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

Hydrogen atom

Molecule

Electronic and vibrational states
Molecule is a dipole with respect to the external field

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!)

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

$$\mu_{j} = \int \phi_{j} \hat{\mu} \phi_{i} \, dr$$

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

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

Electronic spectra and $\pi$-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.

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 $\pi$ 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:

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

Nodes at $x=0$ and $x=L$:

$$\psi = 0, \text{ when } x = 0, L$$

Energy spectrum:

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

Quantum dots: tiny three dimensional boxes for electrons
\[ \beta\text{-carotene: the “model” carotenoid} \]

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.

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 \( 2\times \frac{1}{2} = 1 \)
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)

The principle of mirror symmetry: fluorescence spectrum is the mirror image of the absorption spectrum. The energy difference between the fluorescence and lowest absorption maxima 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

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Figure 1. Absorption, fluorescence and phosphorescence spectra of benzaldehyde. Phosphorescence is measured at -196°C in 10% D_2SO_4/CH_2Cl_2. The fluorescence and phosphorescence spectra were recorded at 296 K. The phosphorescence intensity is about 10^5 times weaker than the fluorescence. Excitation wavelength is near 360 nm for phosphorescence and 410 nm for fluorescence (Q_x excitation). The emission spectra are corrected for the spectral response of the detection system (shift in the vertical scale).
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)

Quantum yield of fluorescence:

\[ \phi = \frac{k_{\beta}}{k_{\beta} + k_{\alpha} + k_{\text{nw}} + k_{\text{other}}} \]

Other can be:
• Energy transfer,
• Photoreactions,
• Fluorescence quenching...

Measured emission intensity on the molecule:

\[ I \sim \phi |\mu_\beta|^2 \]

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?
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!

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).

\[ n(t), G(t), I(t) \]

\[ \text{Time (ps)} \]

Simple kinetics

Fluorescence consists of single photons

Time resolved fluorescence techniques:

Time-correlated single photon counting
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

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

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

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

ADC with dithering: bit noise reduction

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

- 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 photodiode
- Fast response, no afterpulsing

Actively quenched SPAD detectors

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

Laser sources can be cheap

4000-13000 EUR

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

Wilfried Uhrling*, Chantal-Virginie Zint*, Jeremy Burtranger*

Article in: Proceedings of SPIE - The International Society for Optical Engineering - September 2004
DOI: 10.1117/12.545038
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.

- 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

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)

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.

Time resolved fluorescence techniques:
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 techniques:

upconversion
\[ I_{\text{sum}}(t) = I_{\text{gate}}(t) \times I_{\text{fluoresc.}}(t) \]

\[
\frac{1}{\lambda_{\text{sum}}} = \frac{1}{\lambda_{\text{gate}}} + \frac{1}{\lambda_{\text{fluoresc.}}}.
\]

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 techniques:

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

\[ n = n_0 + n_2 I \]

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

Phase fluorimetry (a.k.a. frequency domain fluorescence lifetime measurement)
Molecules are acting as an integrating filter in electronics

Phase fluorimetry (time domain lifetime measurements)

- Current modulation techniques allow wideband sweeping of the modulation frequency to deconstruct 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.

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.
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**

\[
k_{DA} = \frac{9 \ln(10) \kappa^2 c^4 \phi_D}{80\pi n^4 N_0 \tau_D R^6} \int \frac{E_D(\omega) A_A(\omega)}{\omega^4} d\omega
\]

**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.

**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

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

- 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
Solvation dynamics

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

Figure 2. Representative time-resolved emission spectra of C163 (296
K) in DMSO-d6 showing the continuous red shift with time charac-
teristic 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
at ref 31 for more details.)

Leaf response to prolonged illumination –
reduction of fluorescence

Figure 3. Off-fluorescence measurement from an Arabidopsis leaf. In the presence of only weak exciting light the maximal fluorescence probe
is small. When a saturating light pulse is given, the photosynthetic light reactions are saturated and fluorescence (the maximum of first
probe) is obtained. Upon cooling the fluorescence with weak exciting light (2% probe photon flux) the fluorescence (camel) of
probe can be seen in the difference between Fm and the measured maximal fluorescence after a saturating light pulse during illumination (Fm).

Fluorescence quenching in plants

Carotenoids: colorful pigments of nature

Figure 4. Carotenoid absorption spectra of plant extracts. Carotenoids absorb in the UV-visible region.
Carotenoid functions:

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

Evolution is blind and not very smart

Excitation energy transfer

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

\[ ^1\text{Chl}^* \rightarrow ^3\text{Chl}^* \]

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

\[ ^3\text{Chl}^* + ^3\text{O}_2 \rightarrow ^1\text{Chl} + ^1\text{O}_2^* \]

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:

\[ ^3\text{Chl}^* + \text{Car} \rightarrow ^3\text{Car}^* + ^1\text{Chl} \]

Carotenoid triplet relaxes within several \(\mu\)s, and is harmless. Carotenoids also effectively quench singlet oxygen after it has been formed:

\[ ^1\text{O}_2^* + \text{Car} \rightarrow ^3\text{O}_2 + ^3\text{Car}^* \]

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

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

**Excitation annihilation concept: the ‘Highlander’ story**

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-singlet annihilation:**

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**Singlet-triplet annihilation:**

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

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

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. 690 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.

Local environment probing: Ca\textsuperscript{2+} imaging

Fig. 513: Boltry root tip stained with Oregon green. Courtesy of Fei Lei Wong, Zhonghua Chen & Anju Salih, University of Confocal Bioimaging Facility, University of Western Sydney, Australia. Leica SP5 MP with bh SPC-150 FLIM module.

Local environment probing: pH imaging

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

Fig. 514: FLIM image of cultured neurons stained with Oregon green OGB-1 AM. Colour range from tma = 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 Iain Shlosky and Samuel Ferrer, Tel Aviv University, Sackler School of Medicine.
Bacteriorhodopsin, summary of:

- Widely investigated:
  - 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

![Graph showing absorption and emission spectra.]

Absorption
Emission

Wavenumber (cm\(^{-1}\))

Energy

Isomerization coordinate

Absorbance

Ground state
Excited state

trans

cis

Time (ps)

Amplitude (s.v.)


Green fluorescent protein

*Green fluorescent protein (GFP) is a marker for molecular biology derived from the jellyfish Aequorea victoria. Its chromophore acts as a fluorescence tag for various biological applications.*
Fluorescence from GFP originates from ‘I*’: Excited-state proton transfer

Energy transfer in LH2 of purple bacteria

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!
Photoselection of molecules in the sample

Returining to energy transfer in LH2 of purple bacteria

Application: photosynthetic reaction centre
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...