Importance of and Features in Time-Resolved Fluorescence Spectrometry

Traces in HORIBA's Time-Resolved Fluorescence Spectrometer

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Time-resolved fluorescence measurements reveal intermolecular interactions depending upon the environment of the fluorophore. This paper describes the basic background and some examples of how the fluorescence lifetime is more informative than steady-state fluorescence measurements. HORIBA has many fluorescence technologies and resources.

Introduction

Fluorescence is an emission which occurs as the electric dipole transition from the lowest single excited electronic state to the ground state with a typical lifetime of 10^{-10} to 10^{-7}s . The fluorescence lifetime is sensitive to the local environment of the fluorophore, and it reflects dynamic behavior, process and binding characteristics of fluorophores, and probed macromolecules. Therefore, time-resolved fluorescence applications have expanded extensively into biotechnology, life sciences, medical diagnostics, material sciences, and so on, thanks to the remarkable technical advances of the related equipment, including Quantum Dots,[1] a new type of bio-probe.

Fluorescence Lifetime Imaging Microscopy (FLIM) reveals locally distinct environmental regions detected with different lifetimes, even though fluorescence intensity images would not distinguish them.[1-3]

Through steady-state fluorescence measurements, it is possible to observe or evaluate fluorescence spectra, excitation spectra, fluorescence quantum yield (the number of emitted photons per the number of absorbed photons) and polarization/anisotropy.

On the other hand, the following measurements can be gained through time-resolved fluorescence: lifetime obtained by fluorescence intensity decay, time-resolved fluorescence spectra, and fluorescence anisotropy decay.

This article focuses on the benefits of time-resolved fluorescence measurements along with some of HORIBA's history in this field.

How is the Fluorescence Lifetime Described?

The excited-state population decays with the rate constant $k = (k_r + k_{nr})$ of all de-excitation processes according to

$$\frac{dn(t)}{dt} = -kn(t) \quad \cdots\cdots (1)$$

where $n(t)$ is the number of excited molecules at time t following the excitation, k_r is the radiative decay rate, and k_{nr} is the non-radiative decay rate. (2) is

derived by rearranging the equation of (1) and integrating.

$$\int_{n_0}^{n(t)} \frac{dn(t)}{n(t)} = -\int_0^t k dt. \quad \cdots\cdots (2)$$

The Equation 2 results in an exponential decay of the excited state population, $n(t) = n_0 \exp(-kt)$, where n_0 is an initial excited-state population of the fluorophores excited by a pulsed light. Since the fluorescence intensity is proportional to $n(t)$, the time-dependent fluorescence intensity $I(t)$ is described with single exponential decay by

$$I(t) = I_0 \exp(-kt) \quad \cdots\cdots (3)$$

where I_0 is the fluorescense intensity at $t = 0$ immediatly following the exitation pulse. Since the lifetime τ is the inverse of the total decay rate, i.e. $\tau = k^{-1}$, $I(t)$ is expressed as the familiar form:

$$I(t) = I_0 \exp(-t / \tau) \quad \cdots\cdots (4)$$

The lifetime τ is the time it takes for the fluorescence intensity to decay to 1/e or 36.8%.

The non-radiative process includes many de-excitation path ways, such as internal conversion k_{ic}, intersystem crossing k_{isc}, dissociation into reaction products k_d and bimolecular quenching process k_m [M] with the molecule M:

$$k_{nr} = k_{ic} + k_{isc} + k_d + k_m [M] \quad \cdots\cdots (5)$$

k_r and k_{nr} are obtained with the quantum yield Φ_F and lifetime τ as

$$\tau = 1 / (k_r + k_{nr}) \text{ and } \Phi_F = k_r / (k_r + k_{nr}). \quad \cdots\cdots (6)$$

Time-Correlated Single-Photon Counting

Time-correlated single-photon counting (TCSPC) may be regarded as the most sensitive method for time-resolved fluorescence measurements (TRFM). It has a wide dynamic range by detecting a single photoelectron following pulsed excitation.

In a TCSPC system, a time-to-amplitude converter (TAC) measures the time interval between pulsed excitation and arrival of the fluorescence photon detected as a photoelectron by a photomultiplier or microchannel plate. With many excitation repetitions, a histogram of the photoelectron events frequency vs. the time forms fluorescence decay curve. Details on TCSPC are available.[4-6]

HORIBA's Time-Resolved Fluorometers

HORIBA's first product as a TCSPC apparatus was launched in Kyoto, Japan in 1983. The model was named as **NAES**-1100, taken from "Nanosecond Emission Spectrometer", and also because it sounds like the word "nice". HORIBA launched the NAES-500/550 and NAES-700F/700L in 1985 and 1990, respectively, as versions with a more affordable price and higher performance.

The NAES-700L had an N_2 laser and an N_2 pumped-dye laser, and these were

Figure 1 NAES-1100

developed as an excitation source for expanding applications for weak and sub-nanosecond photoluminescent materials. It achieved the highest repetition rate of 1 kHz,[7] whereas the conventional N_2 laser was generally around 50 Hz at the time. Although the 1-kHz pulse light source was still too low repetition for TCSPC, multi-TAC overcame this issue as stated below.

The data shown in this article were all obtained using the NAES Series almost three decades ago, but they are useful in understanding basic ideas on importance of TRFM.

Advantages of the NAES Series

The conventional TCSPC system counts only a single photoelectron using one TAC scan. Considering photoelectron generation as being a statistical event, the detected fluorescence intensity has to be weakened so that only one photoelectron is generated. This means that data acquisition takes a long time. To overcome the inefficient measurement time, the multi-TAC invented by Shindo et al.[8] was adopted to detect multiple photoelectrons using one start scan.

The NAES actually had 8 TACs. Simultaneously, two of them were used to monitor the profiles of the excitation sources, and six of them were used to monitor fluorescence profiles.[7]

The NAES was designed as user-friendly TCSPC apparatus with full operation at the touch of a key for measuring transient fluorescence phenomena and data processing. No NIM (Nuclear Instrument Modules) and no large mainframe computers were required.

The NAES can process data for up to 3 exponential decays using least-squares curve fitting and deconvolution for stored data, even while simultaneously acquiring data for fluorescence decays and excitation profiles.

The Importance of Time-Resolved Fluorescence Measurements

Time-Resolved Fluorescence Measurements (TRFM) has the following four advantages compared to steady-state fluorescence measurements:

First, **time-resolved fluorescence spectra** reveal the real dynamics in molecular interactions that occur in the excited state. Figure 2 shows one of the examples of time-resolved florescence spectra. After pulsed excitation, excimer emission (470 nm) increased and monomer emission (387 nm) decreased at 10 ns (green), 20 ns (red), 30 ns (purple), 40 ns (yellow), and 50 ns (blue).

Second, **time-dependent anisotropy** is very informative, as will be described in the next paragraph along with the theoretical background. Time-dependent anisotropy elucidates the size/volume, rotational information (rotational correlation time θ), local viscosity, and conformation change of the probed macromolecule. Figure 3 shows an example of the anisotropy decay of biological protein, apomyoglobin labeled using ANS[*1]. As a result of analyzing the anisotropy decay, under the test conditions of $T = 290$ K, $\eta = 0.106$ poise, we found that the rotational correlation time of the biological protein (apomyoglobin labeled using ANS) was $\theta = 9.3$ ns, and $V = 3.5 \times 10^4$ A^3. The basic theory on anisotropy decay is

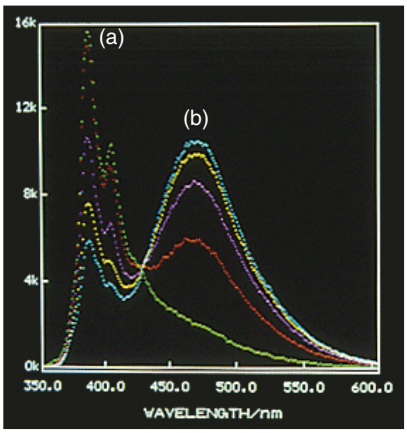

Figure 2 Excimer formation in de-gassed sample: Pyrene in cyclohexane (2.2 mMol).
(a) Monomer Emission, (b) Excimer emission.

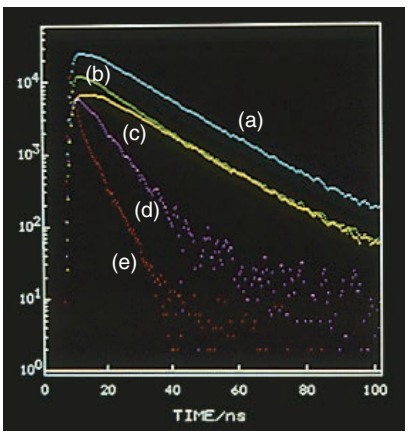

Figure 3 Anisotropy decay: (a) $I_{\parallel}(t) + 2I_{\perp}(t)$, (b) $I_{\parallel}(t)$, (c) $I_{\perp}(t)$, (d) $I_{\parallel}(t) - I_{\perp}(t)$, (e) Lamp profile. Sample was provided by Dr. Tunehisa Araiso, Hokkaido Univ.

described in the paragraph "Time-resolved fluorescence anisotropy".

*1: ANS: 1-Anilino-8-naphthalene sulfonate

Third, **fluorescence lifetime** (FL) is an intrinsic property of the fluorophore itself in a fixed environment, whereas fluorescence intensity (FI) with steady-state measurements very much depends on the various test conditions, such as the fluorophore concentration, photobleaching, and changes in equipment conditions like excitation intensity and optical systems (sample cells, optical mirrors, optical lenses, etc.) over time. Then many corrections and calibrations are required for steady-state FI tests. Therefore, although steady-state measurements are often used, FL is more suitable for quantitative analysis to elucidate the dynamic characteristics of molecules that are in an electronically excited state. FL is highly sensitive to changes in the localized sample environment, such as viscosity, polarity, dielectric constant, and pH. Also, depending on the type of intermolecular interaction, it is actively used for studying molecular interaction with other molecules or different conformational states of macromolecules. These give rise to non-single exponential decay, which is expressed as follows in double exponential case:

$$I(t) = A_1 \exp(-t / \tau_1) + A_2 \exp(-t / \tau_2) \quad \cdots\cdots (7)$$

Even in such a case with overlapped fluorescence wavelengths, FL analysis discriminates between multiple components with a weighted ratio of $A_1 : A_2$, whereas steady-state fluorescence measurements (integration of Equation 7) cannot distinguish the existence of multiple components.[9] Figure 4 shows the results as an example.[10] Cancerous DNA and normal DNA labeled using acridine orange (AO) are known to show different lifetimes. In the modeling binding mode of DNA and AO, AO is dissolved in a solution of sodium dodecyl sulfate (SDS). The 535 nm (shorter lifetime) and 630 nm (longer lifetime) fluorescence components were considered to be due to AO monomers and dimers respectively binding to SDS. The monomers and dimers are localized with distinct interaction. FL measurements play an important role for obtaining the distance and energy transfer efficiency between donor and acceptor molecules. The theoretical background will be described in the next paragraph.

Fourth, the **scattered pulse excitation** light can be eliminated in time-resolved measurements by excluding the excitation light profile time data. Removing the scattered pulse excitation light is especially important for FLIM and surface measurements for non-solution samples.[3]

Photoluminescence lifetime

The photoluminescence lifetime is used for evaluating semiconductor materials. Photoluminescence is the emission upon the re-combination of electrons and the holes in conduction and valence bands, respectively. These occur following light excitation. In some cases, impurity levels are involved in the energy relaxation process. Figure 5 shows photoluminescence decay curves of gallium indium phosphide (GaInP) grown in different temperatures. Sample (b) showed a shorter lifetime, which is due to more contributions from non-emitting re-combination processes. Then sample (a) is a better laser material.

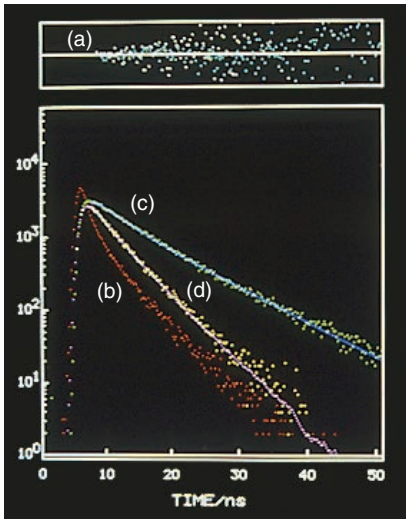

Figure 4 Excitation: 470nm, SDS conc.: 5 nM
(a) Residual, (b) Excitation profile
(c) Obs.630 nm, τ_1=2.48 (41.2%), τ_2=9.03 ns (58.8%)
(d) Obs.535 nm, τ_1=1.41ns (54%), τ_2=3.48 (45.5%)

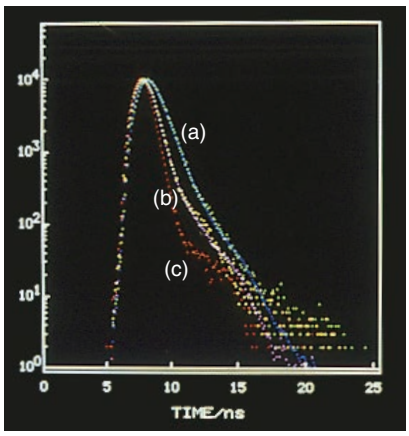

Figure 5 Excitation 337 nm(N_2 laser)
Observation 660 nm.
Sample (a) τ_1=680 ps(83.1%), τ_2=1.26 ns(16.9%)
Sample (b) τ_1=199 ps(83.8%), τ_2=1.16 ns(16.2%)
(c) Laser pulse profile. Sample: provided by Dr. Shigekazu Minagawa, Central Research Laboratory, Hitachi Ltd.

Förster Resonance Energy Transfer

Förster resonance energy transfer (FRET) elucidates molecular interaction and conformal changes using donors (D) and acceptors (A) for evaluating the energy transfer efficiency and the distance between D and A. FRET refers to the non-radiative transfer of an electronic excitation from D to A.

The Förster distance in Å is given by

$$R_0 = 0.211[\kappa^2 n^{-4} \Phi_D J(\lambda)]^{1/6} \quad (8)$$

where n is the refractive index, Φ_D is the donor quantum yield, and κ is the orientation factor for the transition dipoles, typically κ^2 is 2/3 for a random orientation. $J(\lambda)$ is an overlap integral expressing the degree of spectral overlap between donor emission $F_D(\lambda)$ and the acceptor absorption $\varepsilon_A(\lambda)$:

$$J(\lambda) = \int_0^\infty F_D(\lambda)\, \varepsilon_A(\lambda) \lambda^4 d\lambda. \quad (9)$$

The rate constant of energy transfer k_{et} can be represented by the Förster equation as

$$k_{et} = (1 / \tau_D)(R_0 / R)^6 \quad (10)$$

where τ_D is the fluorescence lifetime of the donor in the absence of an acceptor. R_0 is the Förster distance at which energy transfer is 50% efficient. The energy transfer efficiency *(E)* and the distance *(R)* between D and A are given as:

$$E = 1 - (\tau_{DA} / \tau_D), \quad R = R_0 [(1 / E) - 1]^{1/6} \quad (11)$$

where τ_{DA} is the lifetime of the donor in the presence of the acceptor.

Time-Resolved Fluorescence Anisotropy

Since molecules are excited along the transition moment vector, pulsed polarized incident light generates excited state molecules with a parallel transition vector to the incident polarized light.

Then depolarization occurs due to energy transfer to other molecules or molecular rotation caused by Brownian motion.

Time-dependent anisotropy *r(t)* is given by[1, 5, 6]

$$r(t) = [I_\parallel(t) - I_\perp(t)] / [I_\parallel(t) + 2I_\perp(t)] \quad (12)$$

where $I_\parallel(t)$ and $I_\perp(t)$ are the time-dependent fluorescence intensity components parallel and perpendicular to the vertically polarized incident light. $I_\parallel(t)$ and $I_\perp(t)$ for single θ (fluorescence rotational correlation time) were derived by Spencer and Weber[5] as

$$I_\parallel(t) = e^{-t/\tau}(1 + 2r_0\, e^{-t/\theta}),\ I_\perp(t) = e^{-t/\tau}(1 - r_0\, e^{-t/\theta}) \quad (13)$$

where r_0 is the anisotropy at pulsed excitation $t = 0$.

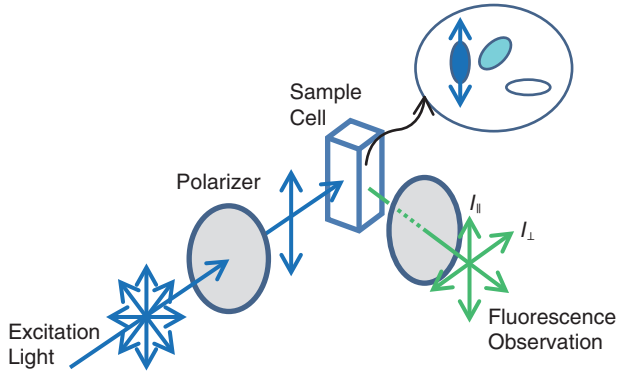

Figure 6 Polarized excitation and fluorescence detection.

Substituting the above equation, $r(t)$ is given by

$$r(t) = r_0 \, e^{-t/\theta}$$
$$\theta \text{ is related to } \theta = 1 / 6D_r = \eta V / k_B T \quad \cdots\cdots (14)$$

where D_r is the rotational diffusion coefficient, η is the local viscosity, V is the effective molecular volume, k_B is Boltzmann's constant, and T is the absolute temperature. Thus θ is evaluated by anisotropy decay, and η or V can be evaluated if either one of them is known. θ is related to the relaxation time of rotation (ρ) as $\theta = \rho / 3$.

In steady-state, the Perrin equation is used as

$$1 / r = (1 / r_0)(1 + \tau / \theta), \quad r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp}) \quad \cdots\cdots (15)$$

where r, r_0, and τ are anisotropy, initial anisotropy, and the fluorescence lifetime. The three parameters r_0, τ, and θ cannot be evaluated by measuring r in a steady state under the one condition of (T, η). In order to evaluate these parameters, a ($1 / r$) vs. (T / η) plot (Perrin plot)[1, 9] is required. Time-resolved anisotropy is easier way than steady-state measurement for evaluating information on molecular rotation and size.
Table 1 shows the summary of the features in TRFM.

HORIBA Group Fluorometers

Before the NAES Series was launched in Kyoto, HORIBA's TCSPC technologies originated from IBH, which was founded in 1977 in Glasgow, Scotland in the UK as a spin-off company from Strathclyde University. IBH joined the HORIBA Group in 2003 as HORIBA Jobin Yvon(JY) IBH. The company celebrated its 40th anniversary this year (2017), and has expertise in developing advanced TCSPC systems. Details on IBH's technology will probably be described in subsequent articles. Using LED devices and diode lasers as pulsed light sources has significantly improved the optical intensity of light sources. The performance of electronics, computers, and software is getting remarkably advanced with modern technical innovations. As the technologies have evolved, we have also expanded our scope of fluorescence spectroscopy applications through collaboration with many partners.

Another fluorescence company PTI (Photon Technology International, Inc.) joined the HORIBA Group in 2014. PTI was founded in 1983 at London, Ontario, Canada, where the original TCSPC company PRA (Photon Research Associate) was funded as a joint-venture company of Western Ontario University. Some key members of PRA moved to PTI. Therefore, HORIBA has now has many fluorescence technologies and

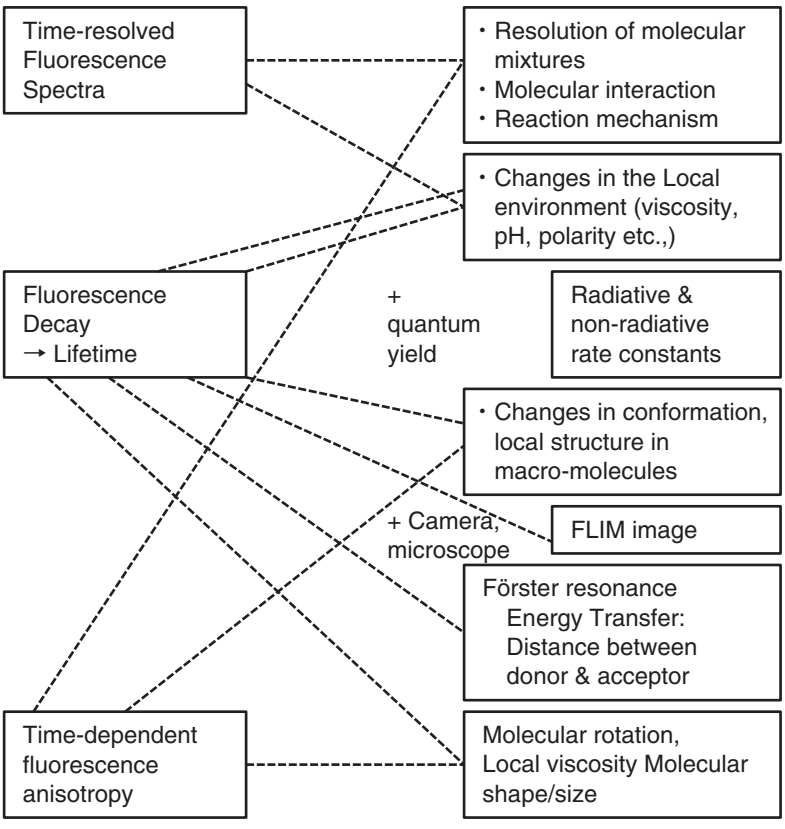

Table 1 Observable and Molecular property in TRFM.

personnel in this field globally, including JY and Spex[*2].

*2: JY acquired SPEX Industries in 1988.

Conclusion

We have emphasized the value of TRFM and the basic theoretical background, and gave some examples of time-resolved spectra and lifetime and anisotropy decay. They reflect the real-time processes and sensitivity of fluorescence lifetimes to the local environment of the fluorophores. However, combining steady-state measurements and the time-resolved fluorescence and phosphorescence methods makes the fluorometry more informative, because they play complementary roles.

HORIBA will continue to use its global human resources and technologies related to fluorometry to provide more valuable technologies and products to the users of fluorometers.

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