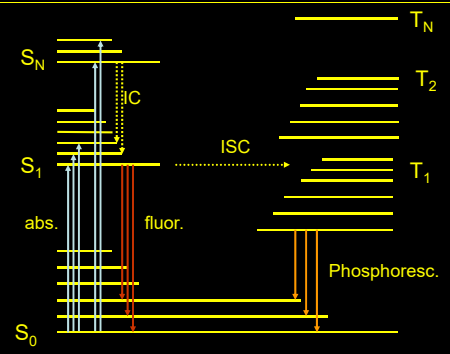
Experimental techniques in time-resolved spectroscopy

(basic methods)

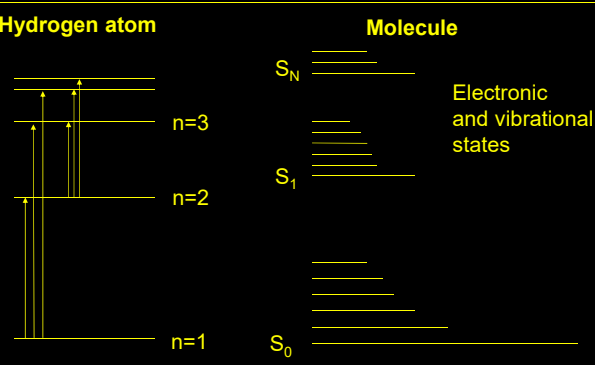
Time-resolved fluorescence spectroscopy

- Explores the time dependence of emission spectra in:
  - Molecules
  - Solids

Molecular energy levels: Jablonski diagram

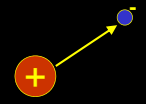


Molecular energy levels

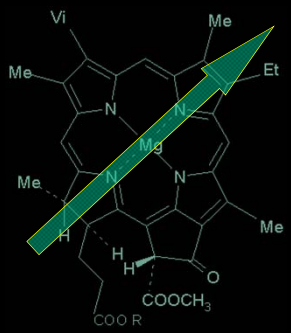


Molecule is a diplole wih respect to the external field

Hydrogen atom



Molecule (cholorphyll a)



In order for the molecule to interact with the light field and change the state, a dipole moment should interact with that field. It is called transition dipole moment. (It is different from a permanent dipole moment!)

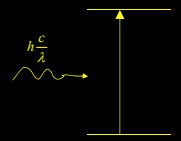
Molecule is a dipole

The magnitude of the dipole moment determines the intensity of absorption/emission line

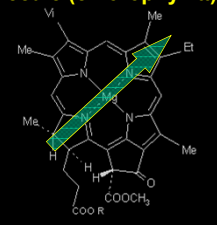
$$\mu_{fi} = \int \varphi_f^* \hat{\mu} \varphi_i \, dr$$

Transition probability (a.k.a. Einstein coefficient)

$$P_{fi} \sim \left|\mu_{fi}\right|^2$$



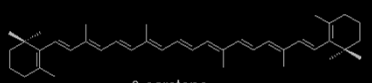
Molecule (chlorophyll *a*)



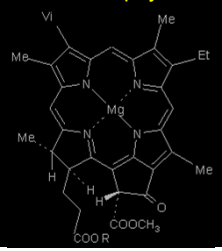
Electronic spectra and π-conjugated systems

General principle of physics: the smaller the box, the further apart are the energy levels. A box can be the extent of the molecular conjugated electron system, or the size of a quantum structure in a semiconductor.

Beta-carotene

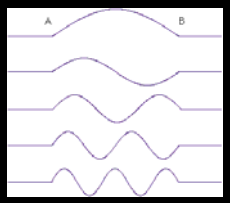


Chlorophyll



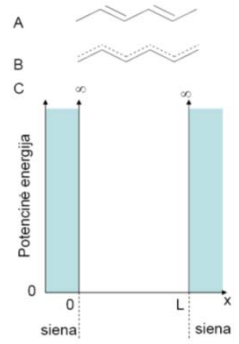
Electronic spectra and π-conjugated systems

The physical basis is the QM problem called particle in a box. Analogy is valid because in pi-conjugated bond system electrons are relatively free to move about.



The energy spacing between allowed states depends on the size of the box!

VIS/nIR spectra and π electron systems



Schrödinger equation for particle in the box:

$$-\frac{\hbar^2}{2m}\frac{d^2\psi}{dx^2} = E\psi$$

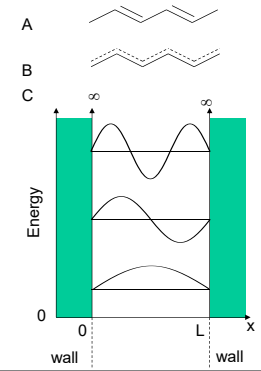
Solution inside the box:

$$\psi = C\sin\frac{p}{\hbar}x + D\cos\frac{p}{\hbar}x$$

The particle is free in the box, and has only kinetic energy:

$$E = \frac{p^2}{2m}$$

VIS/nIR spectra and π electron systems



Boundary condition (value of wavefunction at the start of the box)

$\psi = 0$, when $x = 0, L$

Nodes at x=0 ir x=L:

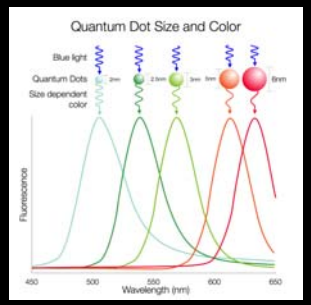
$$\psi = C\sin\frac{p}{\hbar}x$$

$$\frac{p}{\hbar}L = n\pi,\ n = 1, 2\ldots$$

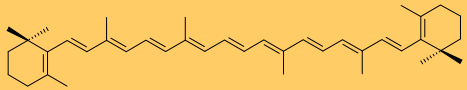
Energy spectrum:

$$E_n = \frac{n^2\hbar^2\pi^2}{2mL^2}$$

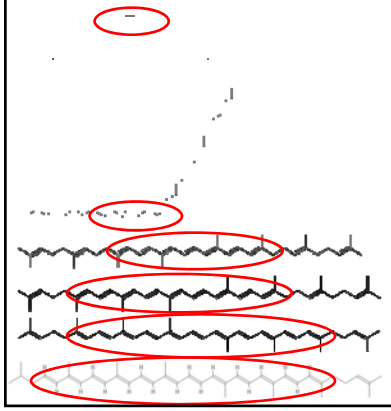
Quantum dots: tiny three dimensional boxes for electrons

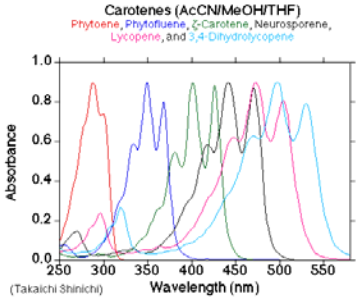


β-carotene: the "model" carotenoid



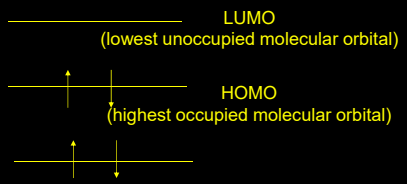
Carotenoids: colorful pigments of nature





Molecular energy levels

Electrons fill in molecular orbitals in such a way that the total energy of the molecule is lowest – two electrons with opposite spins in each molecular orbital.



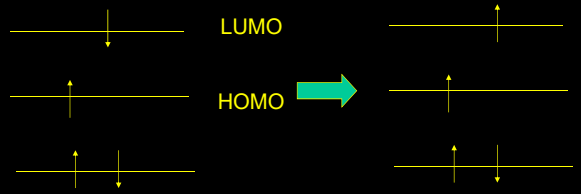
The state with zero net spin are called singlet states

Molecular energy levels

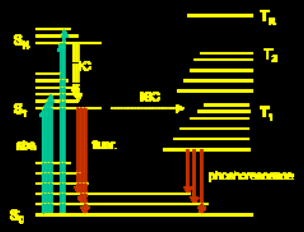
When the molecule becomes excited, there is a non-zero probability for the spin of excited electron to flip. This results in a triplet state (multiplicity=2xspin+1, 2x1+1=3, hence triplet)

Singlet excited state anti-parallel spins

Triplet excited state, parallel spins, net spin is 2x½=1

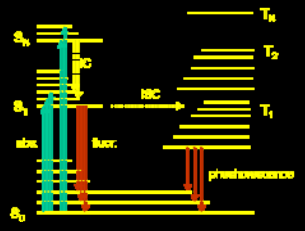


Several general rules about molecular spectra



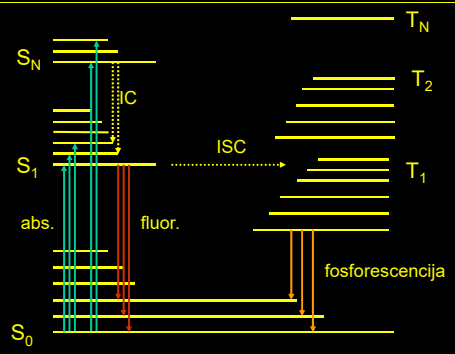
1. Absorption occurs from the bottom level of the ground state (the universal laziness principle ☺)
2. Emission occurs from the bottom of the excited state vibration manifold (Kasha's rule)

Several general rules about molecular spectra



The principle of mirror symmetry: fluorescence spectrum is the mirror image of the absorption spectrum. The energy difference between the fluorescence and lowest absorption maximma is called Stokes' shift.

Jablonski diagram



Each transition between any pair of states has its own transition dipole moment.

Molecular energy levels: Jablonski diagram

Allows a qualitative explanation of absorption, fluorescence and phosphorescence spectra

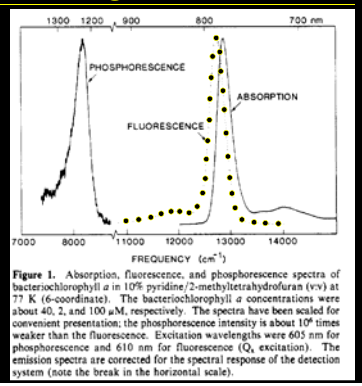


Figure 1. Absorption, fluorescence, and phosphorescence spectra of bacteriochlorophyll *a* in 10% pyridine/2-methyltetrahydrofuran (v:v) at 77 K (6-coordinate). The bacteriochlorophyll *a* concentrations were about 40, 2, and 100 μM, respectively. The spectra have been scaled for convenient presentation; the phosphorescence intensity is about 10^6 times weaker than the fluorescence. Excitation wavelengths were 605 nm for phosphorescence and 610 nm for fluorescence (Q_x excitation). The emission spectra are corrected for the spectral response of the detection system (note the break in the horizontal scale).

Larry Takiff and Steven G. Boxer*

J. Am. Chem. Soc. **1988**, *110*, 4425–4426

Fluorescence spectroscopy

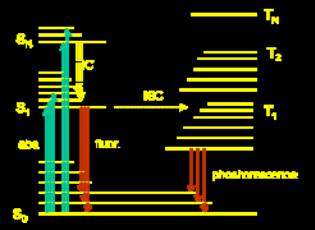
After exciting the sample with light, we detect emitted photons
Advantages:
- High selectivity
- High sensitivity
- Sensitive to the processes in the excited state (relaxation)

Fluorescence spectroscopy

Quantum yield of fluorescence

$$\phi = \frac{k_{fl}}{k_{fl} + k_{ic} + k_{isc} + k_{other}}$$

other can be:
- energy transfer,
- photoreactions
- fluorescence quenching...



Fluorescence spectroscopy

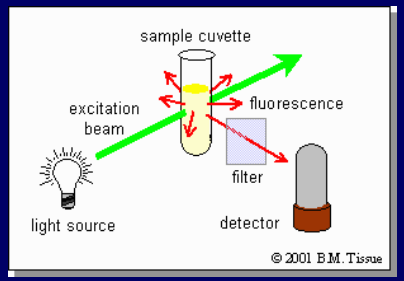
Measured emission intensity on the molecule

$$I \sim \phi \left| \mu_{fi} \right|^2$$

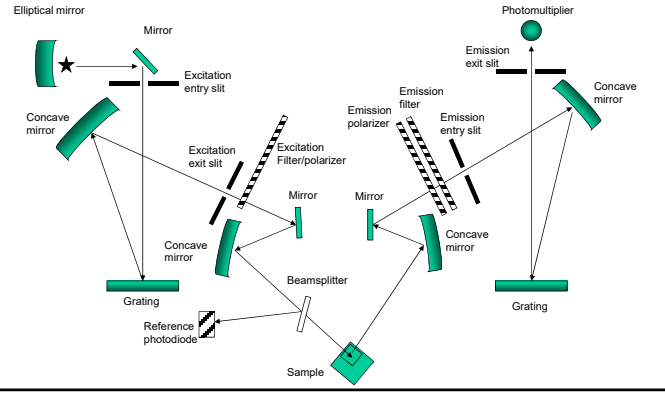
Depends BOTH on the molecular properties (i.e. transition dipole moment), and the processes taking place in the excited state and determining quantum yield.

What is the relationship between the measured fluorescence lifetime and radiative relaxation rate?

Principle of fluorescence experiment



Layout of a realistic spectofluorometer



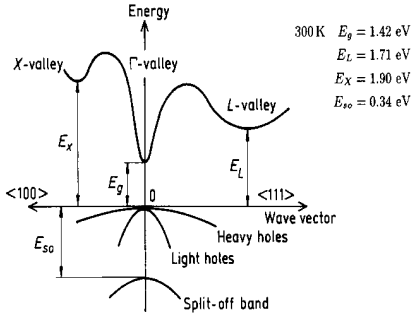
Molecule was excited. What can happen?

- Radiative relaxation
- Internal conversion
- Intersystem crossing
- Excitation energy transfer
- Solvation
- Photoinduced reaction (e.g., isomerization)

When the state of the molecule changes, emission spectrum will change. Therefore, emission is a way of observing all mentioned processes.

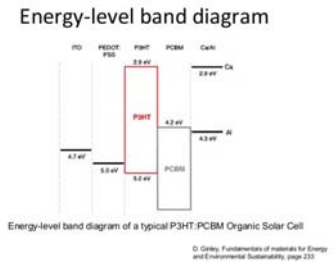
Only useful while excited state is preserved!

Band structures in solid state



300 K $E_g = 1.42$ eV
$E_L = 1.71$ eV
$E_X = 1.90$ eV
$E_{so} = 0.34$ eV

Gallium Arsenide



Organic solar cell (dispersed heterojunction)

A solid-state sample (semiconductor, dielectric, metal, amorphous/organic or crystalline) was excited. What can happen?

- Band-to-band recombination (light output)
- Shokley-Read-Hall (trap-assisted) recombination
- Radiative recombination (light output)
- Auger recombination
- Trap luminescence (light output)
- Non-radiative recombination (light disappearance)

If at least a fraction of carriers recombine in a radiative manner, the carrier dynamics can be observed in time-resolved fluorescence experiments.

Again, when the light output stops, we stop seeing it.

A quantum well/wire/dot was excited. What can happen?

- A hybrid behavior between solid state (bands, state continuum) and molecules (discreet states).

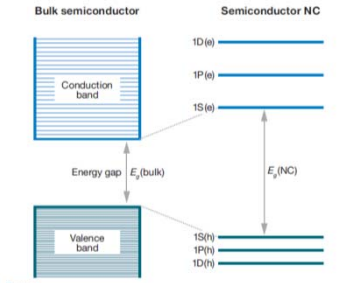
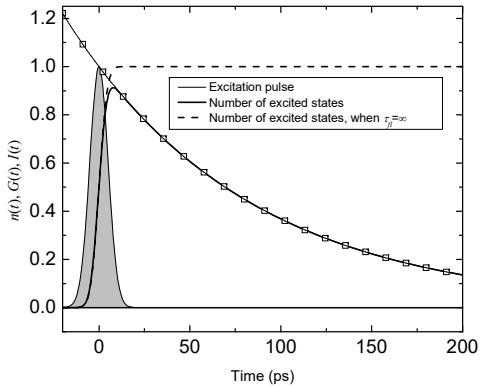
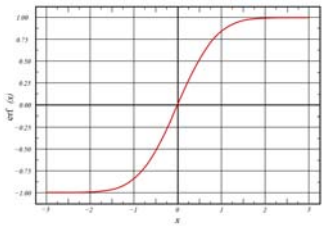


Figure 1
A bulk semiconductor has continuous conduction and valence energy bands separated by a fixed energy gap, E_g, whereas a semiconductor nanocrystal (NC) is characterized by discrete atomic-like states and an NC size-dependent energy gap. In a simple model of a spherical quantum well with an infinite barrier, the NC energy gap, E_g(NC), relates to the bulk semiconductor energy gap, E_g(bulk), by the following expression: $E_g(\mathrm{NC}) = E_g(\mathrm{bulk}) + \frac{\pi^2\hbar^2}{2\mu R^2}$, where R is the NC radius, $m_r = (m_e^{-1} + m_h^{-1})^{-1}$, and m_e and m_h are the electron and hole effective masses, respectively. The NC energy structures are shown for the model case of a two-band semiconductor, which has a single parabolic conduction band and a single parabolic valence band.

V. Klimov Annu. Rev. Phys. Chem. 2007. 58:635–73

Simple kinetics





Time resolved fluorescence techniqes:

Time-correlated single photon counting

Fluorescence consists of single photons

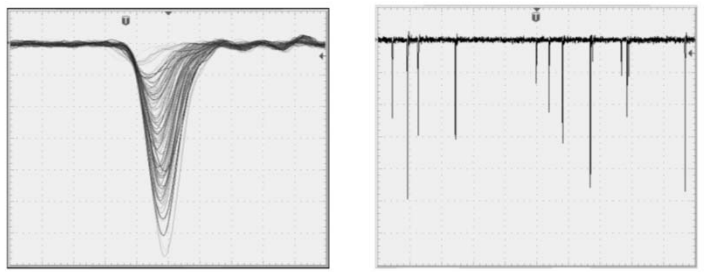


Fig. 118: Single-photon pulses delivered by a R5900 PMT (left, 1 ns / div) and output signal of the PMT at a photon detection rate of 10^7 s^{-1} (right, 100 ns / div). Operating voltage -900V, signal line terminated with 50 Ω.

Excite the sample with high rep.rate laser

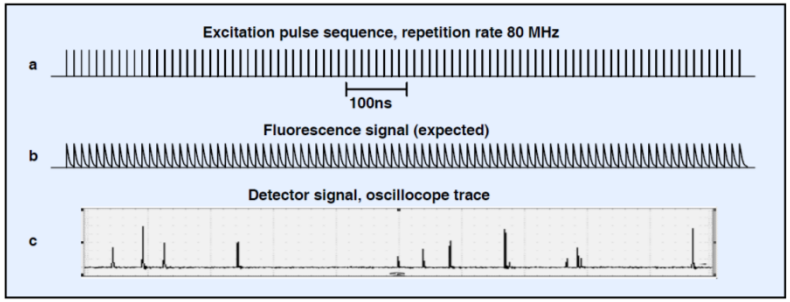
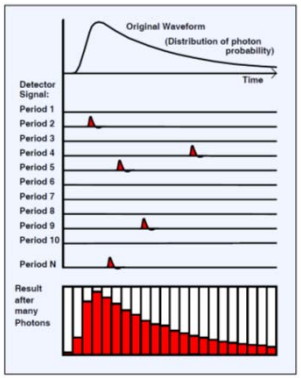


Fig. 119: Detector signal for fluorescence detection at a pulse repetition rate of 80 MHz

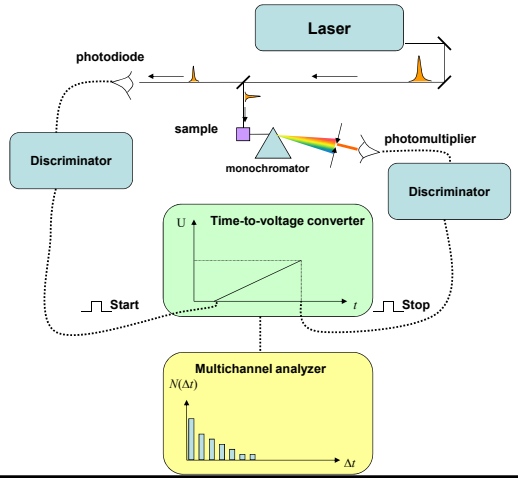
Excite the sample with high rep.rate laser



The histogram of photon arrival times (with respect to the corresponding laser pulses) is the fluorescence decay curve.

The method relies on statistics

Only the timing (not signal amplitude) noise is important



Constant fraction discriminator: a way to avoid timing noise in variable amplitude signal

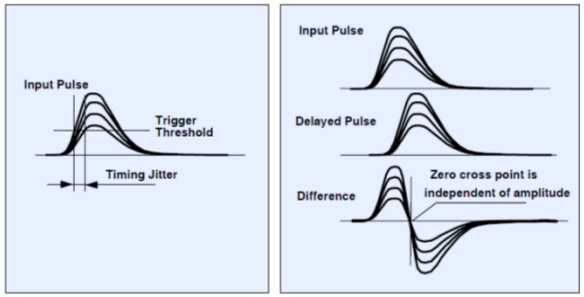


Fig. 158: Leading-edge triggering (left) and constant-fraction triggering (right)

Constant fraction of the total amplitude of a particular pulse. The circuit also discriminates on the total amplitude (threshold) to reject very small spurious pulses.

Constant fraction discriminator: a way to avoid timing noise in variable amplitude signal

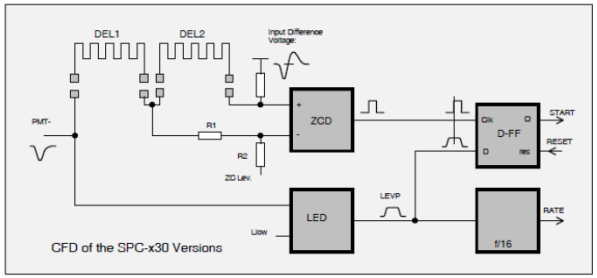


Fig. 162: Principle of the CFD in the detector (start) channel of the -30 SPC versions

ZCD – a comparator (ultrafast infinity gain op-amp); D-FF – a leading edge trigger (similar to oscilloscope); LED – leading edge detector.

Time-to-amplitude converter (TAC): a ramp generator with rudimentary brain

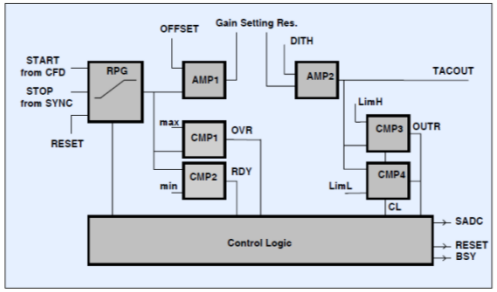


Fig. 165: Principle of the TAC

- Output window control (CMP3, CMP4),
- Dithering for ADC to correct bit noise

ADC with dithering: bit noise reduction

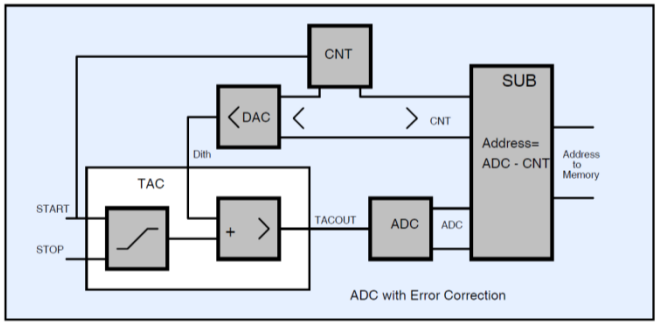
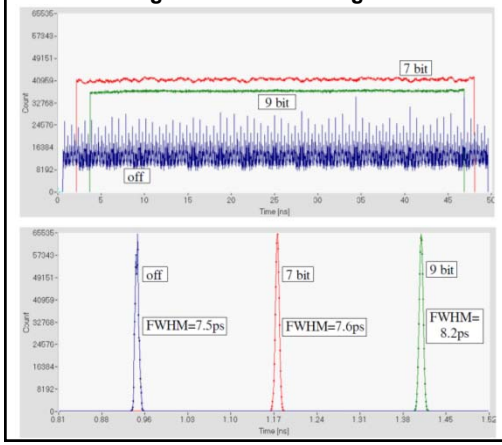


Fig. 166: TAC/ADC principle used in the bh TCSPC modules

- DAC generates an additional periodic signal for ADC. It is later subtracted from the address, because it is known.
- Since the time of different photons with identical arrival time is now randomized, the ADC characteristic becomes smooth instead of step-like

Dithering invalidates the edges of the ADC characteristic, but is worth it

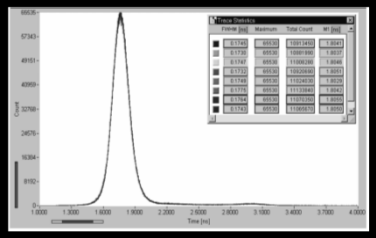


The sides are 'cut out' of the window

No significant effect on electrically measured time resolution

Detectors for TCSPC

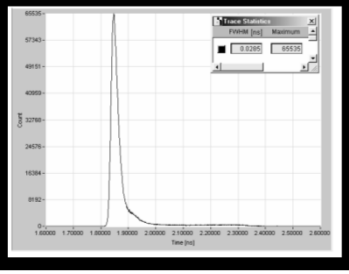
- PMT+HV module + TEC cooling + overload protection



Detectors for TCSPC

Fig. 184: Hamamatsu R3809U MCP

- MCP PMT – best time resolution, but tricky to operate;
- Expensive
- Easy to damage by overload



Hybrid PMTs

- Photoelectrons are accelerated and injected into an avalanche photodide
- Fast response, no afterpulsing

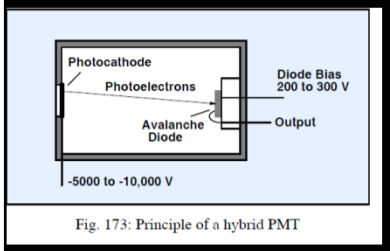
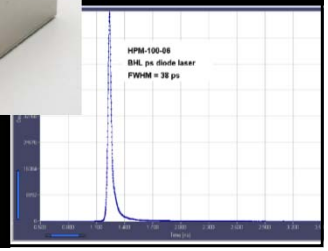


Fig. 173: Principle of a hybrid PMT



Actively quenched SPAD detectors

Very good, but low active area (20-50 um diameter typical)

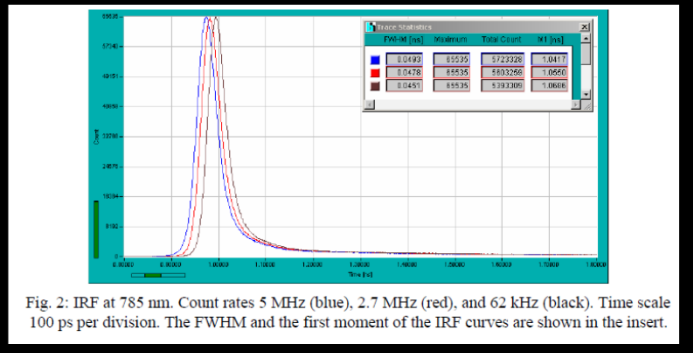


Fig. 2: IRF at 785 nm. Count rates 5 MHz (blue), 2.7 MHz (red), and 62 kHz (black). Time scale 100 ps per division. The FWHM and the first moment of the IRF curves are shown in the insert.

TCSPC spectral measurement

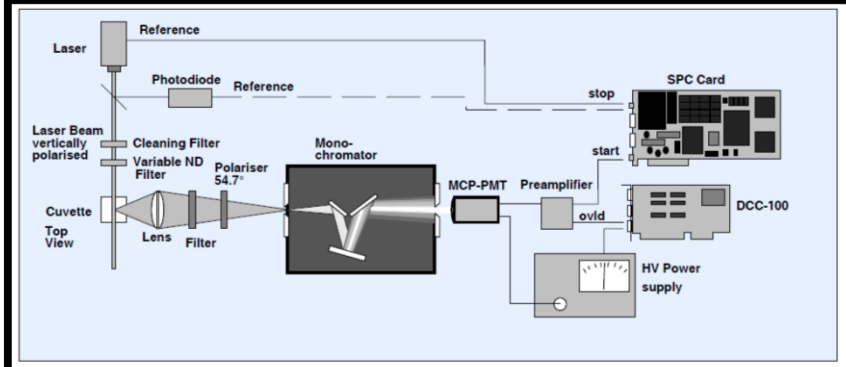
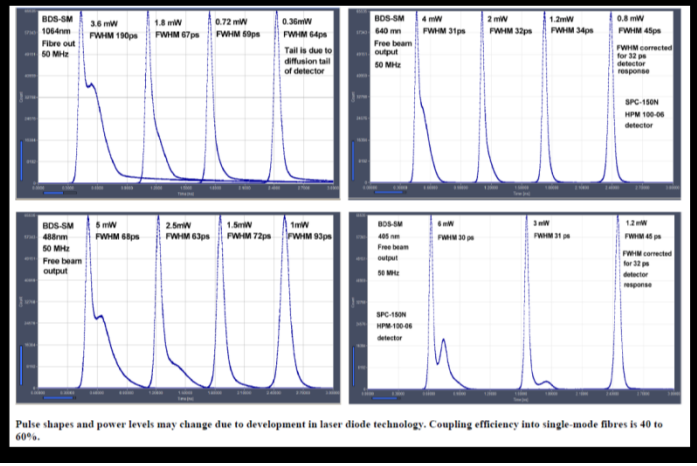


Fig. 354: Typical setup of a fluorescence lifetime spectrometer

Laser sources can be cheap

- 40 mm x 40 mm x 110 mm
- Free-beam or fibre output
- Pulse width down to 60 ps
- Repetition rate 5 MHz / 50 MHz (switchable)
- All electronics integrated
- No external driver unit
- Simple +12V supply

4000-13000 EUR



Pulse shapes and power levels may change due to development in laser diode technology. Coupling efficiency into single-mode fibres is 40 to 60%.

A low cost high repetition rate picosecond laser diode pulse generator.

Wilfried Uhring*[a], Chantal-Virginie Zint[a], Jeremy Bartringer[a]

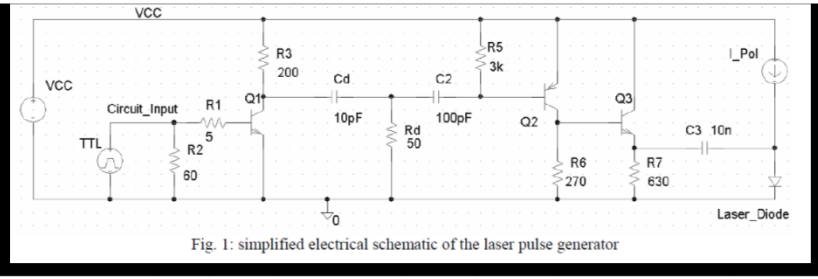


Fig. 1: simplified electrical schematic of the laser pulse generator

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A low cost high repetition rate picosecond laser diode pulse generator.

Wilfried Uhring*[a], Chantal-Virginie Zint[a], Jeremy Bartringer[a]

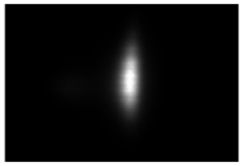


Fig. 6(a). Streak image of the optical pulse with optimal polarization current at 830 nm.

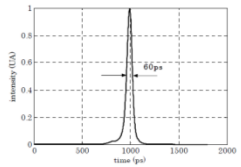
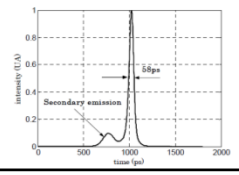
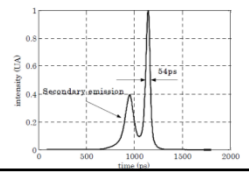


Fig. 6(b). Horizontal profile of the streak image. The pulse width is 60 ps FWHM with optimal polarization current.

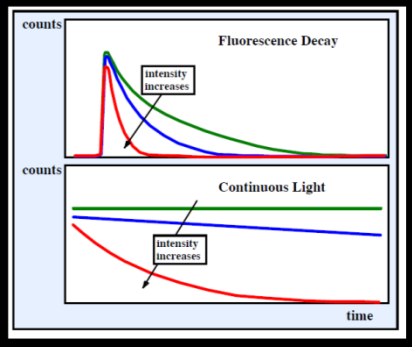




Common problems in TCSPC

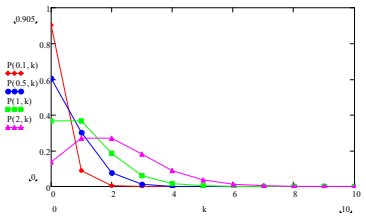
Classic pile-up

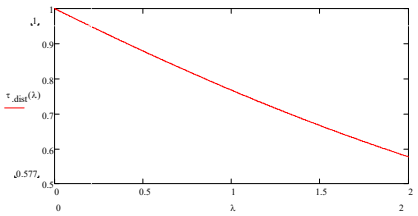
- The TCSPC data is only correct when significantly less than one photon is detected during each measurement. Otherwise the first photon of the two, three etc. will be registered and the apparent lifetime will be shorter.



Classic pile-up

- How much is too much?





- If 5% error in lifetime is acceptable, the upper limit on the counting rate is roughly 0.2 photons per detection period, or the counting rate should be less than 20% of the laser repetition rate.

Inter-pulse pile-up in high rep. rate measurements

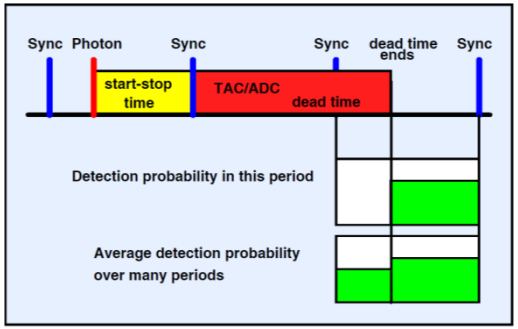
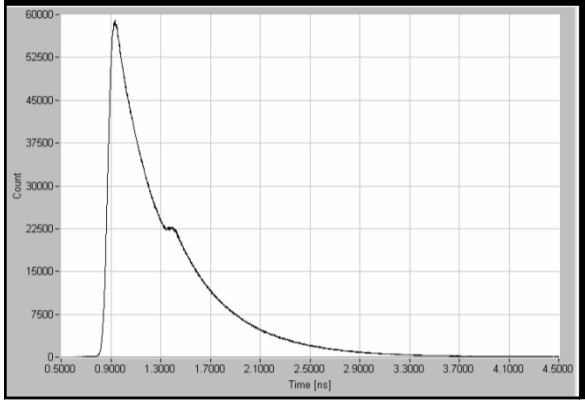


Fig. 342: Mechanism of inter-pulse pile-up

Optical reflections



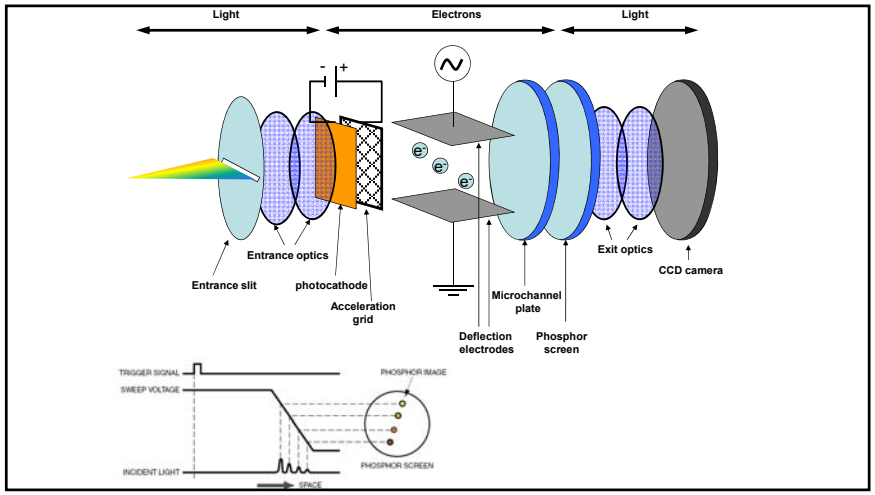
Looks like inter-pulse pile-up, Hard to avoid, but can be minimized by tilting the suspect component, or placing it in converging/diverging beam

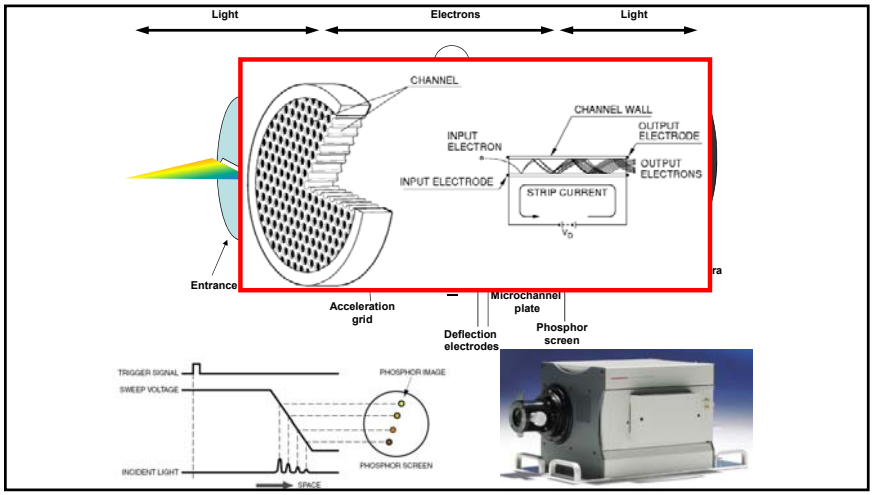
TCSPC: features

- Time resolution 50-250 ps (limited by electronic jitter of the detector)
- 'Cheap'
- Good signal-to-noise (as good as you are willing to wait)
- No intense lasers necessary (semiconductor lasers are enough)
- Single-color
- Suitable for imaging (FLIM)

Time resolved fluorescence techniqes:

Streak camera



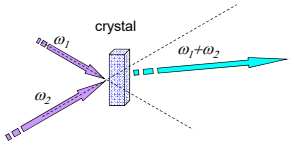


Streak camera

- Simultaneous measurement of the spectrum and the kinetics.
- Very sensitive
- Time resolution of synchroscan cameras down to 1-2 ps.
- Expensive (~500 k€) ;

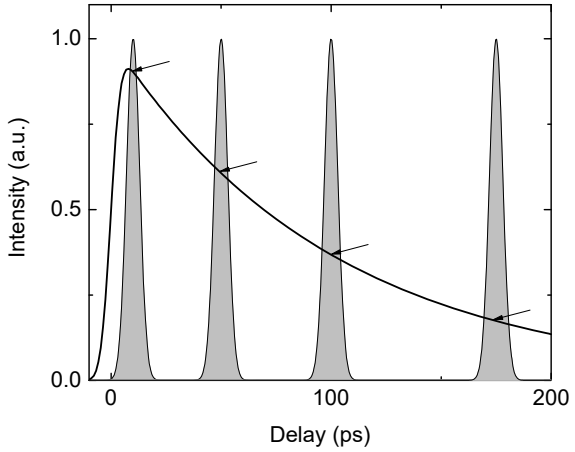
Time resolved fluorescence techniqes:

upconversion

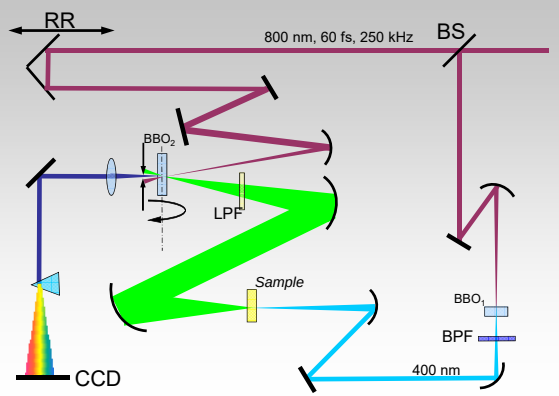


$$I_{sum}(t) = I_{gate}(t) \times I_{fluoresc.}(t)$$

$$\frac{1}{\lambda_{sum}} = \frac{1}{\lambda_{gate}} + \frac{1}{\lambda_{fluoresc.}}$$



Dispersed Fluorescence Upconversion Setup



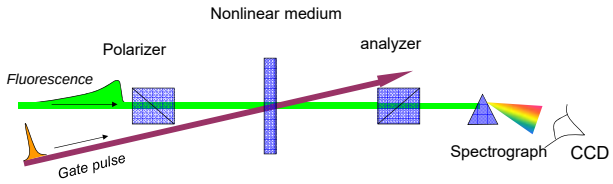
Fluorescence upconversion

- Problem: calibration of spectral sensitivities at different wavelengths
- Time resolution down to 50fs!
- A lot of excitation light required (bad for the samples)
- Experiments take time (one wavelength is phasematched at a time)
- Wavelength resolution limited by the spectral width of the gate pulse.

Time resolved fluorescence techniqes:

Optical Kerr shutter

Kerr shutter



$$n = n_0 + n_2 I$$

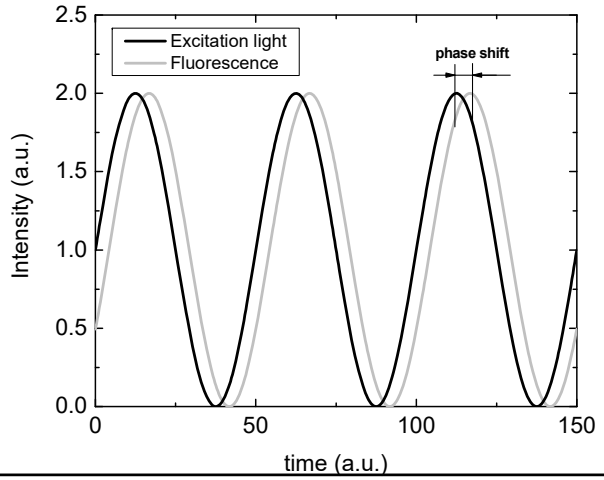
Optical Kerr effect – birefringence induced by the (polarized) electric field.
Nonlinear medium operates as a shutter that is open only during the gate pulse.

Optical Kerr shutter

- Entire fluorescence spectrum measured at a time
- Time resolution down to 50 fs
- Extremely high laser intensities required
- Materials with large Kerr effect have intertial response (CS_2, water)
- Troublesome experimental implementation

Time resolved fluorescence techniques:

Phase fluorimetry (a.k.a. frequency domain fluorescence lifetime measurement)



More frequencies – more complex decays can be disentangled

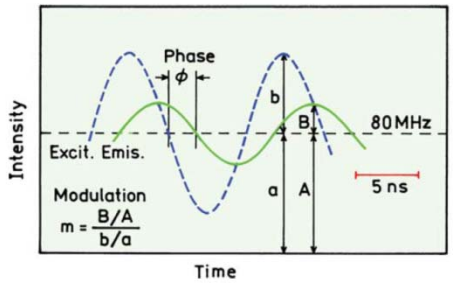
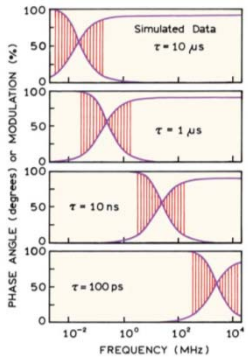


Figure 5.2. Definitions of the phase angle and modulation of emission. The assumed decay time is 5 ns and the light modulation frequency is 80 MHz.

Molecules are acting as an integrating filter in electronics



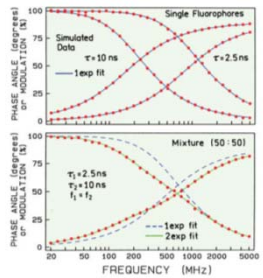


Figure 5.5. Simulated frequency-domain data for single- (top) and double- (bottom) exponential decays. The phase angle increases and the modulation decreases with increasing modulation frequency. The datapoints indicate the simulated data. **Top**: The solid lines show the best fits to a single decay time. **Bottom**: The dashed and solid lines show the best single- and double-exponential fits, respectively.

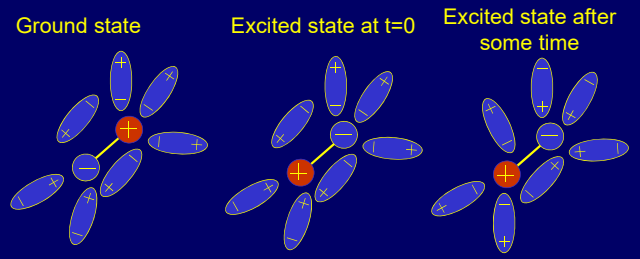
Phase fluorimetry (time domain lifetime measurements)

- Current modulation techniques allow wideband sweeping of the modulation frequency to de-construct response curves.
- Non-intuitive artifacts.
- Decay recovery is based on the assumptions on the (exponential?) decay of emission.
- No expensive equipment required (?)
- Good choice for ‘quick and dirty’ analysis of multiple samples.

Processes we will look at:

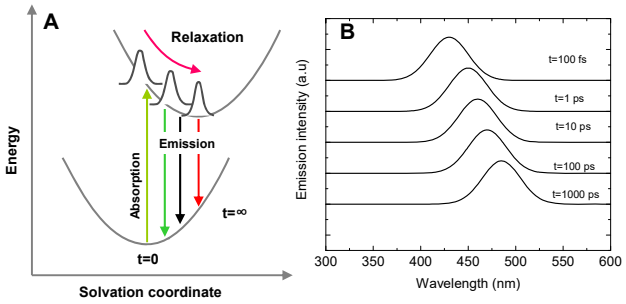
- Solvation
- Excitation energy transfer (EET)
- Vibrational relaxation
- Charge transfer (electron, proton)

Conceptual example: solvation

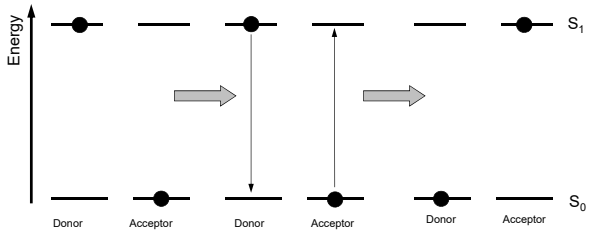


Adjustment of solvent molecules around the solute to minimize the overall system energy.

Conceptual example: solvation

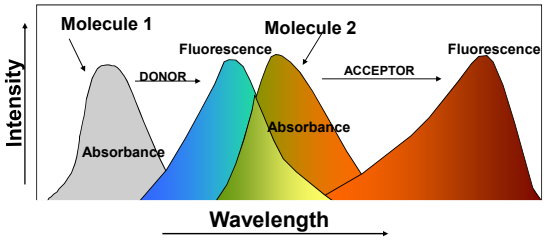


Förster energy transfer



Förster Resonance Energy Transfer

$$k_{DA} = \frac{9\ln(10)\kappa^2 c^4 \phi_D}{80\pi n^4 N_{av} \tau_D R^6} \int \frac{F_D(\omega) A_A(\omega)}{\omega^4} d\omega$$



Assumptions

- When transfer is over, the correlation between donor and acceptor state is lost
- No orbital overlap between donor and acceptor (large distances)
- Dipole-dipole coupling
- Donor has relaxed to the bottom of its emissive state

After all these assumptions it is almost a miracle that the model works, but it does, and does it amazingly well.

FLIM and FRET

- Optical ruler for ~10 nm distances.

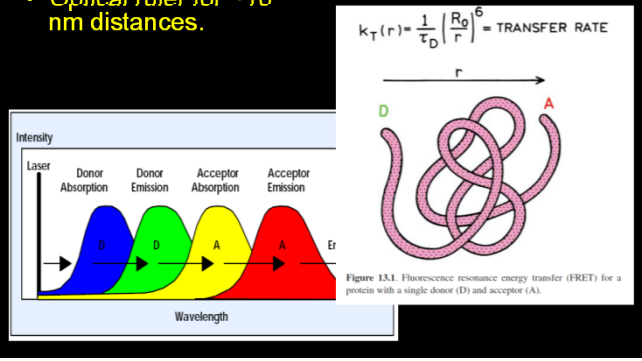
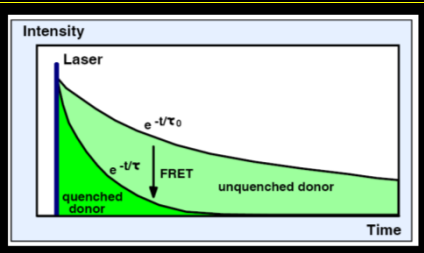


Figure 13.1. Fluorescence resonance energy transfer (FRET) for a protein with a single donor (D) and acceptor (A).

Energy transfer and donor lifetime



Donor fluorescence lifetime is reduced, because the acceptor is "sucking away" excited states

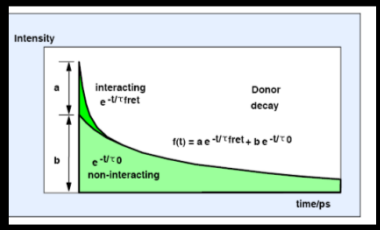
You could just look at the acceptor intensity, but

- Donor and acceptor concentrations in the cells are not known precisely
- Absorption spectra may overlap
- Calibration and control is tricky

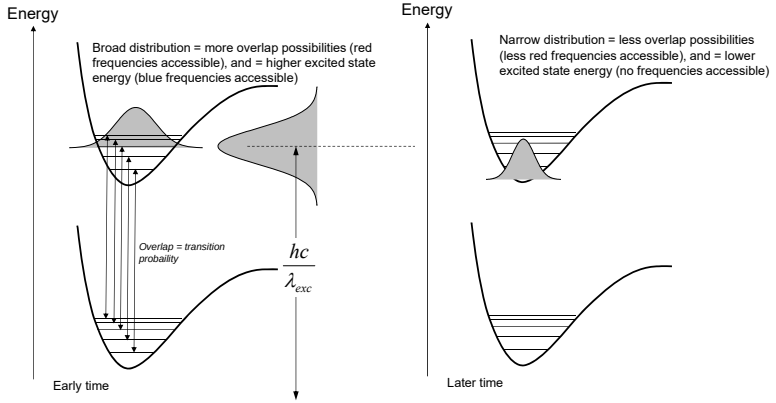
Time-resolved FRET

- Direct measurement of the reduction in donor lifetime

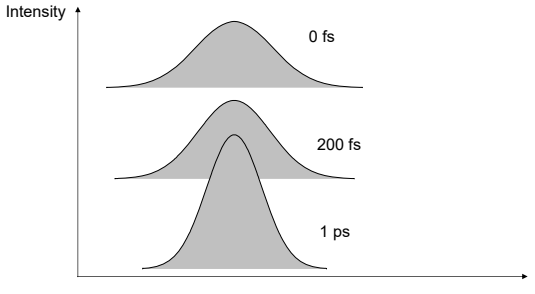
1. No FRET – slow donor decay
2. Yes FRET – part of the donors disappear quickly.



Vibrational relaxation

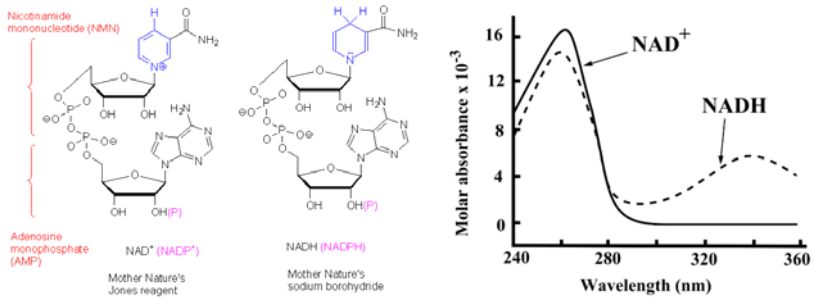


Vibrational relaxation



In simplest case, IVR will result in the narrowing of the emission spectrum. For typical molecules, the lifetimes of vibrationally excited states are of the order of a few ps. Overall (when many modes combine, the typical lifetimes can be 100 fs or less, depending on the density of vibrational states coupled to the electronic transition)

Charge transfer = production of different molecule



Charge transfer = production of different molecule

- Large shifts in spectral positions
- Large changes in dipole strength
- Spectra impossible to predict, but the dynamics will be sensitive to external electric fields, e.g. solvent polarity

Time resolved fluorescence: applications

Solvation dynamics

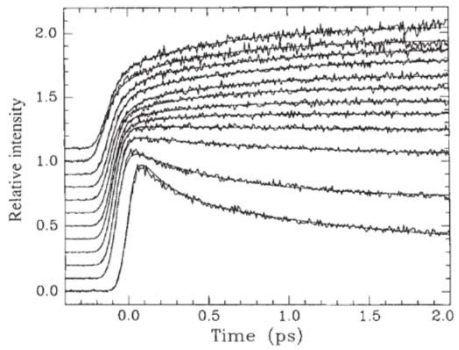


FIG. 1 Femtosecond fluorescence up-conversion data for coumarin 343 anion (sodium salt, 10^{-4} M) in aqueous solution. The traces show data obtained at wavelengths ranging from 460 nm (bottom) to 570 nm (top) in steps of 10 nm.
METHODS. A coherent Mira mode-locked Ti:sapphire laser operating at 850 nm was used. The second harmonic (425 nm) was generated in a 0.4 mm BBO (β-barium borate) crystal and used to excite the coumarin dye, which flowed through a 1 mm quartz cell. The remaining 850-nm light was used to gate the fluorescence by mixing the fluorescence emission in another 0.4 mm BBO crystal. The cross-correlation of the 425 nm and 850 nm pulses was measured as 100–110 fs (full width at half maximum). The intensity of the sum frequency was measured as a function of time delay between the 425 nm and 850 nm pulses, controlled with a stepper motor. By angle tuning the mixing crystal, 12 emission decays were collected at 10-nm intervals. The data plotted here are the relative intensities of upconverted light as a function of delay time. These data were fitted using global analysis, thereby constructing a set of time-resolved emission spectra which reproduce the measured data. The solvation response function S(t) (equation (1)) was then calculated from the set of spectra.

R. Jimenez et al.

NATURE · VOL 369 · 9 JUNE 1994

Solvation dynamics

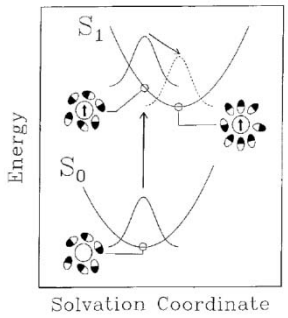


Figure 1. Schematic diagram of how an electronic transition in a solute can be used to study the dynamics of solvation. Shown here is a

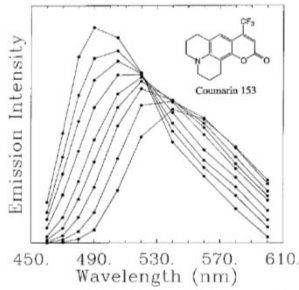


Figure 2. Representative time-resolved emission spectra of C153 (296 K) in formamide showing the continuous red shift with time characteristic of solvation dynamics. The times represented are 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 50 ps in order of decreasing peak intensity. (See ref 51 for more details.)

Fluorescence quenching in plants

Leaf response to prolonged illumination – reduction of fluorescence

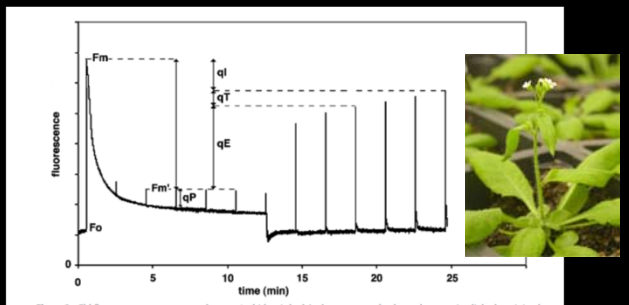
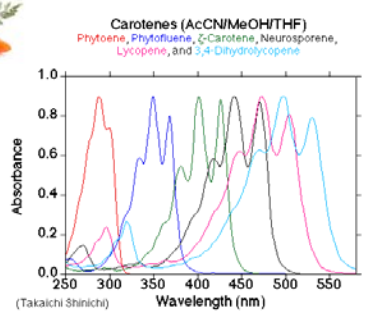


Figure 2. Chl fluorescence measurement from an Arabidopsis leaf. In the presence of only weak measuring light the minimal fluorescence (F_o) is seen. When a saturating light pulse is given, the photosynthetic light reactions are saturated and fluorescence reaches a maximum level (F_m'). Upon continuous illumination with moderately excess light (750 μmol photons m^{-2} sec^{-1}; growth light was 130 μmol photons m^{-2} sec^{-1}), a combination of qP and NPQ lowers the fluorescence yield. NPQ (qE + qT + qI) can be seen as the difference between F_m and the measured maximal fluorescence after a saturating light pulse during illumination (F_m'). After switching off the light, recovery of F_m' within a few minutes reflects relaxation of the qE component of NPQ.

P. Mueller et al. *Plant Physiology*, April 2001, Vol. 125, pp. 1558–1566, www.plantphysiol.org © 2001 American Society of Plant Physiologists

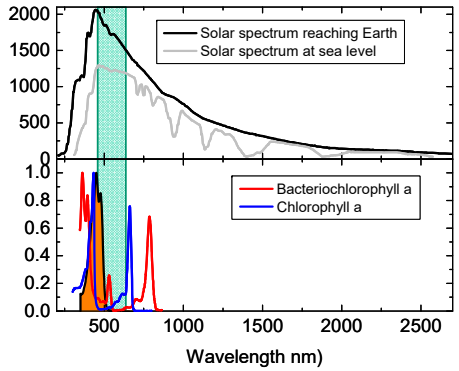
Carotenoids: colorful pigments of nature



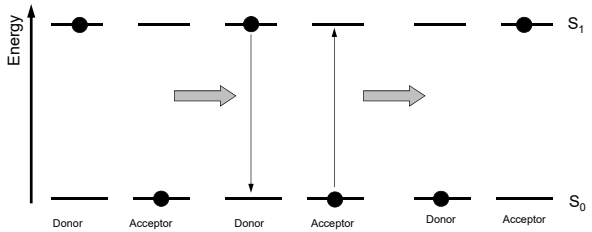
Carotenoid functions:

1. Structural
2. Light harvesting
3. Photoprotection
4. Regulation

Evolution is blind and not very smart



Excitation energy transfer



Photoprotection

Excited chlorophylls (in solution) have ~64% probability of ISC:

$$^1Chl^* \rightarrow {}^3Chl^*$$

This state is dangerous because it can excite an oxygen molecule via T-T annihilation:

$$^3Chl^* + {}^3O_2 \rightarrow {}^1Chl + {}^1O_2^*$$

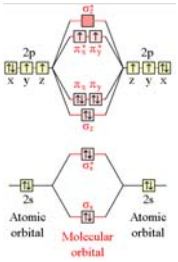
Singlet oxygen will take electrons from anything, and is dangerous.
Carotenoids have a triplet state lying below that of the excitation of oxygen molecule. It can quench Chl triplets:

$$^3Chl^* + Car \rightarrow {}^3Car^* + {}^1Chl$$

Carotenoid triplet relaxes within several μs, and is harmless.
Carotenoids also effectively quench singlet oxygen after it has been formed:

$$^1O_2^* + Car \rightarrow {}^3O_2 + {}^3Car^*$$

Incidentally, singlet oxygen generation is the major mechanism employed in photodynamic cancer therapy. No carrots for cancer patients!

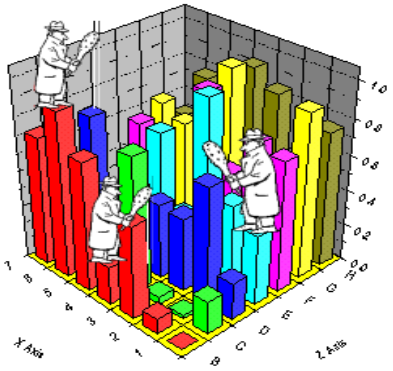


Oxygen ground state is a triplet (Hund's rules)

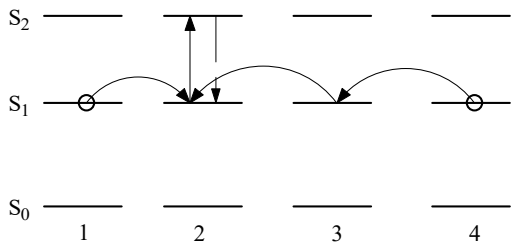
Lessons to learn:

- Triplet states are sensitive to oxygen!
- Typically, they relax on micro-to-millisecond time scale.

Excitation annihilation concept: the 'Highlander' story

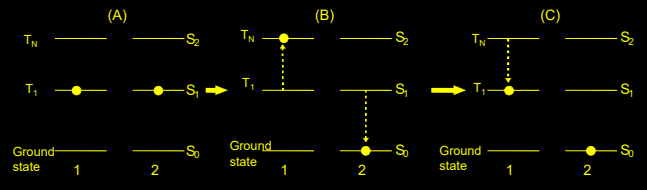


Singlet-singlet annihilation:



When two excitations migrating in the molecular assembly occasionally visit the same molecule, the latter is promoted to the higher excited state (S_2). The higher excited states usually relax very fast (dashed line) back to the first excited state (S_1). The net result of such process is one excitation left out of the two that have collided.

Singlet-triplet annihilation:



Identical to singlet-singlet annihilation, but the triplet does not migrate, it acts as a trap for singlet excitations.

10468 *Biochemistry* **2000**, *39*, 10468−10477

Generation of Fluorescence Quenchers from the Triplet States of Chlorophylls in the Major Light-Harvesting Complex II from Green Plants†

Virginijus Barzda,*,‡ Mikas Vengris,‡,§ Leonas Valkunas,§ Rienk van Grondelle,‖ and Herbert van Amerongen‡

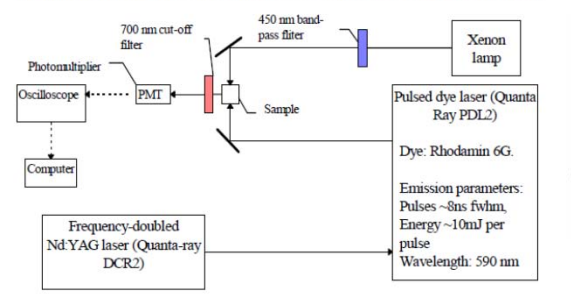
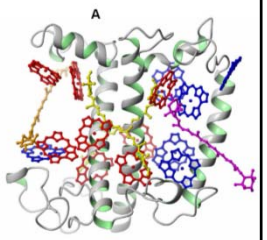


Fig. 6. The general block scheme of experimental setup for laser-flash induced transient fluorescence measurements.



10468 *Biochemistry* **2000**, *39*, 10468−10477

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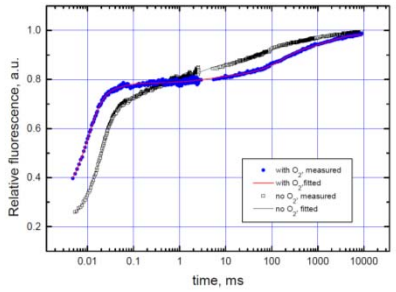
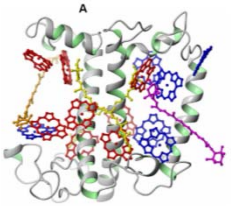




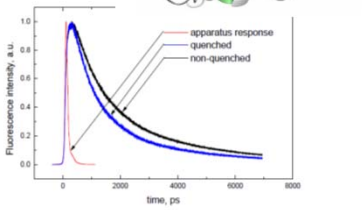
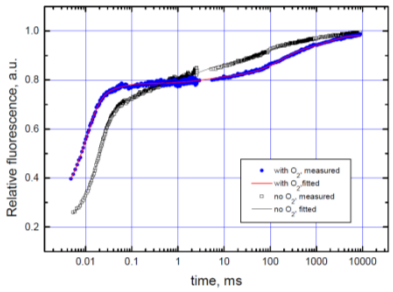


Fig. 15. Fluorescence kinetics at 77K before and after the excitation by a series of laser pulses. Apparatus response function of the setup measured on scattering non-fluorescent sample is also shown. The kinetics were normalised to the maximum.

Stern-Volmer equation

$$\frac{I}{I_0} = \frac{1}{1 + k_q \tau_0 [Q]}$$



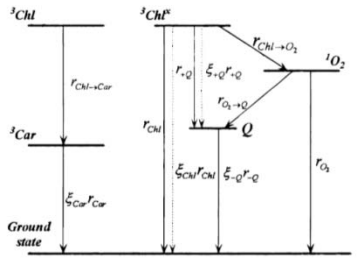
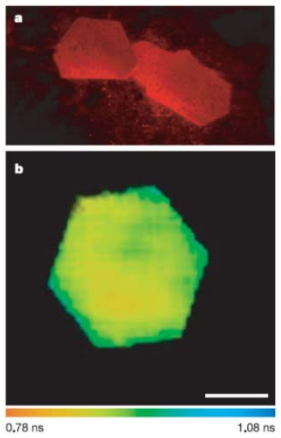


FIGURE 6: Working scheme of Chl triplet decay and generation of long-lived quencher *Q* in LHCII. ^{3}Chl and ^{3}Chl* represent triplets located on Chls with and without neighboring Car, respectively. ^{3}Car and $^1O_2$ symbolize triplet states of Car and singlet oxygen, respectively. Solid arrows and corresponding rates show the relaxation pathways of the Chl triplets. Dashed lines depict the "effective" reactions to which we change the ^{3}Chl* reaction with oxygen by renormalizing with the phenomenological coefficients ξ_{Chl} and ξ_{+Q}.



nature
LETTERS

Molecular basis of photoprotection and control of photosynthetic light-harvesting

Andrew A. Pascal[1], Zhenfeng Liu[2], Koen Broess[3], Bart van Oort[1], Herbert van Amerongen[3], Chao Wang[2], Peter Horton[1], Bruno Robert[1], Wenrui Chang[2] & Alexander Ruban[1]

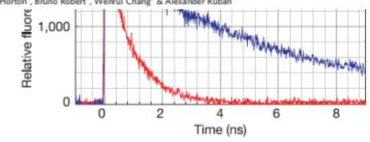
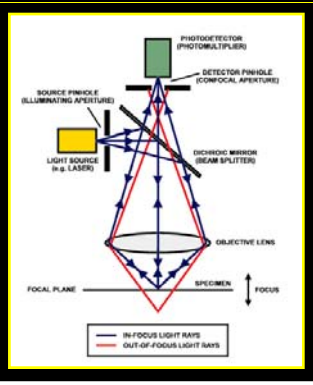


Figure 2 | Quenching of chlorophyll fluorescence in LHCII. a, Confocal fluorescence image of LHCII crystals. **b**, Fluorescence lifetime image of an LHCII crystal. The false colours indicate the fluorescence lifetimes in the corresponding pixels, and the colour code is presented below the panel. One picture consists of 64 × 64 pixels. Scale bar, 50 μm. **c**, Fluorescence decay curves of trimers (blue trace) and crystals (red trace), the latter obtained by averaging decay curves from an area of 11 × 11 pixels within the crystal.

FLIM for FRET

Technical implementation of FLIM:



In your confocal microscope, replace excitation laser by a picosecond diode laser, and use a photon counting detector connected to TCSPC electronics

Time-resolved FRET – detailed spatial information on molecular interactions

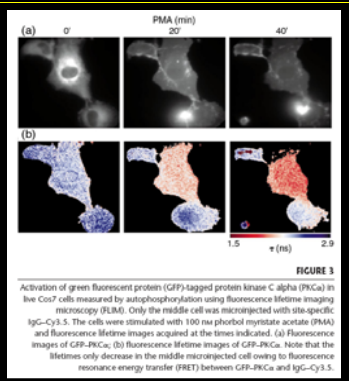


FIGURE 3

Activation of green fluorescent protein (GFP)-tagged protein kinase C alpha (PKCα) in live Cos7 cells measured by autophosphorylation using fluorescence lifetime imaging microscopy (FLIM). Only the middle cell was microinjected with site-specific IgG–Cy3.5. The cells were stimulated with 100 nM phorbol myristate acetate (PMA) and fluorescence lifetime images acquired at the times indicated. (a) Fluorescence images of GFP–PKCα; (b) fluorescence lifetime images of GFP–PKCα. Note that the lifetimes only decrease in the middle microinjected cell owing to fluorescence resonance energy transfer (FRET) between GFP–PKCα and IgG–Cy3.5.

P.I.H. Bastiaens and A. Squire, *Trends in Cell Biology*, 1999. **9**(2): p. 48-52.

FLIM of autofluorescence for cancer imaging: FAD

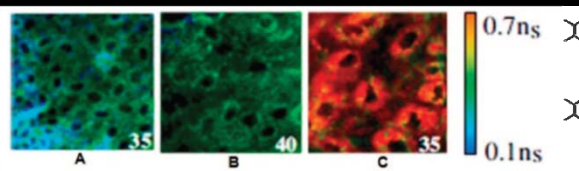


Figure 50. Fluorescence lifetime images of FAD in vivo, normal (A), low-grade precancer (B), and high-grade precancer (C) squamous epithelium tissues (ex. 890 nm, 400−600 nm emission). The number in the corner indicates depth in μm. Reprinted with permission from ref 488. Copyright 2007 National Academy of Sciences, U.S.A.

Skala, M. C.; Riching, K. M.; Gendron-Fitzpatrick, A.; Eickhoff, J. Eliceiri, K. W.; White, J. G.; Ramanujam, N. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 19494.

Local environment probing: Ca^{2+} imaging

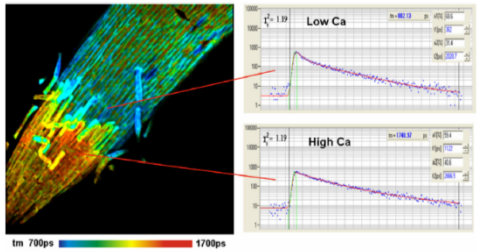


Fig. 513: Barley root tip, stained with Oregon green. Courtesy of Feifei Wang, Zhonghua Chen & Anya Salih, University of Confocal Bioimaging Facility, University of Western Sydney, Australia. Leica SP5 MP with bh SPC-150 FLIM module

Local environment probing: Ca^{2+} imaging

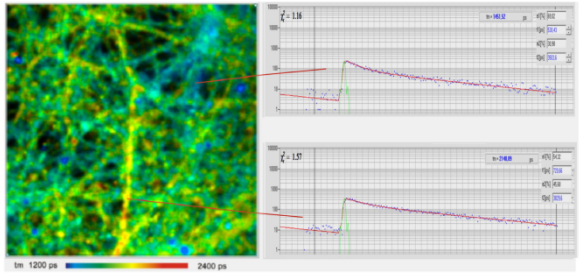
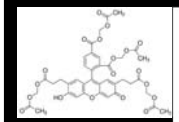


Fig. 514: FLIM image of cultured neurons stained with Oregon green OGB-1 AM. Colour range from τm = 1200 ps (blue) to 2400 ps (red). Decay curves of regions with low Ca (top) and high Ca (bottom) shown on the right. Data courtesy of Inna Slutsky and Samuel Frere, Tel Aviv University, Sackler School of Medicine.

Local environment probing: pH imaging



BCECF

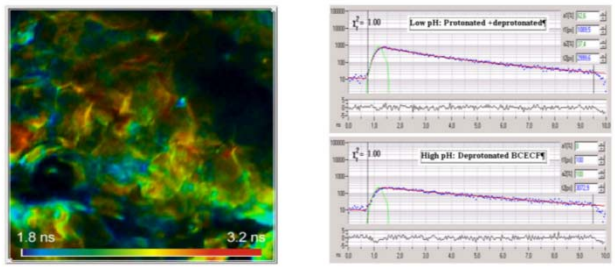


Fig. 512: Left: Lifetime image of skin tissue stained with BCECF. The lifetime is an indicator of the pH. Right: Fluorescence decay curves in an area of low pH (top) and high pH (bottom)

Isomerization of retinal in bacteriorhodopsin

Bacteriorhodopsin, summary of:

- Widely investigated:
  - Nature – 35 papers (1990 – 2018)
  - Science – 46 papers(1990 – 2018)

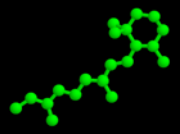
  (Source: Web of science)

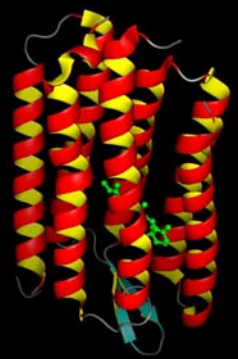
Function:

- Light drive proton pump that pushes protons across the membrane against the direction of the electric field.
- Found in purple membranes of *Halobacterium salinarium, up to 90% of membrane mass.*

Structure: membrane protein

- Structure resolved to 1.65 Å.
- 7 alpha helices containing a retinal chromophore

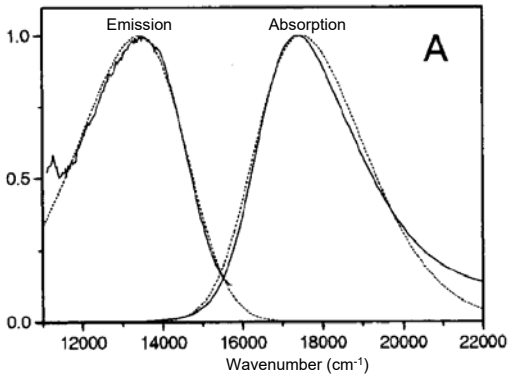


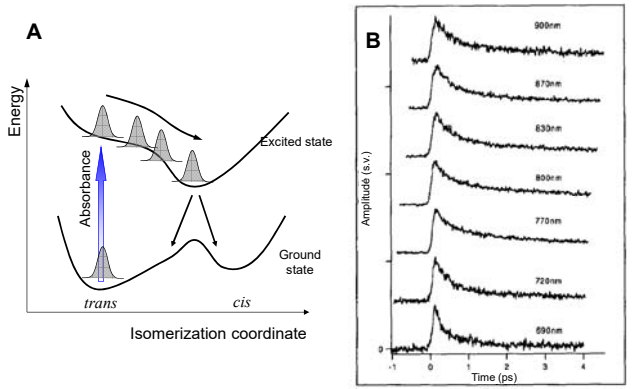


Advantages:

- Chemically stable and photostable
- Well known structure, easy to crystalize
- Bacteria grow a lot of it
- Fast, photoactive and therefore interesting
- Can be used as biomolecular tool or a model system for photoreactions

Absorption and emission





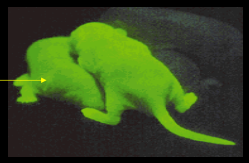
M. Du and G.R. Fleming, *Biophysical Chemistry*, 1993. **48**(2): p. 101-111.

Green fluorescent protein

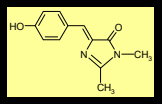
Aequorea victoria: jellyfish

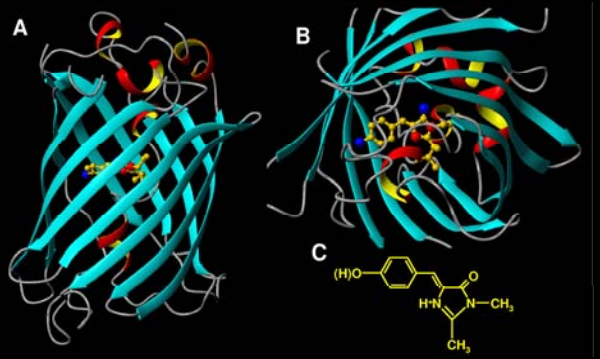
GFP protein

Marker for molecular biology

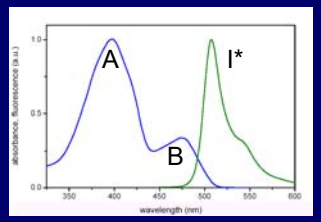


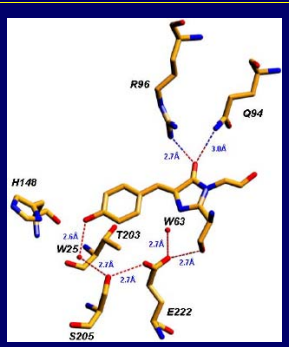
GFP chromophore

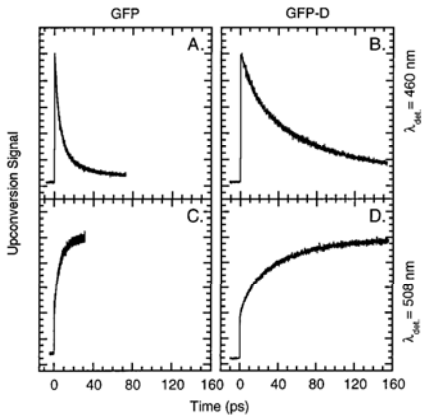




Fluorescence from GFP originates from 'I': Excited-state proton transfer*

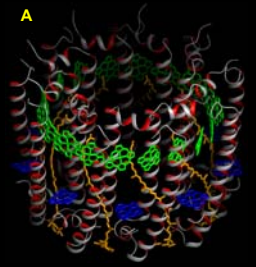


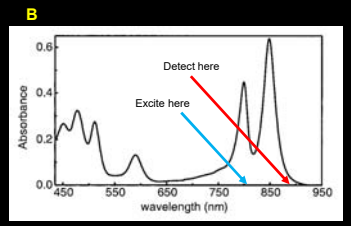




M. Chattoraj, B.A. King, G.U. Bublitz, and S.G. Boxer, *Proceedings of the National Academy of Sciences (USA)*, 1996. **93**: p. 8362-8367.

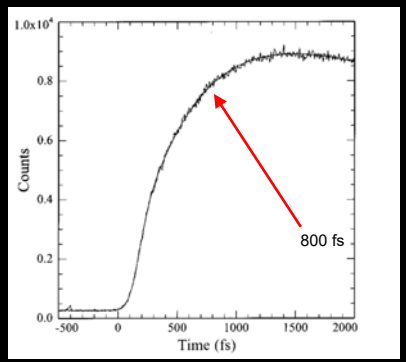
Energy transfer in LH2 of purple bacteria

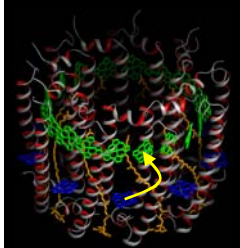




R. Jimenez, S.N. Dikshit, S.E. Bradforth, and G.R. Fleming, *Journal of Physical Chemistry*, 1996. **100**(16): p. 6825-6834.

Fluorescence of the acceptor pigment develops with the delay after the excitation. The delay is equal to energy transfer time.





Energy transfer between isoenergetic pigments

- If the excitation energies of different pigments are equal, emission wavelength does not change due to energy transfer;
- Therefore, the fluorescence appears at that wavelength without any delay.
- However, the polarization of the emission will change!

Fluorescence anisotropy

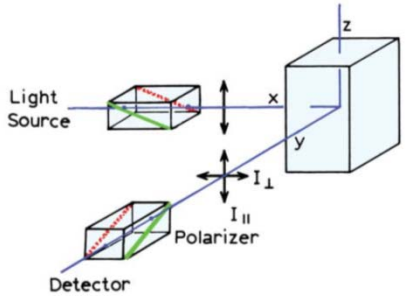


Figure 10.1. Schematic diagram for measurement of fluorescence anisotropies.

Dipole radiation diagram

355

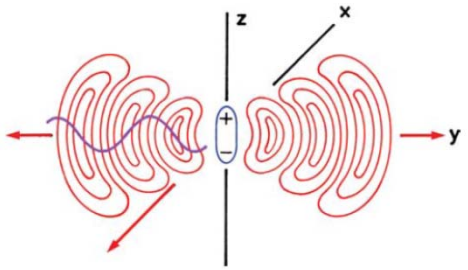


Figure 10.3. Electric field from a radiating dipole oriented along the z-axis. The thin arrows on the lines indicate the direction of the electric field E. The wide arrows indicate the direction of energy migration, which is symmetrical around the z-axis.

Molecular dipole moment

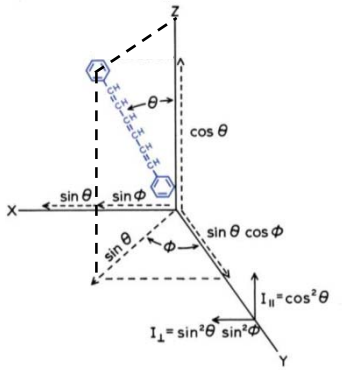


Figure 10.4. Emission intensities for a single fluorophore in a coordinate system.

Photoselection of molecules in the sample

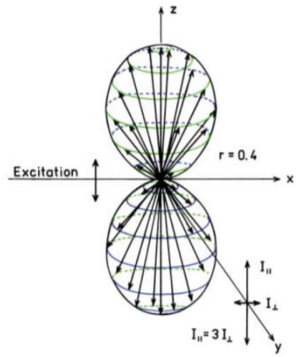
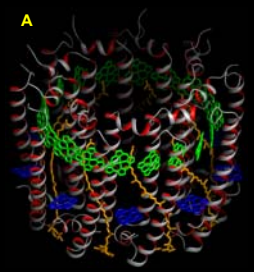
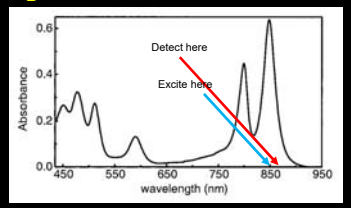


Figure 10.6. Excited-state distribution for immobile fluorophores with $r_0 = 0.4$.

Returining to energy transfer in LH2 of purple bacteria





R. Jimenez, S.N. Dikshit, S.E. Bradforth, and G.R. Fleming, *Journal of Physical Chemistry*, 1996. **100**(16): p. 6825-6834.

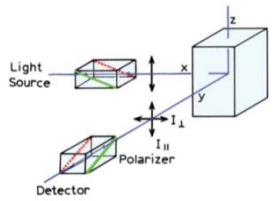
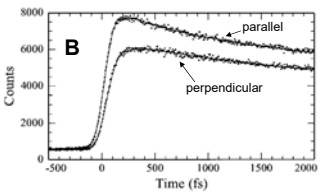
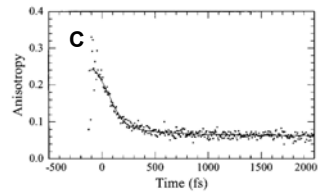
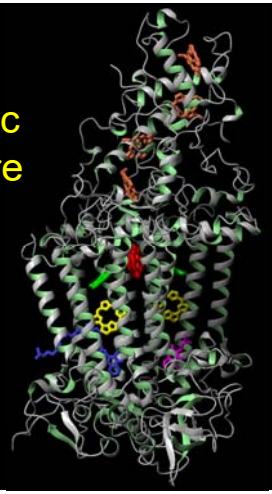


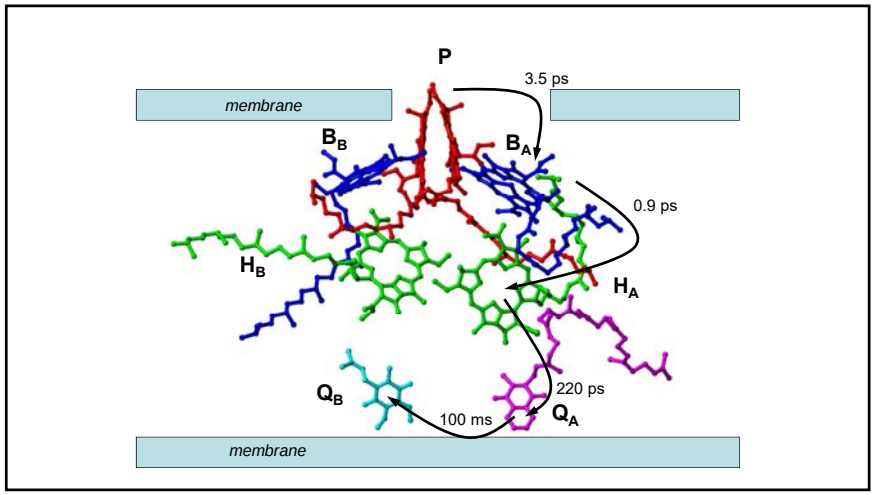
Figure 10.1. Schematic diagram for measurement of fluorescence anisotropies.

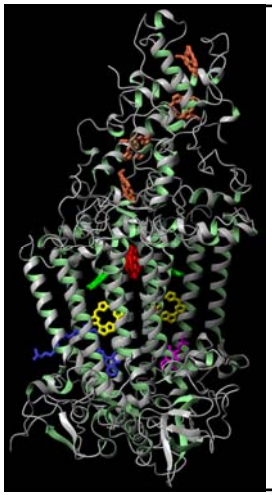




Application: photosynthetic reaction centre







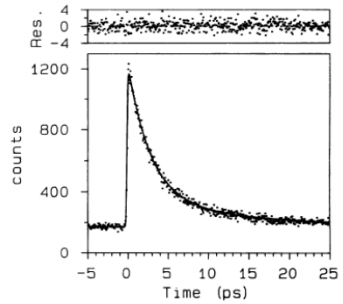


FIG. 1. Spontaneous emission decay of P* in *Rb. sphaeroides* R26 with excitation at 850 nm and emission at 940 nm. Solid line is a double exponential fit with $a_1 = 80.8\%$, $\tau_1 = 2.7$ ps, $a_2 = 19.2\%$, and $\tau_2 = 12.1$ ps. Res., residual.

Emission of the special pair in the reaction center decays mostly with 2.7 ps lifetime, corresponding to the first step in the charge transfer chain.

Fluorescence is good, but...

- Contains only the information about the excited states, whereas interesting things happen in ground state as well...

Therefore we switch to...