EXPERIMENTAL METHODS TO STUDY BIOLOGICAL STRUCTURES II

LUMINESCENCE, LASER

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Definitions of luminescence

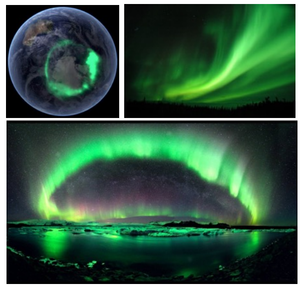
- Relaxation from excited state followed by light emission
- Radiation emitted by matter in excess of thermal emission
- "Cold light"
- Processes of fluorescence and phosphorescence

Emission by luminescence: everywhere

Photoluminescence

Luminescence everywhere

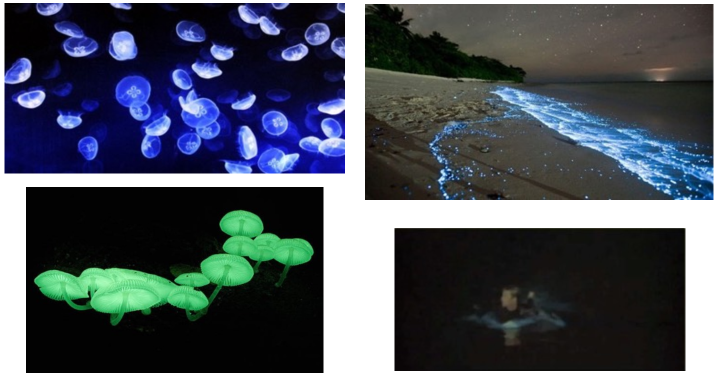
Display lights



Aurora borrealis

Radioluminescence

Luminescence everywhere



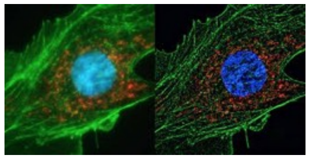
Bioluminescence

Luminescence everywhere

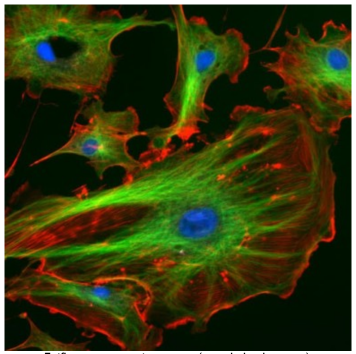
Bioluminescence
Firefly

Luminescence everywhere

GFP-mouse (green nude mouse)



Superresolution microscopy (Nobel-prize 2014)



Epifluorescence microscopy (cytoskeletal system)

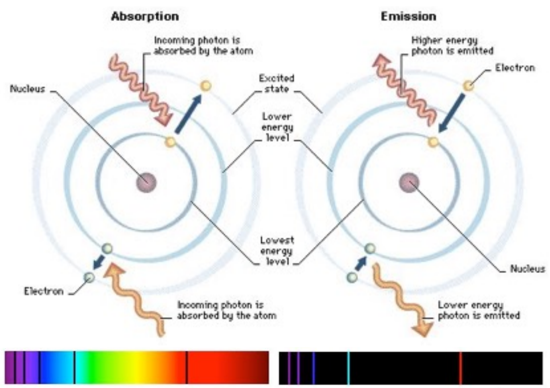
Fluorescence

Types of luminescence

(a) Excitation Mode	Luminescence Type
absorption of radiation (UV/VIS)	photoluminescence
chemical reaction	chemiluminescence, bioluminescence
thermally activated ion recombination	thermoluminescence
injection of charge	electroluminescence
high energy particles or radiation	radioluminescence
friction	triboluminescence
sound waves	sonoluminescence

(b) Excited State (Assuming Singlet State)	Luminescence Type
first excited singlet state	fluorescence, delayed fluorescence
lowest triplet state	phosphorescence

Absorption and emission by an atom

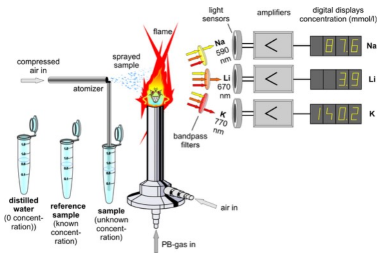


Absorption line spectrum   Emission line spectrum

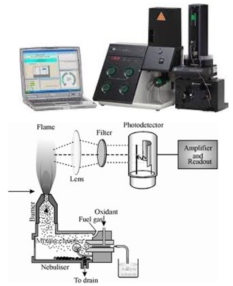
Application of emission sectroscopy

Flame photometry

Qualitative and quantitative analysis of alkali metals

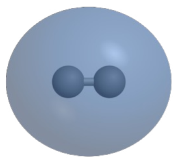


Clinical practice: determination of serum ions (Na^{+}, K^{+})



State of a molecule is affected by its motional modes

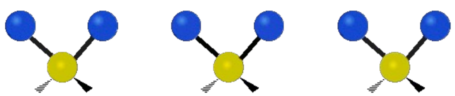
Molecule: atoms connected by chemical bonds
Simplest case: diatomic molecule (e.g., hydrogen molecule)



Molecules ***vibrate*** and ***rotate***!

Vibration: periodic motion ***along*** the axis of the covalent bond
Rotation: periodic motion ***around*** the axis of the covalent bond

Examples of vibrational motion in the triatomic methylene group ($-CH_2-$):



Asymmetric stretching   *Symmetric stretching*   *Scissoring*

Energy of a molecule

Max Born (1882-1970)

J. Robert Oppenheimer (1904-1967)

Born-Oppenheimer approximation:

$$E_{total} = E_e + E_v + E_r$$

Iportant notions:

- Types of energy states are independent (not coupled)
- Energy states are non-continuous, but discrete
- Transition between states involves packets (quanta) of energy
- Scales of transition energies between different states are different:

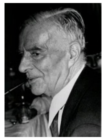
$$E_e \overset{\sim 100x}{>} E_v \overset{\sim 100x}{>} E_r$$

~3x10^{-19} J (~2 eV) > ~3x10^{-21} J > ~3x10^{-23} J

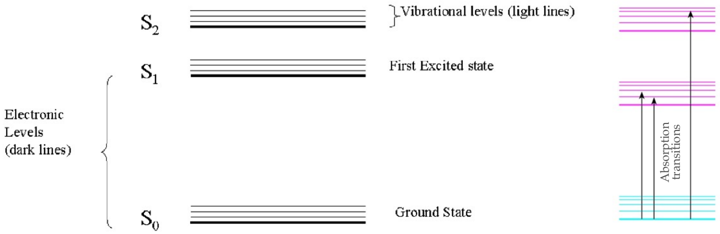
("Rule of thumb": ultraviolet > visible > infrared)

Representation of energy states

Jabłoński diagram:
illustrates the electronic states of a molecule and the transitions between them (with arrows)



Alexander Jabłoński
(1898-1980)



Spin states

Wolfgang Pauli
(1900-1958)

Pauli's exclusion principle:

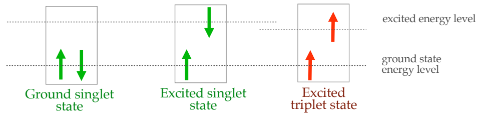
- Each quantum state can be occupied by a single electron.
- Within an atom there cannot be two electrons for which all four quantum numbers are identical.

fully occupied subshell: spin pairing (opposite-spin electrons pair)

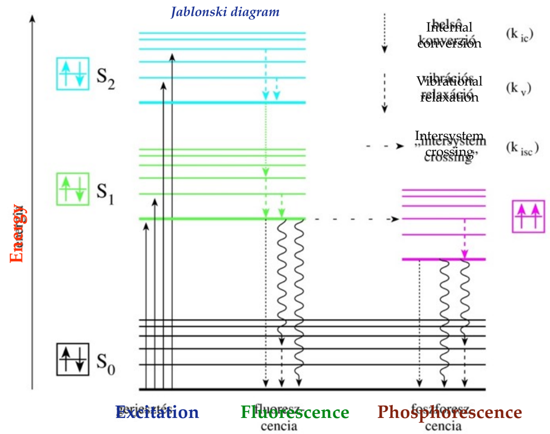
Singlet and ***triplet*** states:
number of orientations of magnetic moment associated with net spin state (in magnetic field) = 2S+1 = 1 (*singlet*) or 3 (*triplet*). (S = net spin, e.g., in fully occupied subshell (+1/2)+(-1/2) = 0)

S: singlet state: paired electrons with opposite spins, net spin (S) = 0, number of orientations (2S+1) = 1.

T: triplet state: there are identical spin-state electrons in the molecule, net spin = 1 (e.g., (+1/2)+(+1/2) = 1), number of orientations (2S+1 = 2+1) = 3.



Processes of luminescence



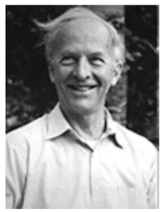
Internal conversion: non-radiative transition between electron energy states (e.g., $S_2 \rightarrow S_1$)

Vibrational relaxation: de-excitation process within the same electron energy state

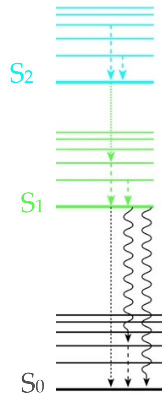
Intersystem crossing: transition between singlet and triplet states accompanied by spin change (e.g., $S_1 \rightarrow T_1$)

Kasha's rule

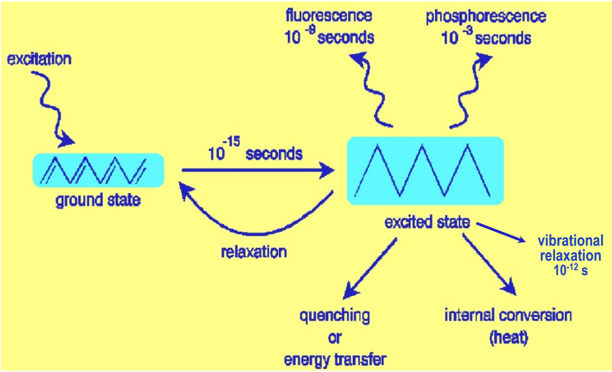
Photon emission (fluorescence or phosphorescence) occurs only from the lowest-energy excited electronic state (i.e., S_1 or T_1) of a molecule.



Michael Kasha (1920-)
American physicist

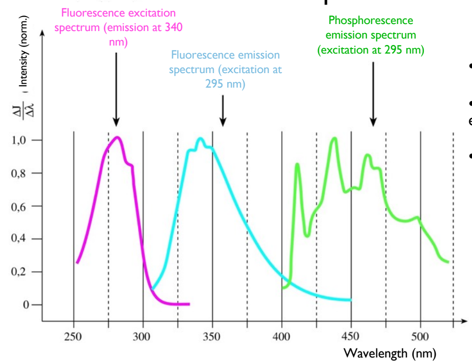


Time scale of transitions



Characterization of luminescence

Luminescence spectra



- Band spectra
- Fluorescence excitation and emission spectra are symmetric
- Stokes shift

George Stokes (1819-1903)

Fluorescent dyes: "fluorophores"

By the specific attachment fluorophores, non-fluorescent molecules may also be studied (fluorescent labeling)

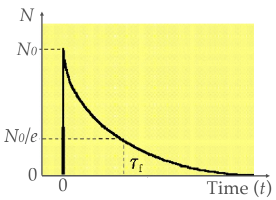
Chracterization of luminescence II.

Quantum yield

$$\Phi = \frac{\text{number of emitted photons}}{\text{number of absorbed photons}} \leq 1 \qquad \Phi = \frac{k_f}{k_f + k_{ic} + k_{isc} + k_Q}$$

k_{nr}=non-radiative transition rate constants

Excited-state lifetime (τ)



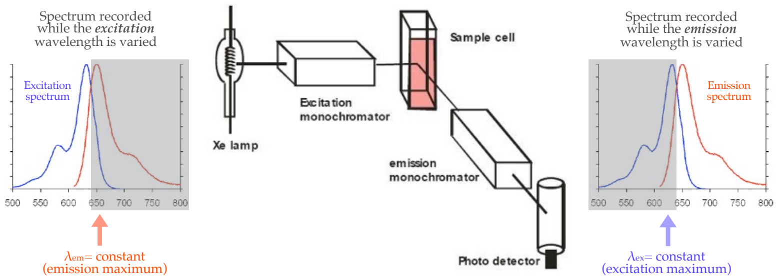
$$N = N_0 e^{-(k_f + k_{nr})t}$$

$$\tau = \frac{1}{k_f + k_{nr}}$$

N = molecules in excited state
t = time
k_f = fluorescence rate constant
k_{nr} = non-radiative transition rate constant
τ = fluorescence lifetime

Measurement of fluorescence

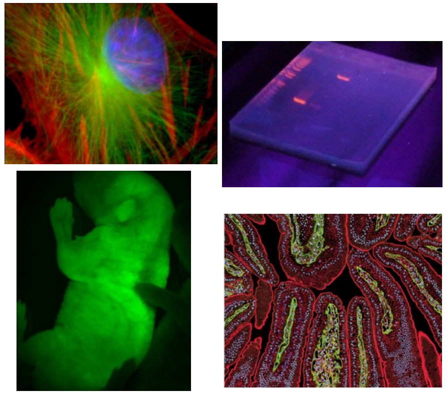
Fluorescence spectrometer
("Steady-state" spectrofluorometer)



Biomedical applications of fluorescence

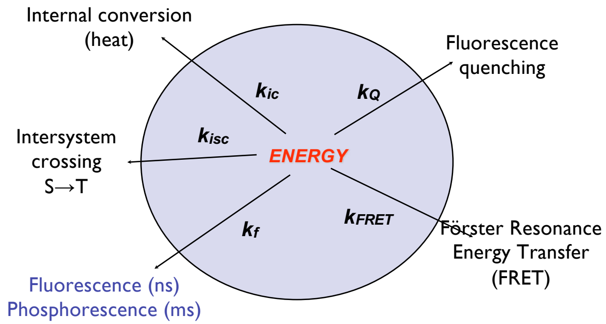
A few examples:

- Fluorescence microscopy
- DNA sequencing (chain termination method)
- DNA detection (EtBr)
- DNA microarray
- Immunofluorescence
- Fluorescence-activated cell sorting (FACS)
- Förster resonance energy transfer (FRET)
- Fluorescence recovery after photoleaching (FRAP)
- Fluorescent protein conjugation technologies
- Quantum dots
- etc...



Spectroscopic applications

Fate of absorbed energy

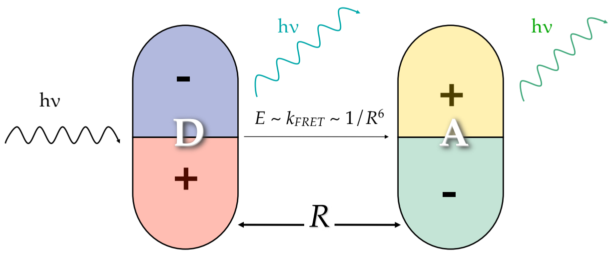


Radiative *and* non-radiative transitions!

Förster Resonance Energy Transfer (FRET)

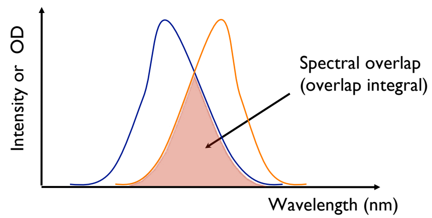
- Occurs by non-radiative dipole-dipole interaction between an excited ***donor (D)*** and an proper ***acceptor (A)*** molecule under certain conditions (spectral overlap and close distance).
- ***Fluorescence Resonance Energy Transfer (FRET):*** if the participants of the transfer are fluorophores.

Theodor Förster (1910-1974)



Conditions of FRET

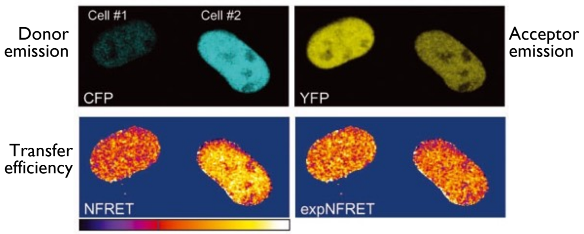
- **Fluorescent** donor and acceptor molecules.
- The distance **(R)** between donor and acceptor molecules is 2-10 nm!
- **Overlap** between the emission spectrum of the **donor** and the absorption spectrum of the **acceptor**.



Applications of FRET

- **Molecular ruler:** distance measurement on the nm (10^{-9}m) scale.
- High sensitivity (see sixth-power dependency)!
- **Applications:**
  - Measurement of ***interactions*** between molecules.
  - Measurement of ***structural*** changes on molecules.

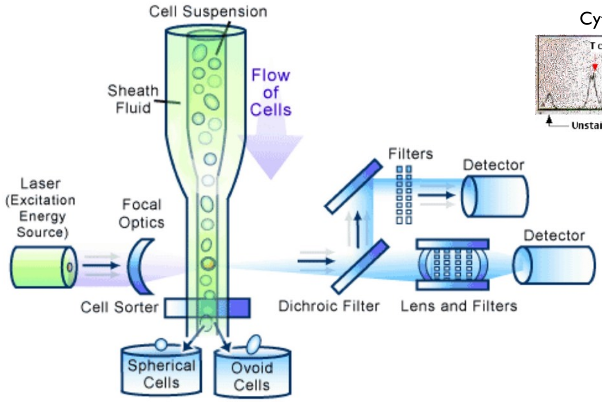
FRET microscopy



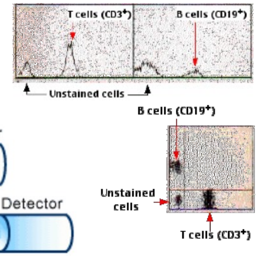
Fluorescence activated cell sorter (FACS)

Flow cytometry

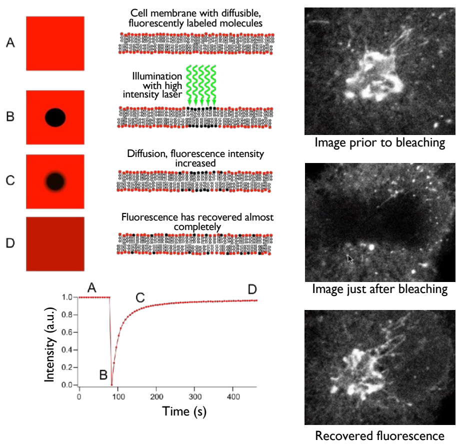
- A cell suspension, fluorescently labeled by using specific antibodies, is analyzed cell by cell
- Numerous parameters are measured simultaneously (fluorescence intensity at several wavelengths, small- and large-angle scatter)
- Statistical analysis
- If needed, cells can be separated according to their fluorescence



Cytometry statistics



Fluorescence Recovery After Photobleaching (FRAP)

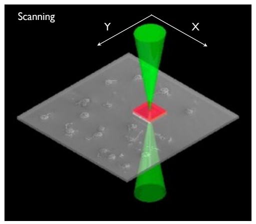


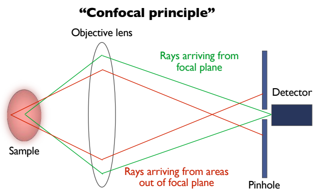
The diffusion coefficient can be determined from the time-dependent recovery of fluorescence as:

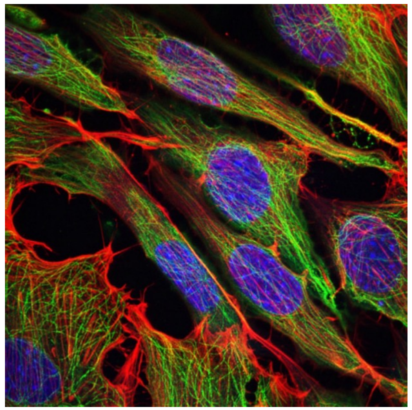
$$D = \frac{w^2}{4t_D}$$

D = diffusion coefficient
w = width of bleached area
t_D = time constant

Laser scanning confocal microscopy



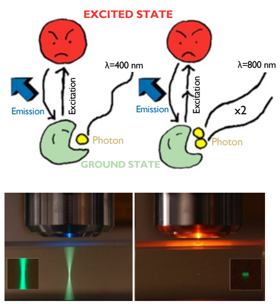




Green: microtubules; Red: actin; Blue: nuclei

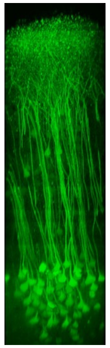
Multiphoton microscopy

- Energy of two (or more) photons are added during excitation
- Excitation (hence emission) only in the focal point (limited photodamage)
- Excitation with long wavelength (near-IR), short (fs) light pulses
- Large (up to 2 mm) penetration due to long wavelength

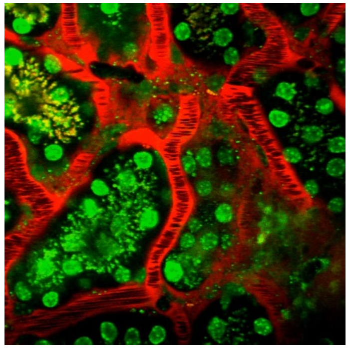


Single-photon fluorescence

Two-photon fluorescence



Cortical pyramidal cells

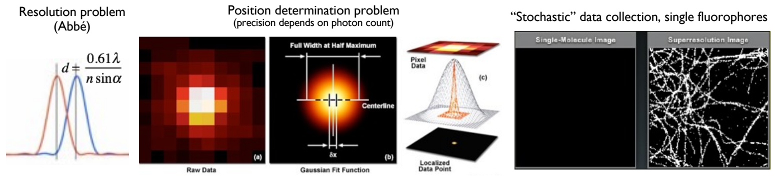


Green: proximal kidney tubules; Red: albumin (plasma)

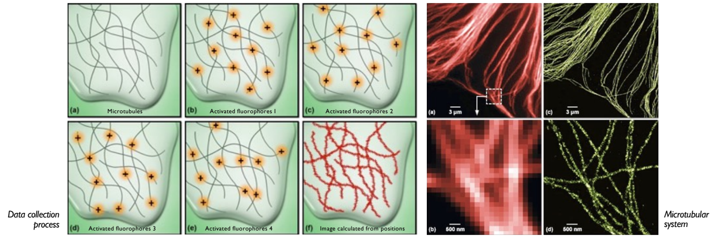
Super-resolution microscopy

Chemistry Nobel-prize, 2014

Resolution problem is converted into position-determination problem



STORM ("stochastic optical reconstruction microscopy"); PALM ("photoactivated localization microscopy")

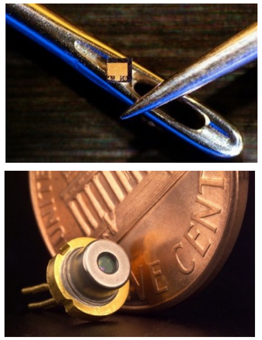


Laser

Lasers are everywhere

Laser: "Light Amplification by Stimulated Emission of Radiation"

Luminescent light source based on light amplification.



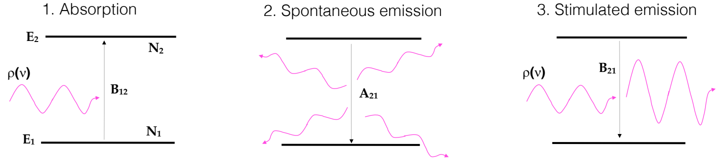
5 mW diode laser
few mms

Terawatt NOVA laser - Lawrence Livermore Laboratories
Size of a football field

Laser history in a nutshell

- 1917 - Albert Einstein: theoretical prediction of stimulated emission.
- 1946 - G. Meyer-Schwickerather: first eye surgery with light.
- 1950 - Arthur Schawlow and Charles Townes: emitted photons may be in the visible range.
- 1954 - N.G. Basow, A.M. Prochorow, and C. Townes: ammonia maser
- 1960 - Theodore Maiman: first laser (ruby laser)
- 1964 - Basow, Prochorow, Townes (Nobel-prize): quantum electronics
- 1970 - Arthur Ashkin: laser tweezers
- 1971 - Dénes Gábor (Nobel-prize): holography
- 1997 - S. Chu, W.D. Phillips and C. Cohen-Tanoudji (Nobel-prize): atom cooling with laser.
- 2013, october 8: NIF (National Ignition Facility, USA): launching nuclear fusion with 192 laser beams, positive energy balance.
- 2017 - ELI (Extreme Light Infrastructure), Szeged, Hungary. generation of attosecond (10^{-18} s) light pulses.
- 2018 - Nobel-prize in physics: Arthur Ashkin (laser tweezers), Gérard Mourou and Donna Strickland (ultrashort laser pulses)

Principles of laser I. stimulated emission



1. Absorption

Frequency of transition: $n_{12}=N_1B_{12}\rho(\nu)$

$\Delta E= E_2-E_1=h\nu$ energy quantum is absorbed.

2. Spontaneous emission

Frequency of transition: $n_{21}=N_2A_{21}$

E_2-E_1 photons travel independently in all directions.

3. Stimulated emission

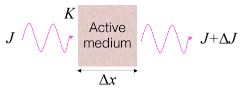
Frequency of transition: $n_{21}=N_2B_{21}\rho(\nu)$

In the presence of exernal field. Field energy is increased. Phase, direction and frequency of emitted and external photons are identical.

Explanation: two-state atomic or molecular system.
E_1, E_2 : energy levels, $E_2>E_1$
$\rho(\nu)$: spectral energy density of external field.
N_1, N_2 : number of atoms or molecules on the given energy level.
B_{12}, A_{21}, B_{21}: transition probabilities (Einstein coefficients), $B_{12} = B_{21}$

Principles of laser II. Population inversion

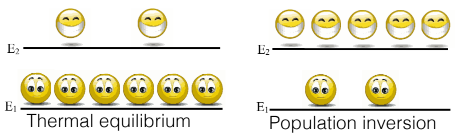
Light amplification depends on the relative population of energy levels.



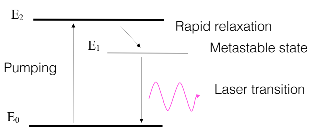
$$\Delta J=JK(N_2-N_1)\Delta x$$

J = intensity
K = constant
x = distance travelled within medium
N_1, N_2 = number of atoms on the E level

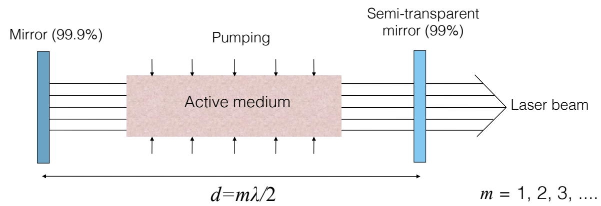
N.B.: If ΔJ is positive, more light exits the medium, than has entered!



- Population inversion only in multiple-state systems!
- Pumping: electrical, optical, chemical energy



Principles of laser III. Optical resonance



Resonator:

- two parallel (or concave) mirrors
- part of the exiting light is coupled back into the medium
- positive feedback -> self-excitation -> resonance

- Optical switch in the resonator: Q-switch, pulsed mode

Properties of laser light

1. Small divergence
   Parallel (collimated) beam

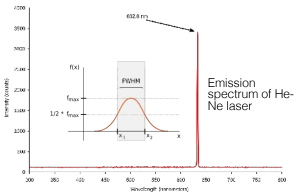
2. Large power
   In continuous (CW) mode, tens, hundreds of W (e.g., CO_2 laser)
   In Q-switched mode, momentary power is enormous (GW)
   Because of small divergence, large spatial power density.

3. Small spectral bandwidth
   "Monochromaticity"
   Large spectral energy density

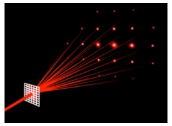
4. Often polarized

5. Possibility of extremely short pulses
   ps, fs

6. Coherence
   phase identity, interference tendency; temporal coherence (phase identity of photons emitted at different times); spatial coherence (phase identity across beam diameter). Application: holography, optical coherence tomography



Emission spectrum of He-Ne laser



Types of lasers

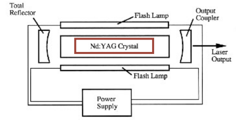
Based on **active medium**:

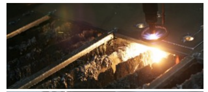
1. Solid state lasers
Metal doping in crystals or glasses; Ruby, Nd-YAG, Ti-sapphire
Red-infrared spectral range; CW, Q-switched mode, large power

2. Gas lasers
Best known: He-Ne laser (10 He/Ne). Small energy, wide use
CO_2 laser: CO_2-N_2-He mixture; $\lambda \sim 10$ µm; Huge power (100 W)

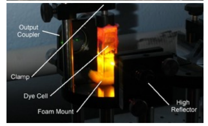
3. Dye lasers
Dilute solution of organic dyes (e.g., rhodamine, coumarine);
Pumped by another laser.
Large power (Q-switched mode); Tunable

4. Semiconductor (diode) lasers
On the boundary of p- and n-type doped semiconductors.
No need for resonator mirrors (total internal reflection)
Red, IR spectral range. Huge CW power (up to 100W)
Beam characteristics are not very good. Wide use because of small size.

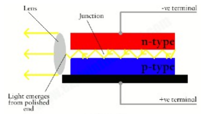




CO_2 laser (cutting a metal sheet)



Dye laser



Diode laser

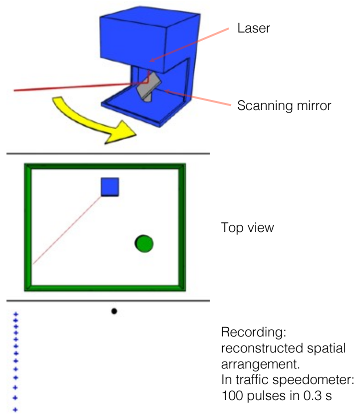
Today: laser lines (wavelengths) are available from X-rays to infrared light!

Factors to be considered in laser applications

- Steerability (small divergence)
- Monochromaticity
- Coherence
- Possibility of short pulses
- Power:
  - 5 mW – CD-ROM drive
  - 5–10 mW – DVD player or DVD-ROM drive
  - 100 mW – high-speed CD-RW writer
  - 250 mW – DVD-R writer
  - 1–20 W – solid-state laser for micromachining
  - 30–100 W – surgical CO_2 laser
  - 100–3000 W – industrial CO_2 laser (laser cutter)
  - 1 kW – 1 cm diode laser bar

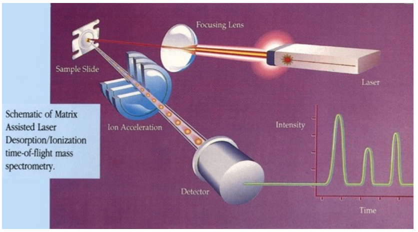
Speed measurement with laser

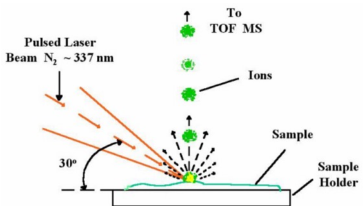
LIDAR: "Light Detection and Ranging"



Laser property utilized: Steerability

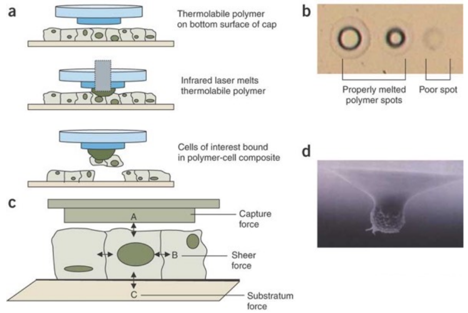
MALDI-TOF:
matrix-assisted laser desorption/ionization time of flight mass spectrometry



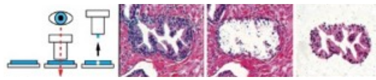


Laser property utilized: Power density

"Laser capture microdissection"



Laser property utilized: Power density, steerability

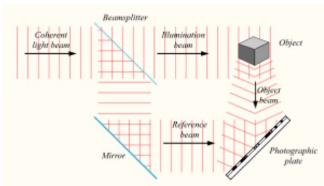


Significance: local analytics are possible
analitika lehetősége
(chemistry, genetics)

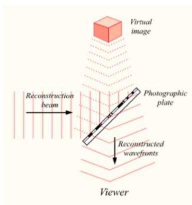
Holography

Laser property utilized: Coherence

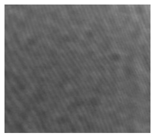
Dénes Gábor
(1900-1979)



Recording a hologram



Visualization of a hologram



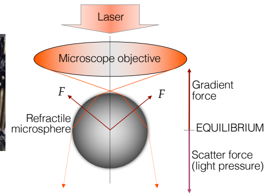
Surface of a hologram recording

Holograms

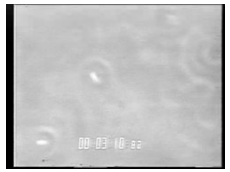
Laser tweezers

Laser property utilized: power density, steerability

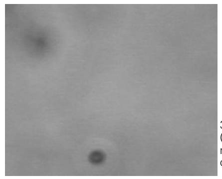
Arthur Ashkin (1970)



In the laser tweezer, momentum change occurs between the photons and the trapped particle.

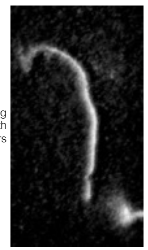


Trapping bacterial cells with laser tweezers

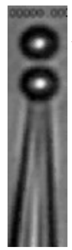


3 μm latex (polystyrene) microspheres in the optical trap

Manipulating molecules with laser tweezers



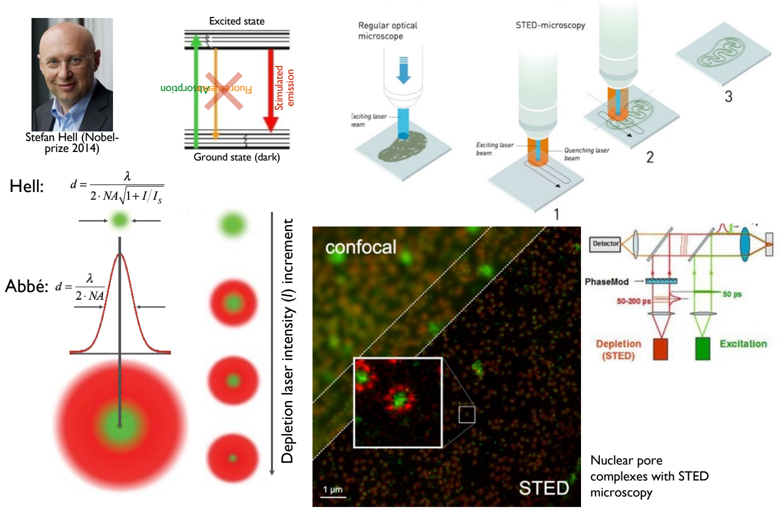
Actin filament



dsDNA

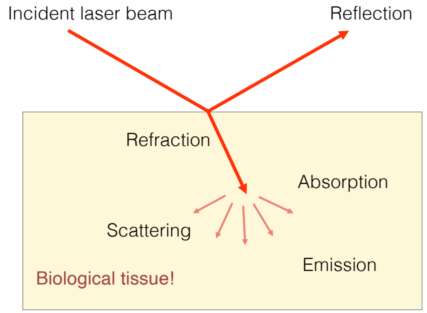
Molecular force measurement!

STED microscopy
(STimulated Emission Depletion)



Nuclear pore complexes with STED microscopy

Medical applications of lasers



Laser properties to consider:

- Steerability (small divergence, surgeries)
- Power (surgical applications)
- Monochromaticity (tissue absorbance)
- Coherence (interference, image formation)

The effects depend not only on the properties of the laser, but also on those of the biological tissue: absorbance, transmittivity, light-induced reactions.

Medical applications of lasers

- Surgical disciplines: “laser knife”, coagulation, blood-less surgery.
- Tumor removal, tattoo removal: CO_2 and Nd:YAG lasers, holmium laser lithotripsy (urology).
- Dermatology: wide-spread uses (tattoo removal, naevus removal, etc.)
- Dentistry: caries treatment (caries absorbs preferentially).
- Photodynamic tumor therapy: laser acivation of photosensitive chemicals preferentially taken up by the tumor.
- Opthalmology: Retina lesions, photocoagulation, glaucoma, photorefractive keratektomy (PRK).

Dermatological applications
Hair removal

Phototricholysis, photoepilation

Mechanism: selective photothermolysis, selective absorption by chromophores

Employed chromophores:
1. Carbon (exogenous, carbon or graphite-containing creams)
2. Hemoglobin (endogenous)
3. Melanin (endogenous)

Before treatment After treatment

Dermatological applications

Tattoo removal

Before treatment After treatment

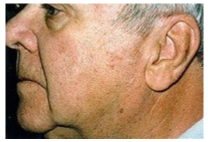
Naevus removal

Before treatment After treatment

Dermatological applications

Removal of superficial blood vessels

Before treatment



After treatment

Resurfacing

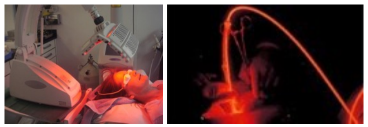
Wrinkle removal

Rhinophyma (sebaceous gland hypertrophy, fibrosis)

Oncological applications: Photodynamic therapy

Photodynamic therapy (PDT):
Roswell Park Cancer Institute 1970's.

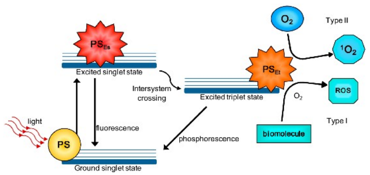
Three-component tumor therapeutic method:
1. Photosensitizing agent, 2. Light, 3. Oxygen.



Delivery of light into the patient: surface exposure, optical fiber

Steps:

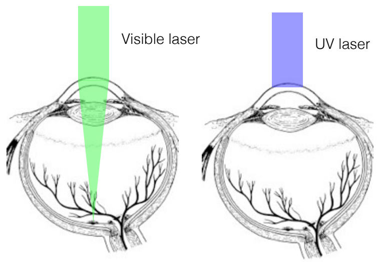
1. Administration of photosensitizing agent (aminolevulic acid, ALA).
2. Incubation for few hours. ALA is transformed into protoporhyrin IX.
3. Illumination of target area with diode laser (few minutes).
4. Protoporphyrin absorbs ➛ excited singlet state ➛ triplet state ➛ energy transfer with triplet oxygen ➛ excited, reactive oxygen ➛ tissue reaction. The illuminated area necrotizes in a few days.

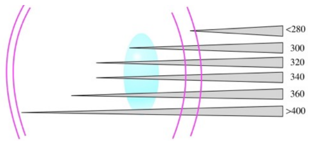


Ophthalmologic applications:

Considerations

Transmittivity of optical media is wavelength-dependent



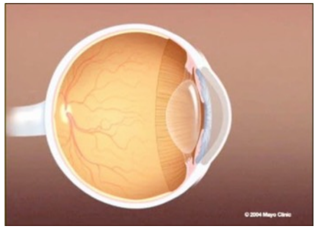


Ophthalmologic applications
LASIK

“Laser-assisted In Situ Keratomileusis”
A type of refractive laser eye surgery

History:
Jose Barraquer, 1970: construction of a microkeratome, with which he was able to cut lines and lobes in the cornea with laser (keratomileusis).
Lucio Buratto (Italian) and Ioannis Pallikaris (Greek), 1990: combination of keratomileusis photorefractive keratectomy.
Thomas and Tobias Neuhann (Germany), 1991: automated microkeratome.

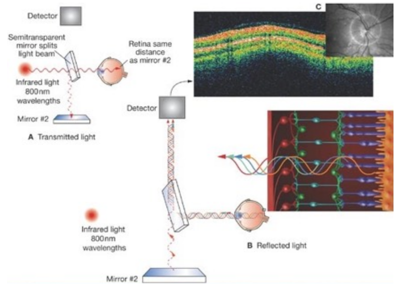
Steps:
1. Removal of contact lens (7-10 days prior to treatment)
2. Scanning the topography of the cornea with low-power laser.
3. Cutting and lifting a layer of the cornea with femtosecond laser.
4. Removal of material from the corneal stroma (few tens of microns). Excimer laser (193 nm).

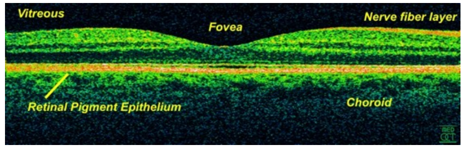


Ophthalmologic applications
Optical Coherence Tomography (OCT)

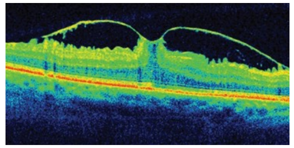
- Non-invasive
- Contrast-agent free
- Near microscopic resolution

Principles:
light rays reflected in deeper tissue layers can be separated from scatter by using interferometry. The spatial position of the reflecting layers can be determined. The structure of the illuminated sample can be resolved within 1-2 mm depth.





Normal retina



Macula degeneration