Instrumentation for Fluorescence Lifetime Measurement using Photon Counting

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Abstract

We describe the evolution of HORIBA Jobin Yvon IBH Ltd, and its time-correlated single-photon counting (TCSPC) products, from university research beginnings through to its present place as a market leader in fluorescence lifetime spectroscopy. The company philosophy is to ensure leading-edge research capabilities continue to be incorporated into instruments in order to meet the needs of the diverse range of customer applications, which span a multitude of scientific and engineering disciplines. We illustrate some of the range of activities of a scientific instrument company in meeting this goal and highlight by way of an exemplar the performance of the versatile *DeltaFlex* instrument in measuring fluorescence lifetimes. This includes resolving fluorescence lifetimes down to 5 ps, as frequently observed in energy transfer, nanoparticle metrology with sub-nanometre resolution and measuring a fluorescence lifetime in as little as 60 µs for the study of transient species and kinetics.

Keywords TCSPC · Fluorescence lifetime · Photon counting · *DeltaFlex* · HORIBA Jobin Yvon IBH

1
Introduction

Fluorescence is a phenomenon that finds application across numerous disciplines, from life sciences and biomedicine to nanotechnology and materials. Fluorescence is not only a powerful molecular research tool, but also underpins critically important breakthroughs in techniques [1,2]. This is perhaps most notable in healthcare, where fluorescence is ubiquitous in disease diagnostics and 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. This 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, the fluorescence lifetime τ being the exponential constant describing the decay of fluorescence following δ-function impulse excitation.

The company is one of the very earliest spin-out companies from a Scottish University, celebrating the 40th anniversary of its founding in 2017. Now, as part of HORIBA, it plays a key role in HORIBA's present-day market-leading position in sales of fluorescence spectrometers [3]. The global market for analytical instrumentation exceeds $50Bn and within this figure fluorescence spectroscopy and microscopy total $500m with fluorescence lifetime spectroscopy the most rapidly growing part of the whole fluorescence market, increasing at 8% compound annual growth rate [3]. The reason for this is because fluorescence lifetime measurements bring with them not only the salient properties of fluorescence such as a high sensitivity (down to the single molecule limit), spectral specificity and non-destructive nature, but additional advantages which include:

1. Time-resolved capability for revealing dynamic information and kinetic rate parameters.

2. Increased specificity by means of temporal discrimination against 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 absolute fluorescence intensity and quantum yield measurements.

Put simply the benefits of fluorescence lifetimes compared to steady-state fluorescence are analogous to the motion picture compared to the still photograph, the former providing everything the latter does and much more besides.

It was with these opportunities in mind that in 1978 co-author David Birch moved to a lectureship in physics at the University of Strathclyde in Glasgow hoping to bridge between what was then a quite large separation globally between the university and industry sectors. He joined former colleagues from the Physics Department at the University of Manchester, Bob Imhof and Tony Hallam, having together formed IBH Consultants Ltd in 1977. At the time two techniques for fluorescence lifetime measurement were starting to emerge from early research [4,5]; time-correlated single-photon counting (TCSPC) and phase-modulation.

IBH worked on developing TCSPC [6,7] to the level where today it is now firmly established as the world's favourite. However, in 1977 it was not clear that either of the techniques would *"stand the test of time"* and support a business as back then fluorescence lifetime measurement was still the preserve of the specialist. Certainly there was not the established market there is today. A NATO ASI meeting in St Andrews in 1980 [8] brought many of the major exponents of the two techniques together without resolving the best way forward for the field. This was partly because few applications at the time were exposing any deficiencies in either technique by stretching them to their limits and in particular single molecule detection had yet to be demonstrated and become dominated by photon counting. Originally applications were clustered around chemistry, but these expanded a long time ago to embrace areas as diverse as the life-sciences, medicine, nanotechnology, materials and energy, this widening of the customer base and doing much to support our business growth.

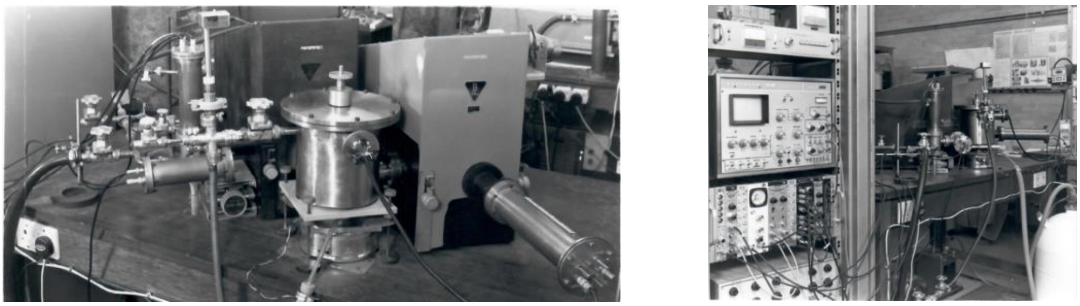
The fact that there was no established TCSPC market back in the late 1970s and early 1980s also had advantages for us in that we were in the enviable and unusual position of being able to build the market the way we thought it should look and naturally this reflected our beliefs and experience. The fact that our founders were also active researchers meant we had a good knowledge and empathy with the needs of many of the applications of interest to our customers. With PhD theses in excited state decay time measurement, and experience of working in industry behind them, our founders set about looking for a manufacturer to turn their ideas into products. Initially Edinburgh-based Nuclear Enterprises, who manufactured exactly the NIM (Nuclear Instrument Modules) needed for TCSPC, showed interest. However, it was by attracting Edinburgh Instruments to the market that the early IBH designs and decay analysis software became available to customers for the first time in the *Model 199*. Other companies producing TCSPC instruments around that time, and who subsequently departed the TCSPC market, included Photochemical Research Associates Inc., a spin-out from the University of Western Ontario and Applied Photophysics Ltd, a spin-out from the Royal Institution in London. Around 1990 our company became a manufacturer of complete systems in its own right and then in 2003 joined HORIBA to form HORIBA Jobin Yvon IBH Ltd. This brought a TCSPC range to HORIBA to complement its steady-state fluorimeters and provided increased global exposure for our products.

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. The most important thing for any company is to survive as without survival nothing is possible. However, good staff, products, marketing, customer support etc can then become the means, not only for survival, but for growth. In the following sections we try to bring out the meaning of "good products" from the standpoint of choosing a fluorescence lifetime spectrometer and aspects to look out for. We will cover the major considerations involved in this decision making and illustrate them with examples in fluorescence lifetime spectroscopy, while keeping in mind that they can be easily adapted across many other areas of scientific instrumentation, particularly those in other areas of spectroscopy. Decision making criteria naturally include performance specifications, but of comparable standing are ease of use, functionality, versatility, reliability and in some cases modularity and the flexibility to upgrade so as to keep up with new developments. The latter in particular align perfectly with our company philosophy of making the latest cutting-edge techniques available so that customers can then *"explore the future"* with confidence.

2
Fluorescence Lifetime Systems
2.1 From our Instrument Origins to Today

Like many instrument companies our origins lie in University research, initially in the Schuster Laboratories at the University of Manchester and subsequently in the Department of Physics at the University of Strathclyde in Glasgow. From these origins our products continue to have a close relationship with research requirements. Figure 1 shows a photograph of the TCSPC fluorescence lifetime spectrometer system which started us out in the 1970s on this *"journey of a lifetime"* [9,10]. Features that were then novel included the use of optical monochromators to select both the excitation and emission wavelengths and an all-metal thyratron-gated coaxial nanosecond flashlamp, the latter designed and constructed in-house. The use of an emission monochomator, rather than cut-off filters used previously to select fluorescence, opened the door to improved spectroscopic capabilities, such as time-resolved emission spectra and reduced stray light detection. A bespoke cryostat included a temperature regulated, manually controlled 4-sample turret, which was kept under vacuum to eliminate oxygen quenching and frost formation at low temperatures. The all-metal flashlamp generated a spark, was usually filled with nitrogen or hydrogen, operated stably at 30 kHz and had the salient advantage over earlier designs based on glass or ceramic enclosures in containing the bugbear of spark-induced radio-frequency oscillations in the decay curves. Subsequent versions of the flashlamp [11] were commercialised and became the affordable pulsed source of choice for over two decades until semiconductor diodes emerged around 2000. General purpose NIM timing electronics, originally developed for nuclear physics and combined with a multichannel analyser, were used for timing and decay acquisition respectively. These were precise and fairly linear, but not at all user-friendly owing to the many cables and manually selected settings.

(a)



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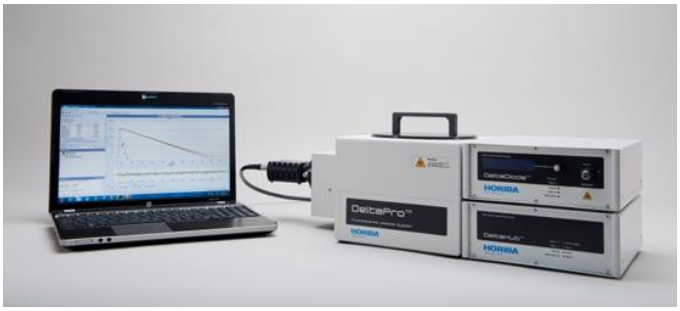


Fig.1. (a) TCSPC fluorescence lifetime spectrometer from the early 1970s developed in John Birk's Photophysics Group in Manchester University [9,10] and incorporating the all-metal coaxial flashlamp, two optical monochromators and NIM timing electronics. (b) The clean-cut lines of one of its descendants, the *DeltaPro*, with its on-line data analysis, epitomises the ease of use of present-day versions.

A lifetime determination then, as now, involved recording the fluorescence decay and the excitation pulse in separate measurements in order to perform reconvolution analysis to extract lifetime values that would otherwise be corrupted by the finite temporal instrumental pulse of typical full width at half maximum (FWHM) of ~ 2 ns. The non-linear least squares data analysis algorithm written by Bob Imhof had a number of rigorous features [12]. These included termination of the iterations to find the lifetime only when the chi-squared goodness of fit criterion had found a true minimum rather than when its rate of descent had slowed, as is common in the widely used Marquardt algorithm. The algorithm also included the capability to iteratively shift the function fitted to the decay data in order to correct for the photo-electron transit time dependence on wavelength in the photomultiplier and the finite width of the discrete timing channels. Needless to say in the early years data was stored on paper tape and analysed on a central institutional main frame computer. The subsequent introduction of the microprocessor changed all this by allowing, for the first time, on-line computation and almost immediate answers. How times change!

In terms of pulsed sources mode-locked lasers offered an expensive alternative to flashlamps in the early days, but they were clearly a *tour-de-force* and both types were subsequently replaced for general use by the advent of low cost semiconductor sources. However, trade-offs and optimization of the source repetition rate, dead time and the inherent time resolution of the overall system are still sometimes required. This is largely governed by the application and discussed further in Section 2.2.

The mainstay of our success in the early manufacturing years was the IBH *Model 5000F* version of the all-metal coaxial flashlamp shown in Fig. 2(a). Typically operating at up to 50 kHz repetition rate this design was gold plated as an additional measure to further reduce 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. When filled with nitrogen the 337 nm line proved to be quite intense. With hydrogen (or deuterium for extra intensity) the flashlamp came into its own as a very workable and affordable alternative to frequency-doubled mode-locked lasers for UV excitation of protein intrinsic fluorescence. However, it was in the visible where flashlamps often proved inadequate. The introduction of semiconductor light-emitting diodes and lasers spanning the UV to near infra-red changed all this. Our *NanoLED* range provided the first UV LED excitation of protein fluorescence decay [13], and more recently the *DeltaDiode* range (Fig. 2(b)), operating at repetition rates up to 100 MHz, has a unique place in the field having set a new benchmark in data acquisition rates [14]. The combined advantages of the concomitant ~ 1000x higher data collection rate, narrower pulses, maintenance-free operation, higher stability and monochromatic nature of their laser variants quickly led to semiconductor sources superseding flashlamps in TCSPC.

(a) (b)

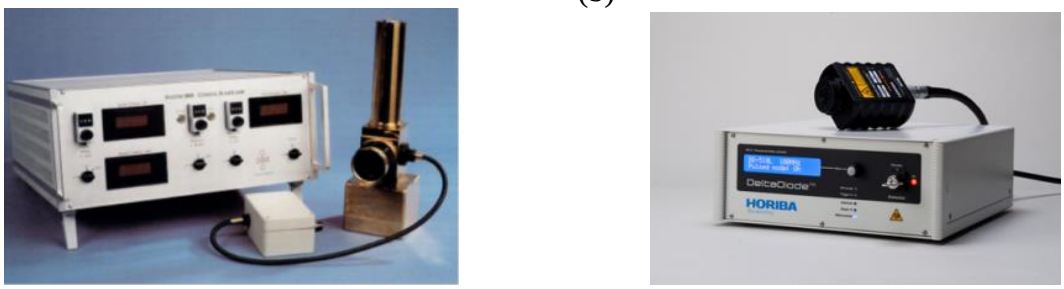


Fig.2. (a) *Model 5000F* coaxial nanosecond flashlamp and (b) the picosecond *DeltaDiode*.

Quite early on in the company's development we recognized the need and opportunities in accessing as much as possible of the fluorescence fingerprint (Eq. 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. The approach aims at retrieving simultaneously the maximum information content available in the multidimensional contour defining the fluorescence signature of a sample, which can be conveniently expressed as a function F:

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

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.

Initially we worked on a two-channel approach [15] to correct for any temporal change in the excitation pulse by means of simultaneous acquisition of fluorescence and excitation (SAFE). Coincidentally around the same time Kiyoaki Hara was developing the *NAES* lifetime system at HORIBA to achieve a similar goal. The main difference between our approaches was the *NAES* system used multiple time-to-amplitude converters (TAC) for timing whereas we used a single TAC, which ensured a common time calibration. Our two channel implementation was also ideal for simultaneously recording the two orthogonal planes of polarization needed for anisotropy decay measurements [16]. Soon we extended the approach to 4 channels by using fibre optic coupling to a spectrograph to demonstrate for the first time simultaneous decay measurements at multiple fluorescence wavelengths [17]. This pointed the way towards much faster measurement of time-resolved emission spectra (TRES) than the sequential wavelength-stepping approach used previously. Nevertheless, measurement times were still measured in hours because of 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 combine the SAFE and dual fluorescence channels in the IBH *Model 5000W SAFE* (Fig. 3(a)). This instrument was an expansion of the popular space-saving folded geometry of the *Model 5000U* which had a single fluorescence channel. The *5000W SAFE* rapidly became the most versatile fluorescence lifetime system yet produced as it included not only three channels, but the capability to multiplex up 16 TCSPC channels simultaneously [18]. This was achieved by virtue of the first application specific integrated circuit (ASIC) designed for fluorescence. Based on complementary metal-oxide-semiconductor (CMOS) technology the ASIC is illustrated in Fig. 3(b) in a NIM module which were still commonly used at the time. The ASIC provided the first commercial readout system for multi-anode microchannel plate photomulitpliers, thus opening the door to various forms of multiplexed imaging. Moreover the device broke through the 1-2% "stop" to "start" rate barrier of conventional TCSPC and permitted count rates up to 37% [19]. Joining the company in 1995 co-author David McLoskey led this research out of his PhD [20] and into the marketplace.

Together these developments provided a pointer for future technologies aimed at accessing more of the fluorescence signature by means of simultaneous measurement. This goal has still to be fully achieved and is a fertile area of research today. CMOS single-photon avalanche diode (SPAD) array detectors combined with individual diode timing offer

significant potential for rapid TCSPC multiplexing, particularly in fluorescence lifetime imaging microscopy (FLIM) where the micron size SPAD pixels are less of a limit on sensitivity [21] as they are in TRES [22]. Conventional photomultiplier variants still have advantages over SPADs in spectroscopy where the highest time resolution and sensitivity are required. This is because photomultipliers have a more constant temporal response with photon wavelength, much larger detection area offering several orders of magnitude higher sensitivity and wider wavelength spectral response including below 400 nm [7].

(a) (b)

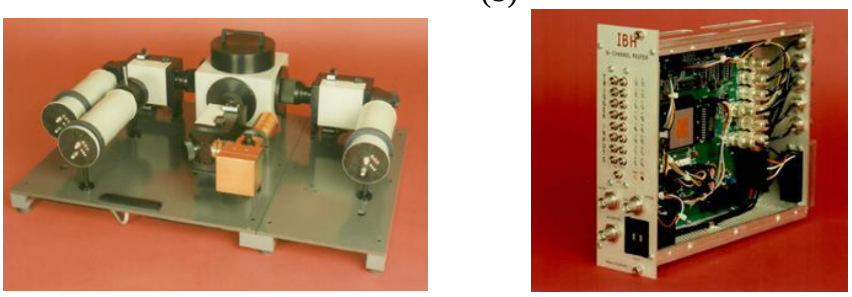


Fig.3. (a) Model *5000W SAFE* and (b) its 16 channel TCSPC multiplexing module.

As can be seen from the foregoing much of our work has been in close collaboration with university research, the application awareness of which helping to shape the innovation and specifications which our customers require. The UK's present market leadership in TCSPC was initiated by members of the University of Strathclyde's Photophysics Research Group in forming its spin-out IBH back in 1977 and this successful collaboration is ongoing. Our Glasgow facility also works closely with our sister company HORIBA Instruments Inc. based in New Jersey who are pursuing the goal of "total spectroscopy" in steady-state fluorimeters. Widely used for liquid analysis the HORIBA *Aqualog* combines a CCD detector with a spectrograph to give absorption, transmission and excitation-emission matrix (A-TEEM^{TM}) in a single measurement at up to 4000x the rate of conventional single channel fluorimeters. The HORIBA *Duetta* is a more general purpose version with extended response up to 1100 nm.

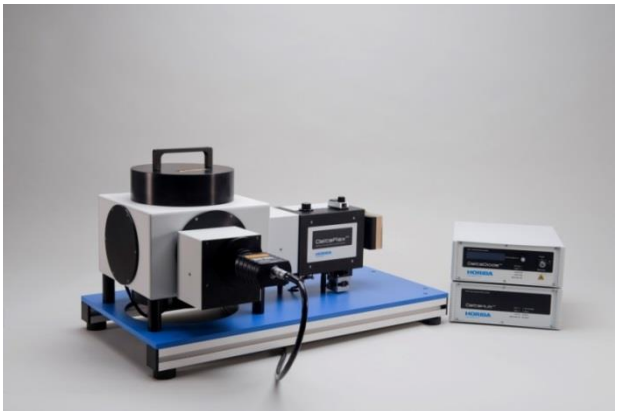
2.2
The *DeltaFlex*

Moving rapidly to 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 plug-and-play when swapping components, auto-calibration, retro-fit and upgrades with such as excitation sources and sample compartment accessories. These features are greatly facilitating the broadening of the user base away from the TCSPC specialist and opening up new applications across the disciplines. Our TCSPC journey has come a very long way from the confines of NIM electronics.

Monolithic Time-to-Digital Converters (TDCs) have largely replaced TACs and ADCs in recent systems such as the *DeltaFlex*. While these do not 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 1 ms - 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 over 20,000 histograms per second are opening-up whole new applications for TCSPC in 3D imaging, light detection and ranging (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 at the instant of their detection and subsequently streamed to disk for sorting and manipulation - each retaining their own "individuality" within resultant files that range from a few hundred megabytes to potentially terabytes in size.

(a)



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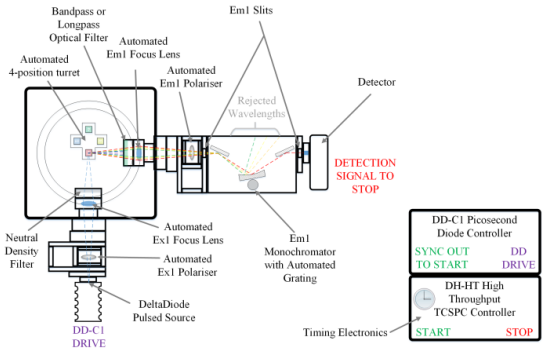

Fig.4. The *DeltaFlex* – a truly modular instrument which satisfies the wide range of fluorescence lifetime applications and overcomes the fear of obsolescence by facilitating upgrade. Photo (a) and schematic (b).

The *DeltaFlex* (Fig.4) offers all-round spectroscopic tuning and fully automated operation under software control with flexibility for ease of upgrade. In the *DeltaFlex* the fluorescence emission is selected using a monochromator as opposed to a long pass optical filter in the *DeltaPro*. This offers the capability for TRES with fluorescence decays measured sequentially at incrementing fluorescence wavelengths which can then be temporally sliced in software to reveal the TRES. Global analysis of the resultant datasets can also determine the decay associated spectra. When required an excitation monochromator can be added. The Seya-Namioka monochromator used in the *DeltaFlex* is ideal for fluorescence decay studies as it contains only 2 mirrors and a single concave holographic diffraction grating in a compact construction. This minimises the temporal dispersion due to differences in the light path to a level where it is not the limiting factor in time resolution when exciting with LED

and diodes lasers combined with photomultiplier detection. Hence there is no need to incur the sensitivity loss of subtractive dispersive monochromators caused by them having an additional grating and more mirrors (see section 3.1).

In terms of pulsed sources for the UV; 250-370 nm sub-nanosecond LED sources, which can be pulsed up to a repetition rate of 25 MHz, are used. It should be noted that LED sources are not polarized and if maximum sensitivity is required there is no need to use polarizers with these sources. In the visible to NIR regime 375-1310 nm picosecond *DeltaDiode* laser sources are used with repetition rates of up to 100 MHz depending on the time range required (10 ns-100 μs). The selected repetition rate depends on the lifetime to be measured and is typically equivalent to the reciprocal of the measurement time range, which is selected to avoid re-excitation during the fluorescence decay. High throughput *DeltaHub* timing electronics with their short (< 10 ns) dead time are optimum in utilizing the 100 MHz repetition rate of the *DeltaDiodes*. These laser sources are vertically polarized so should be used with at least a polarizer in the emission arm at an angle of the magic angle 54.7° to prevent polarization artefacts from distorting the measured fluorescence decay [1].

For the much longer time ranges needed for phosphorescence decay measurements (100 μs – 10 s) *DeltaDiode* lasers may be operated in a "burst mode" by fast gating of their 100 MHz pulse train. Hence the same excitation source can be used to cover picosecond to second time scales and with no operator intervention required. Longer-pulse LEDs, *SpectraLEDs*, are also available from 265-1275 nm for phosphorescence decay measurement using multi-channel scaling rather than TCSPC. The UV capability of LEDs, as first demonstrated in protein fluorescence decay [13], has also made protein phosphorescence decay more accessible as a structural tool [23]. *SpectraLEDs* are an alternative to the xenon flashlamp commonly used for phosphorescence excitation and offer numerous advantages such as a sharper turn-off (near absence of tail), software control of duration (and thus average power), and operation at a wider range of repetition rates - the combination of which allows the excitation parameters to be optimised to suit the sample in order to minimise the time required for measurement.

In the *DeltaFlex* all the optical components and sample turret can be motorised and directly controlled with the *EzTime* software, which is particularly useful when used in conjunction with the *EzTime's* scripting capabilities. Detector options include the near infrared (NIR) and a T-format can be easily added if required. The spare ports on the sides and underneath the sample chamber help ensure optimum optical alignment for a wide range of sample environments including cryostats and front face illumination. Fig.5 shows some of the optional configurations available with the *DeltaFlex* and also its compact version, the *DeltaPro*. The high-throughput *DeltaHub* timing electronics and *PPD* detection module are recommended for 95 % of fluorescence lifetime applications. However should higher timing resolution be required a microchannel plate photomultiplier (MCP-PM) or hybrid (*HPPD*) detector and (TAC-based) *FluoroHub A+* timing electronics can be selected. With a narrow optical pulse excitation source such as the *DD-375L* or *DD-405L* the temporal FWHM can be routinely reduced down to below 50 ps enabling lifetimes down to 5 ps to be recovered. The *DeltaDiode* lasers implement novel synchronisation circuits that adapt to further optimise performance, when used in such high resolution measurements, by automatically correcting for electronic jitter in the signal path.

The trade-off incurred by the higher timing resolution offered by a MCP-PM is that the system data acquisition rate is reduced owing to the increased dead time, firstly by the MCP-PM, which can typically only support count rates of 20 kHz as compared to the standard

PPD-850 detector where the upper-limit is ~ 10 MHz; secondly by the larger dead time of the matching timing electronics (~1 μs for the *FluoroHub A+* as compared to 10 ns for the *DeltaHub*) and concomitant matching source repetition rate (20 MHz *FluoroHub A+* vs 100 MHz *DeltaHub*); and finally, it is common practice to increase the number of data channels when seeking the highest time-resolution measurements, which again increases the measurement time. Conversely, at the other end of the scale run-times with the *DeltaFlex* can be programmed to record 1 to 26,000 decays measured sequentially in as little as 1 ms to 1 min per decay.

(a) (b)

(c) (d)

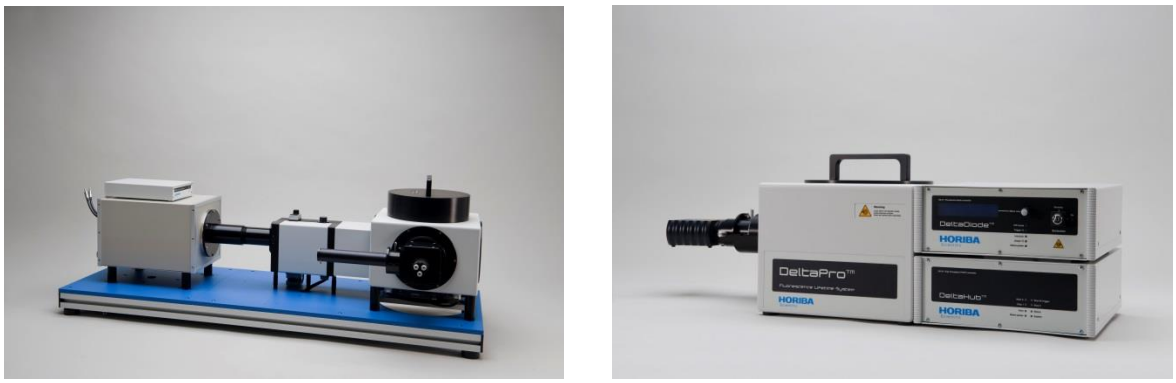

Fig. 5. The *DeltaFlex* fluorescence lifetime system incorporating (a) ps diode laser excitation and near infra-red detection up to 1700 nm, (b) continuum laser excitation, excitation monochromator and microchannel plate photomultipler detection, (c) coupling optics for an ultra-fast Ti:Sapphire laser for use in such as multi-photon excitation, (d) with filter rather than optical monochromator fluorescence wavelength selection in the *DeltaPro*.

Historically MCP-PMs were the only alternative offering a faster impulse response than conventional photomultipliers. However hybrid photomultipliers, with the metal dynode stages of a traditional photomultiplier replaced with a silicon avalanche photodiode, are finding increasing use. These can also be used in conjunction with low timing jitter electronics, such as the *FluoroHub-A+*, with a time resolution of a few hundred fs per bin.

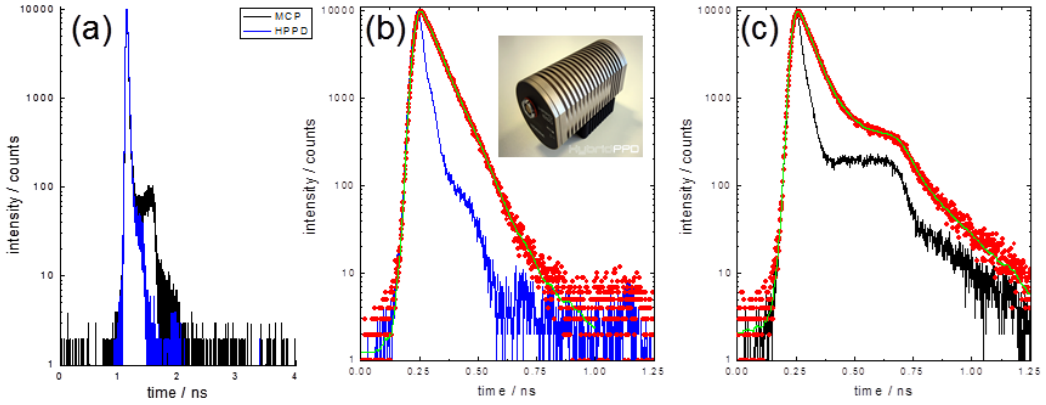

Fig. 6. Comparison of (a) MCP-PM and *HPPD* detector responses measured directly using a *DeltaDiode-405L*. A FWHM of 38ps was obtained for both detectors. Measured decay of DASPI (lifetime ~ 60 ps) with a (b) *HPPD* (Inset) and (c) MCP-PM detector.

Although the transit time spread of the MCP-PM is shorter, the fact that the *HPPD* hybrid version of our *PPD* detector range exhibits a reduced after-pulse, as illustrated in Fig. 6(a), and can sustain higher count rates (MHz compared to the 20,000 cps for the MCP-PM), is making these devices more popular. Certain versions have transit time spreads that enable lifetimes up to the limit of the TCSPC technique to be recovered, while others have high quantum efficiencies. The reduction in after-pulse helps reconvolution (Eq.2) when determining shorter-lived decays, as shown in Fig. 6(b) and (c), which compare a typical MCP-PM with a *HPPD* when determining a 60 ps decay time.

Finally it should be noted that the flexibility of our TCSPC range extends beyond the stand-alone capabilities of the *DeltaFlex* and *DeltaPro.* Key components, including diode sources, detectors, and timing electronics are readily available separately for upgrade of existing instruments in the field such as the *FluoroLog* and *FluoroMax*. Indeed hybrid TCSPC-steady-state systems are available as factory orders to give an all-round spectral-temporal performance in a space-saving system (Fig.7). These instruments provide a full research capability for accessing the fluorescence contour offered by Eq.1 including quantum yield, steady state polarization and the excitation-emission matrix (EEM) in addition to offering the measurement of lifetimes. TCSPC components such as the *DeltaDiode* can also be added to microscopes for FLIM applications.

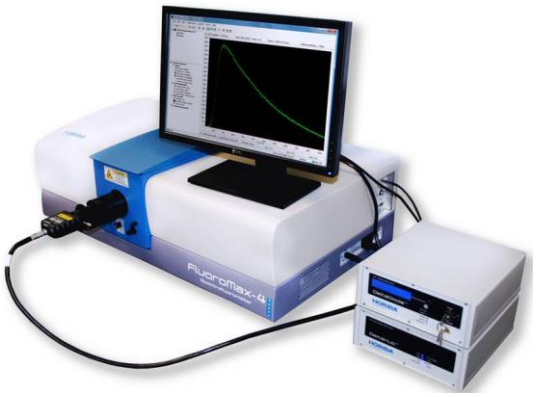

Fig.7. The *DeltaTime* hybrid steady-state and TCSPC lifetime system.

2.3
Software

Software for fluorescence lifetime spectroscopy fulfils three roles: instrument control, decay analysis and data display. Along with the hardware required to make the measurements, major advances have occurred in the associated software. The purpose of the software has expanded to control and optimise the hardware for data acquisition, as well as its original use in analysing the resultant data. It should assist the user by being intuitive and simplify the measurement process; enabling lifetime data to be obtained with ease. However, although the aim is the smooth collection and determination of fluorescence parameters, the interpretation of these data is very dependent on the sample under study and specific knowledge relating to this is still needed for the user to put the outcome in context and draw sensible conclusions.

The fact that microprocessors and memory can be added to almost all of the individual components making up a TCSPC system means that a degree of "intelligence" can be incorporated. The TCSPC system can then be thought of as a collection of intelligent nodes. The software can interrogate the system to ascertain the status of these nodes, such as the wavelength of the monochromator, lens and polariser positions, as well as the time range and repetition rate of the excitation source. This "knowledge" enables the use of scripting (a language akin to a computer program) to be used to automate data collection and fitting. The software is now able to exert a high degree of control over the hardware and is integral to the measurement system. Overall this means that software can be employed to assist the novice user from the simple matching of the excitation source repetition rate to the measurement time range (to avoid more than one optical excitation pulse arriving in the histogram time window) to estimating the data range over which to perform the data analysis. On the other hand, for more experienced users it allows access to equipment settings and control over the data analysis parameters to tailor the measurement process to the sample.

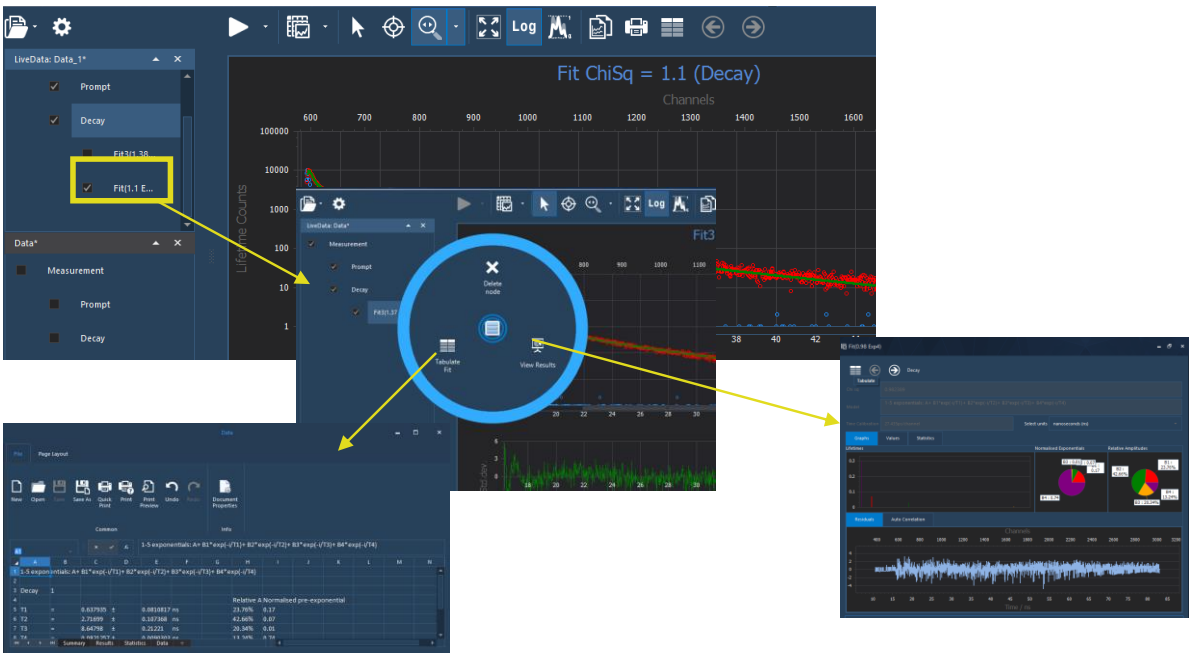

Fig.8. Screen shots of the user interface for the *EzTime* software package**.**

Advances in computer operating systems and hardware means that the software should take account of the latest versions (at the point of writing, Windows 10) and be optimised for use with touch screen monitors allowing for equipment settings and data collection to be performed with a simple "tap" on the screen. An example of such software is the new *EzTime* package. Screen shots showing aspects of the *EzTime* user interface are shown in Fig. 8. This software package controls the hardware and performs data collection and analysis. With just one "tap" on the screen it is possible to collect data and analyse it as the sum of exponentials and other models (1, 2 and 3 exponential models can be automatically fitted as soon as data acquisition stops). The output of the analysis (as well as any selected graph data) can be output as a spreadsheet that can be opened by most popular spreadsheet / graphing programs. A simple scripting language combined with the "intelligent nodes" in the *DeltaFlex* optical system enables the tailoring of the measurement to the sample under study, whether it is the scheduling of repetitive measurements, the control of temperature, polarisation or wavelength throughout the data acquisition process. An autoscript assistant also enables users unfamiliar with scripting to obtain scripts for common measurement types without realising that they are writing one. These can be further optimised using an "on-board" script editor if required.

2.4 The Role of the Manufacturer

The role of a spectroscopy instrument manufacturer overlaps with the research of the customer in satisfying the latter's requirements, but in fact the manufacturer's role is much broader. This is true even if we compare it to a researcher designing and developing an instrument for laboratory use. Design and development are on the face of it common to both, but in reality the manufacturer has to take on-board many additional considerations. These include testing prior to CE marking and compliance with other regulatory requirements such as WEEE and RoHS, scaling-up the number of systems to satisfy orders, maintenance of stock levels, factoring into design the continuity of component supply, implementing quality control systems, ease of use in order to support a user-base with varying levels of skill, meeting delivery deadlines, installation, attaining test specifications, and customer support, which includes manuals, training, applications advice and ensuring the availability and reproducibility of replacement parts for the anticipated lifespan of the product. Hand-in-hand with manufacturing goes the marketing and global sales network and all the usual functions familiar to most companies such as human resource management, ensuring a safe and attractive working environment, and finance operations such as payroll and accounting. In order to achieve all of this the company has to ensure the funds for financing the work required are always in place. Last, but by no means least, the company needs to sell the products at prices that are attractive yet still make a profit for the business to continue, invest in new developments, and grow! The necessity for products to be affordable and yet of high performance inevitably brings with it compromises at times. As such it is equally inappropriate for products to be over-designed as well as under-designed. The term *"horses for courses"* comes to mind in such a context!

The manufacturer-customer landscape has changed considerably, and very much for the better, in recent years. The relationship is now much more of a partnership which strives to serve mutual interest. Companies are increasingly aware of the need to invest in the market and give something back to the community they serve. With this in mind we initiated the FluoroFest series of international workshops in Prague in 2009, and the 12th in the series was held in Glasgow in April 2017 as part of our 40th anniversary celebrations (Fig. 9). These didactic workshops offer a programme designed to be of interest to multidisciplinary

delegates and span an introduction to the basics of fluorescence to more advanced applications presented by world-leading experts. All delegates receive copies of the lectures and support material. FluoroFest differs from the usual academic research conference because of the opportunity for hands-on time and small group training on a wide range of fluorescence instruments covering steady-state spectroscopy, lifetimes, EEM and microscopy. These all support themes taken from some of hottest topics in fluorescence world-wide, such as instrumentation/techniques, life sciences/biomedical and nanotechnology/materials. In addition to plenary and invited lectures, contributed talks, student flash presentations and posters are included. For the Glasgow event generic skills for post-graduate and post-doctoral delegates were included with publication advice given in a lecture by the Institute of Physics Publishing, who also received papers from the meeting for a special issue of *Methods and Applications in Fluorescence*. In-keeping with the partnership between the community and vendor FluoroFest always includes a forum on shaping the future of fluorescence wherein industry listens to delegates outlining their needs and aspirations for new technology and techniques.

(a) (b)

Fig.9. (a) Delegates at the 2017 12th FluoroFest dinner at Ross Priory on the banks of Loch Lomond. (b) Co-author Philip Yip demonstrating the *DeltaPro* at the Glasgow FluoroFest.

3
Demonstrating Time Resolution through Applications

In this section we illustrate some of the principles of fluorescence lifetime measurement by focusing on the high time resolution of the *DeltaFlex* in a few of its many applications. In an application-focused scientific instrument company it is important to have applications specialists with diverse user-experience from a customer perspective. Co-author Kulwinder Sagoo joined our company in 2002, with a chemistry background and a Masters degree from Brock University. Her research making use of time-resolved spectroscopy for biomolecule characteriation then continued at McMaster University prior to joining IBH. Co-author Graham Hungerford obtained his PhD in the Photophysics Group at the University of Strathclyde in 1991 for work on near-infrared TCSPC and then continued in research involving fluorescence at the Universities of Strathclyde, Leuven and Minho (Chemistry and Physics Departments) before joining the company in 2008. Together they work with the rest of the team to ensure that the company continues to deliver on getting the ultimate in performance into customers' laboratories.

The push for reliable limits of time resolution embodies our corporate philosophy. Although time resolution lies at the heart of fluorescence lifetime spectroscopy it can in fact take on several interpretations. For example:

1. The fastest fluorescence decay time which can be resolved.
2. The smallest difference in fluorescence lifetime which can be resolved.
3. The minimum time needed to measure a fluorescence decay.

3.1
Fastest Decay Times

Interpretation number 1 concerns measuring the decay constant associated with the difference between the fluorescence decay *F(t)* of a sample and the instrumental pulse (response) profile $P(t')$ recorded by tuning the emission monochromator to the excitation wavelength and replacing the sample with a scattering medium of negligible temporal broadening such as a silica colloid. $P(t')$ incorporates the temporal broadening associated with the excitation pulse, spread in optical path length, the detector and the timing electronics. In principle the narrower $P(t')$ the better the ultimate time resolution which can be achieved. The two measurements are then handled in software using iterative convolution analysis whereby the excitation pulse is considered to be composed of a series of δ-functions and the fluorescence decay a linear superposition of the corresponding fluorescence impulse responses [2] i.e.

$$F(t) = \int_0^t P(t')\, i(t-t')dt' \quad = P(t) \otimes i(t) \tag{2}$$

Where the sample's fluorescence δ-function impulse response $i(t-t')$ contains the fluorescence lifetime τ_f according to

$$i(t) = i(0) \quad \exp\left(-\frac{t}{\tau_f}\right) \tag{3}$$

The variable t' is a moving time delay that defines the instant at which each δ-function component of the instrumental pulse generates the start of a fluorescence response. On iteration the best fit of *F(t)* from Eqs. 2 and 3 to the measured fluorescence decay data D_t defines τ_f by means of minimizing the sum of the squares of the weighted residuals W_t using the chi-squared (χ^2) goodness of fit criterion as illustrated in Fig. 10, where

$$W_t = \frac{D_t - F(t)}{\sqrt{D_t}} \tag{4}$$

and

$$\chi 2 \quad = \sum_{\substack{Data \\ channels}} {W_t}^2 \tag{5}$$

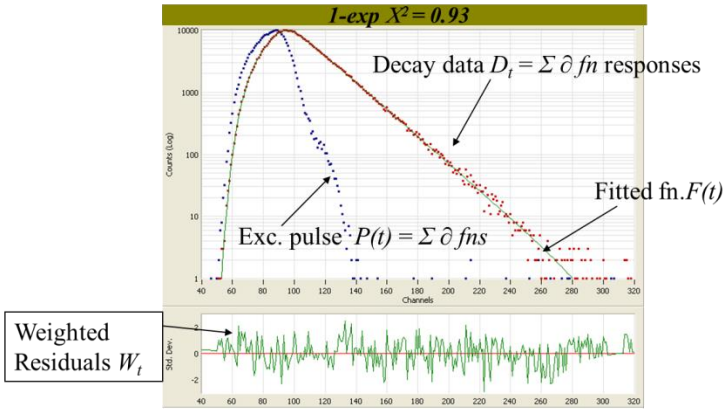

Fig. 10. Fitting to a fluorescence decay using reconvolution analysis [2].

A χ^2 value from Eq. 5, that is normalized according to the number of detection channels and the number of fitted parameters, should approach 1 if the correct model is being applied.

Clearly many systematic errors can limit the time resolution. For example the detection of stray excitation light which mirrors $P(t')$, temporal instabilities in the light source and timing electronics, non-linearity in the time base etc. These have an increasingly detrimental effect on the goodness of fit as the number of counts in the data (i.e. the statistical precision) increases. If we take for example time dispersion in optical monochromators, this can be eliminated by the use of two monochromators configured such that the time dispersion is subtracted. However this comes at a heavy price in terms of loss in throughput due to reflections from all the additional gratings and mirrors required. We use the proprietary *TDM* time domain monochromator optimized for fluorescence lifetime measurements and based on a Seya-Namioka geometry with only one grating and two mirrors in order to maximize throughput and minimize stray light. Moreover, other sources of temporal broadening, such as that of the detector exceed any optical transit time dispersion and, given such contributions add in quadrature as the sum of the square of their values, the monochromator time dispersion is a second order effect [7]. The minimal change in instrumental pulse for the *TDM* is illustrated in Fig. 11 at zero, 1st and 2nd order grating positions for a *DeltaDiode* wavelength of 378 nm and MCP-PM detection.

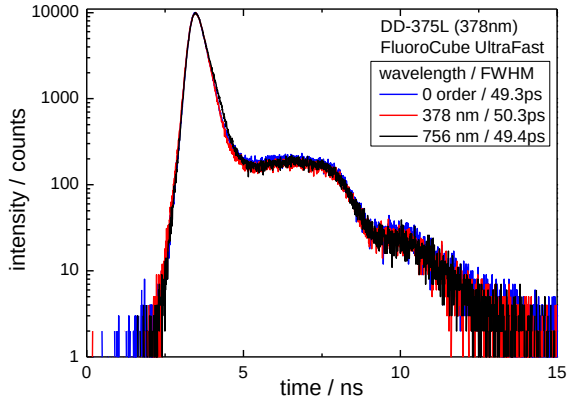

Fig.11. Instrumental pulse profiles demonstrating minimum wavelength transit time dispersion of ~ 1 ps recorded using the *TDM* time domain monochromator and a *DeltaDiode* laser at 378 nm.

A generally accepted *"rule of thumb"* in reconvolution analysis using Eqs. 2-5 is that it is possible to extract a decay component ~ 1/10th of the instrumental FWHM. Hence from Fig. 11 it should be possible to extract ~ 5 ps decay time. Indeed this was confirmed in a recent study of lycopene, a carotenoid commonly found in tomatoes, other red fruit and some vegetables [24]. Commercially available lycopene (LYC) was compared with a control lycopene sample extracted from tomato and lycopene extracted from tomato following ultrasonic treatment at 584 kHz for 15 min (T15) and 60 min (T60) [25]. Fig. 12 shows the raw data obtained for these 4 samples and a typical fit.

(a) (b)

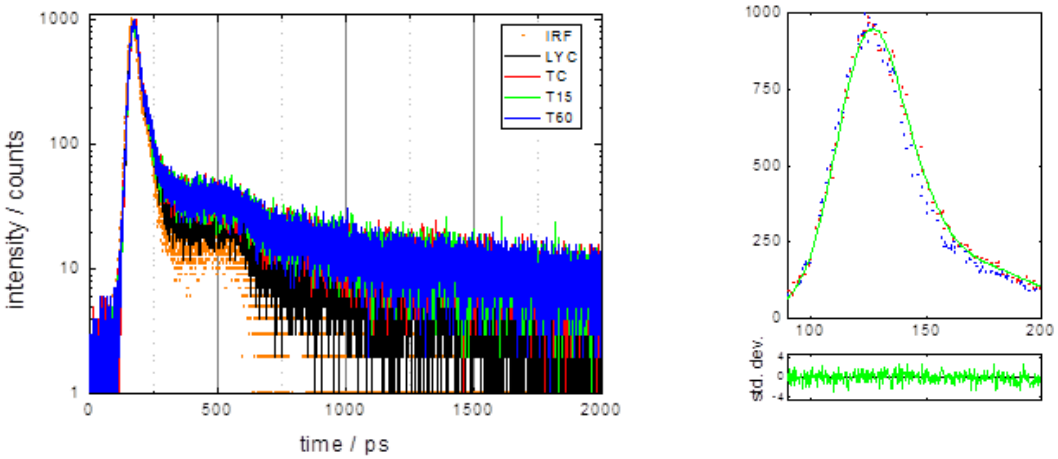

Fig.12. (a) Time-resolved fluorescence decay of various lycopene samples [25] monitored at 550 nm, with excitation at 409 nm using a *DeltaDiode*. These are for commercially available lycopene (LYC) extracted from tomato without ultrasonic treatment (TC) and with ultrasonic treatment for 15 mins (T15) and 60 min (T60). The instrumental response function (IRF) is also shown and has a FWHM = 36 ps. (b) Decay, IRF and fitted function, along with weighted residuals for LYC.

Table 1 shows the results of decay analysis for the 4 lycopene samples according to

$$i(t) = \sum f_n \exp\left(-\frac{t}{\tau_n}\right), \quad \text{for } n = 3 \qquad (6)$$

Table 1. Results of the 3-exponential fluorescence decay analysis of the 4 lycopene samples according to Eq.6 and including average decay time <τ> [25]. Details are provided in the caption to Fig 12.

	Decay time / ps			Fraction / %				
Sample	τ_1	τ_2	τ_3	f_1	f_2	f_3	$\langle\tau\rangle$	χ^2
LYC	5.1±0.8	807±20		84	16		6.1	1.15
TC	4.4±0.4	313±69	2014±183	62	8	30	7.1	1.21
T15	4.8±0.9	322±69	1764±123	60	8	32	8.0	1.18
T60	5.1±1.1	277±57	1758±123	62	7	31	8.2	1.19

The results in Table 1 confirm a dominant decay component of around 5 ps and this is consistent with work previously reported from a calculated value of 5 ps [26] and measured value of 4.7 ps

obtained using ultrafast absorption spectroscopy [27]. Given that light which travels 0.3 mm in 1 ps it can be seen that care is needed to maintain geometric consistency between recording the instrumental response function and the fluorescence decay.

3.2
Resolving Differences in Decay Curves

In the previous example time resolution was demonstrated through finding the minimum decay component which could be measured. Equally important is resolving the difference between decay curves. This problem is manifest in quenching, energy transfer, binding etc and is particularly demanding in the case of fluorescence polarization anisotropy decay, where it is highly dependent on the precision of the instrument.

It is informative to consider an analogy between resolving power in optical spectroscopy and mass spectrometry in resolving small difference in measurands, the equivalent expressed here as:

$$\text{Resolving Power} = \frac{\tau_f}{\Delta_f} = \frac{1}{\text{Resolution}} \qquad (7)$$

For a fluorescence lifetime $\Delta\tau_f$ is essentially described by the statistical error calculated from the reconvolution analysis. This is influenced by the number of counts (statistical precision) recorded and systematic errors such as non-linearity in the time base or the detection of stray excitation light. All such factors are reflected in the goodness of fit.

We can similarly think about the resolution of parameters derived from measuring the fluorescence lifetime, such as distance resolution between donors and acceptors undergoing fluorescence resonance energy transfer (FRET) or the radius resolution from the fluorescence anisotropy decay of a rotating fluorophore. Together these approaches offer complementary distance measurement and resolution. FRET typically offers an effective range of ~ 1 nm to 10 nm and fluorescence anisotropy decay ~ 0.1 nm to ≥ 10 nm [1]. This complementarity comes about because FRET falls off rapidly as ~ r^{-6}, and the rotational correlation time of anisotropy decay of a rotating fluorophore at an equivalent rate of ~ r^{-3}. Both offer sub-nanometre resolution, even down to 0.1 nm, and this is well-illustrated by the application of anisotropy decay to nanoparticle metrology.

Synthetic nanoparticles have joined with nature's indigenous varieties to create an urgent need for their size measurement. This is particularly true for the 1 – 10 nm range which can easily traverse cellular membranes and are of increasing concern in respect of the associated implications for human health. It is perhaps surprising that, although there are a wide range of available methods for this task, such as electron microscopy and different scattering approaches [28], at this point in time there are no universal standards with which to compare nanoparticles. Here we describe how fluorescence decay measurements offer a comparatively low-cost and portable solution to address this need. Although the underlying theory of fluorescence polarization anisotropy decay is relatively straight forward, and has been extensively reviewed for fluorophores in solution [29-31], from a measurement point of view it does present some additional demands on measurement precision and accuracy to that of determining a simple fluorescence lifetime from a decay. This is because the latter effectively compares a fluorescence decay with a zero baseline, whereas anisotropy decay, as can be seem from Eq.8, depends on, and compares, the difference between two decay curves. Here is

a brief summary of the theory of the simplest case of relevance. This relates to a nanoparticle labelled with a dye, and the linear addition of other rotational kinetics, such as free dye rotation or dye wobbling on the nanoparticle, which can also be successfully analysed.

By recording vertically (*V*) and horizontally (*H*) polarized fluorescence decay curves, $I_{VV}(t)$ and $I_{VH}(t)$, orthogonal to vertically (*V*) polarized excitation, a time-resolved anisotropy function $r(t)$ is generated i.e.

$$r(t) = \frac{[I_{VV}(t) - GI_{VH}(t)]}{[I_{VV}(t) + 2GI_{VH}(t)]} \tag{8}$$

Where *G* is a factor ($G = I_{HV}(t)/I_{HH}(t)$) which corrects for transmission efficiencies and

$$I_{VV}(t) = exp\left(-\frac{t}{\tau_f}\right)\left[1 + 2r_0\, exp\left(-\frac{t}{\tau_c}\right)\right] \tag{9}$$

$$I_{VH}(t) = exp\left(-\frac{t}{\tau_f}\right)\left[1 - r_0 exp\left(-\frac{t}{\tau_c}\right)\right] \tag{10}$$

Leading to

$$r(t) = r_0\, exp\left(-\frac{t}{\tau_c}\right) \tag{11}$$

Where is r_0 the initial anisotropy at $t = 0$, which has a maximum value of 0.4, and τ_c the rotational correlation time describing the rate of depolarization due to isotropic rotation.

The simplest depolarization occurs for a fluorophore that can be treated as a spherical rigid rotor undergoing Brownian rotation in an isotropic medium such as a solvent. In this case τ_c can be expressed by the Stokes-Einstein equation

$$\tau_c = \frac{\eta V}{kT} = \frac{1}{6D} \tag{12}$$

where η is the microviscosity, *V* the hydrodynamic volume = $4\pi R^3/3$ prescribed by the rotor of hydrodynamic radius *R*, *T* the temperature, *k* the Boltzmann constant and *D* the rotational diffusion coefficient such that

$$R_p = \left(\frac{3kT\tau_c}{4\pi\eta}\right)^{1/3} \tag{13}$$

The adaptation of this simple theory to a dye attached electrostatically or covalently to a nanoparticle is straightforward as it is then the nanoparticle hydrodynamic radius which is prescribed as depicted in Fig. 13(a). Ideally the fluorescence lifetime τ_f is ~ τ_c [32]. If τ_f >>

τ_c the depolarization is too fast to measure accurately during the fluorescence lifetime and if $\tau_f << \tau_c$ too little depolarization occurs during the fluorescence lifetime. However, these theoretical constraints have to a large extent been circumvented by modern high-repetition rate sources such as the *DeltaDiode* combined with high-throughput *DeltaHub* timing electronics. This is because they facilitate the accumulation of significant number of counts, with even several exponents down from the peak of a fluorescence decay still providing a useful monitor of the particle rotation such that $\tau_f << \tau_c$ can still produce a viable measurement, as we will illustrate here.

In the case where the dye partitions between being bound to the nanoparticle and free rotation

$$r(t) = (1-f)r_0\, exp\left(-\frac{t}{\tau_{c1}}\right) + f r_0 exp\left(-\frac{t}{\tau_{c2}}\right) \qquad (14)$$

where f is interpreted as the fraction of fluorescence due to dye bound to silica nanoparticles rotating with a correlation time τ_{c2} and $1-f$ due to dye molecules unbound in the colloid and rotating faster with a correlation time τ_{c1}.

Silica colloids of well-defined size and produced by the Stöber process [33,34] are readily available under the LUDOX tradename (Grace). Indeed they are widely used in fluorescence lifetime spectroscopy to scatter the excitation pulse when recording the instrumental response for reconvolution analysis using Eq.2.

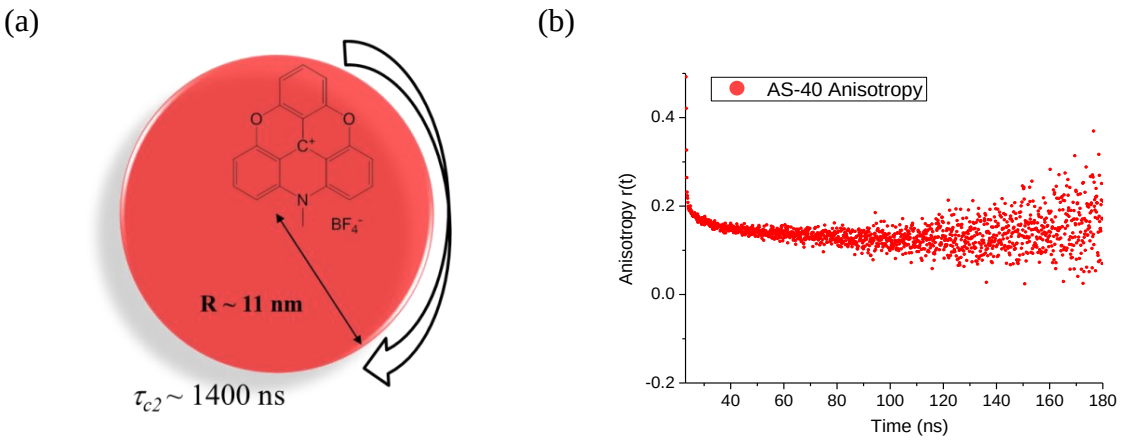

Fig.13. Rotating 11 nm radius LUDOX AS-40 nanoparticles (a) labelled with Me-ADOTA (b) their anisotropy decay measured with a *DeltaFlex* using *DeltaDiode* excitation at 503 nm, fluorescence detected at 570 nm [38].

Early work [35] with fluorescence anisotropy for the metrology of LUDOX nanoparticles used 6-Methoxy quinolinium labels that require excitation in the ultraviolet. Unfortunately, these suffered from the likelihood of data contamination caused by the excitation of background fluorescence in what is not a spectroscopically pure sample. Most dyes emitting in the visible part of the spectrum (thus circumventing background fluorescence problems), have too short a fluorescence lifetime (i.e. $\tau_f << \tau_c$) to be optimum for use with nanoparticles. Recently a range of triangulenium dyes with ~ 20 ns fluorescence lifetime have been synthesized [36,37], which seem to be ideal for the task and bring the spectral range within that of the *DeltaDiodes* with their high-repetition rate ps pulses. Me(thyl)-ADOTA (Fig.

13(a)) is one such example [38]. Its anisotropy decay (Eq. 8) when electrostatically bound to silica particles in LUDOX AS-40 at pH 9–10 is shown in Fig. 13(b).

Table 2. Fluorescence anisotropy decay analysis of various LUDOX colloids labelled with Me-ADOTA using Eqs 13 and 14 [38]. The anisotropy data was acquired in typically ~ 20 min. The microviscosity was taken to be 10^{-3} Pa s (1 cp). The measured average particle radius R_p, the manufacturer's values R_m and the particle radii obtained previously [35] using 6-methoxyquinoline (6-MQ) are shown. The latter were measured in ~ 10 hr with 10^5, $5x10^5$ and 10^6 counts in the peak D_p of the difference curve $I_{VV}(t) - GI_{VH}(t)$ for the 3.5 nm, 6 nm and 11 nm particles respectively, at a channel width of 28 ps, whereas only $\leq 10^4$ counts were required in D_p at a channel width of 104 ps for a better precision in R_p to be obtained with Me-ADOTA excited by a *DeltaDiode* at 503 nm**.** τ_{c2} defines the particle radii and τ_{c1} most probably describes the ADOTA dye wobbling on the nanoparticle. The data were analysed using impulse reconvolution analysis [7] analogous to that described in Section 3.1 for fluorescence lifetimes.

<	With Me-ADOTA [38]			>	<	With 6-MQ [35]	>
LUDOX	$D_p/10^3$ **(cts)**	τ_{c1} **(ns)**	τ_{c2} **(ns)**	χ^2	$R_p \pm \Delta R_p$ **(nm)**	R_m **(nm)**	$R_p \pm \Delta R_p$ **(nm)**	$D_p/10^5$ ***(cts)***
SM-AS	3.0	3.48 ± 0.93	100.0 ± 18.0	1.18	4.6 ± 0.3	3.5	4.0 ± 0.4 (SM-30)	1.0
AM	3.5	4.08 ± 1.30	210.0 ± 19.5	1.09	5.9 ± 0.2	6.0	6.4 ± 0.5 (AM-30)	5.0
AS-40	12.0	12.1 ± 2.06	1424 ± 471	1.18	11.1 ± 1.1	11.0	11.0 ± 1.6 (AS-40)	10.0

Table 2 shows that over the range studied the errors determined demonstrate a Resolving Power ($R_p/\Delta R_p$) for these kind of measurements of at least 10 (Eq.7) and it is clear that sub-nanometre resolution is being obtained on the smallest particles. In fact increasing D_p by ~ 26 x gives sub-nanometre resolution for all the particles and ~ 0.1 nm for the two smallest [38].

The ability to acquire high data rates efficiently becomes essential where samples are changing rapidly such as in a flow-cell or during polymerization. Indeed the formation of silica gel provides a good example of this. In the case of growing silica nanoparticles an average radius of a much larger size distribution as compared to LUDOX is obtained. Both hydrogels and alcogels can be studied. Table 3 illustrates this for an alcogel of tetraethyl orthosilicate (TEOS).

Table 3. The results of the anisotropy analysis of Me-ADOTA in TEOS under acidic conditions [38]. Over 48 hr a monotonically increasing average nanoparticle radius $< R_p >$ of ~ 1.4 nm to 1.8 nm, as determined from τ_{c2}, is observed. Such measurements provide a stern test of the resolution of the technique and reveal close to 0.1 nm precision with D_p ~ 3,500 cts acquired in ~ 20 min. The τ_{c1} value of ~ 0.3 ns up to 46.5 hr is generally consistent with free dye rotation in water before the gel time t_g (~ 50 hr) is reached, further complicating the kinetics.

Time (hr)	τ_{c1} **(ns)**	$(1-f)r_0$ **(%)**	τ_{c2} **(ns)**	fr_0 **(%)**	χ^2	$< R_p >$ **(nm)**
19.0	0.20 ± 0.19	16.01	3.00 ± 0.70	83.99	1.11	1.42 ± 0.10
21.0	0.34 ± 0.09	18.54	3.14 ± 0.83	81.46	1.02	1.45 ± 0.12
23.0	0.43 ± 0.11	21.51	4.03 ± 1.45	78.49	1.04	1.57 ± 0.17
43.5	0.30 ± 0.12	12.93	4.07 ± 1.14	87.07	1.03	1.58 ± 0.14
46.5	0.32 ± 0.11	14.27	4.50 ± 1.10	85.73	1.04	1.63 ± 0.12
48.5	0.83 ± 0.19	23.40	6.00 ± 2.26	76.60	1.06	1.79 ± 0.20
67.0	0.50 ± 0.14	14.95	6.13 ± 1.59	85.05	0.98	1.81 ± 0.14

What is interesting about Table 3 as compared to Table 2 is that a Resolving Power better than 10 is obtained even for such small (~ 1 nm) nanoparticles, which give < 20 x lower rotational times This reflects the instrument stability, linearity of time base, stray light rejection and reliability of data analysis. Moreover, the small sizes obtained for TEOS with the *DeltaDiode* are consistent with those previously reported for tetramethyl orthosilicate (TMOS) using fs two-photon excitation [39].

Interestingly, although the measurements were performed using the *DeltaFlex*, the versatility and spectral capabilities of this instrument are not always essential for these kinds of measurements and indeed the *DeltaPro* (Fig. 5(d)), with its lower cost, fluorescence selection using cut-off filters and dichroic polarisers, would be adequate for many such applications in nanoparticle metrology. The ultra-precision afforded by the research capabilities of the *DeltaFlex* in analysing complex fluorescence decay kinetics is not necessarily always needed as the fluorescence decay just provides a marker with which to track particle rotation. All that is required is for an adequate representation of the kinetics to be as good as the statistical precision of the data. This can be achieved by first fitting the fluorescence decay (the denominator in Eq. 8) using a series of exponentials and then using impulse reconvolution to iterate the rotational terms (e.g. Eq. 14) to give the best fit to the numerator in Eq. 8 which contains the rotational information [2].

3.3
Minimum Measurement Times

The final interpretation of time resolution concerns the need to detect transient species as occurs in many areas of analysis such as chromatography and flow cytometry. Here we highlight the application of the *DeltaFlex* in bringing some of the advantages of fluorescence lifetime spectroscopy to the study of stopped-flow kinetics. In stopped-flow reactants are expelled from syringes, mixed and injected into a flow cell. The flow is then stopped and the ensuing reaction / interaction monitored by fluorescent labelling of one of the reactants or by using its intrinsic fluorescence.

Typically reactant syringes expel the reactants, which are rapidly mixed and enter a flow cell to be detected. Until relatively recently lifetime instrumentation was not efficient enough to collect sufficient data on the critical ms timescales on which many reactions occur. However, the recent introduction of very low dead time electronics, coupled with high repetition rate excitation sources has made this possible. Moreover, the advantages of lifetime measurements listed in Section 1 can be brought to bear. Specifically the obviation of photochemical bleaching and determination of the absolute measurand of decay time represent demonstrable advantages for stopped-flow. In the TCSPC histogram streaming measurement mode, available in both *DeltaFlex* and *DeltaPro* systems, up to 26,000 time-resolved fluorescence decays can be obtained; with each histogram seamlessly collected in as little as 1 ms. The 100 MHz capability of the *DeltaDiode*, is ideally matched to the very low dead time of the *DeltaHub* (10 ns) and together they have pushed data acquisition rates in TCSPC to new limits. When short data collection times are employed, this efficiency is required in order to obtain data of sufficient statistical precision for accurate decay time determination. The stopped flow accessory fits easily into the standard cuvette (10 mm path length) holder via modification of the sample chamber lid shown in Fig.4. Using the control software, data collection can be started either using an external TTL signal from the stopped

flow accessory or manually, within the software, in the histogram streaming measurement mode.

(a) (b)

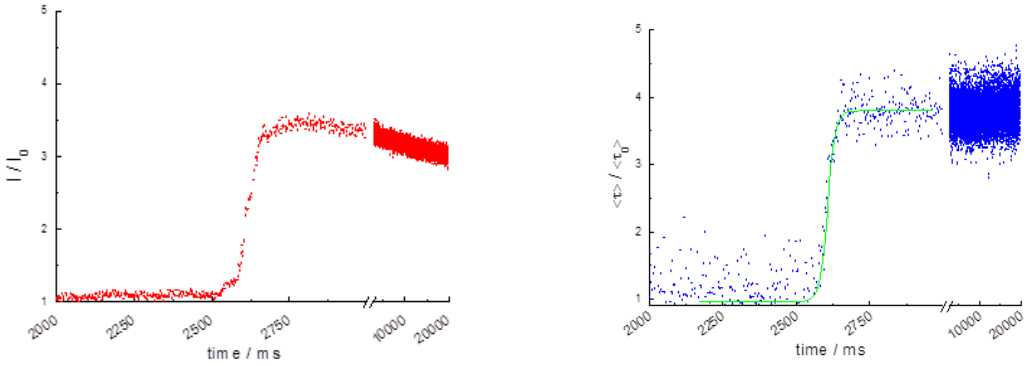

Fig.14. Comparison between (a) relative intensity (I/I_0) and (b) relative average lifetime data ($<\tau> / <\tau_0>$) obtained from a stopped-flow measurement of curcumin binding to bovine serum albumin. Data was collected every 5 ms. The excitation source was a *DeltaDiode-395L* operating at 100 MHz with fluorescence detected at 500 nm.

In order to illustrate this capability we consider the interaction of curcumin with protein [40]. Curcumin is commonly found in turmeric and is of wider interest as it is thought to have potential health benefits due to its antioxidant properties. The role of serum albumin in blood makes it an ideal model protein with which to observe this interaction. For stopped-flow a solution of curcumin in DMSO was placed in one syringe, with the other reactant syringe filled with bovine serum albumin in buffer. In this solvent mixture curcumin is weakly fluorescent and exhibits a short fluorescence lifetime. Upon interaction with the protein both the fluorescence quantum yield and lifetime increase. The data acquisition was started to provide a "background" and the reactants expelled manually. After decay analysis the change in average lifetime during the course of the experiment was plotted and this is displayed in Fig. 14 along with the relative change in fluorescence intensity. Reaction rates can be determined from such plots. However, it is interesting to see how the constancy of average lifetime change is preserved in the face of the gradual photobleaching, bringing to bear a major advantage of lifetime determination (see Section 1), and eliminating a systematic error which would otherwise affect kinetic calculations based on relative intensity.

Hand in hand with such considerations goes the question of how many counts are required to gain the precision needed for a fluorescence lifetime measurement? Of course if it is a static and stable sample, TCSPC offers the opportunity to count as many photons as possible in order to obtain the most stringent test of the kinetic model and maximise precision. However, for a transient sample, such as in a stopped-flow experiment, the question is very apposite. Previous work suggested fewer than 200 counts in a decay were sufficient to obtain 10% precision for a 2.5 ns lifetime [41]. Fig. 15 supports this finding as a total of 233 counts recorded in the fluorescence decay of a BODIPY derivative [42] gave a lifetime of 366 ps, which compares well with a lifetime of 376 ± 12 ps obtained from 14000 counts [14].

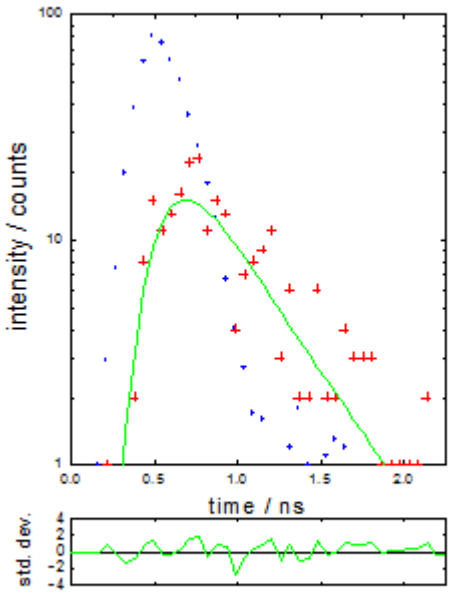

Fig.15. Fluorescence decay (+) of a BODIPY derivative [42] measured in 60 μs, along with the instrumental response (▸), weighted residuals (–) and fitted function (–). The lifetime obtained of 366 ps, compares favourably with that measured for the same sample to a higher precision (~14000 counts) with an MCP-PM. For the latter a value of 376 ± 12 ps was obtained [14].

4
Conclusions

Fluorescence lifetime spectroscopy is now a mature technique, but innovation to improve performance and usability is ongoing. Perhaps the main developments over the past 20 years have been the introduction of semiconductor sources and the faster data acquisition to take advantage of their multi-MHz repetition rate. Together these are enabling more of the multidimensional characteristics of fluorescence to be measured. Our company's early innovations in multiplexed TCSPC helped point the way, but there is still much more to be done before the full spectroscopic contour afforded by Eq. 1 can be realized. Even when this is achieved conventional simplex steady-state and lifetime spectrometers are likely to be just as important as they are today as there are nearly always compromises to be made in new developments and the search for higher performance implies dedicated and specific, rather than general capabilities. We watch and work with keen interest to help the future unfold.

5
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