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Finding the Time for Fluorescence
Its Measurement and Applications in Life Science

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We summarise how developments in technology have brought fluorescence lifetime spectroscopy from being the preserve of the specialist to becoming a major tool for research across many science and engineering disciplines. We highlight the advantages which fluorescence lifetime measurements can bring, not only to underpin research, but also through application in helping to solve real-world problems. We illustrate this with recent examples in cancer and Alzheimer's research, which are aimed at improving disease understanding, diagnosis and therapeutics.

Introduction

Fluorescence is a phenomenon that offers a powerful research tool and finds applications across numerous disciplines. These include the life sciences, medicine, nanotechnology, materials, the environment and energy to name but a few. Crucially fluorescence continues to demonstrate how it underpins critically important breakthroughs in techniques even down to the single molecule level. This is perhaps most notable in healthcare, where fluorescence is ubiquitous in disease diagnostics and has provided the basis for sequencing the human genome. Understanding, controlling and designing fluorescence assays relies on being able to accurately and precisely measure the properties of fluorescence. These properties form a multi-dimensional fingerprint that can be described conveniently by:

$$\text{Fluorescence} = f\,(I, \lambda_{exc}, \lambda_{em}, p, r, t) \quad (1)$$

Where I describes the fluorescence intensity, λ_{exc}, λ_{em} the excitation and emission wavelengths that give rise to their respective spectra, p the polarization that can be used in anisotropy measurements, r the position as used in microscopy and t the time described by the fluorescence lifetime τ, the exponential decay constant following δ function excitation. Accessing this fingerprint quickly comes down to the design and functionality of instrumentation. Our company, HORIBA Jobin Yvon IBH Ltd, specialises in the design, development and manufacture of fluorescence lifetime systems and associated components and software. This year celebrating the 40th anniversary of its founding in 1977 the company is one of the very earliest spin-out companies from a Scottish University and joined HORIBA in 2003 to form together a leading supplier of fluorescence spectrometers. Fluorescence lifetime spectroscopy is the most rapidly growing part of the whole fluorescence market. The reason for this is because fluorescence lifetime measurements bring with them:

1. Time-resolved capability for revealing dynamical information and kinetic rate parameters.
2. Increased specificity by means of temporal discrimination against

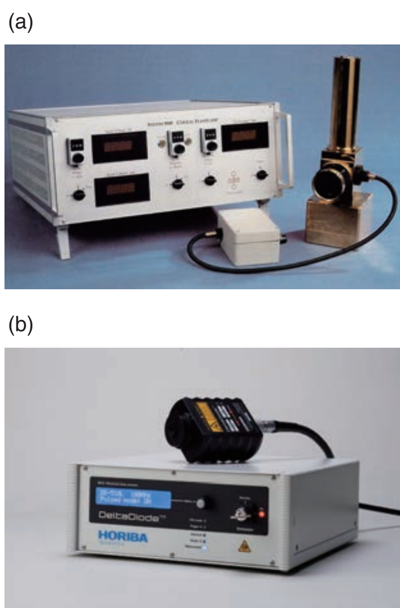

Figure 1 Model 5000F coaxial nanosecond flashlamp (a) and DeltaDiode picosecond LED/LD (b).

background and unwanted fluorescence.

3. Independence from fluorophore concentration changes such as those caused by photo-bleaching.
4. Ease of calibration and comparison between samples, which contrasts with the complexity of fluorescence intensity or quantum yield measurements.

Dr. Kiyoaki Hara HORIBA, Ltd. has provided a very useful description of the basic theory and technique of fluorescence lifetime measurement in an accompanying article in this newsletter so we will not repeat it here, but put simply in terms of information content, the additional benefits which lifetimes bring to fluorescence spectroscopy are analogous to a movie as compared to a photograph.

The Journey to Present-Day Lifetime Capabilities.

Like many instrument companies our origins lie in University research. Initially working together in the Schuster Laboratories at the University of Manchester, and subsequently in the Department of Physics at the University of Strathclyde in Glasgow, co-author David Birch joined with Bob Imhof and Tony Hallam to form in 1977 the basis of what is now HORIBA Jobin Yvon IBH Ltd. Co-author David McLoskey took over from Bob Imhof as IBH Technical Director in 1997 and the company has since grown to employ 18 “Horibarians”. The philosophy which drove the company to success in the early days still inspires it today. This is based on satisfying the need for the latest research capabilities to be made widely available to customers and from the outset we believed the best approach to be that of the digital technique of time-correlated single-photon counting (TCSPC).

The mainstay of our success in the early manufacturing years was the Model 5000F all-metal coaxial flashlamp shown in Figure 1a. Operating typically at 30 kHz repetition rate this design was the first nanosecond flashlamp to reliably overcome distortion on decay curves caused by radio-frequency oscillations generated by the spark discharge. Although the pulse energy was low, typically ~ < 1 pJ, and average power < 1 μW, single-photon timing detection sensitivity made flashlamps workable. Being very competitively priced in comparison to mode-locked lasers, versions of this design[1, 2] became a widely used source for these measurements for over two decades, eventually being superseded by semiconductor sources. The latter became particularly important for UV LED excitation of protein fluorescence lifetime, initially through our NanoLED range[3] and latterly in the DeltaDiode range[4] (Figure 1b) which at repetition rates up to 100 MHz have a unique place in the field. The combined advantages of the concomitant ~ 1000x higher data collection rate, faster pulses, maintenance-free operation, higher stability and monochromatic nature of their laser variants quickly led to semiconductor sources superseding flashlamps in TCSPC.

Quite early on in the company’s development we recognized the need and opportunities in accessing as much as possible of the fluorescence fingerprint (Equation 1) in a single measurement and in the 1980s set about this task by developing TCSPC signal multiplexing and routing techniques for the first time. Initially we worked on a two-channel approach to overcome temporal changes in the excitation pulse by means of simultaneous acquisition of fluorescence and excitation (SAFE).[5] Coincidentally, Dr. Hara (described in an

accompanying Review article in this December issue) was working to develop the NAES lifetime system to achieve a similar goal around the same time. The main difference between our approaches was the NAES lifetime system used multiple time-to-amplitude converters (TAC) whereas we used a single TAC. Our two channel implementation was also ideal for simultaneously recording the two orthogonal planes of polarization needed for anisotropy decay measurements.[6] Soon we extended the approach to 4 channels by using fiber optic coupling to a spectrograph to demonstrate for the first time simultaneous decay measurements at multiple fluorescence wavelengths.[7] This pointed the way towards much faster measurement of time-resolved emission spectra than the sequential wavelength-stepping approach used previously. Nevertheless, measurement times were still measured in hours due to the low repetition rate limitation of the flashlamp, and TCSPC took a while to shake off its reputation as a slow method even after semiconductor diode excitation became widespread.

Our research and development in multiplexing led to us to combining the SAFE and dual fluorescence channels in the Model 5000W SAFE (Figure 2). This instrument was a highly versatile fluorescence lifetime system as it included not only three channels, but the capability to multiplex up 16 TCSPC channels simultaneously.[8] This was achieved by virtue of the first ASIC designed for fluorescence. Based on CMOS technology the device is illustrated in Figure 2 in a NIM module which were commonly used at the time. The combination with multi-anode microchannel plate photomultipliers then opened the door to various forms of multiplexed imaging.

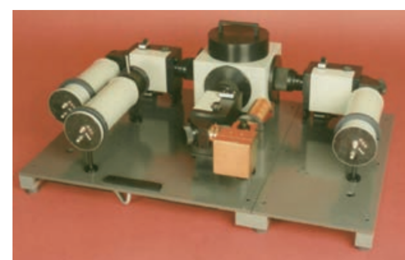

Figure 2 Model 5000W SAFE and its 16 channel TCSPC multiplexing module.

Together these developments provided a benchmark for the future towards accessing more of the fluorescence contour by means of simultaneous measurement. This goal has still to be fully achieved and is a fertile area of research today where single-photon avalanche diode array detectors promise progress in some applications despite their much reduced detection area as compared to conventional photomultipliers.

Today our instruments have evolved from the early days to be much more user-friendly, while still retaining the cutting-edge research capabilities required by our customers. For example the recently released EzTime software with its optimised touchscreen interface is unique for the field and dramatically simplifies the process of fluorescence lifetime measurement via script automation and "one-click" automated fitting and presentation of results. In addition, the instrument auto-identification and configuration provided by our F-Link bus technology ensures easy retro-fit and upgrades with such as excitation sources and sample compartment accessories. Monolithic Time-to-Digital Converters (TDCs) have replaced TACs and ADCs in recent systems such as the DeltaFlex, and while these don't offer the extreme electrical time resolution achievable with TACs (a few hundred femtoseconds is readily achievable with a TAC nowadays), all-digital TDCs in combination with 100 MHz diode light sources can attain previously undreamt of measurement times of less than 1ms - a long way from the several hours that we endured in the early days. Such short measurement times allow TCSPC measurements to be continuously streamed to disk, rates as high as 20,000 histograms per second opening-up whole new applications for TCSPC, not only in fluorescence lifetimes, but also in 3D imaging and LIDAR. Personal computers are now so powerful that photons need not be counted and logged in fixed size histograms, but may now be "tagged" with information about conditions existing

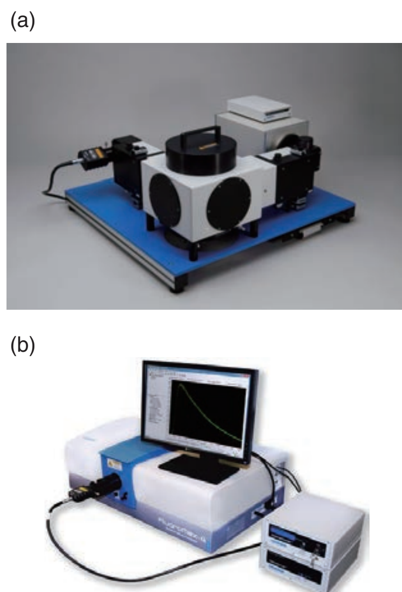

Figure 3 DeltaFlex dedicated lifetime system (a) and a hybrid FluoroMax-TCSPC lifetime system (b).

at the instant of their detection and subsequently streamed to disk for sorting and manipulation - each retaining their own “individuality” within the terabyte files that result.

Our range covers both stand-alone lifetime systems (Figure 3a) and hybrid steady-state lifetime systems (Figure 3b). The provenance from our early systems (Figure 2) in retaining a modular construction is clearly evident in the DeltaFlex. This flexible configuration permits simple expansion to accommodate a whole range of options including additional detection channels, microchannel plate photomultipliers, infra-red detection, continuum lasers, cryostats etc. Together their ease of use and high performance continue to widen the appeal of fluorescence lifetime measurement to new users across many disciplines.

Life (time) Science Examples Related to Healthcare

As already mentioned the impact of fluorescence is perhaps never more important than when it is helping to improve healthcare. Here we choose three diverse examples, each making use of the DeltaFlex, to illustrate the power of simple exponential fluorescence decay analysis in providing new molecular insight.

Melanin structure: Placing the time

The skin pigment melanin is our body’s protection against ultra-violet (UV) light and is also present in the brain and eyes while occurring in nature in many different forms including fungi, marine life and fruit. However, it might surprise the reader to learn that the structure of melanin is unknown. Its constituents are known to be dihydroxyindoles, and these are thought to arrange in a sheet-like structure akin to graphite. Indeed recent fluorescence probe studies of the sheet-sensing probe Thioflavin T (ThT) using the Fluorolog have confirmed this,[9] but how the basic constituents are arranged at the molecular level remains somewhat of a mystery. There are several reasons why determining the structure of melanin is important. Although melanin protects us from the harmful effects of UV light it is also thought to play a role in melanoma, the most aggressive form of skin cancer. In addition melanin has potential as a biomimetic since it possesses a very unusual combination of physical properties. It has an extremely broad absorption spectrum from the UV to the near infra-red, is paramagnetic and has an electrical conductivity that is hydration-dependent for over 8 orders of magnitude.

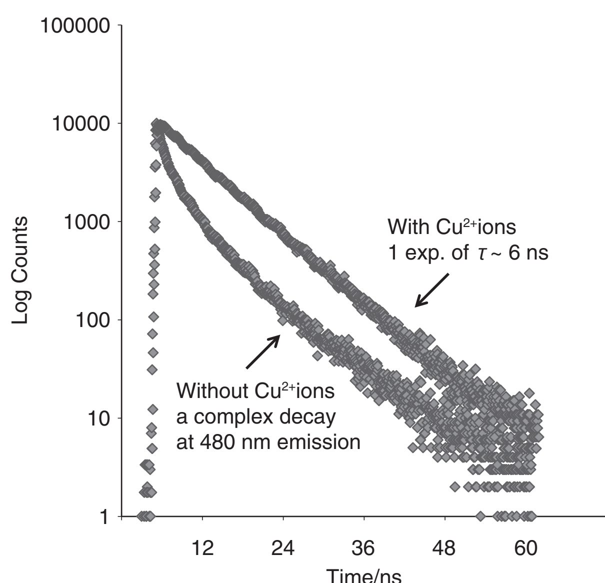

Figure 4 Fluorescence decay of melanin. In the presence of 4 μM Cu^{2+} ions the decay becomes a single exponential of τ ~ 6 ns at 480 nm emission, suggesting a simplified structure.

The fluorescence of melanin is weak and for many years was thought too difficult to study. However, advances in powerful and stable sources such as the DeltaDiode range are opening up new avenues in melanin research. Melanin’s fluorescence decay is complex and certainly not a single exponential, which suggests a complex structure. Copper ions usually quench fluorescence, but Figure 4 shows how the laboratory synthesis of melanin using the oxidation of L-3,4-dihydroxylphenylalanine (L-DOPA) in water in the presence of copper ions (to mimic the natural synthesis in melanosomes) increases the fluorescence decay towards a single exponential of ~ 6 ns at 480 nm emission.[10]

A mono-exponential decay implies a single excited state and therefore the presence of a distinct constituent present in melanin's structure. There are likely to be other structures present, but being able to place the origin of this 6 ns decay time could be a very useful step towards revealing melanin's structure.

Aggregation of beta-amyloid: Alzheimer's at time zero

When protein misfolds it tends to aggregate, leading to a range of diseases described as amyloidosis. This group includes some of the most debilitating diseases in modern society such as Alzheimer's (AD), type II diabetes, Creutzfeldt-Jakob disease and Parkinson's disease. AD is the most common of all neurodegenerative disorders, there is no cure and no simple clinical diagnosis. Amyloidosis is characterised by the aggregation of particular proteins, specific to each disease, that form stable and insoluble amyloid fibrils, which are retained as deposits and not cleared from the body. In the case of AD, the amyloid fibrils are formed by aggregation of the beta-amyloid ($A\beta$) protein. $A\beta$ is a cleavage product of the beta-amyloid precursor protein forming a 38 to 42 amino acid long residue of APP. Originally it was thought that $A\beta$ fibril deposits in the brain as amyloid plaques were the main pathogenic factor leading to neuron death. However, recently it has become clear that neuronal dysfunction and cell death are caused by the smaller soluble $A\beta$ oligomers, which are formed in the initial stages of aggregation and which disrupt the cellular membrane. Therefore detecting $A\beta$ aggregation in its early stages is crucial for a full understanding of the causes and the development of better therapeutics for AD.

Fluorescence spectroscopy lends itself to the study of aggregation processes, and indeed extrinsic probes like ThT are widely used to study $A\beta$ fibril formation. However, ThT has been shown to report only on the later-stage formation of beta-sheets leading to fibrils, not on the early oligomer formation where if anything ThT can disrupt the early stages of aggregation. However, a fortunate quirk of nature is that $A\beta$ has only one tyrosine and no tryptophan to receive energy from the excited tyrosine. In photo physical terms this represents a relatively simple system to what is usually found in protein research and is ideally placed for exciting with a NanoLED or DeltaDiode at ~ 285 nm; the hypothesis being that early-stage aggregation can be monitored via the fluorescence decay. This indeed turns out to be the case,[11] but interesting enough, not by means of changes in decay parameters (which are negligible), but by their relative amplitudes as shown in Figure 5. This behaviour is thought to represent changes in the relative abundance of three tyrosine rotamer orientations.

Hence the intrinsic fluorescence decay of $A\beta$ provides a new opportunity for not only understanding the underlying aggregation kinetics which cause AD, but also developing new therapeutics for its treatment.

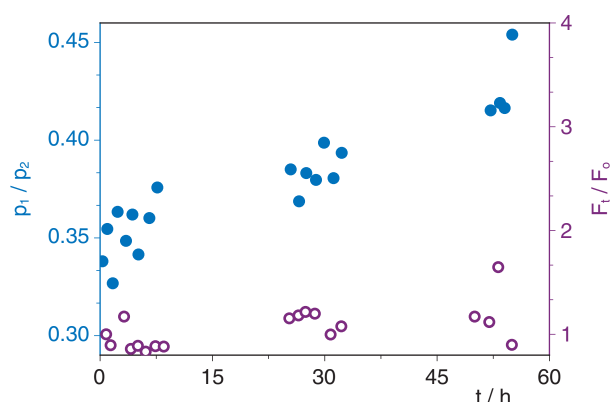

Figure 5 Relative amplitude of two decay parameters (p_1/p_2) when a 3-exponetial decay model $i(t) = \Sigma p_n exp(-t/\tau_n)$, for n=3, is used to describes the tyrosine fluorescence decay of $A\beta$ during the early stages of aggregation. F_t/F_0 describes the fluorescence intensity of the sheet-sensing probe ThT during this time and it can be seen to provide little information on the cytotoxic oligomer formation which occurs as a precursor to Alzheimer's disease.

A bespoke cancer biomarker: time for detection

The previous examples monitored a naturally occurring process, but here we further illustrate the versatility of fluorescence lifetime measurement through research into a bespoke design of nanoprobe capable of detecting messenger RNA (mRNA) cancer biomarkers in cells. mRNA plays a key role

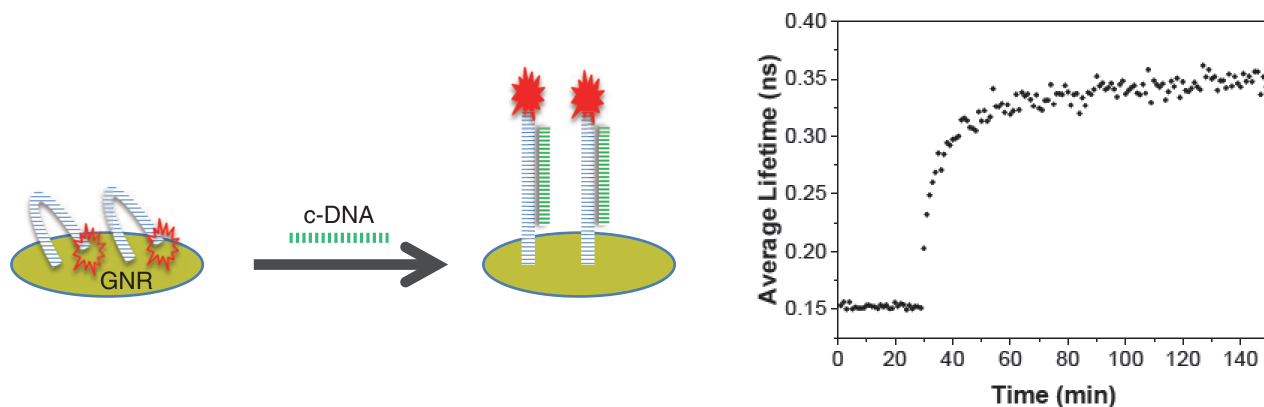

Figure 6 GNR hairpin sensor sprung by binding to c-DNA and the consequential increase in the average fluorescence lifetime of oligonucleotide labelled with 6-carboxyfluorescein as hybridization occurs. Excitation was at 474 nm using the NanoLED laser diode.

in the cellular production of protein. Detection of mRNAs with good specificity and sensitivity will enable an early-stage diagnosis of disease and assist in monitoring and evaluating the efficacy of treatment. Moreover, detection of mRNA provides valuable information for understanding the fundamental mechanisms in cells.

One of the most exciting developments in fluorescence in recent years has been the application of plasmonics.[12] The DeltaFlex has recently been used to research and optimize a plasmonic sensor for mRNA cancer biomarkers whereby a dye-labelled oligonucleotide, bound as a hairpin to a gold nanorod (GNR), forms the basis of the sensing. Initially the fluorescence of the dye is plasmonically quenched by the GNR, but by matching the oligonucleotide to the mRNA of interest the hairpin is sprung by means of hybridization with a complementary sequence in the mRNA and fluorescence enhanced.[13] The sensor is depicted in Figure 6 whereby complementary (c-) DNA simulates the cancer mRNA biomarker. The effect on the average fluorescence lifetime of the 6-carboxyfluorescein label as the hairpin is sprung, and the c-DNA is gradually attached to the GNR, is clearly evident. To obviate the excitation of endogenous cell fluorescence there are clear attractions in exciting in this application in the visible not the UV.

Gold nanorods enter cells with ease and to a varying extent without the need for transfection agents, so in this application all the previously mentioned advantages of fluorescence lifetime measurements, such as being independent of dye concentration, really start to count.

Conclusions

Fluorescence lifetime techniques and applications have clearly come a long way since the early days when the fundamental photophysics of dyes was still being discovered and most applications were centered around chemistry. There is no doubt HORIBA and IBH have made, and continue to make together, key improvements to instrumentation which have enhanced performance and widened applications through ease of use.

How has the market changed? Well the price of systems has fallen in real terms and their capability increased, on-line data analysis and control are now routine, ease of use and software are now as important as specifications,

systems are now more compact and customers now need only be users not experts to benefit. The range of disciplines served has increased considerably and there is now more of a partnership in research between the supplier and the customer. The HORIBA FluoroFest series of international workshops provides a great example of this. FluoroFest brings together our HORIBA specialists and the various scientific communities to exchange ideas and experiences in the common language of fluorescence. The latest and 12th in the series was held in April 2017 in Glasgow to widespread acclaim. Looking to the future innovation is ongoing. We can see major opportunities in miniaturization. Many of these are linked to semiconductor technology in terms of sources, photon detection, data acquisition and processing, all of which will enable more of the fluorescence signature to be determined in a single measurement in a shorter time and eventually lead to new types of sensor systems. Not only sensors for healthcare but also in other sectors such as food, energy, chemicals and the environment. With miniaturization we envisage fluorescence lifetimes moving more “out of the box” and into the hands of users. “Time in hand” as you might say! Of course bench-top spectrometers like the DeltaFlex will continue to be crucial for benchmarking new developments and offering the ultimate in research flexibility and performance.

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