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TIME-RESOLVED SPECTROSCOPY (ABSORBANCE, FLUORESCENCE) IN THE STUDY OF BIOLOGICAL MOLECULES

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Key words : absorption spectroscopy, fluorescence spectroscopy, two-photons microscopy, fluorescence correlation spectroscopy, time-resolved spectroscopy.

The interaction of radiation (ionizing, ultra violet, visible) with biological molecules has beneficial or deleterious effects: it can allow many living organisms to recognize their environment (photosynthesis) but also can damage the biological molecules and lead to an alteration of their activity or to cellular death. A challenge of many scientists is to understand at the molecular level, the processes related to photobiology and radiobiology which generally involved very short-lived species such as excited states and free radicals. One way to approach these short lived chemical intermediates is the use of time resolved absorption and fluorescence spectroscopic techniques which are complementary of the analytical stationary methods. These techniques allow a better understanding of the direct effect of radiation with biological molecules but also help to elucidate biochemical reactivity of molecules by generating specific transient species present in normal metabolic processes. Indeed, a biological process may be extremely complex and composed of an involved sequence of elementary reactions (single bond breaking/-formation reactions, energy and electron transfers etc..). Such processes need to be studied over a wide time domain in order to obtain a full understanding on the molecular level of the elementary mechanisms and how they are related. Different problems such as the function and regulation of biomolecules (proteins, DNA, drugs....).as well as the development of new drugs can thus be aborted

Fast and ultrafast spectroscopy presents a unique tool for studying the dynamics of biological systems. However, the classical time resolved spectroscopic techniques are well

adapted to *in vitro* but not *in vivo* studies. The last years, new quantitative methods derived from fluorescence spectroscopy have emerged for the characterization of the living cells

Our present broader goal is to present the important role of excited states and free radicals in biology and medicine through some -produced excited state and free radicals,

- to study their (photo)physical and (photo)chemical properties by various spectroscopic methods (emission and correlation of fluorescence, transient absorption),
- to determine their spectro- dynamic evolution since few femtoseconds.

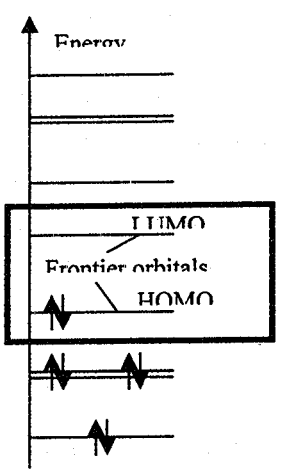
In this text we present also various forms and utilization of dynamic spectroscopy, and in particular, the new developments.

This implies that the fundamental principles of stationary spectroscopy must be previously recalled.

Stationary Spectroscopy

I- Absorption

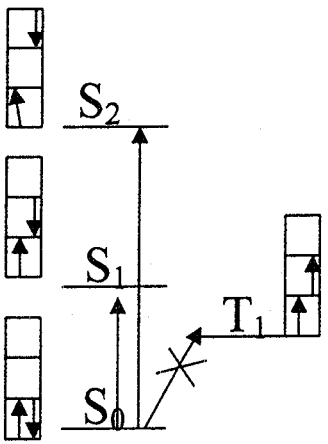
If a molecule is in the path of light (or any electromagnetic radiation) of a range of frequencies, certain energies will be absorbed which correspond to the energy difference between two energy levels of the molecule. Electronic transitions involve energy differences which correspond to the visible and ultraviolet regions of the spectrum, typically 600 $kJ.mol^{-1}$ to 150 $kJ.mol^{-1}$ (800-200 nm).



The lowest energy transition corresponds generally to the transition of the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO).

Five types of orbitals are found in organic molecules; the sigma-bonding (σ) and the sigma-antibonding (σ^*) orbitals involved in single bonds, the pi-bonding (π) and the pi-antibonding (π^*) orbital involved in π or multiple bonds, the non-bonding (n) orbitals localized in heteroatoms (e.g. oxygen, nitrogen and sulphur) without anti-bonding counterpart. To these five orbitals, corres-

-pond five energy levels and several different transitions $\sigma \rightarrow \sigma^*$, $\pi \rightarrow \pi^*$, $n \rightarrow \sigma^*$, $\pi \rightarrow \sigma^*$ and $n \rightarrow \pi^*$. For the organic molecules with double bonds (aromatic core, ethylenic bonds), a great number of π electrons are delocalised and then the transition from the HOMO to the LUMO is a $\pi \rightarrow \pi^*$ transition (noted $\pi\pi^*$). For the molecules which contain heterocycles or heteroatoms, can correspond $n \rightarrow \pi^*$ transition (noted $n\pi^*$).



In the ground state, generally, the HOMO is occupied by two electrons of opposite spin. The total spin is zero and the spin multiplicity M = (2S + 1) is then equal to 1. The ground state is a singlet state noted S_0. The rule of spin conservation indicates that the spin of the promoted electron must be conserved. Thus, the spin multiplicity of the excited state is also one and the excited state is a singlet excited state (S_1, S_2 ... S_n). This rule can be violated. The spin orbit coupling can lead to the spin of the promoted electron being reversed from the direction it had in the ground state. In this case, the two electrons are parallel in the excited state, the total spin is now S = 1 and the spin multiplicity is M = 3; the excited state is called the triplet state (T). At each singlet state corresponds a triplet state of lower energy.

The absorption spectra measured experimentally correspond to the different transitions $S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$, ... $S_0 \rightarrow S_n$.

The Beer-Lambert law

The absorption spectrum of a solute in solution is defined by the spectral distribution of its molar absorption coefficient $\varepsilon(\lambda)$ in M^{-1} cm^{-1} or its efficient section $\sigma(\lambda)$ in cm^2.

Experimentally, the absorption spectra can be obtained using a spectrophotometer. The incident intensity (I_0) provided from a continuous irradiation source (xenon lamp or deuterium lamp depending of the wavelength range); after passing through a monochromator and a photomultiplier, the transmitted intensity I_T was analysed. The incident and transmitted intensities are related by the relation:

$$I_A(\lambda) = I_0(\lambda) - I_T(\lambda) = I_0(\lambda)\,(1 - 10^{-\varepsilon(\lambda)\,c\,l})$$

Where I_A is the absorbed intensity, c the solute concentration (in M) and l the optical path (in cm). The product $[\varepsilon(\lambda)cl]$ is called absorbance A.

The Beer-Lambert law reflects that the transmitted intensity I_T varies linearly with the incident intensity I_0 as:

$$A = -\log[I_T / I_0] = -\log T$$

T is called transmittance.

It must be noted that:

- the Beer-Lambert law is only obeyed for low excitation intensities: in these conditions, the number of excited solute molecules remains very low by comparison to the total number of solute molecules in the solution. If $\varepsilon(\lambda)cl \ll 1$ thus $1 - 10^{-\varepsilon(\lambda)\,c\,l} \sim 2.3\,\varepsilon(\lambda)\,c\,l$ and $I_A(\lambda) \sim 2.3\,\varepsilon(\lambda)\,c\,l\,I_0(\lambda)$ corresponding to a linear variation.

- for an absorbance of A = 2 corresponds a transmittance T= 0.01, that means that only 1% of the incident intensity is transmitted, for A =1, T = 0.1 etc.....

Solvent effects

Polarity is of major importance in many physical, chemical and biological phenomena. The position, intensity and shape of the absorption spectra of organic molecules can be modified by the polarity of the solvent. This effect is called solvatochromism. A hypsochromism (or blue shift) with increasing the solvent polarity is a negative solvatochromism while a bathochromic (or a red shift) is a positive solvatochromism.

This solvatochromic effect reflects the dielectric solute-solvent interactions which result not only from the dipole moments but also from the polarizabilities of the molecules. Indeed, usually organic solutes and solvents have unsymmetrical charge distribution and possesses a permanent molecular dipole moment. The molecule is thus polar and its dipole moment μ measured in Debye (D). A dipole moment formed of two opposite charges e separated by a distance of an 1 Angström has a value of 4.8 D. The polarizability is defined by means of the dipole induced by an external electric field in its own direction.

The four major dielectric interactions are:

- non polar solute in non polar solvent (or polarizability – polarizability)
- non polar solute in a polar solvent (or solute polarizability – solvent dipole)
- polar solute in a non polar solvent (or solute dipole- solvent polarizability)

- polar solute in polar solvent (or dipole-dipole)

The forces between a solute molecule and a solvent molecule are dipole-dipole interactions called Van der Waals or hydrogen forces.

The Van der Waals forces correspond to three different dipole interactions:

- dipole-dipole or Keesom forces when solute and solvent are polar molecules
- dipole-induced dipole or Debye forces between polar and non polar molecules
- induced dipole-induced dipole dispersion forces or London forces between non polar molecules.

All the energies of these different type of attractive forces vary with $1/r^6$; they are short distance interactions.

The hydrogen bonds are specific bonds which appear in protic solvents (water, alcohol..). The chemical bonds (OH, NH, FH...) between an hydrogen atom and an electronegative atom gives a dipolar character to the bond. The hydrogen bonds are formed by dipole-dipole interaction between two molecules.

The solute-solvent interaction by hydrogen bond is specific so the formed complexes can have specific chemical and spectroscopic properties. In this case, it is not a solvent effect.

II-Fluorescence

The fluorescence is largely used to study the structure and the dynamic of the biological molecules at the molecular level. The determination of parameters such as polarity, fluidity, molecular mobility, of the macro-molecules can be obtained from intrinsic or extrinsic fluorescent probes. In general, changes in fluorescence are more readily detected than small changes in absorption. Furthermore, fluorescence is more sensitive to the environment than absorption in part because the process (10^{-11} – 10^{-8} s) is slower than absorption (10^{-15} s) (see below).

An organic molecule in its singlet excited state can deactivate radiatively to the ground state: this radiative relaxation $S_1 \rightarrow S_0$ is called fluorescence.

Experimentally, the fluorescence of a compound is measured using a spectrofluorimeter. The excitation provides from a select spectral range of a continuous

lamp. The fluorescence is collected perpendicularly to the excitation through a monochromator and a photomultiplier system.

The fluorescence of a molecule occurs at longer wavelengths than the absorption. This shift in energy between absorption and fluorescence is called Stokes' shift. In the excited state, vibrational energy losses can occur leading to a rapid decay to the lowest vibrational levels of the first singlet excited state (see Jablonski diagram). The fluorescence occurs from this latter state to vibrational level of the ground state. This corresponds to a lower energy transition (higher wavelength) than that corresponding to the absorption.

It must be noted that:

- in absence of any excited state process, the fluorescence spectra are the mirror image of the absorption spectra.
- if the Stoke shift is dependent of the solvent nature, this corresponds to a reorganization of the solvent around the excited state of the solute.

Acid-base properties and redox potentials of excited states.

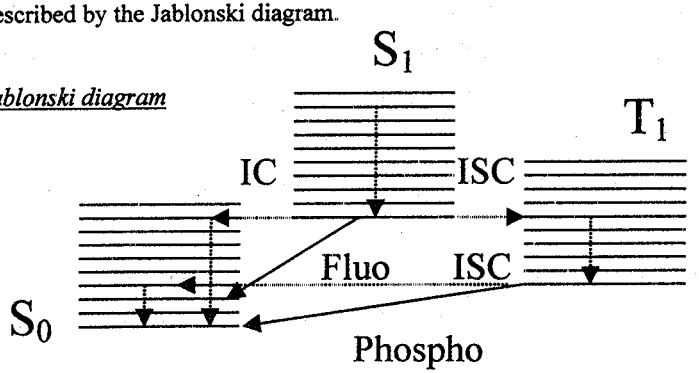
As well as the dipole moment may be changed upon excitation, the acid-base properties of a molecule can be also modified. A change of several units of pK_a can be observed between the fundamental and the excited states.

The excited states are also more easily oxidized and more easily reduced than the ground states molecules.

Transient spectroscopy

The photophysical processes occurring in an organic molecule in solution are generally well described by the Jablonski diagram.

Jablonski diagram



Perrin (1929), Jablonski (1935) and Lewis and Kasha (1944) laid the foundations of a general sequential scheme for the physical activation and deactivation processes of electronically excited states produced by monophotonic excitation. The approximate lifetime of these processes are:

$S_0 \rightarrow S_n$	absorption	10^{-15} s	
$S_n \rightarrow S_1$	internal conversion	$10^{-11} - 10^{-14}$ s	
$S_1 \rightarrow S_0 + h\nu$	fluorescence	$10^{-7} - 10^{-11}$ s	radiative pathway
$S_1 \rightarrow T_1$	intersystem crossing	$10^{-7} - 10^{-11}$ s	non-radiative pathway
$S_1 \rightarrow S_0$	internal conversion	$10^{-7} - 10^{-11}$ s	non-radiative pathway
$T_1 \rightarrow S_0 + h\nu$	phosphorescence	$10 - 10^{-5}$ s	radiative pathway
$T_1 \rightarrow S_0$	intersystem crossing	$10 - 10^{-7}$ s	non-radiative pathway

The internal conversion corresponds to a non-radiative electronic relaxation pathway between two states with the same multiplicity. The inter-system crossing is also a non-radiative deactivation but between two states having different multiplicity. The non-radiative and isoenergetic transitions (IC and ISC) between electronic states are followed by vibrational relaxation with energy transfer to the solvent in each state (ground or excited state).

The two states that have the longest lifetimes and which can be more readily observed usually via absorption and emission are the first excited singlet state S_1 and the lowest triplet state T_1 (The internal conversion from the highest electronic states are too fast to be studied).

The singlet excited state S_1 is characterized by its lifetime τ. The simplest case corresponding to the depopulation of S_1 can be written with k the decay constant (s^{-1}):

$$dN^*/N^* = -kt$$

$$N^*(t) = N^*(0)\, e^{-kt} = N^*(0)\, e^{-t/\tau}$$

τ corresponds to the real lifetime of the excited state and the reciprocal of the lifetime k is the sum of all the rate constants (both radiative and non-radiative) for deactivation processes which can occur from S_1.

$$k = \Sigma k_i = k_{rad} + k_{IC} + k_{ISC} = 1/\tau$$

In some cases, a term must be added representing the additional routes of deactivation due to quenchers such as oxygen.

The greater lifetime of an excited state is its radiative lifetime $\tau_{rad} = (1/k_{rad})$, the lifetime measured in absence of non-radiative processes in competition with fluorescence.

In solution, at room temperature, the radiative lifetime of S_1 of a macromolecule is generally in the nanosecond time scale while its lifetime can be as short as some picoseconds or less. In the same way, the radiative lifetime of the triplet state is very long but its true lifetime is in the nanosecond to microsecond time scale depending of its reactivity.

The fluorescence quantum yield express the proportion of excited molecules that return to the ground state by emitting a fluorescence photon. In other words, the fluorescence quantum yield is the ratio of the number of emitted photons to the number of absorbed photons. Thus,

$$\Phi_f = k_{rrad}/\sum k_i = \tau \times k_{rrd}$$

In the same way, the triplet quantum yield corresponds to the ratio of the excited molecules that convert to the triplet state to the number of absorbed photons.

$$\Phi_{ISC} = k_{ISC}/\sum_i k_i \quad \text{and} \quad \sum_i \Phi_i = 1$$

Many factors can affect the fluorescence or the triplet quantum yield: temperature, polarity, viscosity, H-bonding ability, proximity of heavy atoms or quenchers...

It is worth recalling that the fluorescence quantum yield does not remain always proportional to the lifetime upon an external perturbation. A typical case where the fluorescence quantum yield is affected without any change of the lifetime is the formation of a non-fluorescent complex in the ground state (static quenching).

Reactivity of the excited states; photochemical processes.

The molecular excited states can evolute by different reactivity pathways than the electronic or vibrational relaxations described by the Jablonski diagram. These reactions can be unimolecular or bimolecular.

The bimolecular reactions require the encounter of the excited species with another entity, this means that such reactions occur after diffusion of the molecules. They are slow (nanosecond to second); for example, excimer or exciplex formation, intermolecular electronic energy transfer, intermolecular charge transfer, radical formation etc... Generally, due to their lifetimes, these reactions occur from triplet excited states. The nanosecond to microsecond flash photolysis techniques are well adapted to study these reactions

The unimolecular photo- reactions do not require diffusion of the reactants and thus can be rapid reactions; for example, photodissociation, geminate recombinaison, photoisomerization conformational relaxation, intramolecular charge and proton transfer etc.... These reactions can be studied by femtosecond and picosecond absorption and fluorescence techniques.

Nanosecond Transient absorption spectroscopy

The technique of flash photolysis was introduced in 1949 by Norrish and Porter (Nobel price in 1950). It is a powerful method for studying, as a function of time, transient species produced via electronic excitation of molecules by short pulses of UV or visible radiation. About a decade latter, pulse radiolysis using the same principle was applied to the study of similar species produced by pulses of high energy radiation.

The nanosecond to microsecond flash photolysis or pulse radiolysis are well adapted to the characterization of intermediates implied in slow photochemical reactions, triplet electronic states, radical species, bimolecular process controlled by diffusion, energy transfer, biphotonic processes etc..

Principle of flash photolysis or pulse radiolysis is represented in Figure1. The absorbance change induced in the sample solution by the UV or visible exciting laser flash or the pulse of electrons is monitored by an analysing light beam passing through the sample and reaching a detector (photomumtiplier) at selected wavelengths via a monochromator. The photomultiplier converts changes of the transmitted light intensities into electrical signals which are digitised, displayed, stored and treated by a computer.

The transient absorbances are calculated at each wavelength as a function of time. Corresponding spectra are construct absorbance against wavelength or absorbance against time (kinetic analysis). In the spectral region where the transient species and the original molecule absorbances overlap, a differential spectrum is obtained and the change in absorbance is:

$$\Delta A = (\varepsilon_t - \varepsilon_0)\, c\, l$$

where ε_t and ε_0 are the molar absorption coefficients of the transient species and of the molecule in the ground state respectively, c the concentration of the ground state converted in the excited state and l the optical path length.

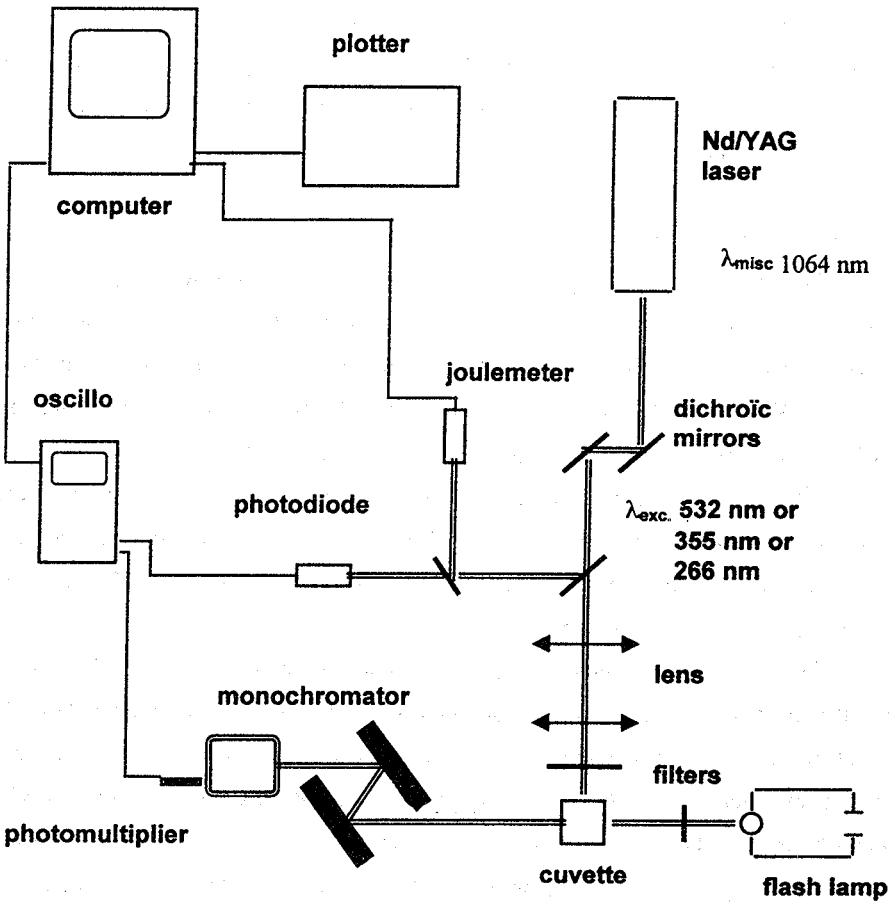


Figure 1

Pump-probe method for transient absorption spectroscopy

This appellation corresponds to femtosecond-picosecond laser photolysis systems. The principle is the same as in nanosecond systems. It consists to excite a significant population of molecules in an excited state by means of a laser pulse and to probe the excited species thus formed with a second light source less intense than the excited source. A typical experimental system is reported in Figure 2.

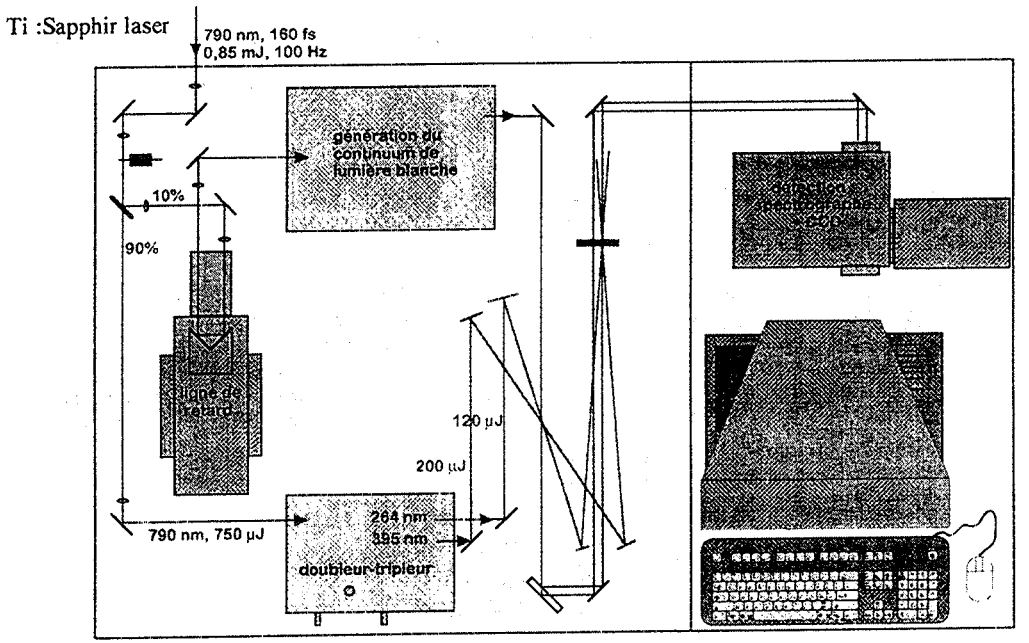


Figure 2

In femtosecond-picosecond experiments, the probing light source provided a fraction of the laser beam which was focussed into water in order to generate a white light continuum. The sample and reference transmitted beams were simultaneously analyzed with a polychromator and a CCD detector.

Single photon counting method

The principle of the single photon counting method is to consider that the probability to detect one photon at time t after an excitation pulse at time 0 is proportional to the fluorescence intensity at this time (t).

A scheme of a single photon counting method is represented in Figure 3. The excitation light pulse source is a laser which must have a high repetition rate (> 1Mhz). Fluorescence emission is detected through a monochromator, by a microchannel plate photomultiplier (or a classic photomultiplier) connected to an amplifier. The excitation light pulse is triggered by a photodiode. Pulse signal is amplified, shaped and connected to the stop input of the TAC (Time Amplitude Converter) and a multichannel analyzer. The photon of fluorescence generates the stop pulse. Thus by detecting one photon by one

photon after successive excitation pulses and by ranging the photons in time it is possible to construct the fluorescence intensity decay.

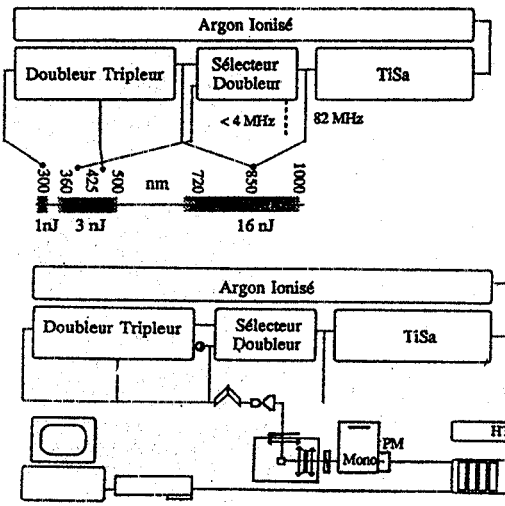


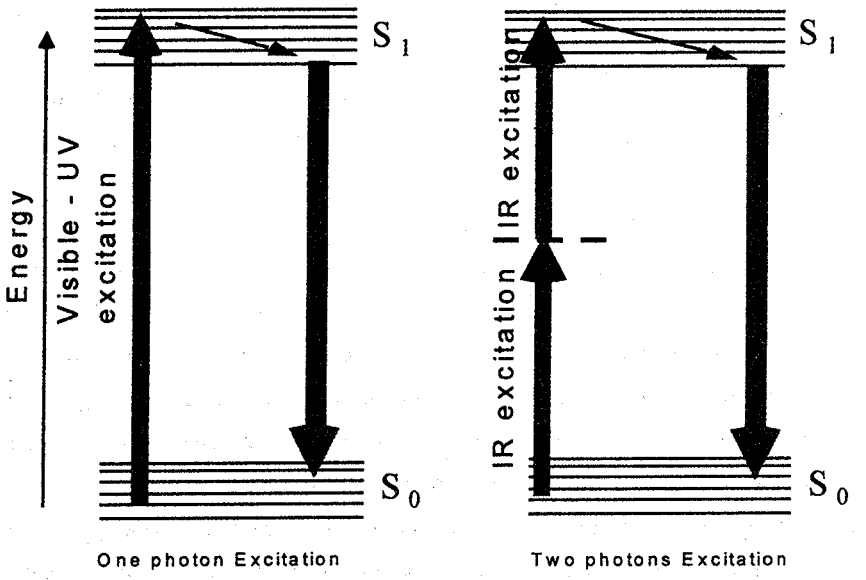
Figure 3

Two photon microscopy

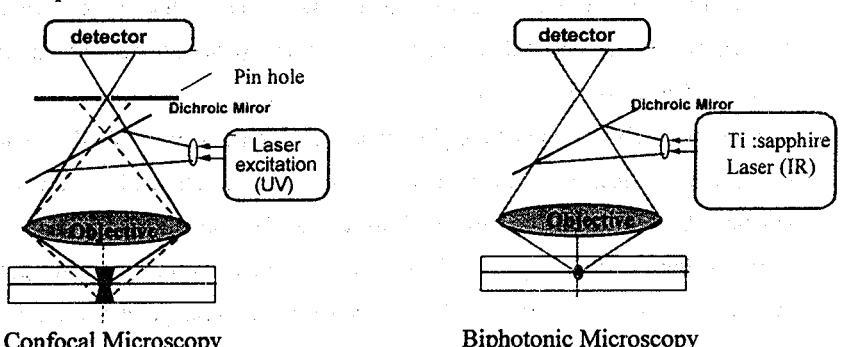
Fluorescence microscopy is arguably one of the most powerful experiment techniques for visualization of biological entities. Fluorescent probes either small molecules or luminescent proteins such green fluorescent protein (GFP), can be attached to biological molecule (protein DNA...) or molecules of interest (calcium ions, heme, drug...) have excellent specificity and sensitivity and can be targeted to particular cell organelles both *in vitro* and *in vivo*. Using fluorescence spectroscopy and microscopy techniques, it is possible to detect fluorescent probes down to the single molecule level, thereby mapping the distribution and functional status of macromolecules and other components mediating cellular processes. Using micro-spectrocopic techniques, including recent developments in imaging technology and excitation methods, it is possible to follow dynamics of biological systems in real time in vivo.

A recent revolution in biological and biomedical imaging stems from the application of multiphoton excitation techniques in microscopy. The basic idea of two photon excitation is to use a long wavelength excitation source such as the energy of a single excitation photon is not sufficient to excite a fluorescent molecule, whereas the combined

energy of two excitation photons is. In other words, fluorescent molecules that are excited by visible or UV light in conventional one-photon excitation are excited by IR light in two-photon excitation.



The probability that two excitation photons are incident on the same molecule at a same time depends on the photon density. In practice, high photon densities are achieved by confining the excitation light both spatially using a microscope objective and temporally using a pulse laser. The must common excitation source in two photon microscopy is the mode-locked Ti:Sapphire laser which emits IR light (wavelength range from 700-1000 nm) in ~100 fs pulses.



Confocal Microscopy

Biphotonic Microscopy

Figure 4

The design of a two-photon microscope strongly resembles (Figure 4) to a confocal system. In both cases, an excitation laser beam is focussed into a sample with an objective. The fluorescence generated from the sample is collected in an epifluorescence configuration and then detected by different methods: single photon counting method for time resolved fluorescence measurements, correlation measurements (see below) and fluorescence imaging. In this latter case, the laser excitation is scanned across the sample by way of beam steering optics typically mirrors mounted on galvanometers or by means of a microplate displacement in the three dimensions.

Two-photon excitation has several advantages:

1- Increased fluorescence collection efficiency: Because of the large difference between excitation and fluorescence wavelengths, they may be easily separated using a simple dichroic mirror without any spectral clipping of the fluorescence. Furthermore in two-photon excitation microscopy, the Raman scattering is always red shifted relative to the excitation wavelength and then does not generate background to the fluorescence as in monophotonic excitation.

2- Localized photoexcitation. In two photon excitation, both the excitation and hence the emission are confined to a small focal volume in 3D (in one photon excitation, the excitation is axially distributed along the laser beam). This leads to several consequences: for fragile molecules which are susceptible to photobleaching, in two photon excitation, the photobleaching is confined to the focal volume whereas in monophotonic excitation the photobleaching occurs also outside the focal volume (the molecules can be photobleached before they ever have the possibility to fluoresce).

3- Deep penetration in turbid media: Biological tissue is usually highly light scattering and then two photon excitation can image significantly deeper than monophotonic excitation (Visible or UV excitation)

4- Easy access to UV energies. Many biological fluorophores required UV excitation energies (tryptophan, tyrosine, nucleic acid bases etc...) Direct UV excitation is difficult , if not impossible, because of damage to the sample. These UV energies (corresponding to wavelengths < 350 nm) can be attained with IR wavelengths by extending the principle of two-photon excitation to three-photon excitation, a method which requires highly concentrated excitation light.

Single molecule spectroscopy

This method has been applied in recent years to detect single molecules in crystalline and amorphous matrices. The basic idea is to excite a very small volume of the sample by focussing a laser beam to a diameter of the order of 1 μm. If the concentration of absorbers is low enough (~nanomole, 10^{-9} M), a single molecule can be selected spatially. For such an experiment to be successful, the molecule should exhibit a strong fluorescence, because its detection takes place by monitoring the fluorescence emission. The technique has been extended to single biological molecules like DNA, RNA, viruses, protein, drug-DNA or drug-protein complexes, etc.....

Fluorescence correlation spectroscopy

The fluorescence correlation spectroscopy (FCS) measures the variations of the fluorescence intensity with time in a small analysed volume. It allows the differentiation of fluorescent molecule populations according to their diffusional rates. FCS is well adapted to study the interaction between a fluorescent probe and its target or between two fluorescent molecules, the conformational modifications, the dynamic of molecules in solution or in cellular medium.

The fluorescence emission is proportional to the concentration of fluorescent molecules. Using a two-photon excitation as described above which corresponds to a very small excitation volume, the variations of the fluorescence intensity around the mean value is related to the diffusion of the molecules through the excitation volume during the analysed time range and to the modifications of the fluorescence properties of the molecules in the excitation volume. The data are analysed by spatial autocorrelation that means by comparison of the data obtained at t and that obtained at $t+\tau$. A typical autocorrelation function is represented in Figure 5. It reflects the fluorescence intensity fluctuations and is a function of the number of fluorescent molecules and of their diffusion rates :

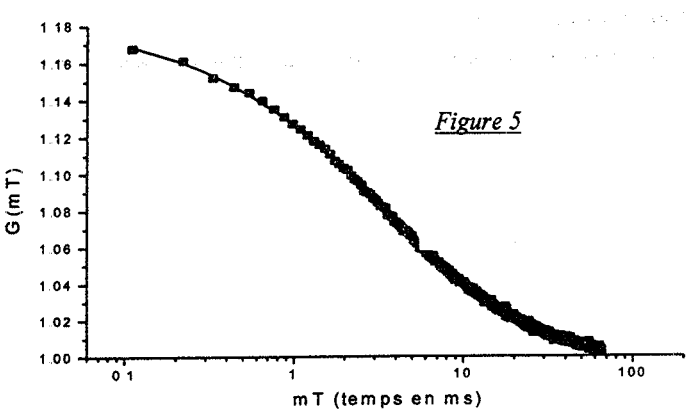


Figure 5

The analytical form of the normalized autocorrelation function for two photon excitation is :

$$g(\tau) = 1 + \frac{(1 - I_b / S)^2}{\sqrt{8} N_m} \left(\frac{1}{1 + (\tau / \tau_d)} \right) \times \left(\frac{1}{1 + (\omega_0 / z_0)^2 (\tau / \tau_d)} \right)^{1/2}$$

I_b background signal, S total signal intensity, τ_d translational diffusion time, N_m number of fluorescent molecules in the excitation volume, ω_0 the beam-waist, z_0 the focal depth along z axis.

The translational diffusion coefficient is calculated as :

$$D = \omega_0^2 / 8\tau_d$$

FCS allows the determination of the excitation volume using fluorescent molecules with known diffusionnal rate constant. The diffusion time depends on the volume of the molecule, the environmental conditions (viscosity, temperature, intracellular medium...).

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Cantor, C.R., Schimmel, P.R. *Biophysical Chemistry*. W.H. Freeman and Compagny, New York (1980).

Bensasson, R.V., Land, E.J., Truscott, T.G. *Excited States and free radicals in Biology and Medecine*. Oxford University press (1993).

Valeur, B. *Molecular Luminescence Spectroscopy*. (S.G. Shulman, ed.)Wiley & Sons (1993).

Cundall, R.B., Dale, R.E. *Time Resolved Fluorescence Spectroscopy in Biochemistry and Biology*. Plenum press, New York (1983).

Lakowicz, J.R., Ed . Topics in Fluorescence Spectroscopy, Volume 1: Techniques, Plenium press, New York (1991).

O'Connor, D.V., Phillips, D. *Time-correlated Single Photon Counting*. Academic Press, London (1984).

Thompson, N.L. *Fluorescence Correlation Spectroscopy*. (J.R. Lakowicz, Ed) Volume 1: Techniques, Plenium press, New York (1991).

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