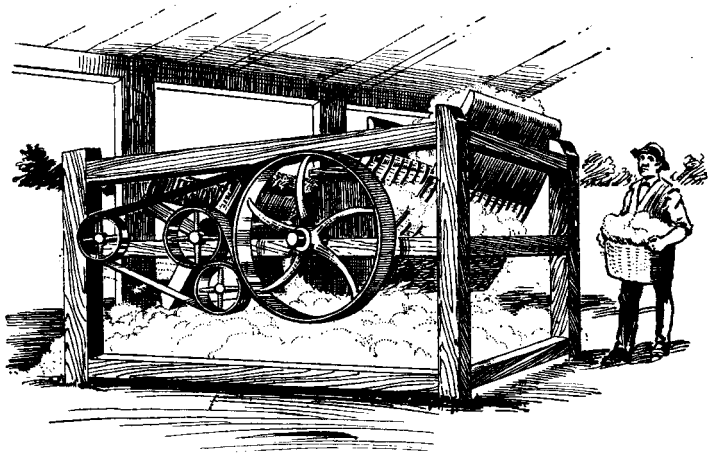
50/(50+0) x 100% = 100%

Contrast in microscopy

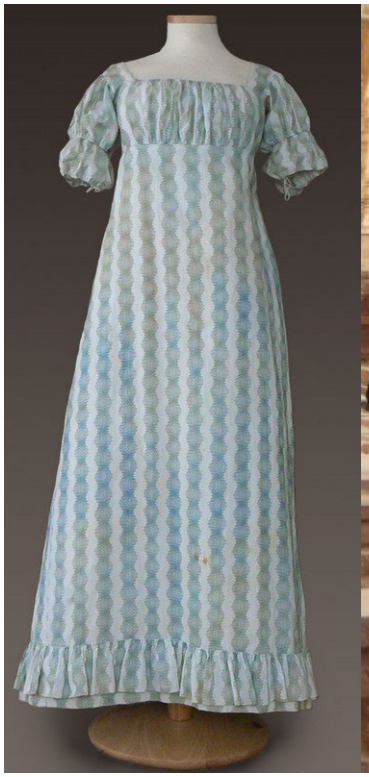
- For transparent specimens, contrast can be improved using various microscopy methods:
  - Bright field microscope (suffers from low contrast)
  - **Stained Specimens (improves contrast)**

Before oil, cotton was the world's one of the most traded (and oldest) commodities

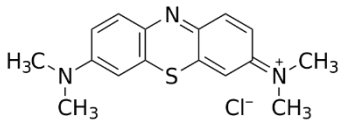
- Cotton



- Clothing & textile



Textile industry drove the development of another industry: **dyes & chemicals**

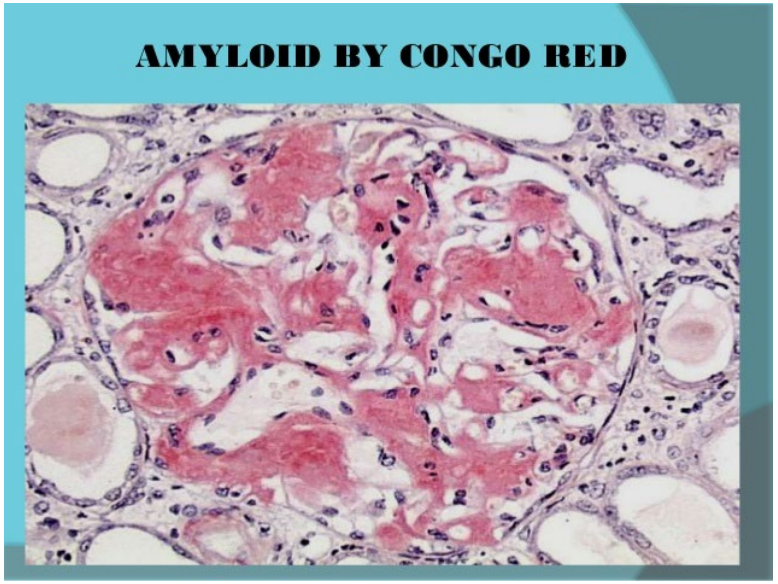


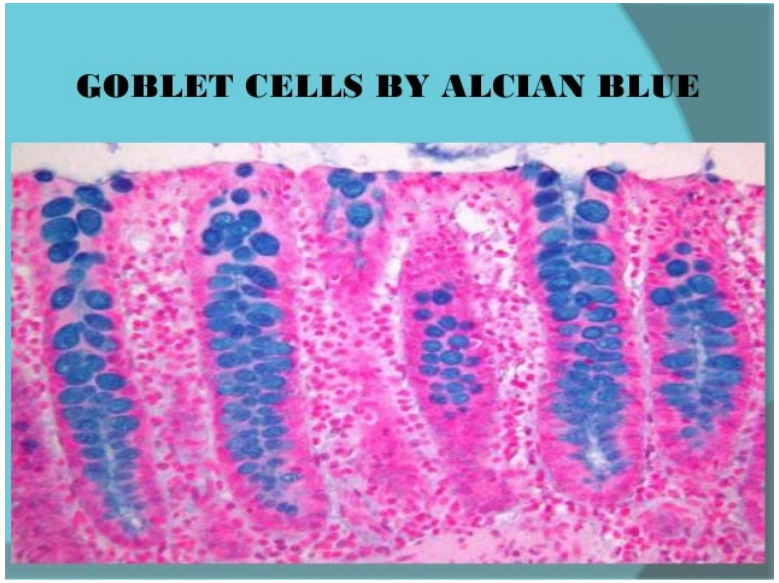
Germany dominated the **chemical/dye industry**

- By the end of the 19th century (late 1800s)

- Historical collection of > 10,000 dyes at Technical University Dresden, Germany.

Side benefits of dyes in microscopy → staining

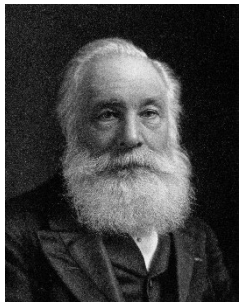




Congo red, which was a cotton dye, used for staining axons & amyloids

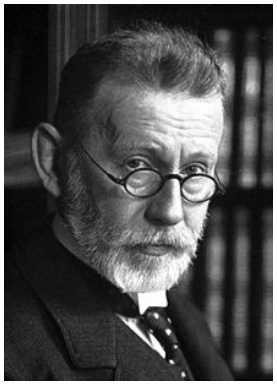
Staining methods for histology were revolutionary in medicine

- Staining provides contrast with high resolution
- While many dyes were initially from natural materials (i.e. haematoxylin from tropical logwood), chemical synthesis starting in 19th century was transformative
- Mauveine – a.k.a aniline purple and Perkin's muave: the first synthetic organic chemical dye discovered by Henry Perkin in 1856, at age 18.



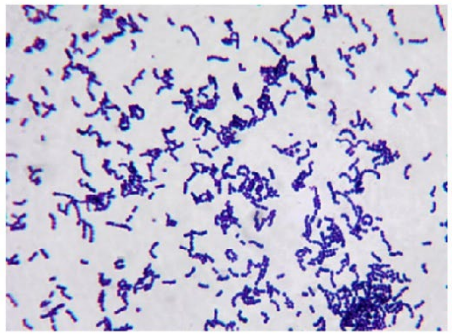
Henry Perkin
1838-1907

- First malaria treatment using synthetic dye methylene blue by Paul Ehrlich.
- Paul Ehrlich won 1908 Nobel prize in medicine for work in immunology

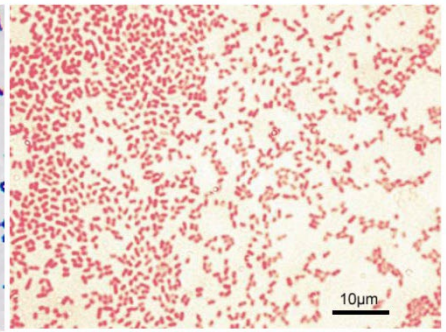


1854-1915

- Invented the precursor technique to Gram staining bacteria.
- His methods for staining the tissue made it possible to distinguish between different types of blood cells, which led to the capability to diagnose numerous blood diseases.



Gram Positive Bacteria



Gram Negative Bacteria

Contrast in microscopy

- For transparent specimens, contrast can be improved using various microscopy methods:
  - Bright field microscope (suffers from low contrast)
  - Staining technique (improves contrast)
  - **Fluorescence microscopy**

  - Dark field microscopy
  - Phase contrast microscope
  - Differential Interference Contrast (DIC) microscopy
  - Polarization microscopy
  - …

- Labelled techniques (uses tags, dyes, stains)

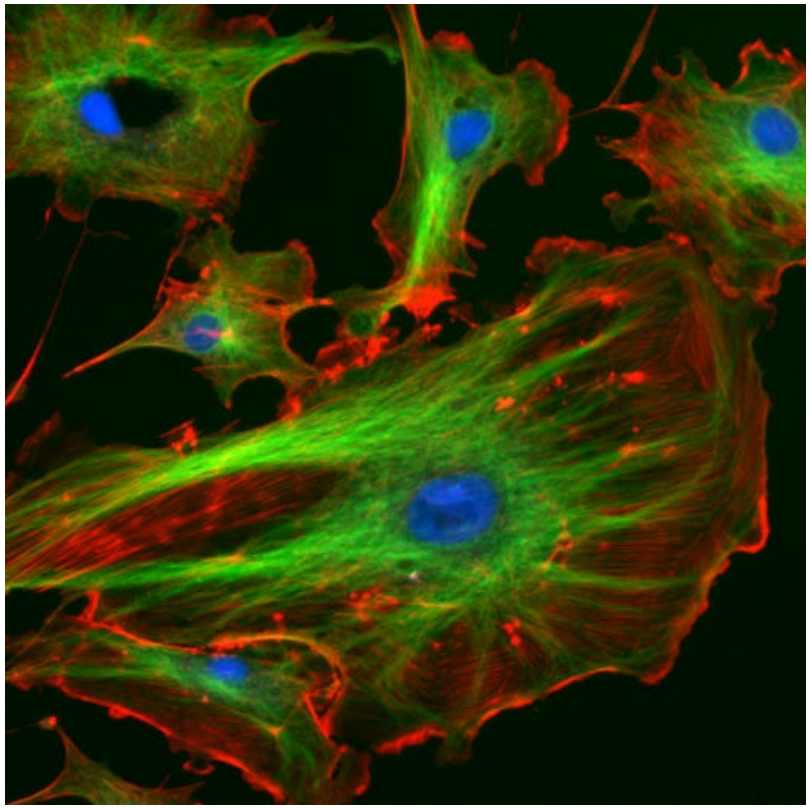
- Label-free techniques
- *covered in* MICRO-562

Fluorescence microscopy: high contrast imaging technique

- **Fluorescence provides:**
  - High contrast
  - High specificity
  - (semi) quantitative monitoring

Implementation
Non-fluorescent molecules & entities are tagged with a fluorescent dye or fluorochrome in order to make them visible.

By fluorescence microscopy:
- Specificity can be achieved at the molecular level.
- The amount, intracellular location, and movement of macromolecules, small metabolites, and ions can be studied.



Bovine pulmonary artery **endothelial cells** under fluorescence microscopy (Wikipedia) :
- Nuclei are stained blue with DAPI
- Microtubules are labelled green by an antibody bound to FITC
- Actin filaments are labeled red with phalloidin bound to TRITC

A variety of fluorescence microscopy technique exists

- **Conventional fluorescence microscopy**
- FRET (Förster resonance energy transfer)
- TIRFM (Total internal reflection fluorescence microscopy)
- FRAP (Fluorescence recovery after photo bleaching)

covered in MICRO-562

- FLIM (Fluorescence lifetime imaging microscopy)
- FLIP (Fluorescence loss in photobleaching)
- FLAP (Fluorescence localization after photobleaching)
- FISH (Fluorescence in situ hybridization)
- FCS (Fluorescence correlation spectroscopy)
- Confocal
- Two photon & multi-photon microscopy
- Super resolution fluorescence microscopy

covered in MICRO-562

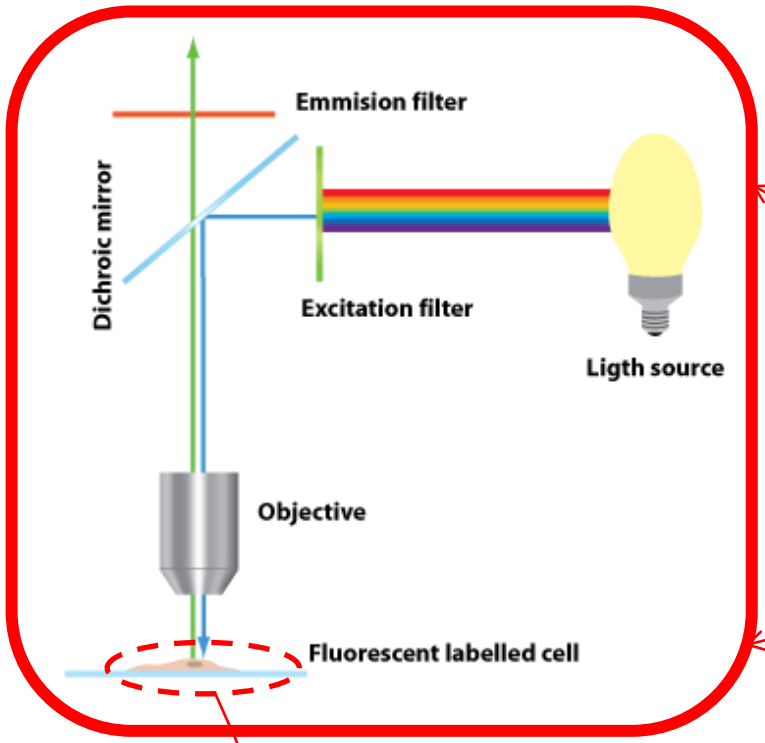
…

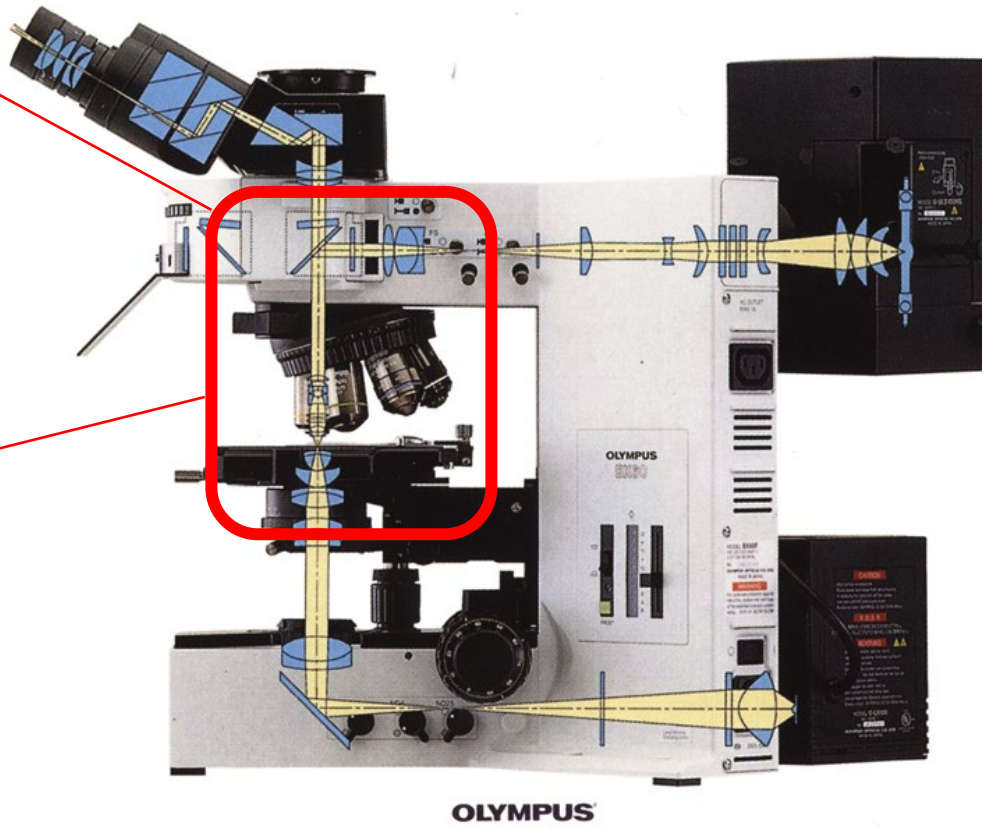
- A strong feature of fluorescence microscopy is that the signals making up an image are “molecule-specific”.
- With the addition of time-lapse methods, it is possible to track time-dependent changes of molecules & dynamic molecular events.

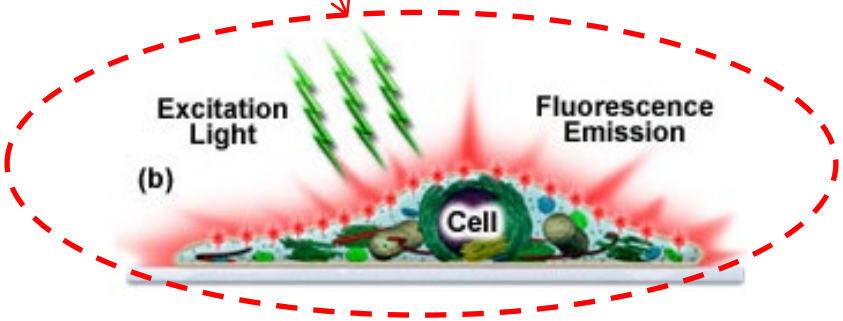
Fluorescence microscopy

- **In order to understand basic fluorescence microscopy, we will discuss:**
  - **Principles of fluorescence emission & excitation**
  - Properties of fluorescent dyes
  - Different types 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 microscopy

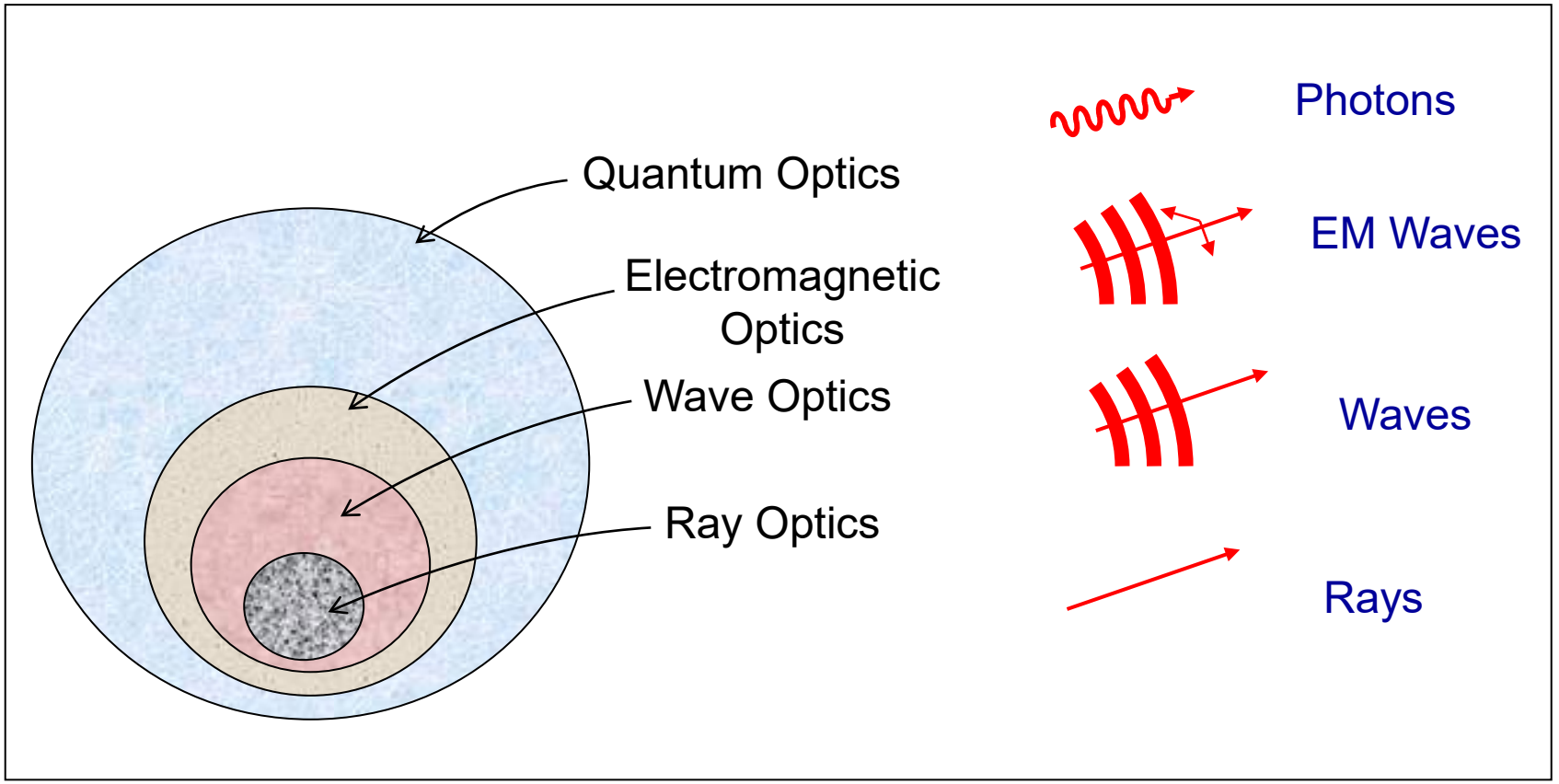






In order to explain fluorescence we have to treat light as "photons"

Hierarchy of Theories in Optics



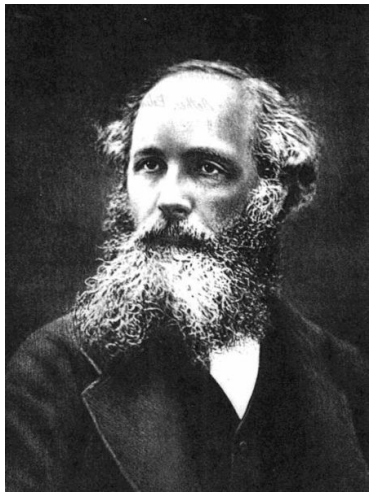
Until about 1900, the wave theory of light described most observed phenomena such as diffraction, interference, etc.

Wave theory & wave phenomena (diffraction, interference …)

- **Thomas Young:** Carried out his original double-slit experiment with light in 1801, showing that the waves of light from the two slits interfere to produce a characteristic fringe (diffraction) pattern on a screen.
- **Wave theory of light** explained well **interference & diffraction phenomena**.

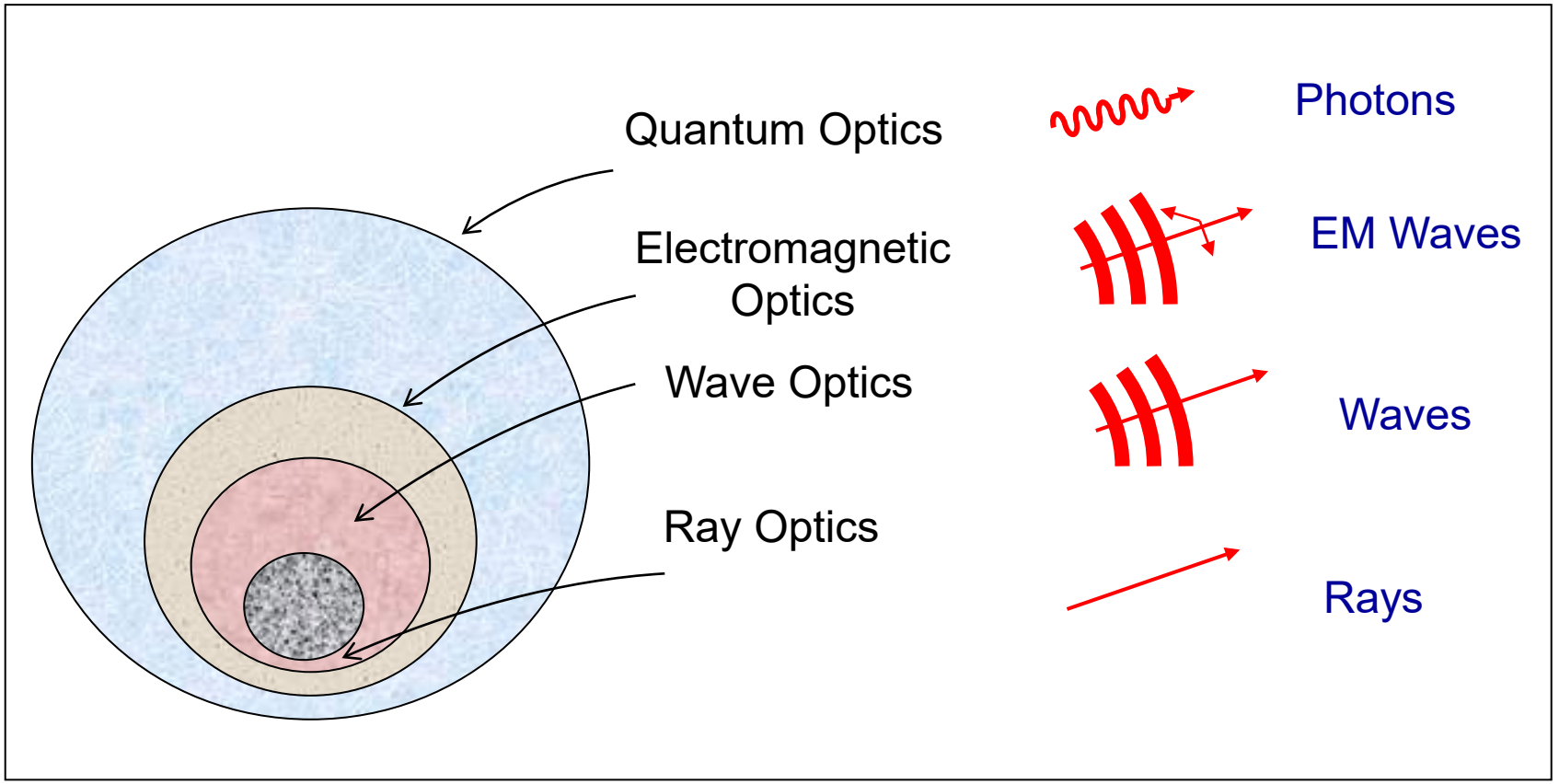
Thomas Young
1773-1829

- **Maxwell's equations:** Unified a set of known experimental laws (Faraday's Law, Ampere's Law) into a set of equations, and formed the foundation of classical electromagnetism (E& M) and classical optics.
- Maxwell was one of the first to determine the speed of propagation of **electromagnetic waves** was the same as the speed of light - hence E&M waves and visible light are the same thing.



James Clerk Maxwell
1831-1879

Hierarchy of Theories in Optics



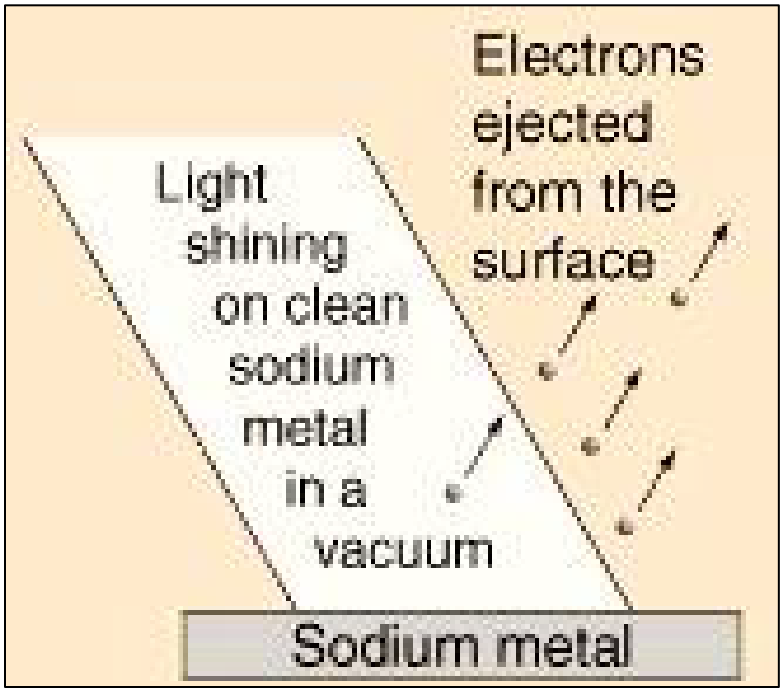
Until about 1900, the wave theory of light described most observed phenomena such as diffraction, interference, etc.

…..but it was not successful in explaining some experimental observations specifically at atomic & molecular level.

Quantum nature of light…

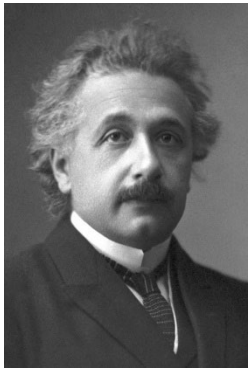
Photoelectric Effect:

- When light is incident on certain metallic surfaces (i.e. sodium), electrons are ejected from the surface.



- The emitted electrons are called *photoelectrons.*
- The effect was first discovered by Hertz.
- The successful explanation of the effect was given by Einstein in 1905. Einstein received Nobel Prize in Physics in 1921 for "his services to Theoretical Physics, and especially for his discovery of the law of the photoelectric effect".

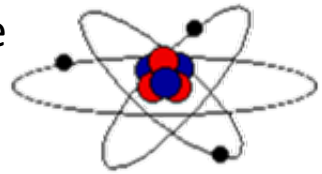
Heinrich Hertz
1857-1894



Albert Einstein
1879-1955

Observation of the Photoelectric Effect

- Electrons are attracted to the nucleus by the electrical force. In metals, the outermost electrons are not tightly bound, and can be easily "liberated".

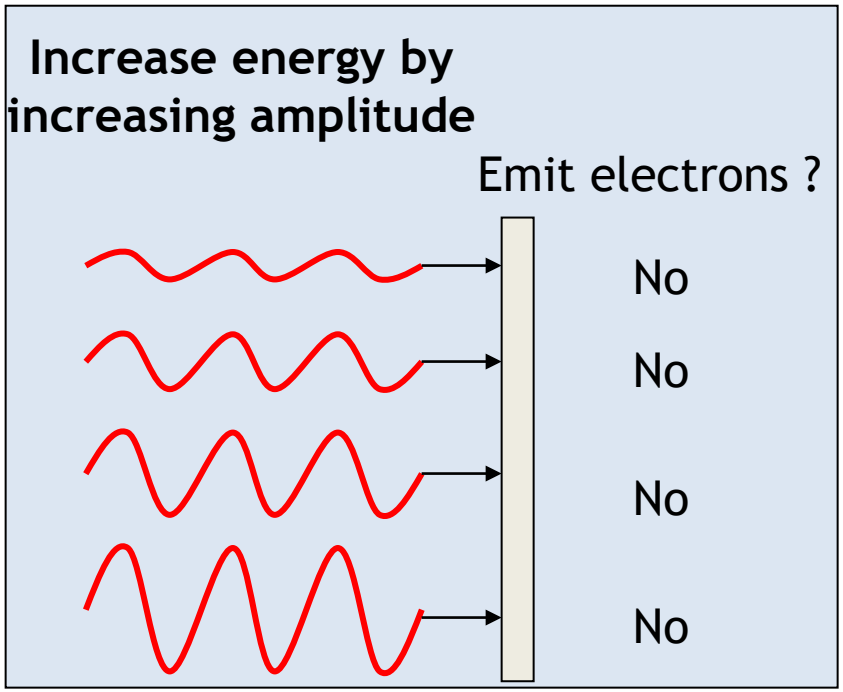


- How can we deliver energy to the metal and "liberate" the electrons (thus generate "photocurrent") with light?

"Classical" **Energy** $\propto |\boldsymbol{E}|^2$

Increase energy by increasing amplitude

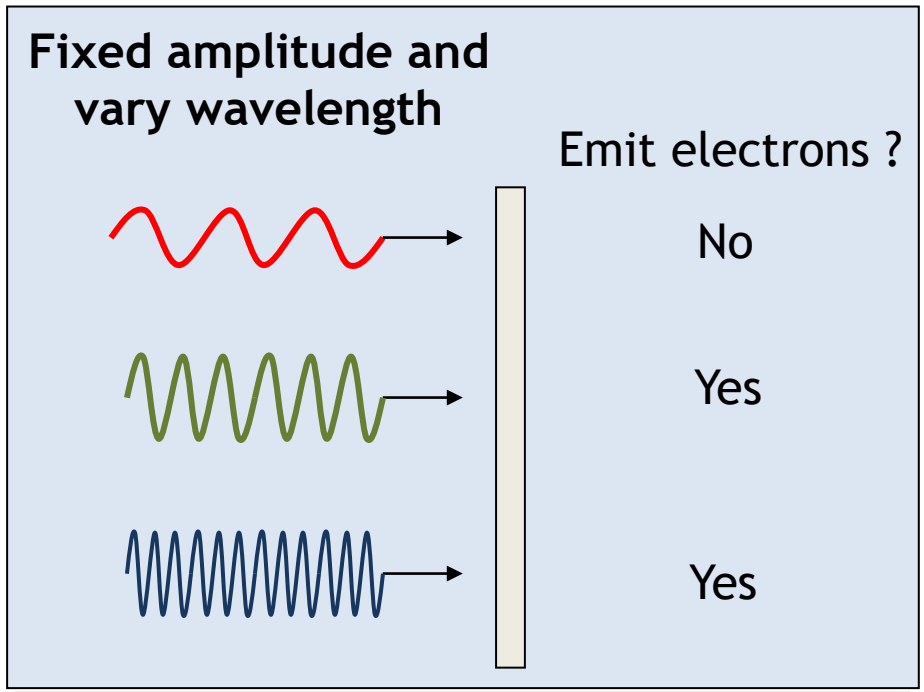
Emit electrons ?



What if ?

Fixed amplitude and vary wavelength

Emit electrons ?



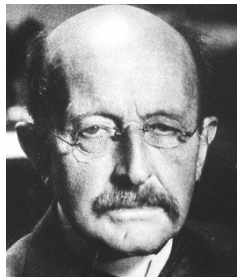
No electrons were emitted until the frequency of the light exceeded a critical frequency!

Quantum Optics

- *Einstein's quantum theory explains this observation:*
  - Light is constituted of **photons**.
  - The photons must have sufficient energy to "free" the electron from the atom in metal.
  - Increasing the field amplitude (i.e. intensity) is simply increasing the number of light particles, but it's NOT increasing the "energy" of each one.
  - The energy of these light quanta is related to their frequency. This is why higher frequency light can knock the electrons out of their metallic atoms, but low frequency light cannot.

Planck–Einstein relation

$$E = h\,\upsilon = h\,c/\lambda = \hbar\omega$$



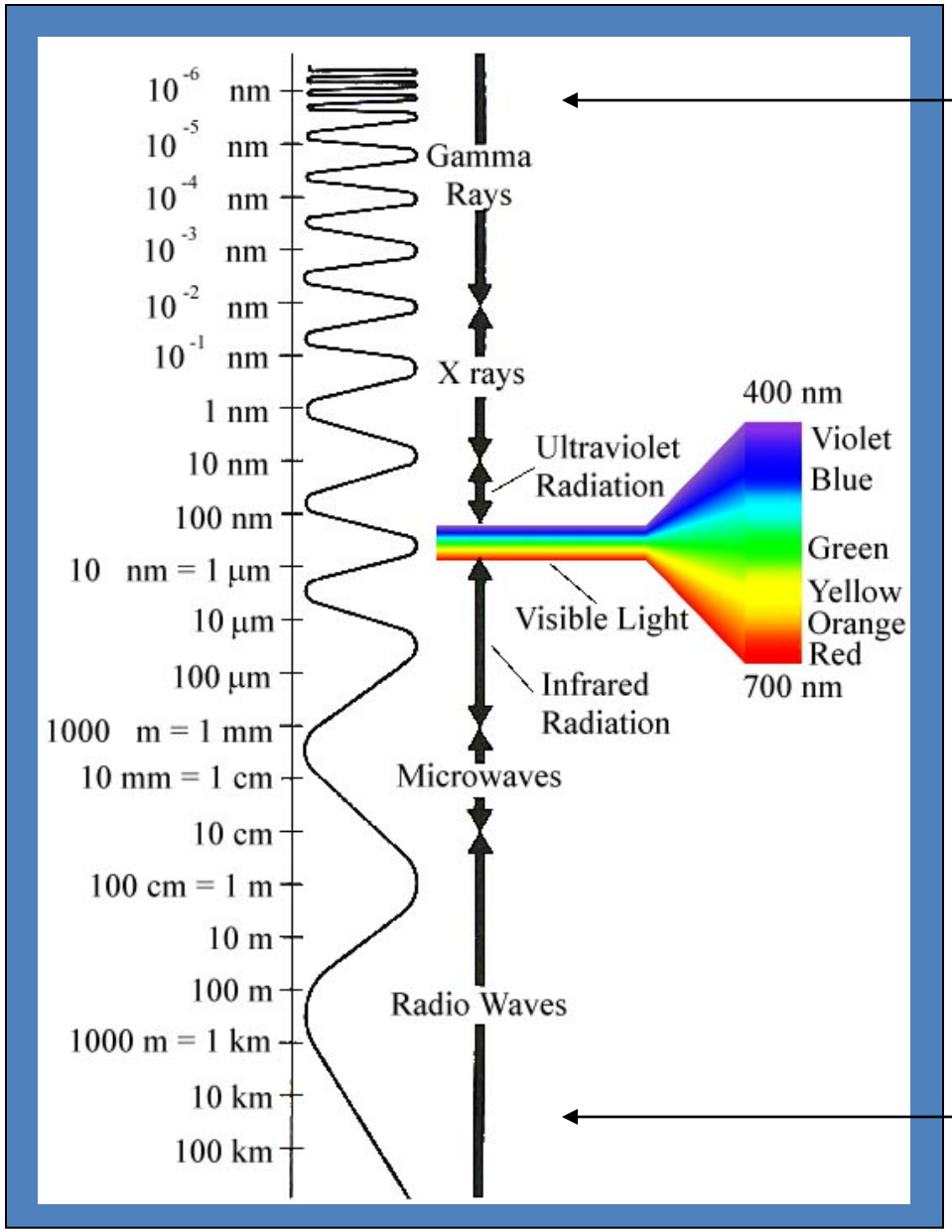
Max Planck
1858-1947

Planck's constant: 6.6×10^{-34} J-s

wavelength

angular frequency

Electromagnetic Spectrum



Shortest wavelengths
(Most energetic photons)

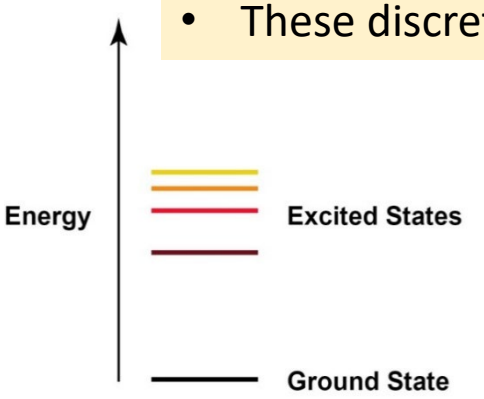
$E = hc/\lambda$

h = 6.6×10^{-34} [J*sec]
(Planck's constant)

Longest wavelengths
(Least energetic photons)

Photon (light) absorption

- A quantum mechanical system can only take on discrete values of energy.
- These discrete values correspond to **energy levels**.



The energy levels are described in **electron volt** (**eV**):

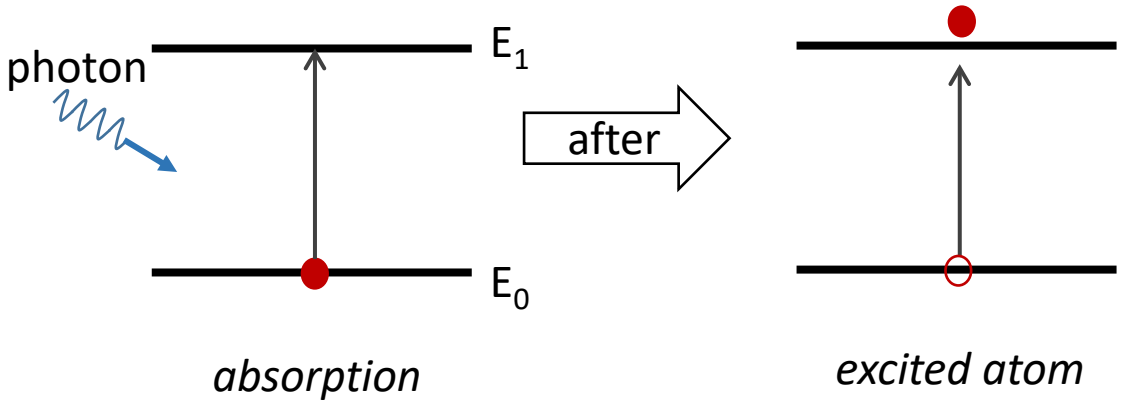
$$\mathbf{E(eV)=}\frac{\mathbf{1239.84}\ (\boldsymbol{eV.nm})}{\boldsymbol{\lambda(nm)}}$$

$1eV=1.602\times10^{-19}$ joule

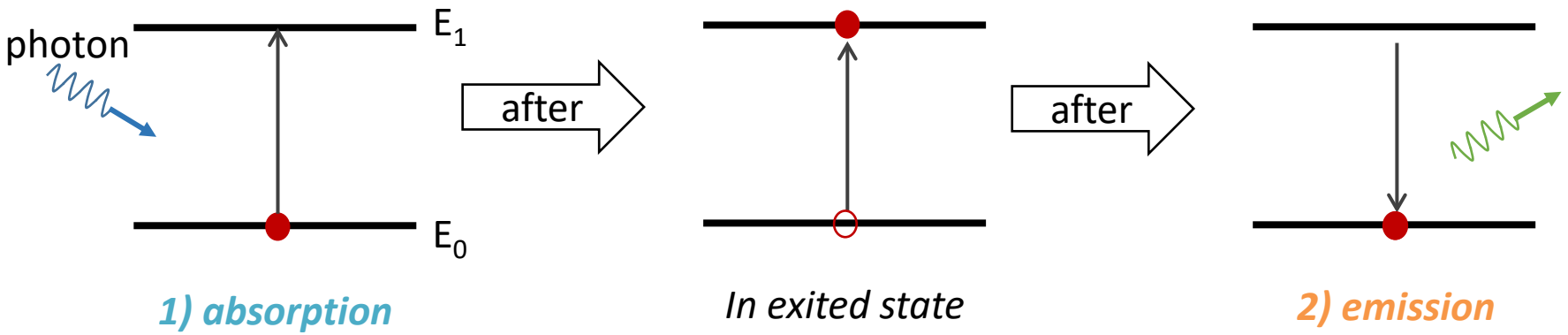
- **After absorbing energy**: An electron can jump from the ground state to a higher energy excited state, hence leaving a hole in the lower energy level.
- Absorbed energy can be delivered optically, electrically, chemically.

In fluorescence microscopy, absorbed energy is provided optically (i.e. by photon absorption)

2-level system



Photon absorption & emission in a 2-level system



1) Absorption: after absorbing energy, an electron jumps from the ground state to a higher energy excited state, hence leaving a hole in the lower energy level.

➔ In fluorescence microscopy absorbed energy is provided optically (i.e. by photon absorption)

2) Emission: If the electron goes back to lower band (or ground state) and combines with the hole, it can radiate the energy by generating a photon.

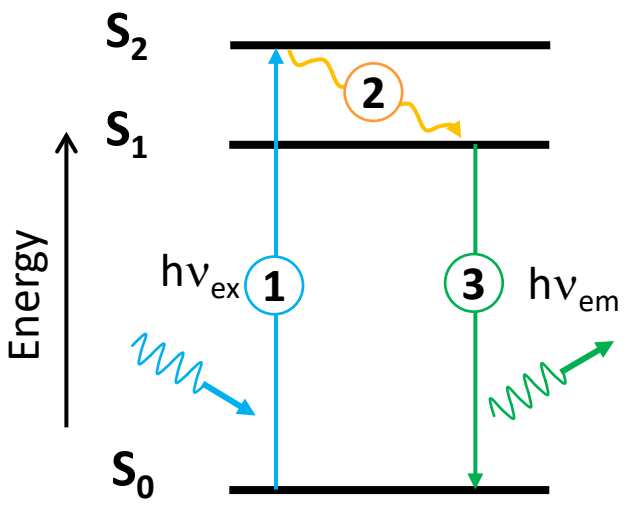
➔ If there is **no loss** in the system, the emitted photon will have an energy of:

$$\Delta E = E_1 - E_0 = h\upsilon = h\,c/\lambda = \hbar\omega$$

Basic Principles of Fluorescence

Fluorescence is a result of a **three-stage process** that occurs in some molecules called **fluorescent molecules (dyes),** which are known as fluorophores or fluorochromes.

1. **Absorption (a.k.a. excitation):** Photon from a source is absorbed by electron in the probe (dye), creating an excited electronic state S_2.
2. **Non-radiative processes:** Rapid decay from excited electronic states of S_2 to emitting energy level S_1 (singlet or triplet excited state)
3. **Fluorescence emission:** A photon with lower energy is emitted while the dye returns to ground state S_0.

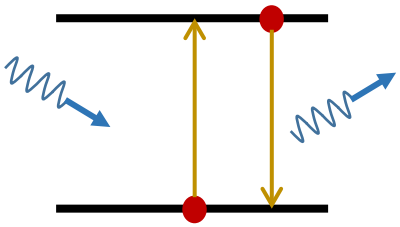


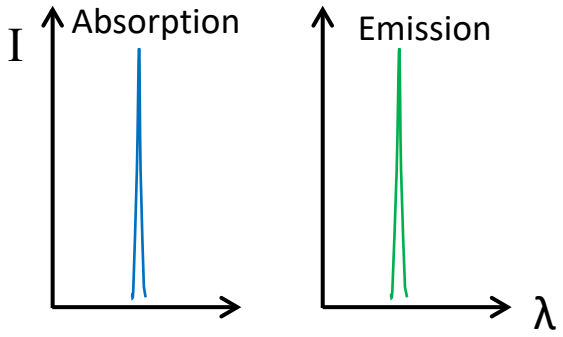
- This scheme simplifies the process.
- In a realistic dye the atomic absorption and emission spectra are broadened into bands.

Photon absorption & emission

- **Atoms**: absorption & emission occurs at specific wavelengths.
- **Molecules:** Electronic and nuclear vibrational motions interact (i.e. couple) with each other
  - ➔ These molecular vibrations **broaden** the spectrum.
  - ➔ Absorption and emission occur over a **spectral band**.

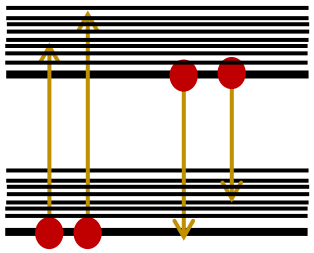
Atoms
(especially at low pressure & gas phase)

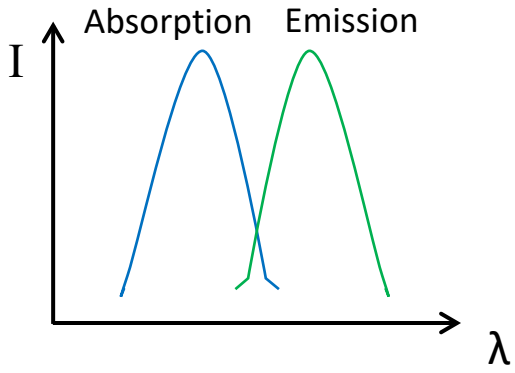




Sharp line spectrum

Molecules

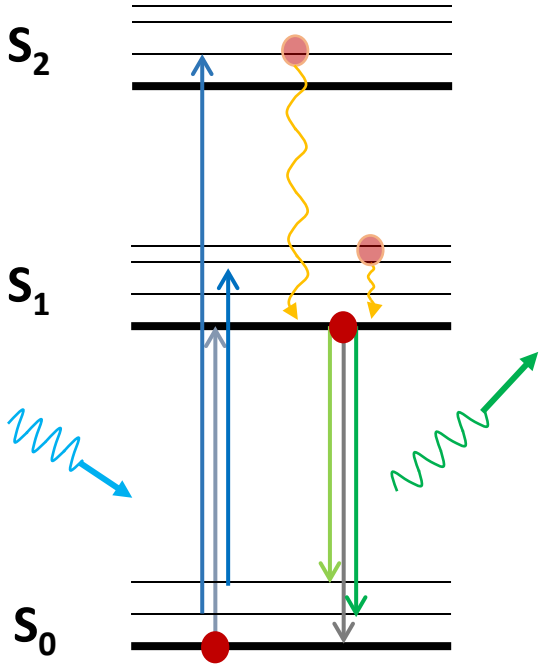




Broadband Spectrum

Processes in Fluorescence

Energy level diagram:



Excited states

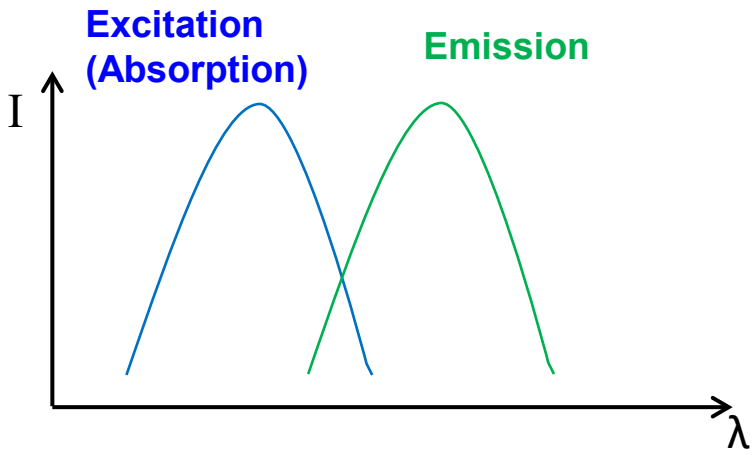
Ground state

Excitation
higher energy

Internal Conversion
Vibrational relaxation
Non-radiative relaxation

Fluorescence Emission
(radiative relaxation/decay)
less energy

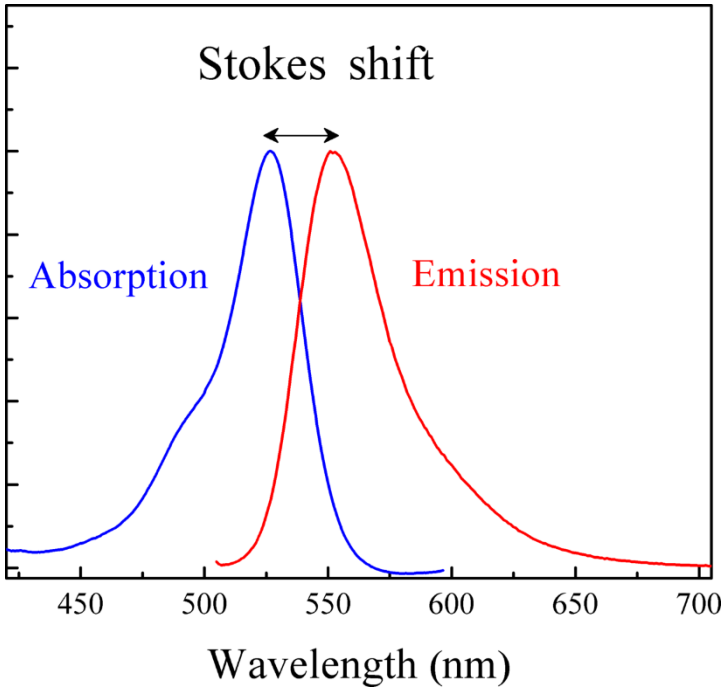
Fluorescence can be excited by a source within the excitation spectra of the dye.



$$E = h\,\upsilon = h\,c/\lambda = \hbar\omega$$

Stokes shift

- There is a difference in energy between the emitted and the absorbed photons.
- The fluorescence light is **red-shifted** with respect to the absorbed light. Therefore, the wavelength of fluorescence light is **longer** than that of the excitation light.
- This shift between the excitation and the emission spectra is called **Stokes Shift**.

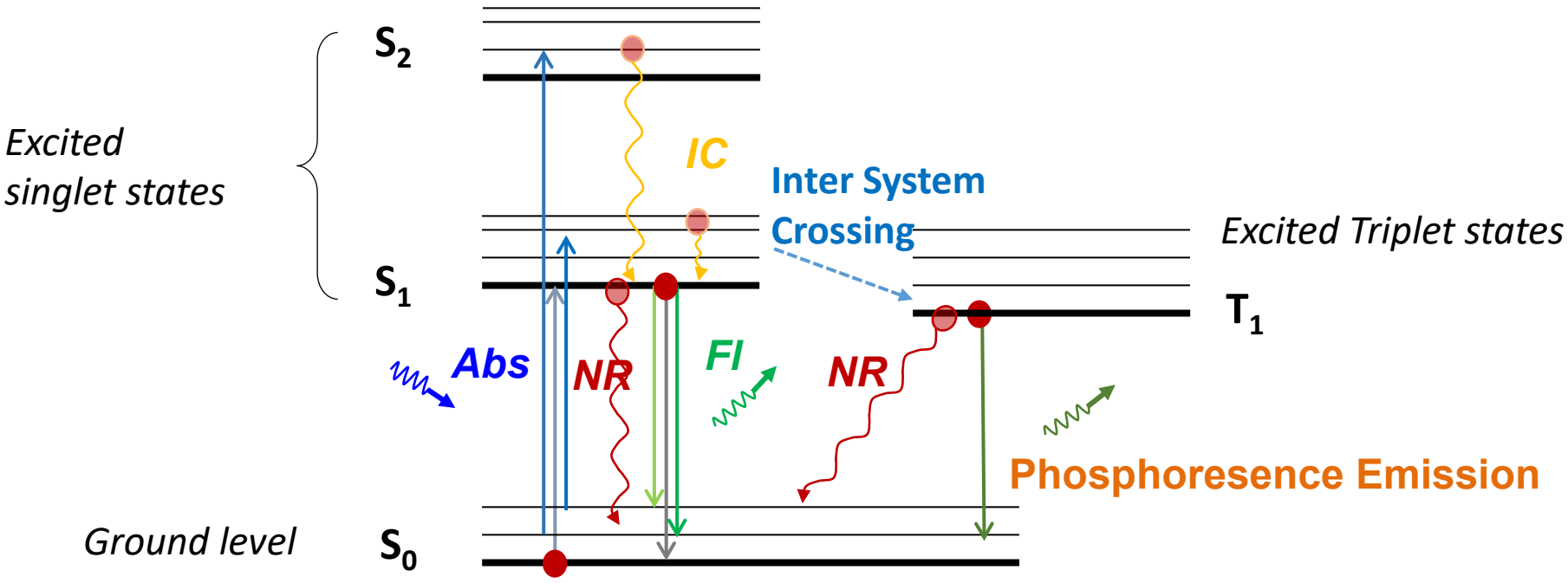


Sir George Stokes
1819-1903

Absorption and emission spectra of Rhodamine 6G has ~25 nm Stokes shift

- A large Stokes shift allows a good wavelength separation of excitation and emission spectra.
- Bigger Stokes shift provides better signal to noise in fluorescence because of filter efficiencies.
- Solvents and excited state reactions can affect the magnitude of the Stokes shift.

Additional processes

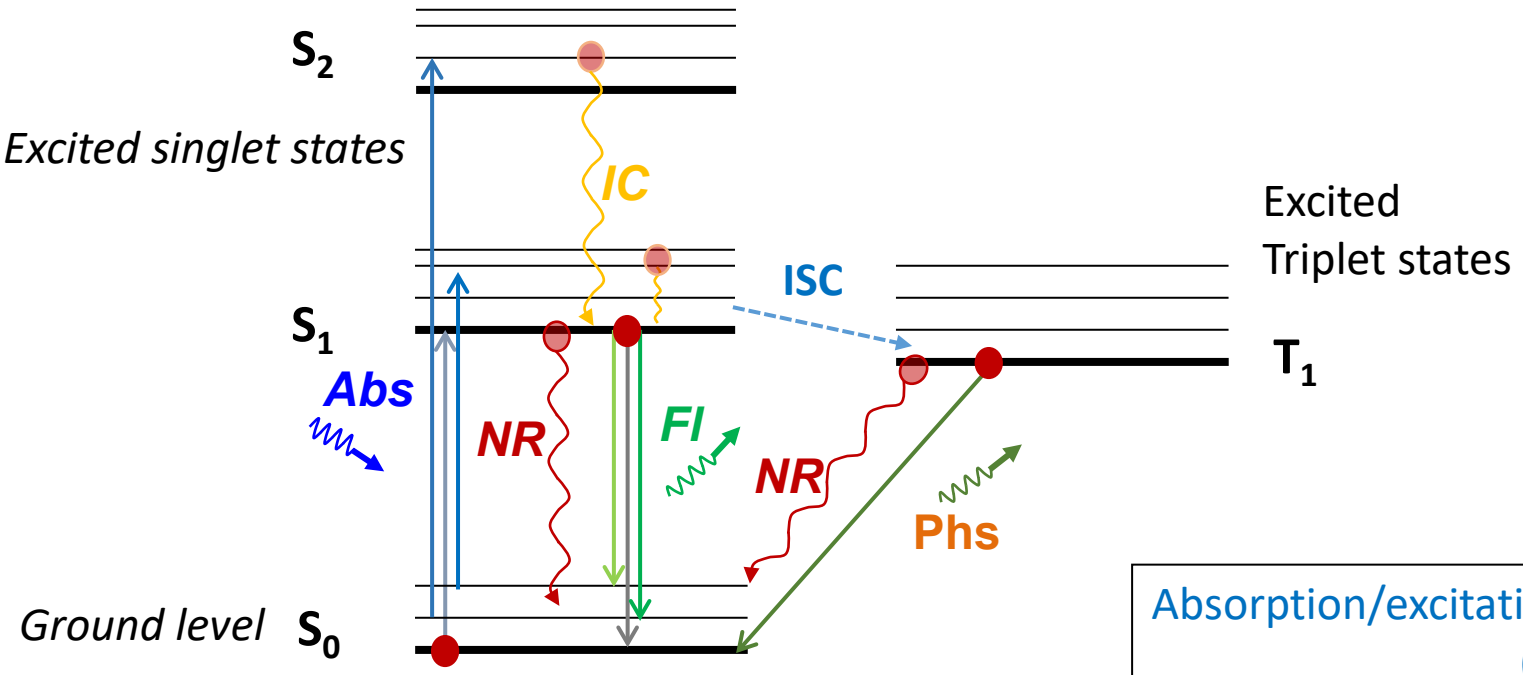


- The excited electron may spontaneously reverse its spin. This process is called **intersystem crossing (IC)**. It results in electron transfer to the triplet states.
- **Phosphoresence:** return from a triplet excited state to a ground state, (electron requires change in spin orientation).
- **Additional non-radiative relaxation:** processes that lead to the energy loss (thus relaxation) without generating radiation (thus creating photon/light).

Jablonski Diagram

- Combination of multiple different processes are presented in Jablonski diagram

Aleksander Jablonski
1898-1980



- *Emission rates of fluorescence are several orders of magnitude faster than that of phosphorescence.*
- *Non-radiative processes may compete with fluorescence if their rate is similar to that of fluorescence.*

Absorption/excitation: **~10^{-15} s** (instantaneous)

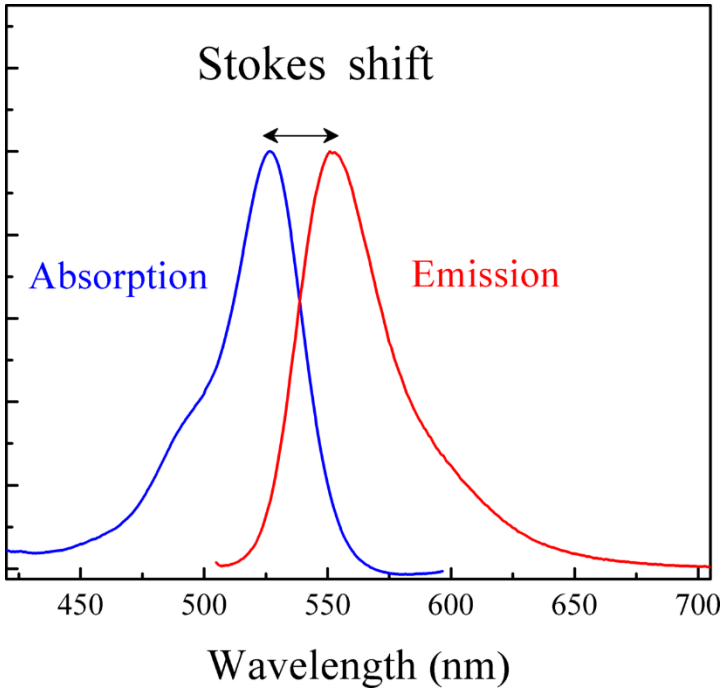
IC & Vibronic Relaxation: ~**10^{-12} s**

Fluorescence: **10^{-9} s most typical**

Phosphorescence: **10^{-3} -10^{-6} s**

Important Fluorescence Terms

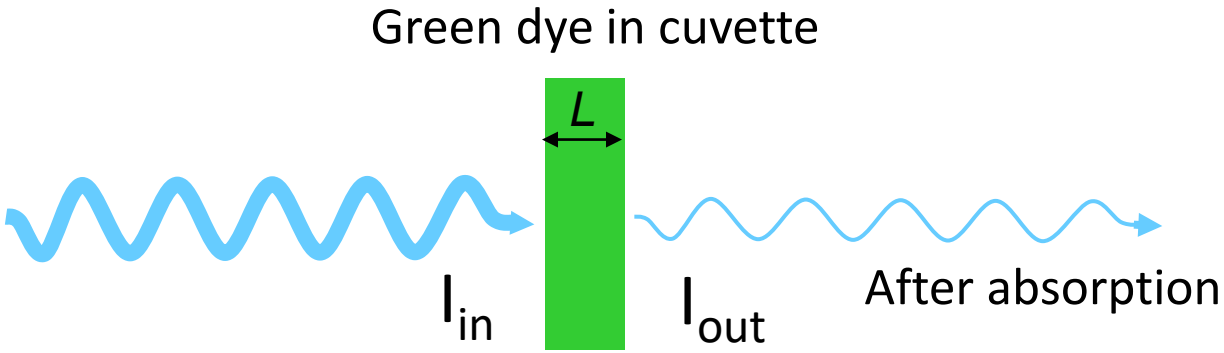
- Excitation (absorption) spectrum
- Excitation (absorption) wavelength
- Emission spectrum
- Emission (fluorescence) wavelength
- Stokes shift



- Extinction (absorption) coefficient
- Quantum efficiency (quantum yield)
- Brightness
- Fluorescence life-time: radiative lifetime & non-radiative lifetime

- Blinking
- Quenching
- Photobleaching

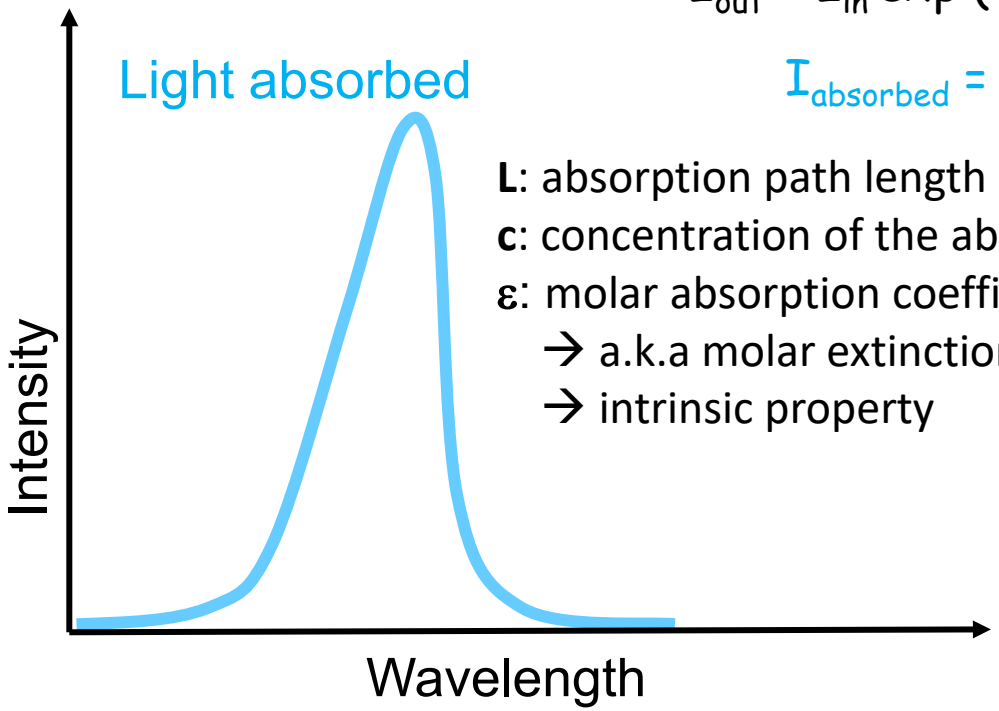
Fluorophore absorption



Beer-Lambert law:

$$I_{out} = I_{in} \exp(-\varepsilon L c)$$

$$I_{absorbed} = I_{in} - I_{out}$$



L: absorption path length (in cm)

c: concentration of the absorber (in M or $mol.L^{-1}$) (molar concentration)

ε: molar absorption coefficient (in $M^{-1}cm^{-1}$ or $mol^{-1}.L.cm^{-1}$)

→ a.k.a molar extinction coeff, molar absorptivity

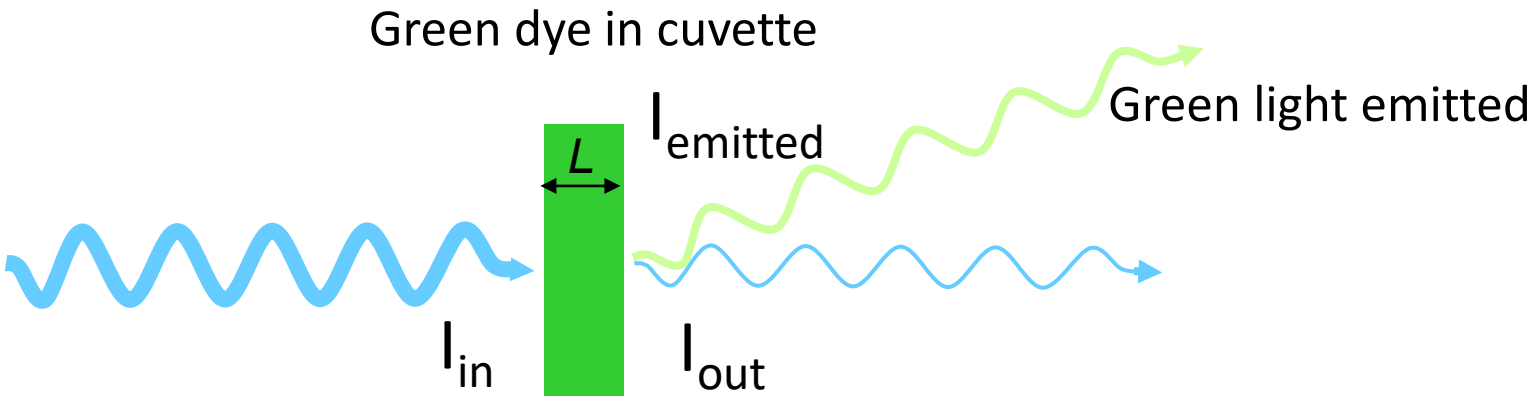
→ intrinsic property

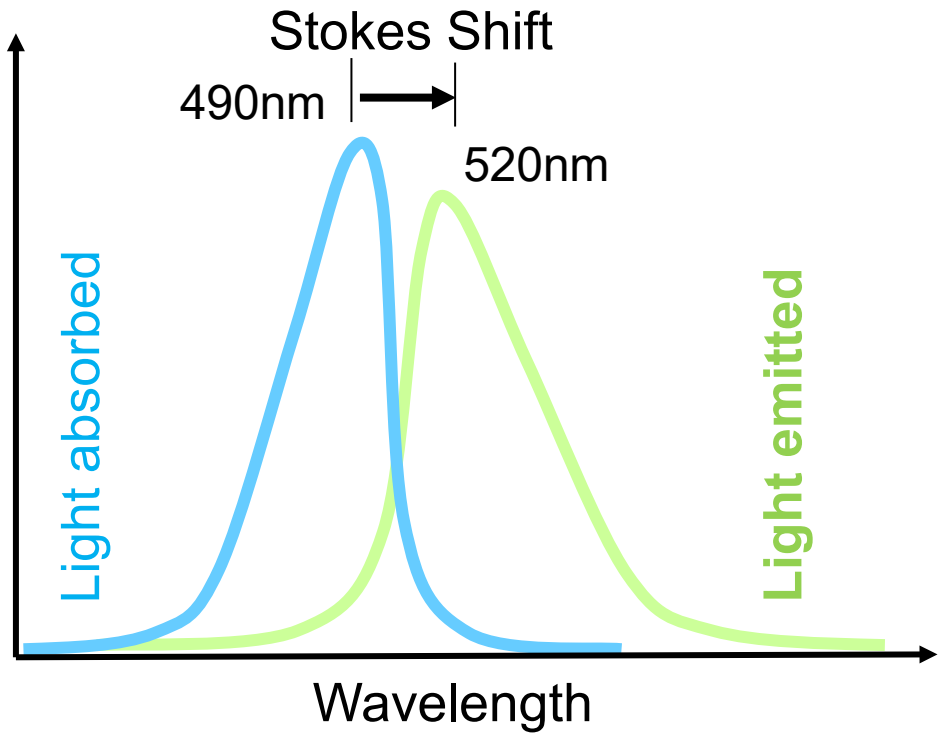
Examples:

Fluorescein: $\varepsilon \sim 70{,}000\ M^{-1}.cm^{-1}$

eGFP: $\varepsilon \sim 55{,}000\ M^{-1}.cm^{-1}$

Fluorophore absorption & emission





Quantum Yield

$Q = I_{emitted} / I_{absorbed}$

$= \#photons_{emitted} / \#photons_{absorbed}$

($I_{absorbed} = I_{in} - I_{out}$)

Examples:

Fluorescein	Q ~ 0.8
Rhodamine B	Q ~ 0.3
eGPP	Q ~ 0.6

Characteristics of fluorescent dyes

- **Quantum yield** of fluorescence, Φ_f, is defined as:

$$\Phi_f = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}} \Rightarrow \Phi_f = \frac{k_{rad}}{\sum k}$$

Here:

k_{rad} is the **radiative rate constant**

$k_{non\text{-}rad}$ is the **non-radiative rate constant**

$\sum k = \sum(k_{rad} + k_{non-rad})$ is the sum of the rate constants that depopulate the excited state

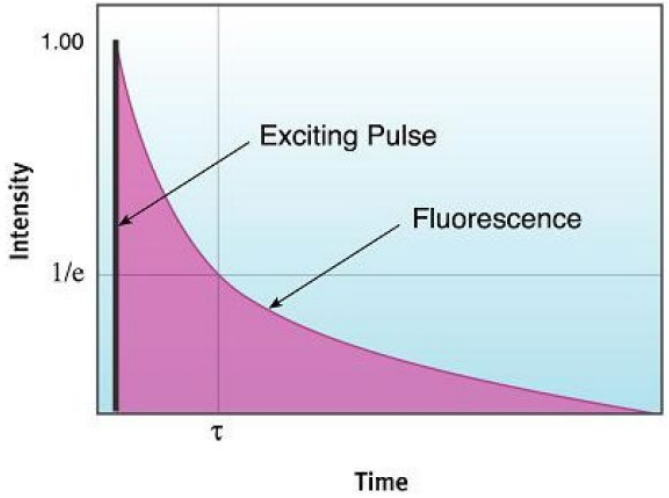
By definition, the **rate constant** is given by $k = 1/\tau$, where τ is the **lifetime**.

Radiative lifetime τ_{rad}, is related to k_{rad} as: $\tau_{rad} = \frac{1}{k_{rad}}$

Fluorescence lifetime:

It is the average time the molecule stays in the excited state (time delay between absorbance and emission) before emitting, and it is **controlled by the total rate** as:

$$\tau_f = \frac{1}{\sum k} = \frac{1}{(k_{non-rad} + k_{rad})}$$



Characteristics of fluorescent dyes

- **Quantum yield** of fluorescence, Φ_f, is defined as:

$$\Phi_f = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}}$$

$$\Phi_f = \frac{k_{rad}}{\sum k} = \frac{k_{rad}}{(k_{rad} + k_{non-rad})} = \frac{\tau_f}{\tau_{rad}}$$

k_{rad} is the radiative relaxation rate
$k_{non\text{-}rad}$ is the sum of all non-radiative relaxation rates

τ_f is the excited state lifetime (fluorescence lifetime)
τ_{rad} is the radiative lifetime

- Quantum yield of fluorescence can be affected by the biological environment

- Lifetime is also sensitive to biochemical microenvironment, e.g. local pH or binding

Fluorophores

Question: Which dye is better?

Answer: The bright dye

Brightness ~ εQ

1 - absorb well (high ε)
2 - emit well (high Q)

Examples:

Fluorescein: 0.8 * 70,000 = 57,000

Rhodamine 0.3 * 90,000 = 27,000

Fluorophore brightness = εQ

Example: Properties of fluorescent protein variants

Table 1 Properties of novel fluorescent protein variants

Fluorescent protein	Excitation maximum (nm)	Emission maximum (nm)	Extinction coefficient per chain[a] ($M^{-1}cm^{-1}$)	Fluorescence quantum yield	Brightness of fully mature protein (% of DsRed)	pKa	$t_{0.5}$ for maturation at 37 °C	$t_{0.5}$ for bleach[b], s
DsRed	558	583	75,000	0.79	100	4.7	~10 h	ND
T1	555	584	38,000	0.51	33	4.8	<1 h	ND
Dimer2	552	579	69,000	0.69	80	4.9	~2 h	ND
mRFP1	584	607	50,000	0.25	21	4.5	<1 h	6.2
mHoneydew	487/504	537/562	17,000	0.12	3	<4.0	ND	5.9
mBanana	540	553	6,000	0.70	7	6.7	1 h	1.4
mOrange	548	562	71,000	0.69	83	6.5	2.5 h	6.4
dTomato	554	581	69,000	0.69	80	4.7	1 h	64
tdTomato	554	581	138,000	0.69	160	4.7	1 h	70
mTangerine	568	585	38,000	0.30	19	5.7	ND	5.1
mStrawberry	574	596	90,000	0.29	44	<4.5	50 min	11
mCherry	587	610	72,000	0.22	27	<4.5	15 min	68

[a]Extinction coefficients were measured by the alkali denaturation method[8,30] and are believed to be more accurate than the previously reported values for DsRed, T1, dimer2 and mRFP1[7]. [b]Time (s) to bleach to 50% emission intensity, at an illumination level that causes each molecule to emit 1,000 photons/s initially, that is, before any bleaching has occurred. See Methods for more details. For comparison, the value for EGFP is 115 s, assuming an extinction coefficient of 56,000 $M^{-1}cm^{-1}$ and quantum efficiency of 0.60 (ref. 30). ND, not determined.

DsRed	εQ ~ 75,000 x 0.79 ~ 59,250 $M^{-1}.cm^{-1}$	**(100%) reference**
mRFP1	εQ ~ 50,000 x 0.25 ~ 12,500 $M^{-1}.cm^{-1}$	(21%)
eGFP	εQ ~ 56,000 x 0.6 ~ 33,600 $M^{-1}.cm^{-1}$	(57%)
Fluorescein	εQ ~ 70,000 x 0.8 ~ 56,000 $M^{-1}.cm^{-1}$	(95%)

DsRed from
Red Discosoma corals

Summary: Important Fluorescence Terms

Excitation (absorption) spectrum → its peak gives the excitation (absorption) wavelength

Emission spectrum → its peak gives emission (fluorescence) wavelength

Stokes shift: The difference in wavelength between the excitation & emission peak wavelengths

Extinction coefficient: A measure of how much light will be absorbed by a given dye/probe concentration and specimen thickness.

Quantum efficiency (yield): Ratio of light absorbed to fluorescence emitted → 0 - 1 (0 - 100%)

Brightness: extinction coefficient * quantum yield

Fluorescence life-time: decay time of a photo-excited fluorophore from excited state to the ground state