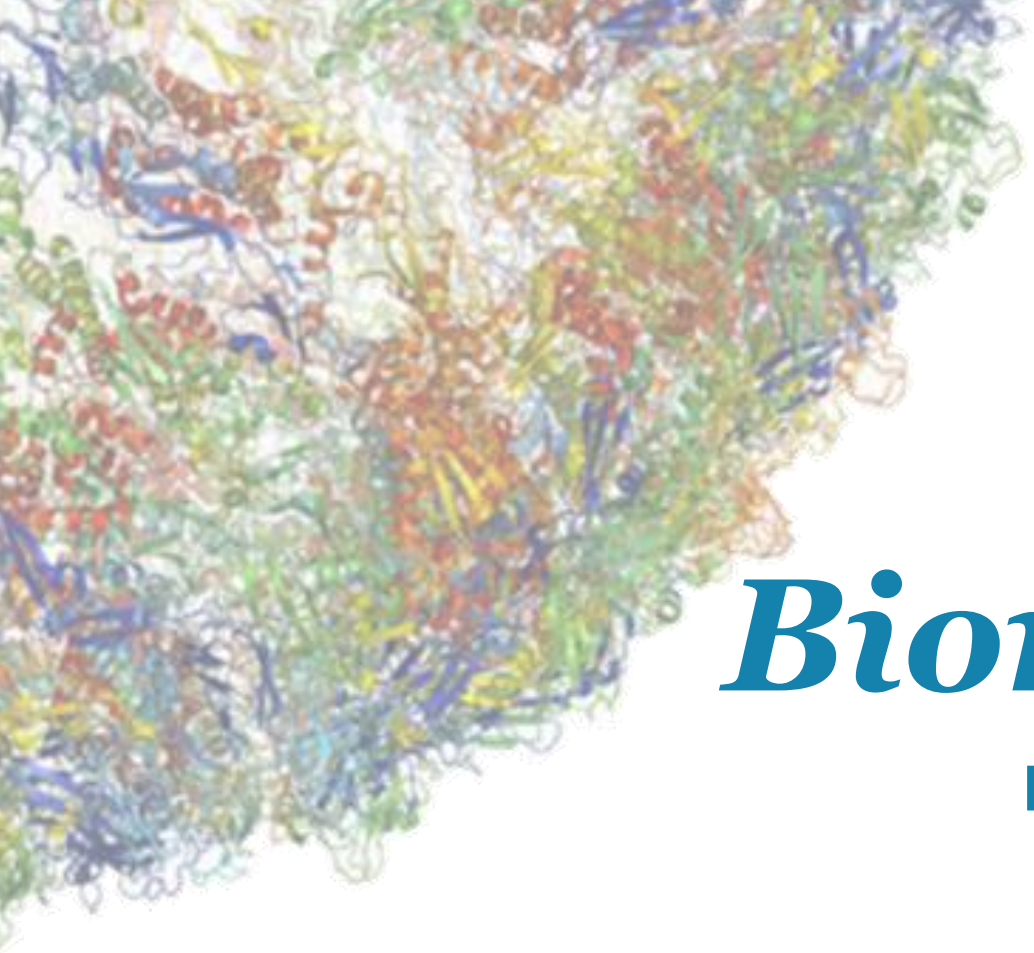
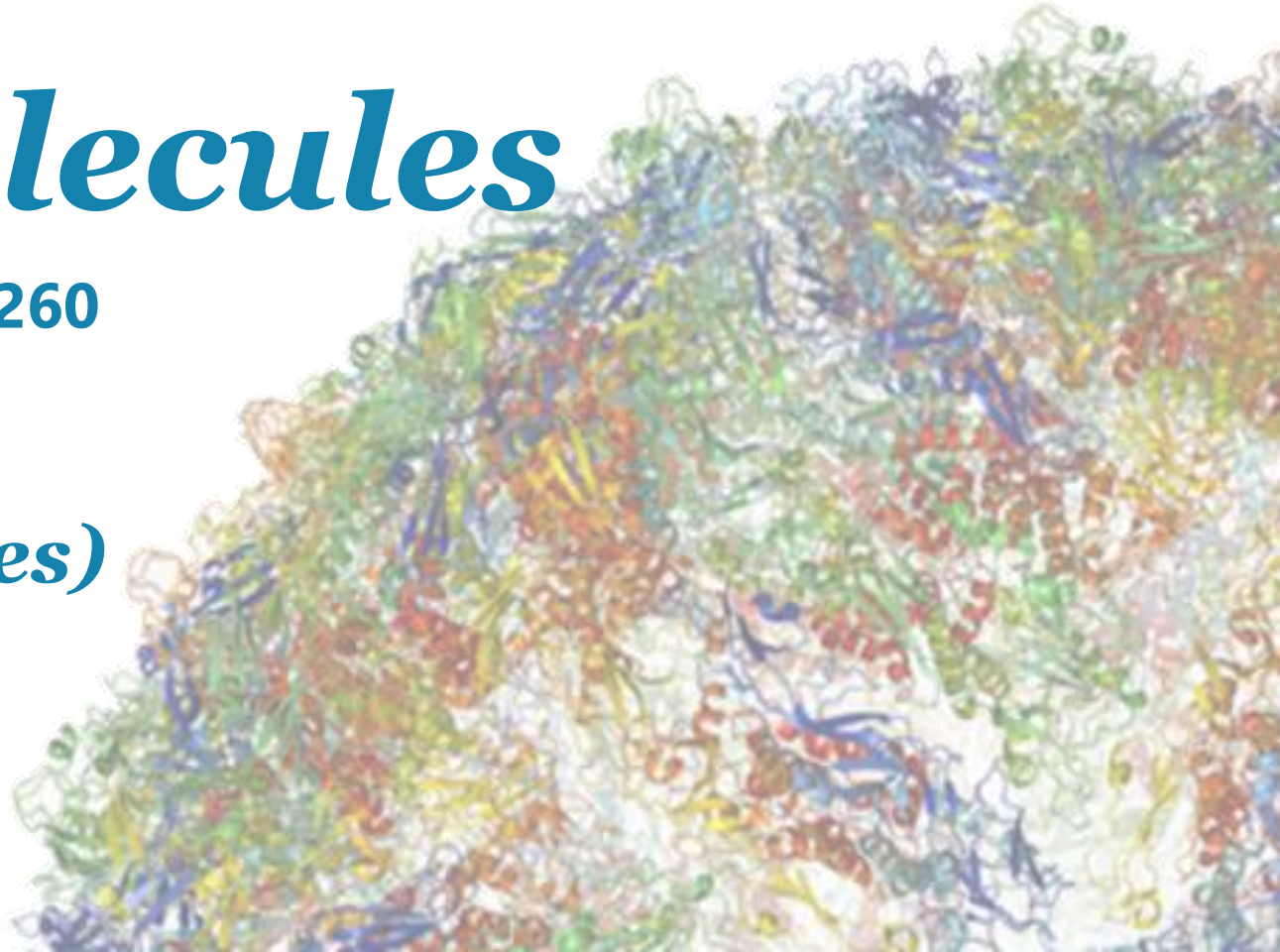


Biomolecules

ELEC-E3260



Spectroscopy (of Biomolecules)

What Will You Learn Today?

Spectroscopy: *working principle and application to biomolecules*

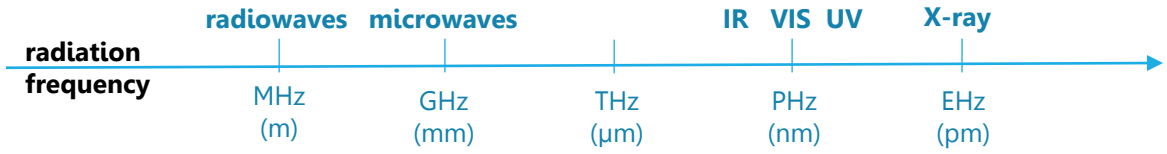
- Light absorption
- Light emission (different luminescent processes)
- Spectroscopy in the THz region (10^{12}Hz)

Spectroscopy: Relevance for Biomolecules

SPECTROSCOPY

how electrons interact with light

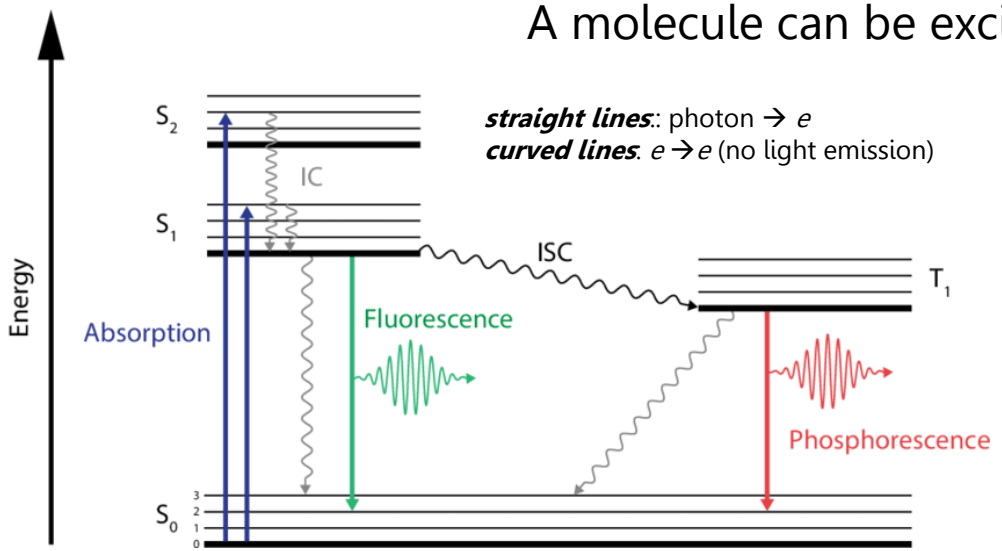
- spectral lines and/or energy of a sample
- to study sample absorbance and/or transmittance and the structure, molecular composition and arrangement



spectroscopic observables	nuclear spins	rotation	vibration	electronic transition	electronic diffraction
relevance to biomolecules	FT-methods, imaging		microscopy		crystal structure

(Optical) Excitation & Jablonski Diagram

A molecule can be excited to its electronic excited state



Absoprtion: *e^- is excited from low energy level to a higher energy level* (energy transfer from a photon to the electron)

- allowed λ = energy difference between the levels ($E > E_G$)
- timescale : 10^{-15}s

$$E = h\upsilon = \frac{hc}{\lambda}$$

Planck´s constant → h; speed of light → c; photon frequency → υ; photon wavelength → λ

non-radiative decay

Internal Conversion (IC): *radiation-less transition between states with the same spin state*

- timescale: 10^{-14}-10^{-11}s

Intersystem Crossing (ISC): *radiation-less transition between states with different spin state*

- timescale: 10^{-8}-10^{-3}s

radiative decay

Fluorescence: *electron in excited states (singlet) decays to a lower energy state*

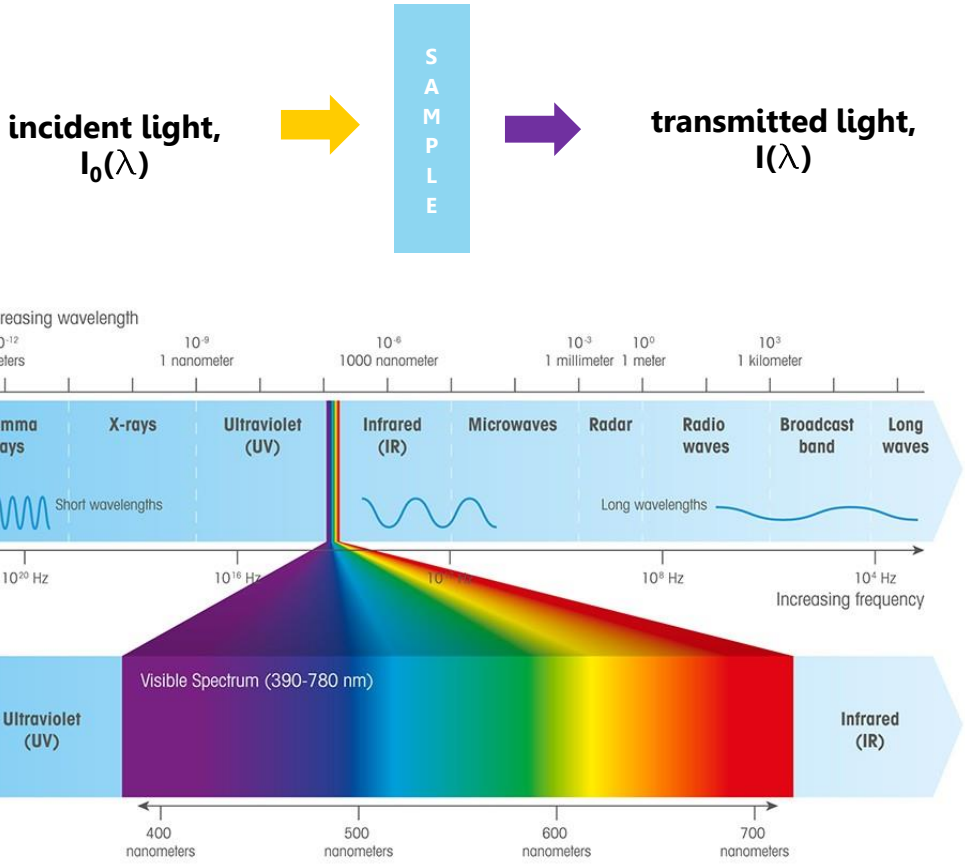
- timescale: 10^{-9}-10^{-7}s

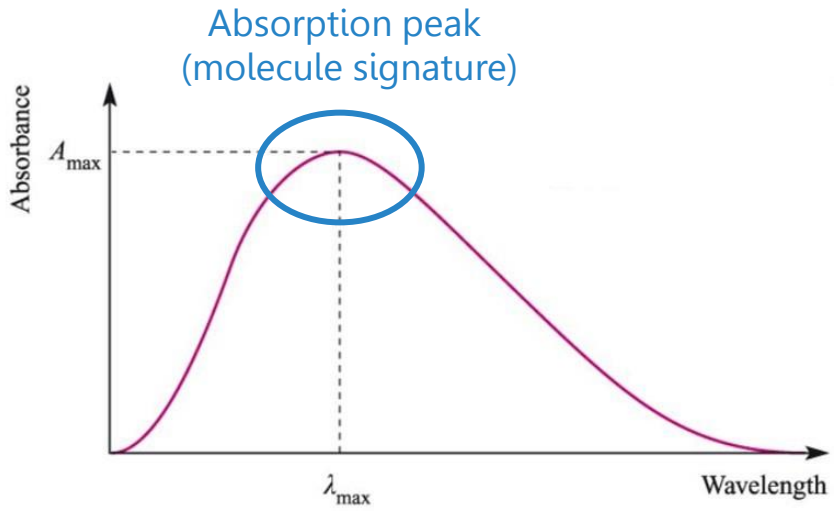
Phosphorescence: *electron in excited states (triplet) decays to a lower energy state*

- timescale: 10^{-4}-10^{-1}s

Molecule Absorption (and UV-Vis)

Lambert-Beer´s Law $I(\lambda) = I_0 e^{-Absorbance}$





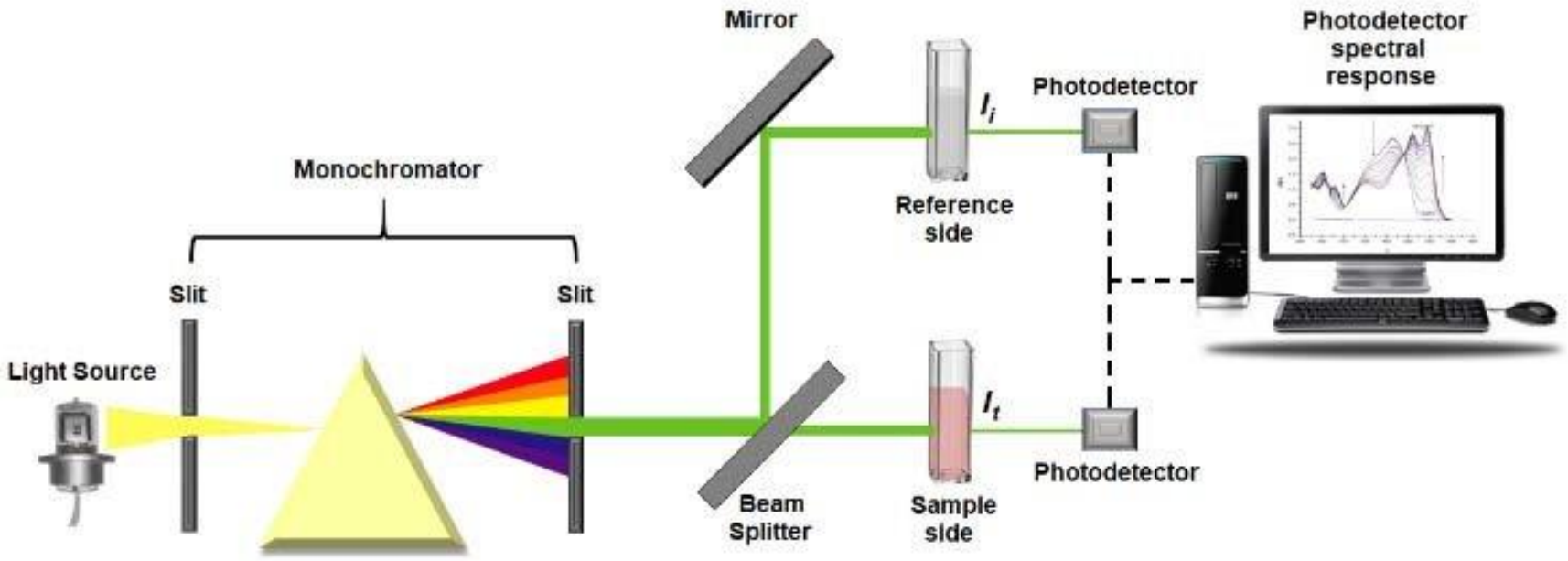
ABSORPTION SPECTRUM:
light passing through the sample v*s.* wavelength

UV: 100nm-400nm
Vis: 400nm-750nm

Why is Absorption Important?

- Absorbance/transmittance of a species
- Identify compounds (through the absorption maximum)
- Color determination
- Impurity concentration
- Reaction dynamics

How to Measure Absorption



$$A\ (or\ Optcial\ Density,\ OD) = \varepsilon\, C\, l$$
(solution)

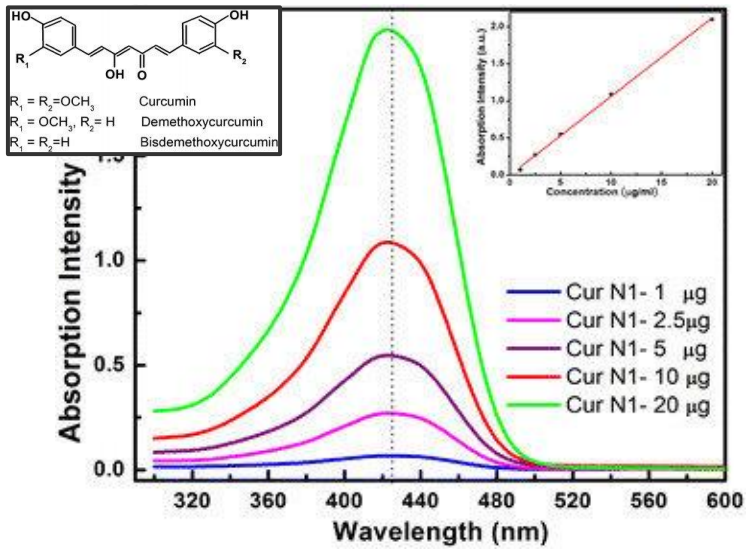
ε =(molar) extinction coefficient
C = concentration
l = path length

$$I = I_0\, e^{-\alpha d}$$
(thin film)

α = absorption coefficient
d = film thickness

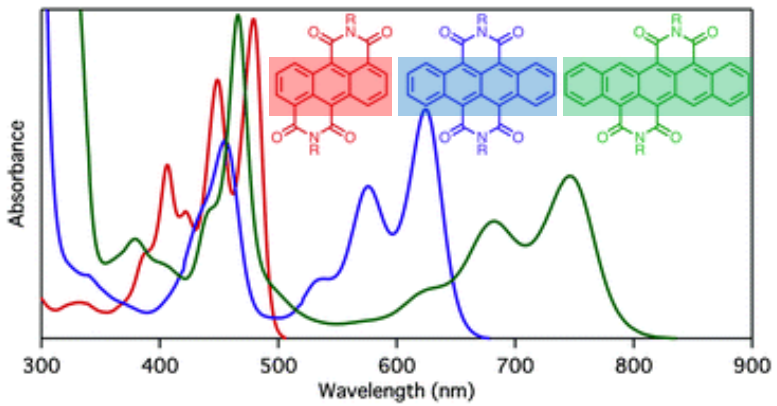
Measuring Absorption…

… as function of molecule concentration



Absorption spectra of curcumin-ethanol solutions for different curcumin concentration. Curcumin solution shows absorbance @λ=425nm and linear increasing absorption with increasing concentration [SpringerPlus **5**, 1147 (2016)]

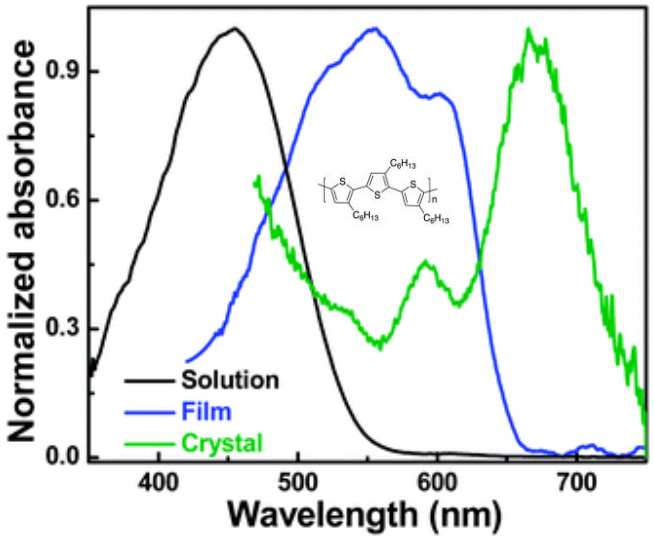
… as function of molecules composition



UV-Vis absorption of different acene-based molecules (anthracene, tetracene, pentacene) [Chem. Comm. **47**, 10112 (2011)]

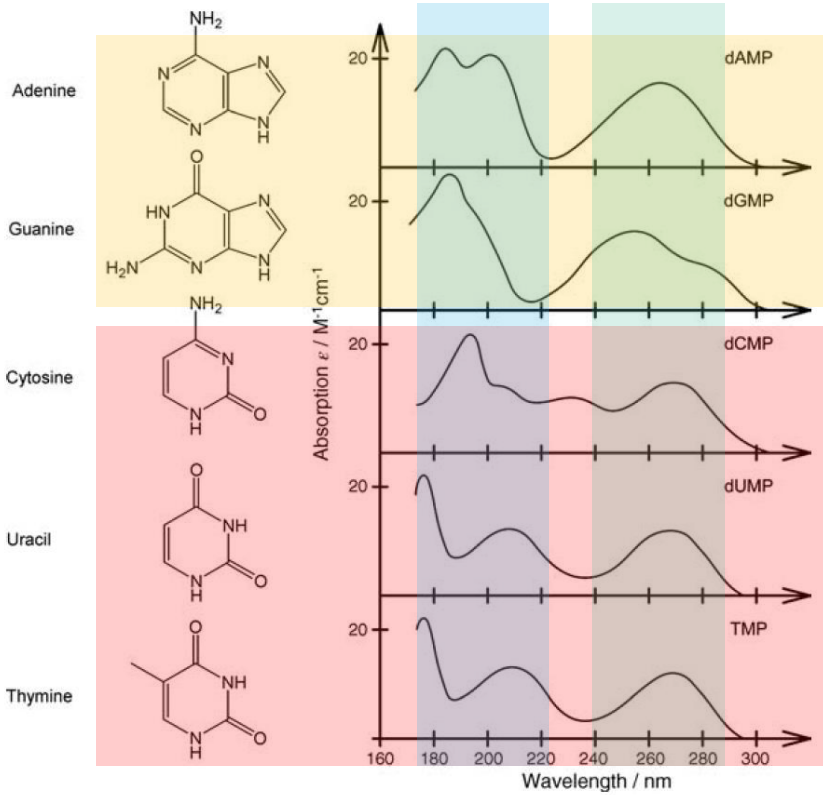
… as function of molecules form

solution *vs.* **film** *vs.* **crystal**

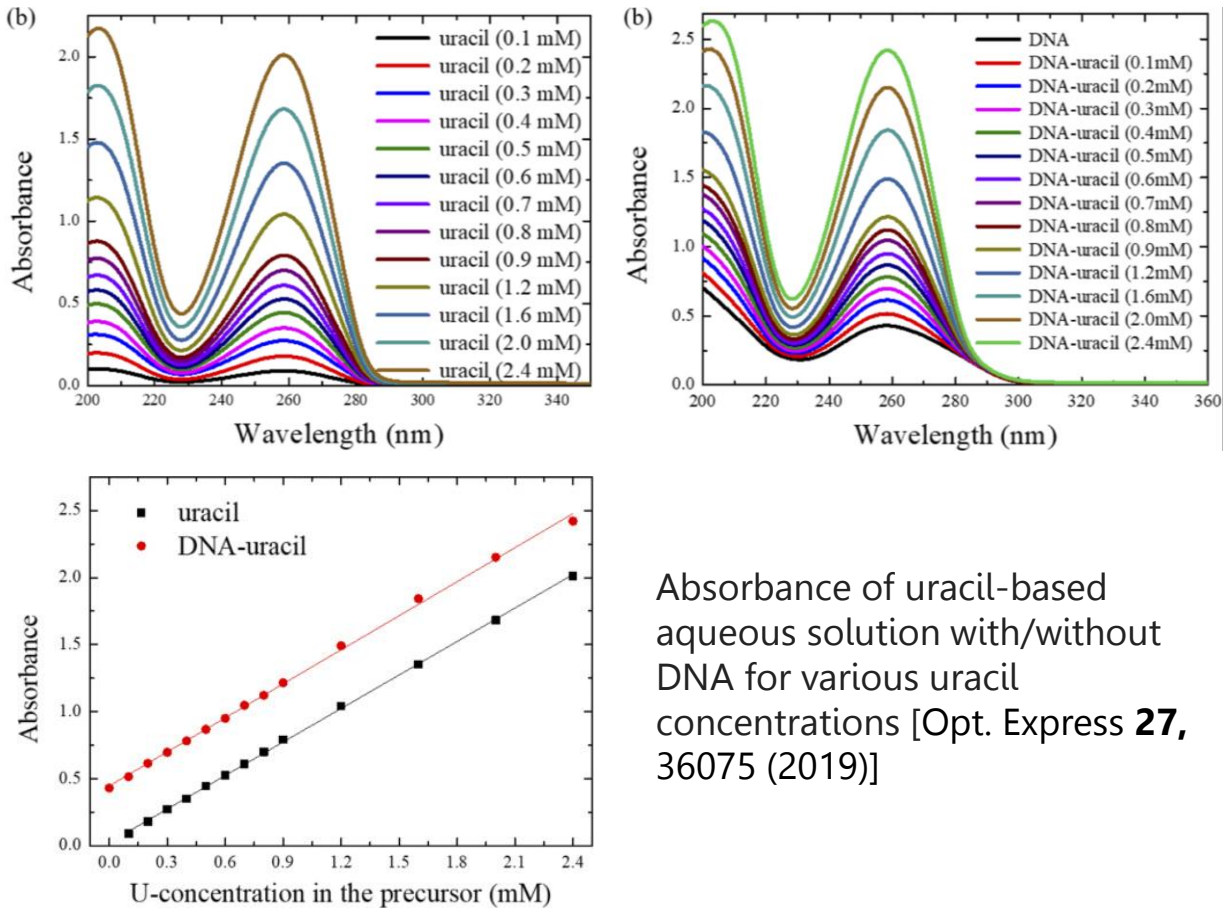


UV-Vis spectra of P3HT in different forms: crystal, solution, thin film on glass. Differences arise from ***aggregation*** (solution), ***molecular packing*** (thin film) and ***crystal structure*** [RCS Adv. **4**, 11121 (2014)]

Absorption of Nucleotides

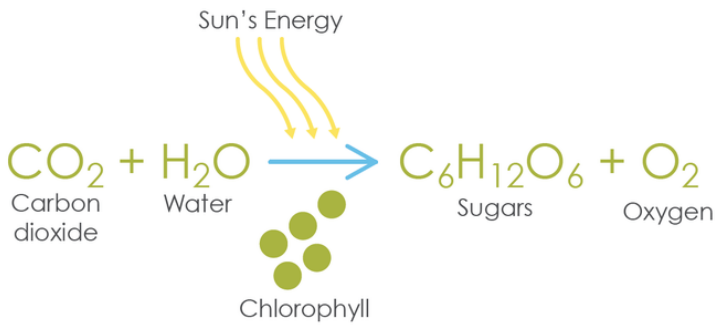


Absorption and structural formula of different nucleotides

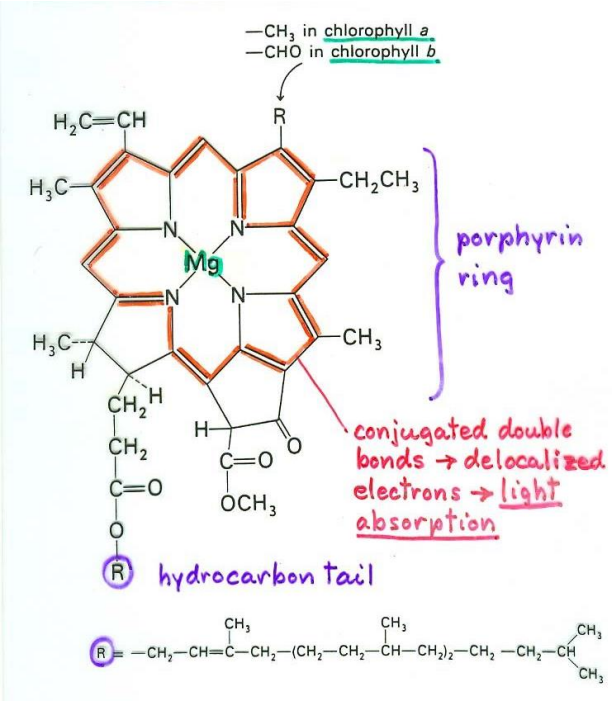


Absorbance of uracil-based aqueous solution with/without DNA for various uracil concentrations [Opt. Express **27,** 36075 (2019)]

Photosynthesis: Absorption in Nature

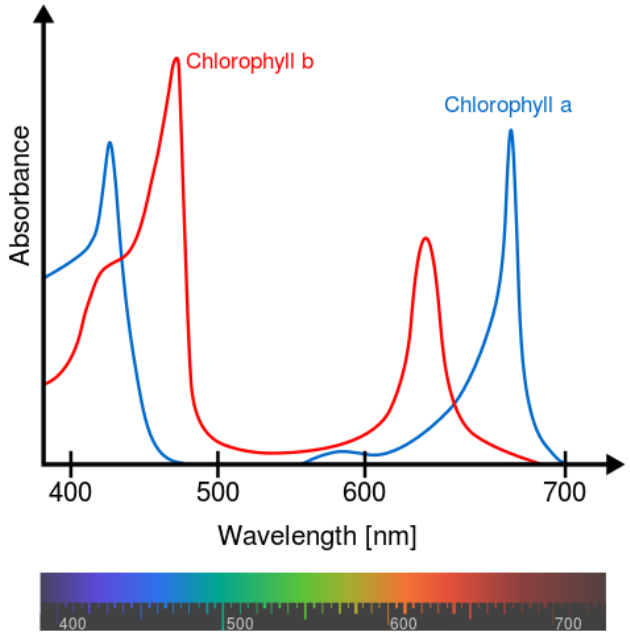


Electronics excitation energy is used for charge separation, to be used to drive all subsequent biochemical reactions



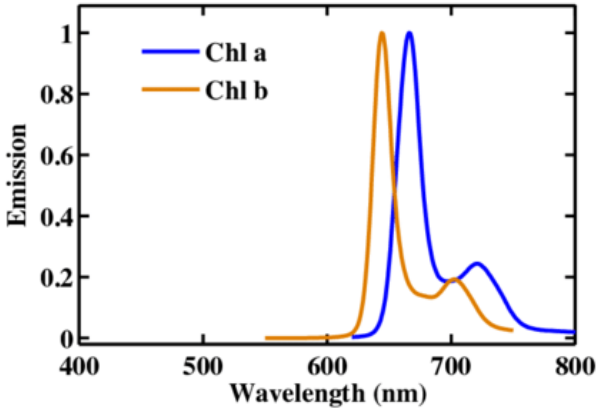
Chlorophylls is based on a porphyrin backbone and represents one of the key pigments for photosynthesis in plants, algae and photosynthetic bacteria.

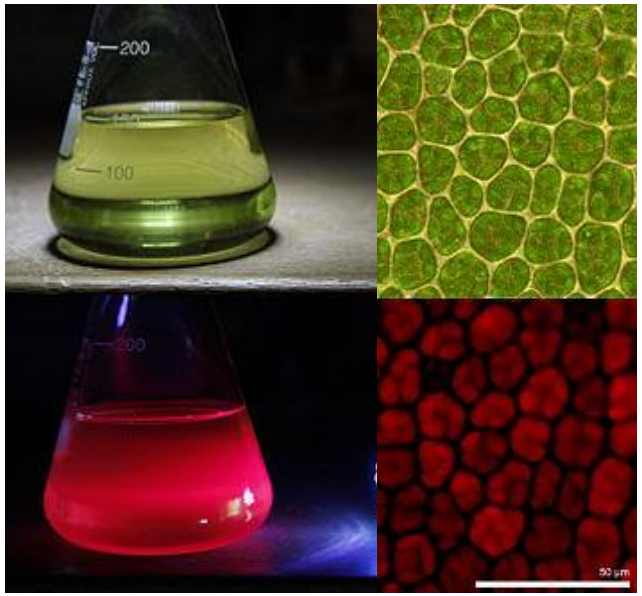
Chlorophyll *a* and *b* are the most common and they exhibit two dominant bands in the blue (400-500nm, Soret band) and red (600-700nm, Q_y band)

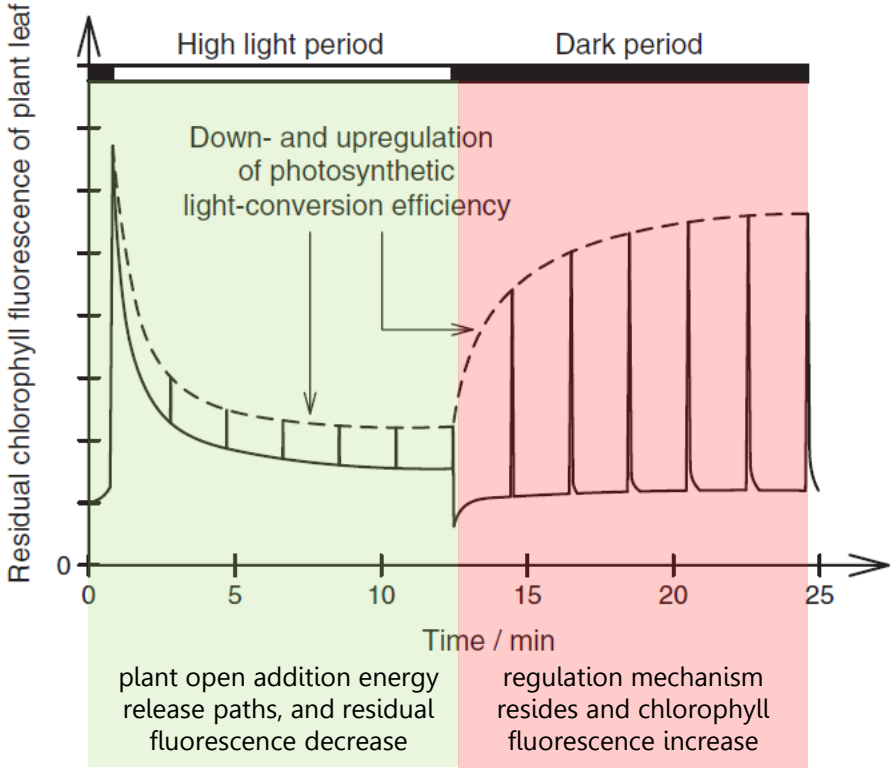


Monitoring Photosynthesis

Upon optical excitation, chlorophyll also exhibits (high) fluorescence (~700nm), which can be used to measure plant parameters







when exposed to different illumination conditions, plants regulate the amount of excitation energy

Fluorescent Molecules

proteins and biomolecules ***do not exhibit intrinsic fluorescence***

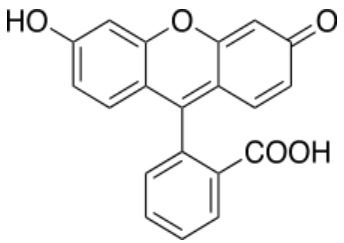
adding fluorescent molecules

fluorescent "markers" are often ***covalently linked*** to the biomolecule of interest

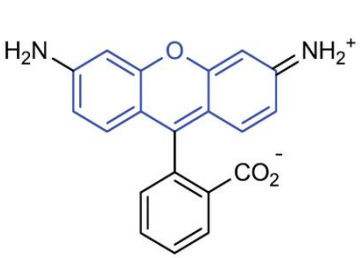
biomolecules can be identified

Fluorescent molecules:

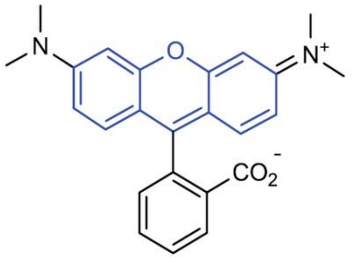
- narrow absorption spectrum
- high extinction coefficient
- high quantum yield ($\Phi = \frac{number\ of\ photons\ emitted}{number\ of\ phtons\ absorbed}$)
- narrow fluorescence spectrum
- soluble in aqueous environment
- chemical active site
- photostability



fluorescin

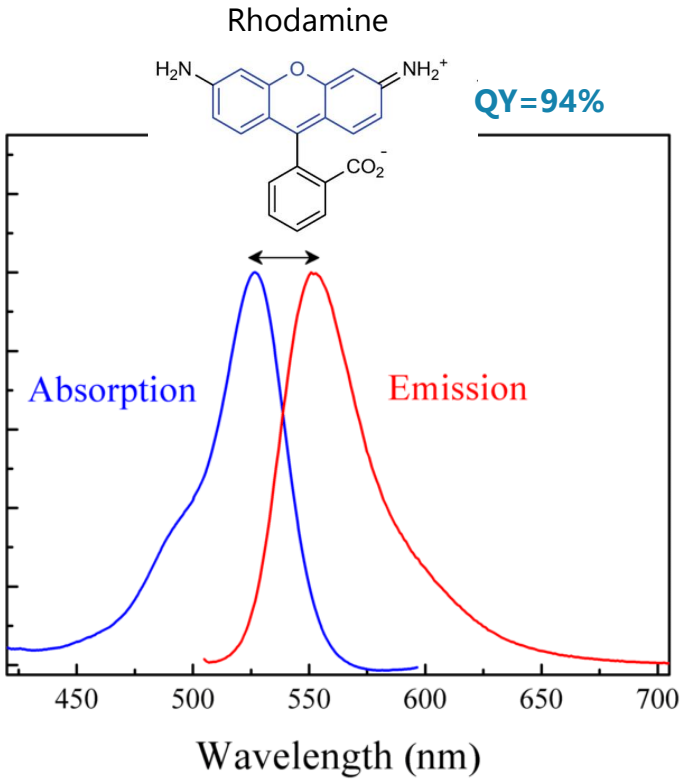


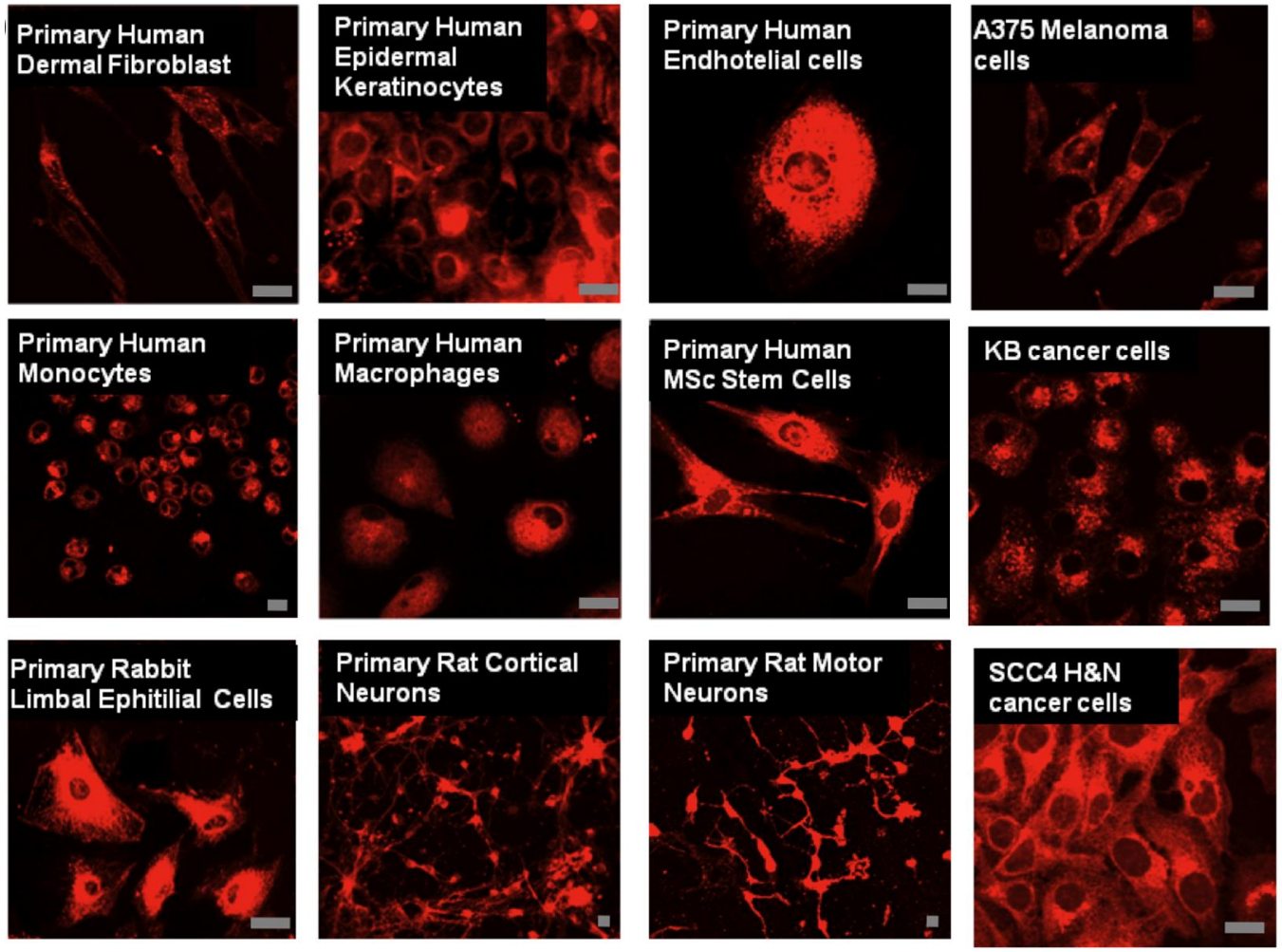
rhodamine green



tetramethylrhodamine (TMR)

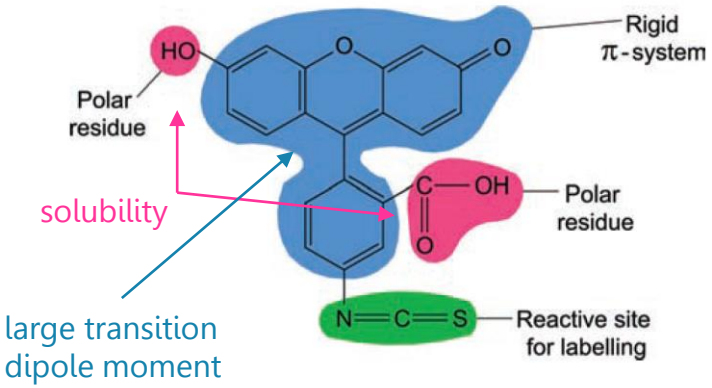
Rhodamine

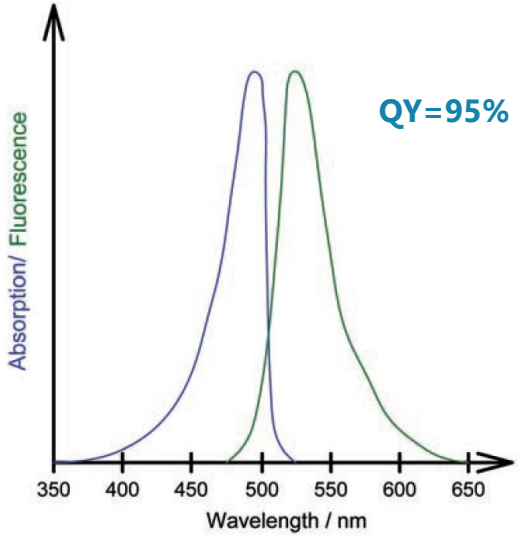




Rhodamine enables visualization of large number of structural details (*i.e.* adhesion filipodia in both HDF and HE cells, the multinuclear nature of SCC4 cancer cells, and the formation of motoneuronal axon on RMN cells) [*PLOS ONE* **5**(5): e10459 (2010)]

Fluorescein

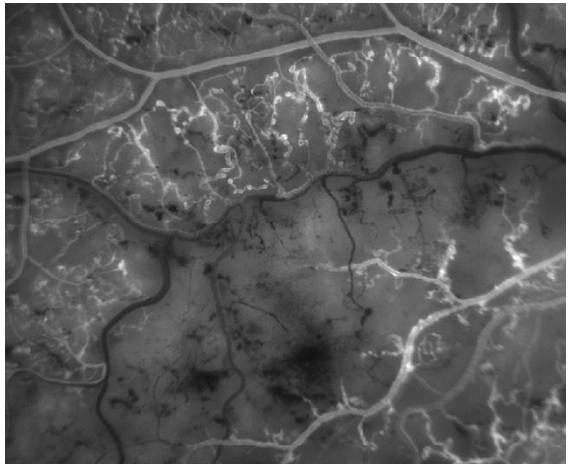




Absorption and fluorescence in the VIS range are separated and do not overlap with absorption and fluorescence of proteins and DNA

Fluorescein angiography picture of ischemic branch retinal vein occlusion in 30-year-old woman.

Images available at RetinalImageBank



High-magnification fluorescein angiogram showing a branch retinal vein occlusion.

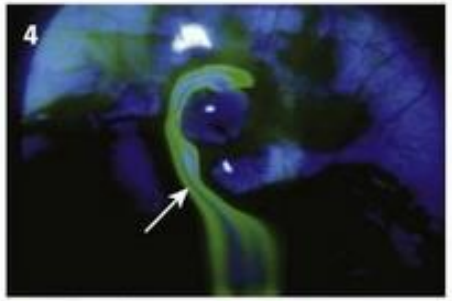
Fluorescein-based Imaging for the Eye

Moisten the fluorescein strip with 1 drop of saline or topical anesthetic. Depress the lower lid and gently place a wetted strip onto the inside of the patient's lower lid so that only the smallest amount is instilled.

detection

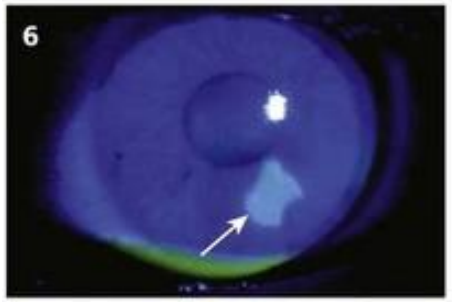
Examine the eye with a Wood lamp or a slit lamp with a cobalt blue filter (shown). Check for areas of bright green fluorescence on the corneal and conjunctival surfaces. Because the naked eyes may not be able to appreciate small defects, magnification should be used.

The Eidolon Bluminator ophthalmic illuminator provides an intense blue LED light with 7× magnification. *(Courtesy of Michael W. Ohlson, OD, FAAO, and Victor J. Doherty, Eidolon Optical, LLC.)*

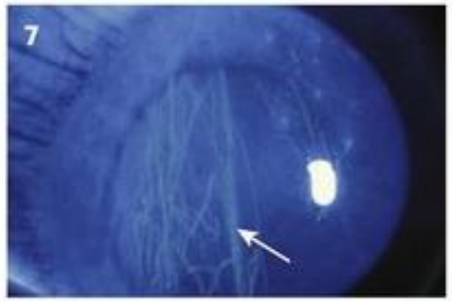


Positive Seidel test. Fluorescein seen streaming down the cornea indicates an open-globe injury. *(From Krachmer JH, Mannis M, Holland E, eds.* Cornea. *3rd ed. St. Louis; Mosby; 2010.)*

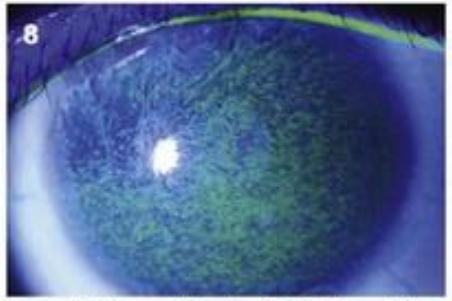
Large corneal abrasion seen with the naked eye. Smaller abrasions or corneal injuries produced by keratitis or a welder's arc flash require slit lamp evaluation to identify minor corneal defects.



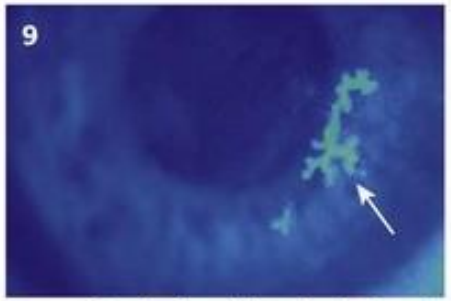
Corneal abrasion as seen via a slit lamp. A moderate-sized abrasion (*arrow*) is revealed by fluorescein staining and blue light. *(From Friedman NJ, Raiser PK, Pineda R.* Massachusetts Ear & Eye Infirmary Illustrated Manual of Ophthalmology. *3rd ed. Philadelphia Saunders; 2009.)*



Vertical linear abrasions. These types of abrasions are typically caused by a foreign body trapped under the upper eyelid. *(From Kliegman R, Stanton B, Behrman R, et al, eds.* Nelson Textbook of Pediatrics. *19th ed. Philadelphia: Saunders; 2011.)*



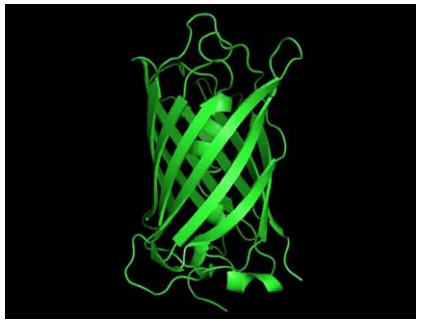
Superficial punctate keratitis. These diffuse, shallow corneal irregularities are caused by chemical irritation, viral illnesses, exposure to bright light, and many other conditions.

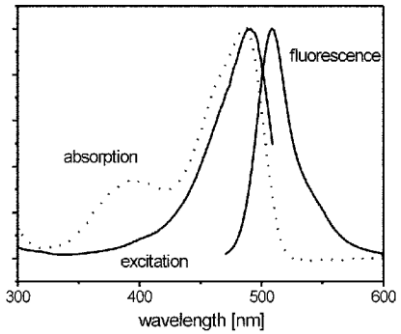


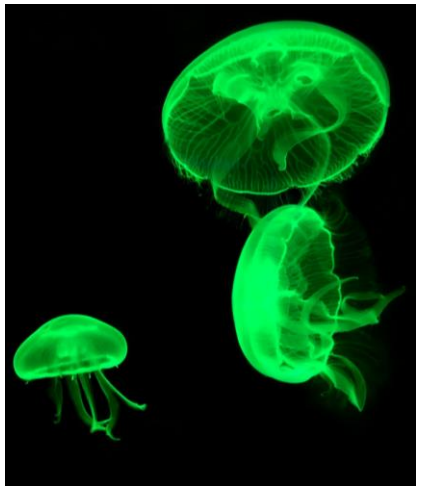
Herpes simplex keratitis. A classic herpetic epithelial dendritic lesion is seen on this fluorescein examination. *(From Palay DA, Krachmer JH, eds.* Primary Care Ophthalmology: Concepts and Clinical Practice. *2nd ed. St. Louis: Mosby; 2005.)*

Fluorescent Proteins

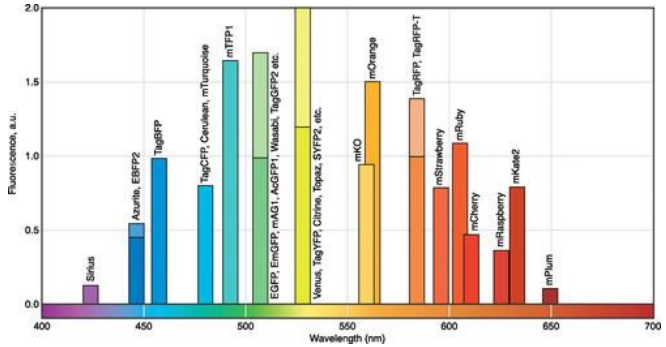
Green Fluorescent Protein (GFP) can be found in jellyfishes and it shows an absorption maximum at ~ 400nm and a fluorescence maximum ~510nm

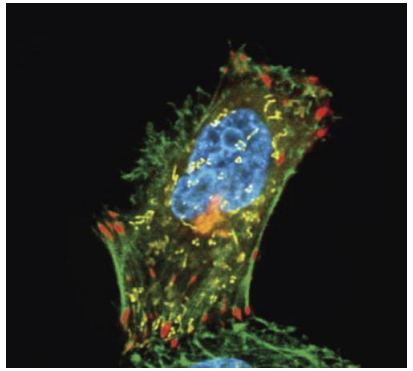






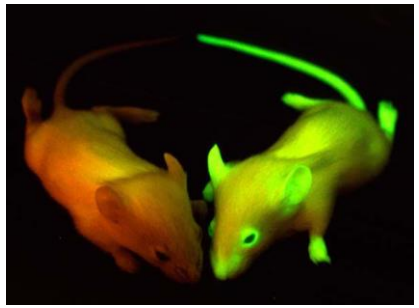
GFP forms from the residues of the serine, tyrosine and glycine, which are in close proximity in the primary sequence of the protein (autocatalytic reaction). GFP can be used as a marker.





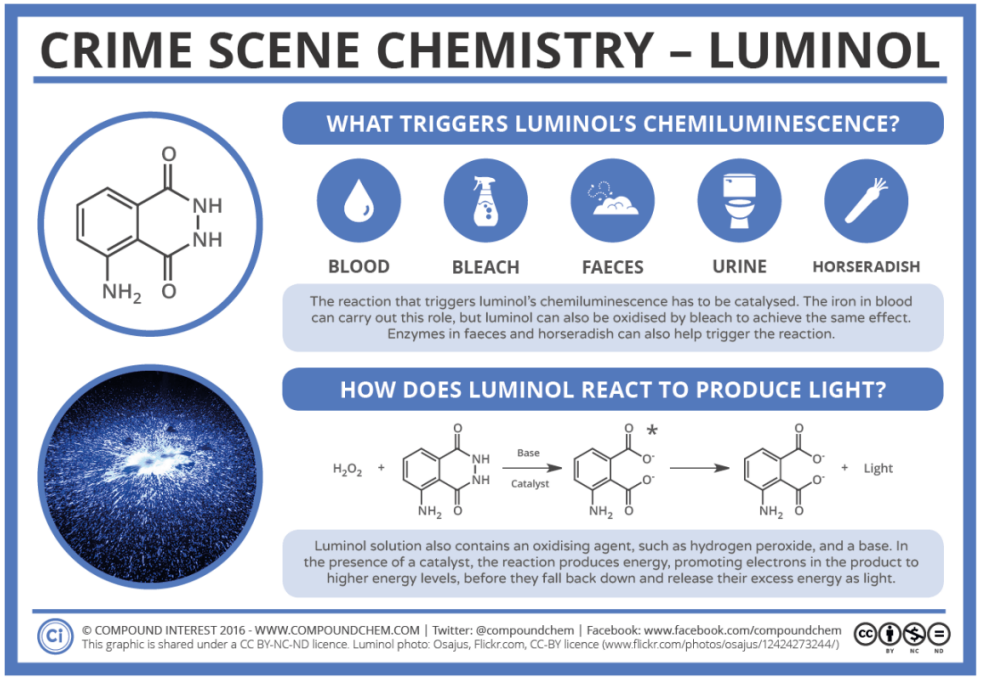
Diversity of proteins and mutations can cover the VIS range. Multicolor labeling, living HeLa cells (TagBFP-H2B, TagGFP2-actin, phiYFP-mito, TagRFP-golgi, mKate2-zyxin). [*Physiol. Rev.* **90**, 1103 (2010)]

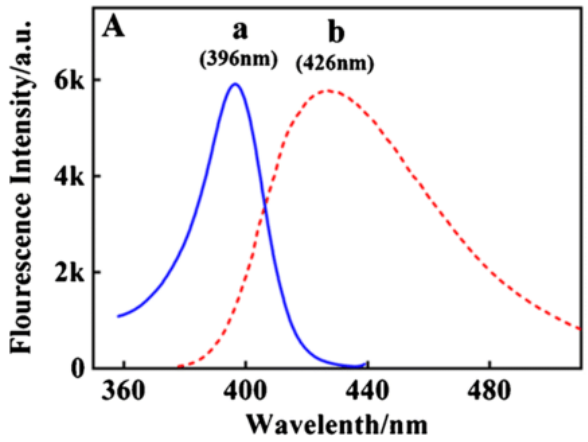
GFP is an excellent "reporter gene," able to attach itself to another gene without interfering with its behavior.



Chemiluminescence & Bioluminescence

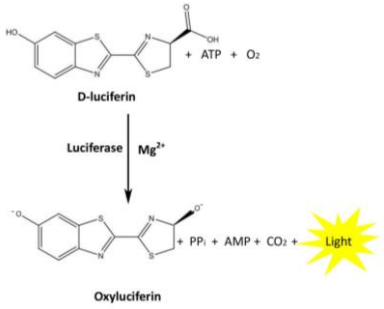
light emission derived from a *chemical reaction* in which, chemically excited molecules decay to the electronic ground state and emit photons



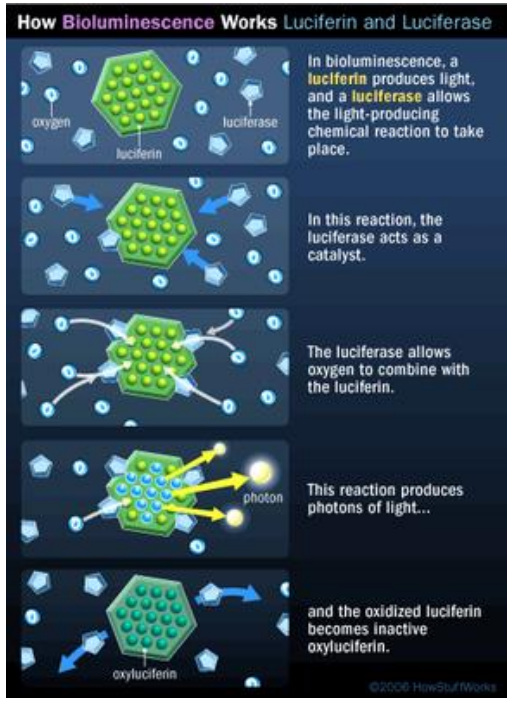


Chemiluminescence & Bioluminescence

form of chemiluminescence naturally occurring in living organisms

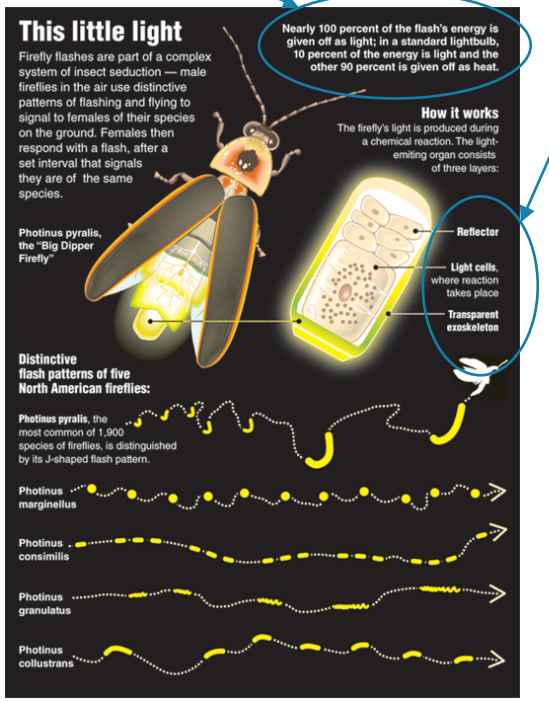


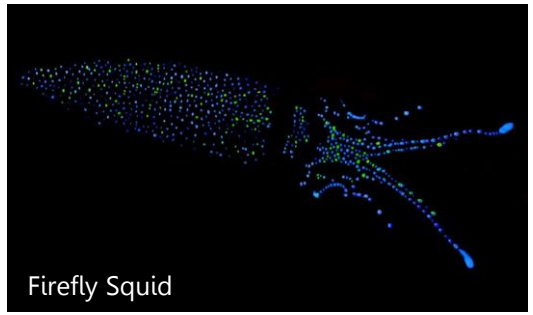
luciferin-luciferase reaction



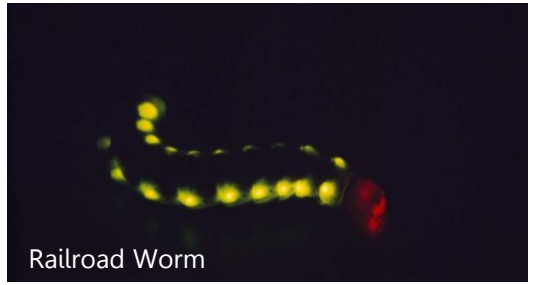
efficient process

complex photonic structure

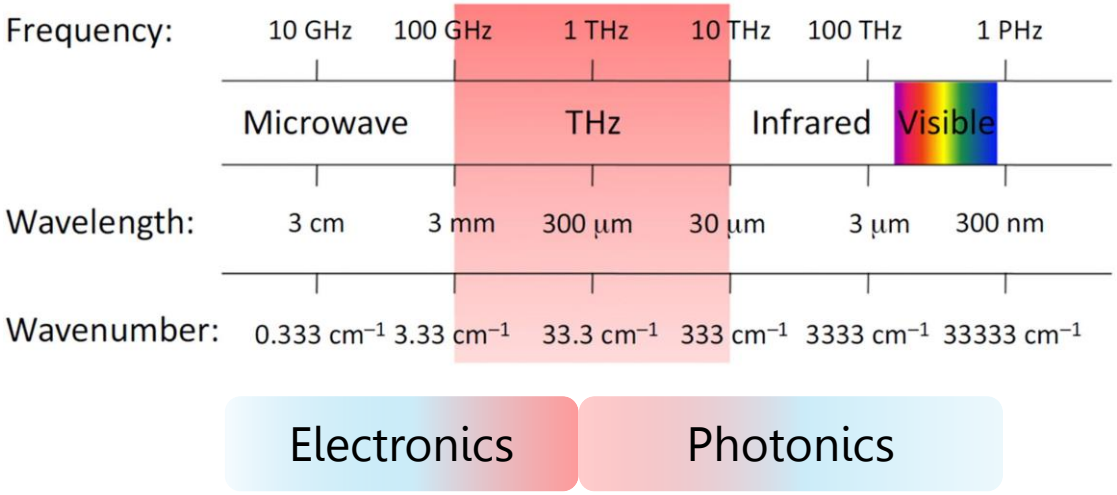




Source:
National Geographic



THz Region [10^{12}Hz]



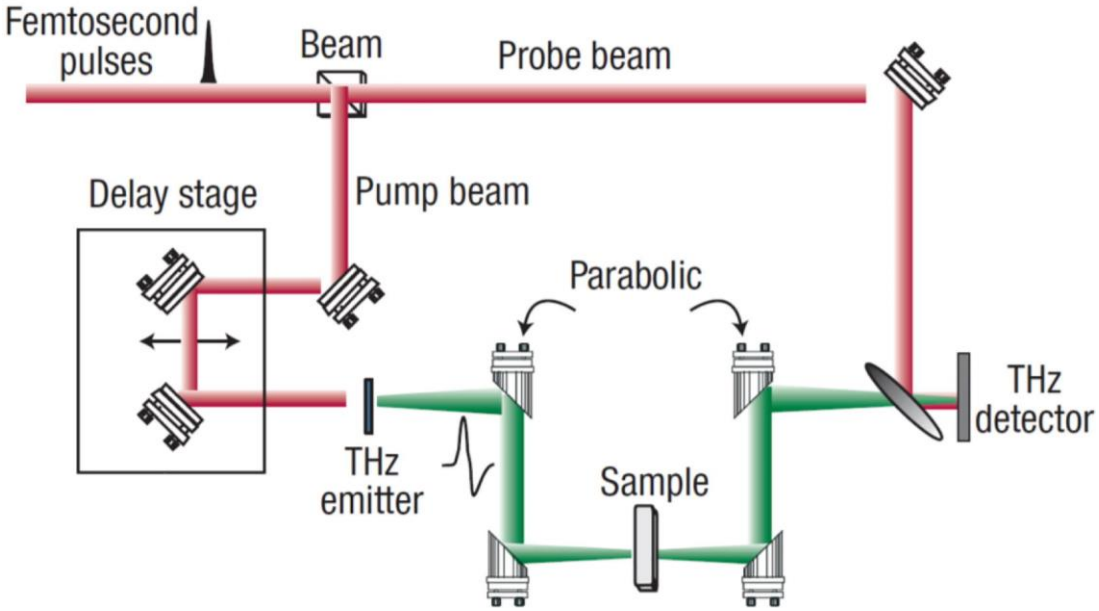
- not perceptible by the human eye
- not ionizing
- can cross many non-conducting common materials (*i.e.* paper, fabrics, wood, plastic, organic tissues)

vibration frequencies of (bio)molecules in THz region are related to ***collective vibrations***, ***distorted vibrations*** and **structural deformation**

THz radiation allows ***contactless*** and ***non-destructive*** analysis of the materials under investigation with resolution higher than micro- and millimeter waves

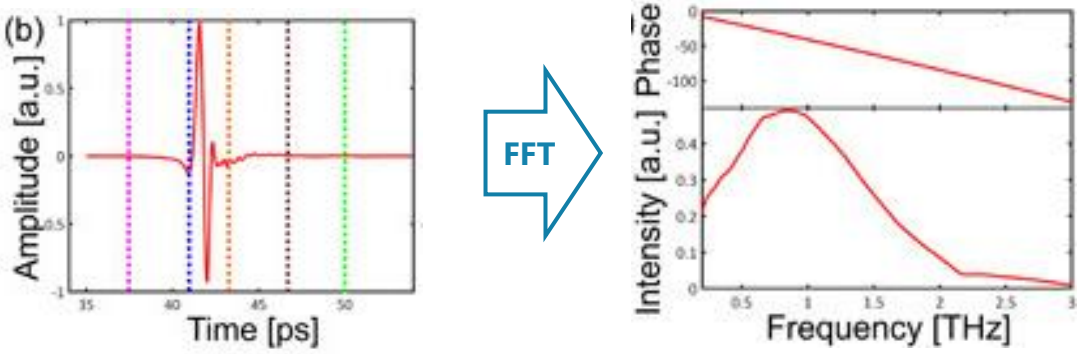
THz Time-Domain Spectroscopy (THz-TDS)

THz-TDS directly measures the ***transient electric field*** (rather than its intensity)



THz-TDS can work in ***transmission*** and ***reflectance*** mode

$$\frac{1}{\sqrt{2\pi}}\int_{-\infty}^{\infty} E(t)e^{-i\omega t}\,dt$$



complex refractive index $\tilde{n} = n + ik$

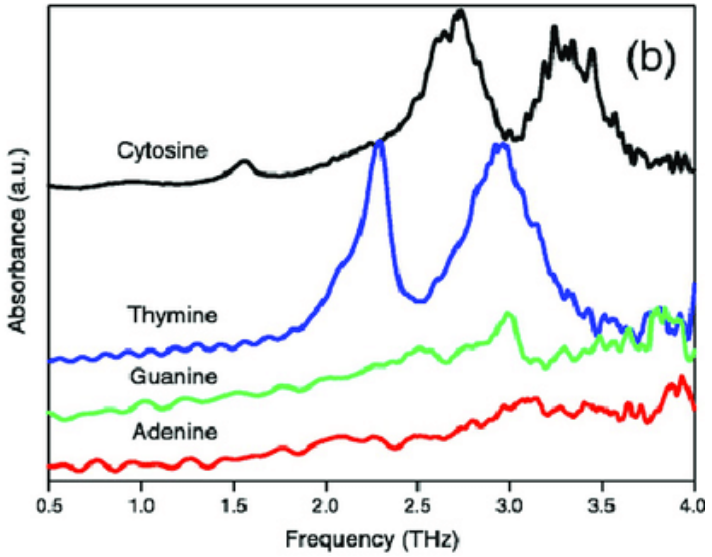
n: refractive index
k: absorption (or extinction coeff.)

dielectric permittivity $\tilde{\varepsilon} = \varepsilon_1 + i\varepsilon_2 \; with \left[\tilde{n} = \sqrt{\tilde{\varepsilon}\tilde{\mu}/\varepsilon_0\mu_0}\right]$

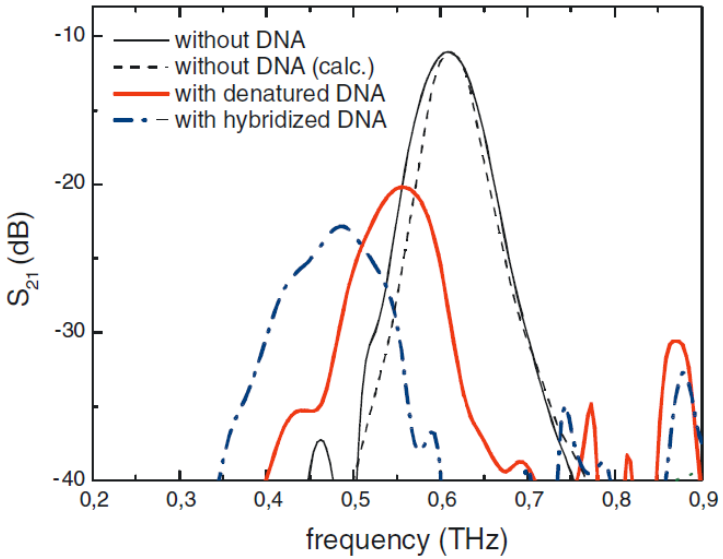
conductivity $\tilde{\sigma} = \sigma_1 + i\sigma_2 \; with \begin{cases} \sigma_1 = \varepsilon_0\omega\varepsilon_2 \\ \sigma_2 = \varepsilon_0\omega(\varepsilon_\infty - \varepsilon_1) \end{cases}$

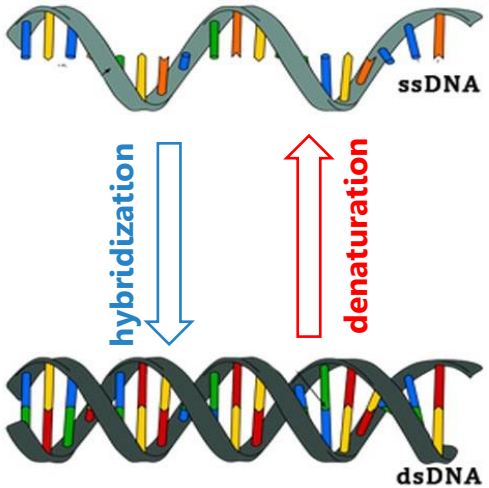
THz-TDS of Nucleotide & DNA

THz spectrum of nucleic acids reflect intermolecular collective vibrations, the lattice vibrations and the configuration characteristics of nucleic acid molecules



Four nucleotides (adenine, cytosine, guanine, thymine) show characteristics bands near 3 THz [*Spectrochim. Acta A: Mol. and Biomol. Spec.* **179**, 255 (2017)]



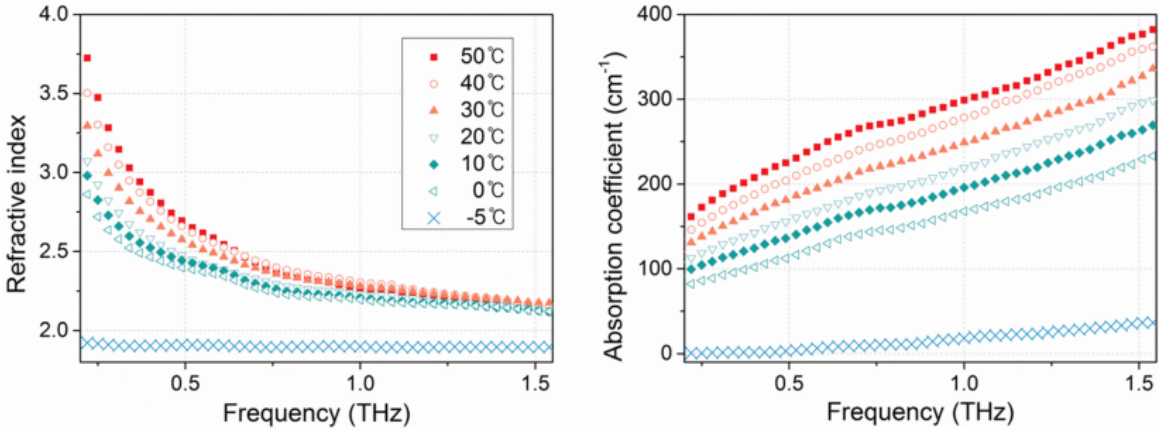


Frequency dependence of THz signal for hybridized, denaturated and without DNA. THz fingerprint enables differentiation between single- and double-stranded DNA [Phys. Med. Biol. **47**, 3815 (2002)].

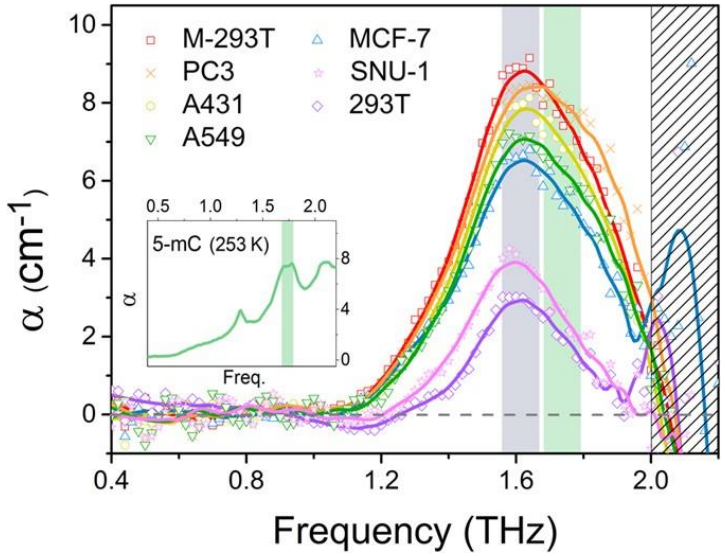
THz Spectroscopy for Tumor Detection

THz-TDS is capable of distinguishing between healthy and malignant tissues, being sensitive to:

- morphology
- water content (dielectric permittivity, refractive index, absorption coefficient, dielectric relaxation)

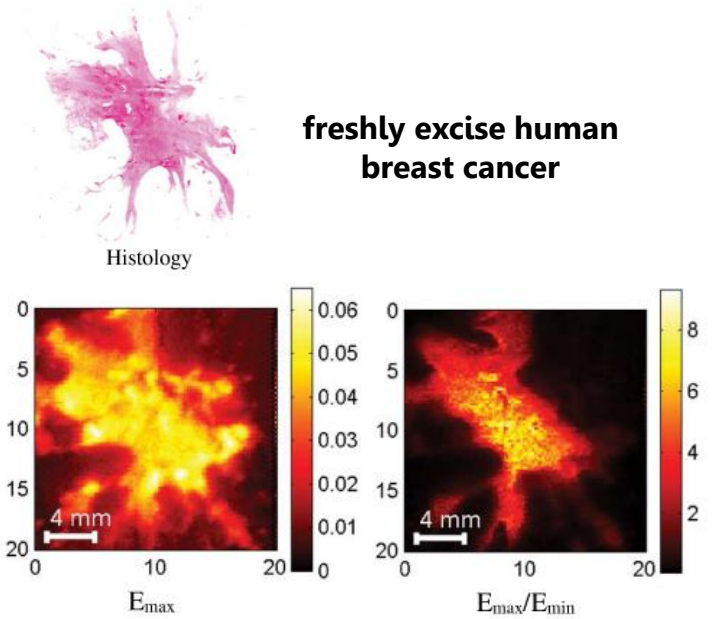


Refractive index and absorption coefficient of water molecules in the THz region, as functions of frequency and temperature.

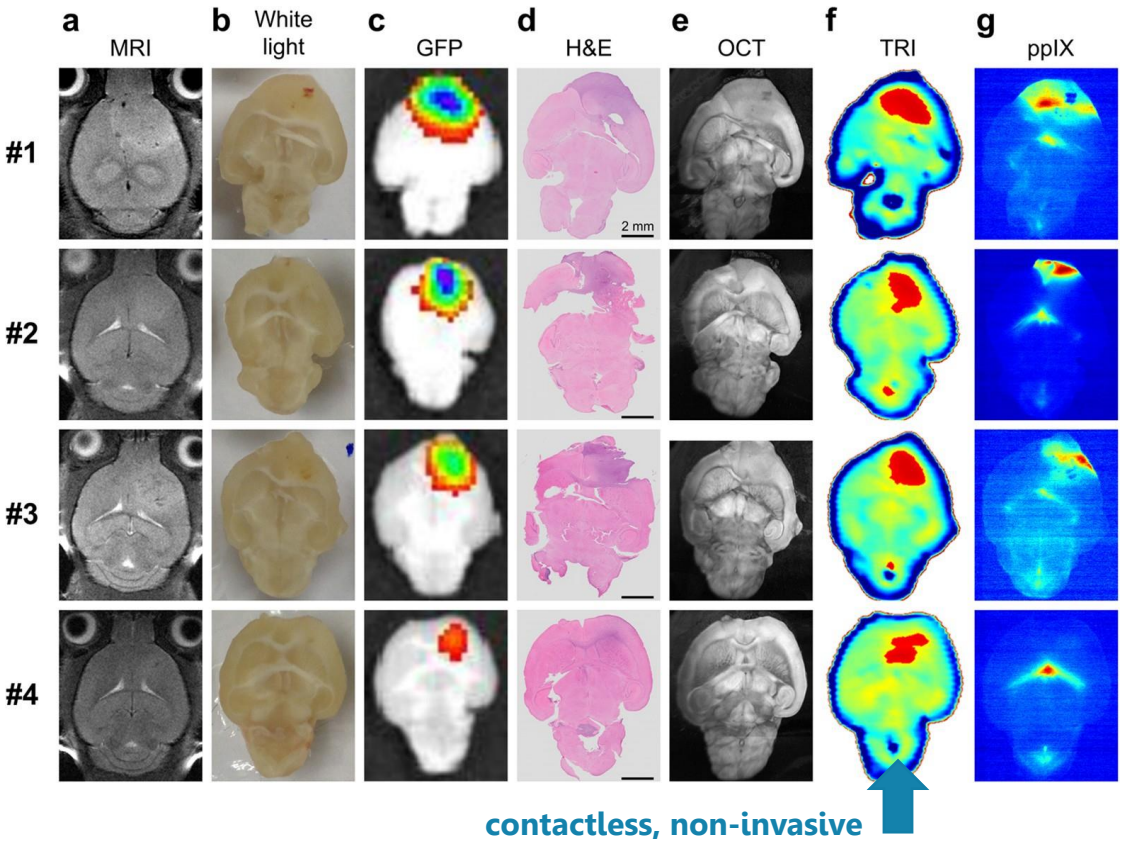


THz absorption coefficient of two control samples of DNA (kidney cell M293T and 293T) and five types of cancer DNA: prostate cancer (PC3), skin cancer (A431), lung cancer (A549), breast cancer (MCF-7) and gastric cancer (SNU-1), originated from methylation measured at -20°C [IEEE J. of Sel. Topics in Quantum Elect. **23**(4), 8600109 (2017)].

THz Imaging for Tumor Detection

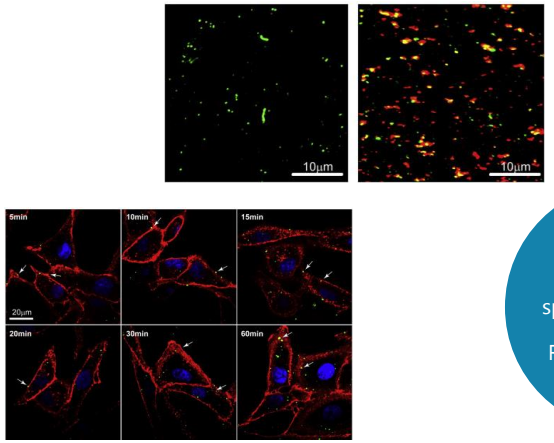


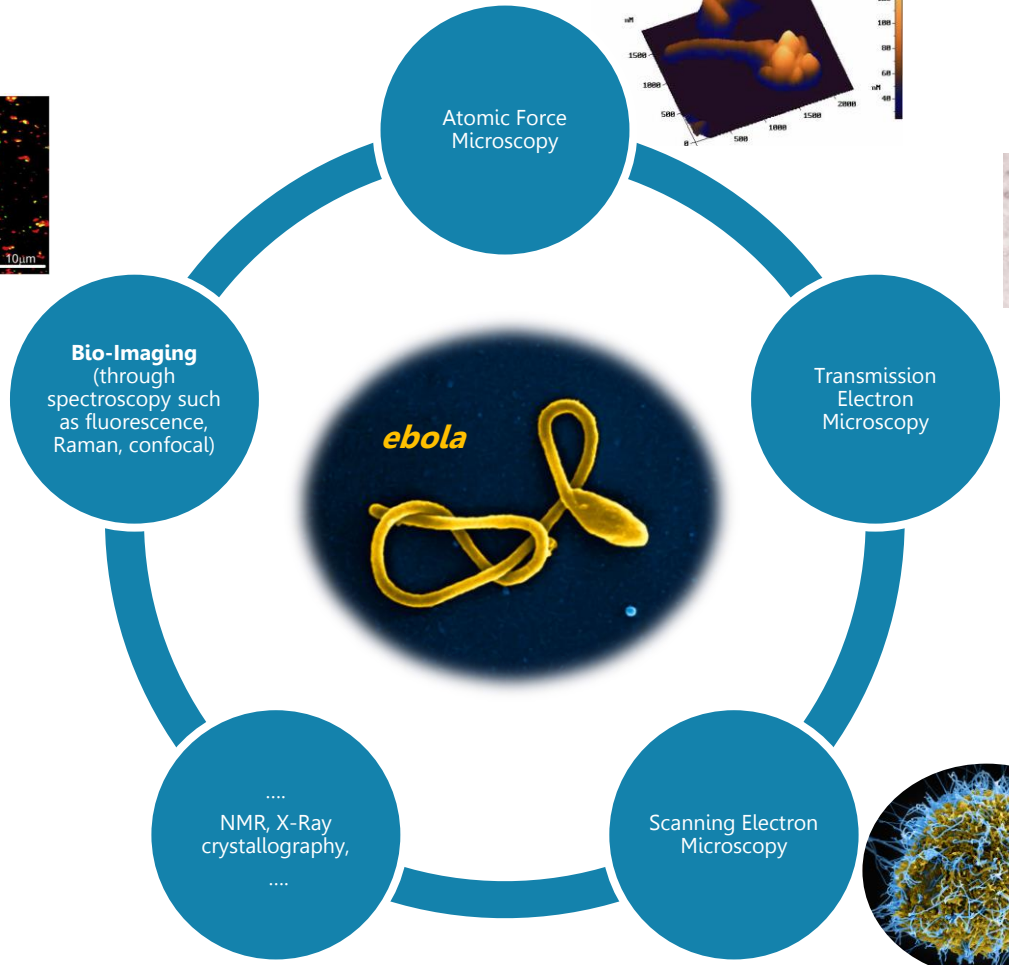
THz images generated using the maximum of the reflected pulse (E_{max}) and the ratio between maximum/minimum of the reflected pulse (E_{max}/E_{min}), along with histology report [Opt. Exp. **17**(15), 12444 (2009)]

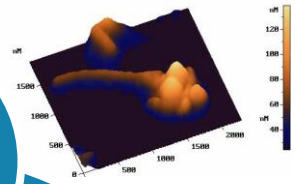


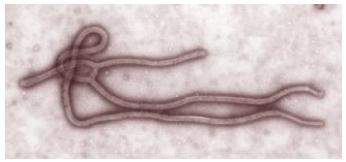
Tumor discrimination in mice using (**a**) MRI, (**b**) white light, (**c**) GFP fluorescence, (**d**) hematoxylin and eosin (H&E) stained images, (**e**) optical coherence tomography (OCT), (**f**) TRI and (**g**) 5-ALA-induced ppIX fluorescence [Sci. Rep. **6**, 36040 (2016)].

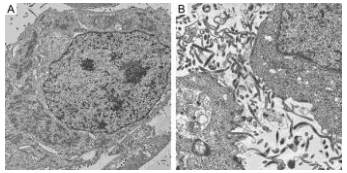
Complementary Techniques

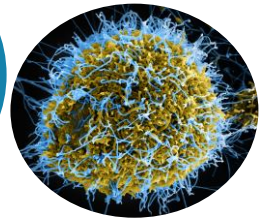












Summary of Today

Spectroscopy: *study structure and molecular arrangement through spectral response*

- excitation and light emission (*i.e.* absorption, fluorescence)
- THz spectroscopy, with biomolecules showing a characteristics fingerprint

If you are interested in THz Spectroscopy
ELEC-E4760 Terahertz Techniques (period V)