

FLUORESCENCE LIFETIME

finally picking up momentum!

Fluorescence lifetime (FLT) largely failed to make a significant inroad as a screening technology when it was first introduced nearly a decade ago, despite the fact that intrinsically it has many benefits, particularly in dealing with assay interferences. Market studies undertaken in the intervening period have consistently uncovered a latent appetite within drug discovery for FLT assays. Recent publications have successfully addressed target class applicability, assay validation and assay reagent availability issues using FLT. The FLT reader situation has also changed with the launch of a new dedicated FLT plate reader from Edinburgh Instruments able to generate a large signal window with excellent precision using Almac's FLEXYTE® assay kits. There is an undoubted sense that the momentum around FLT is picking up and the recent advances in FLT discussed in this article make FLT a more attractive assay technology option today.

Fluorescence lifetime (FLT) was first offered as a detection modality on a multi-mode plate reader by Tecan back in 2002. Not long after its introduction we discussed assay interference as a limiting factor in HTS¹. In this article we concluded that FLT had the potential to deliver the greatest signal robustness (ie ability to circumvent assay interferences) of the then available assay technologies, but its HTS fitness (ie suitability of an assay technology for screening) was inadequate. At that time (Summer 2003) this inadequacy centred around the paucity of publications supporting the use of FLT against a diversity of target classes; few, if any, examples of validated FLT applications citing assay development experience; and limited availability of specific probes and assay kits optimised around their FLT characteristics. During the

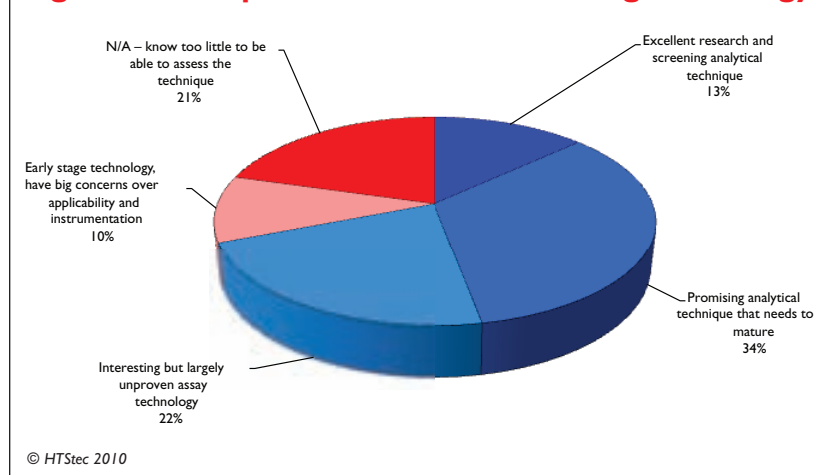
interim period HTStec has undertaken two surveys^{2,3} to investigate the disconnect in FLT adoption. In both cases we uncovered a latent interest and an appetite within the drug discovery community to consider FLT assays.

How is FLT perceived?

Survey respondents' perception of FLT as a screening technology in 2007 are presented in **Figure 1**. This shows that only a small minority thought FLT was an excellent research and screening analytical technique. The majority perceived FLT as a promising analytical technique that needs to mature, followed by an interesting but largely unproven assay technology. This figure nicely illustrates the need for more end user references supporting the successful application of FLT.

By Dr John Comley

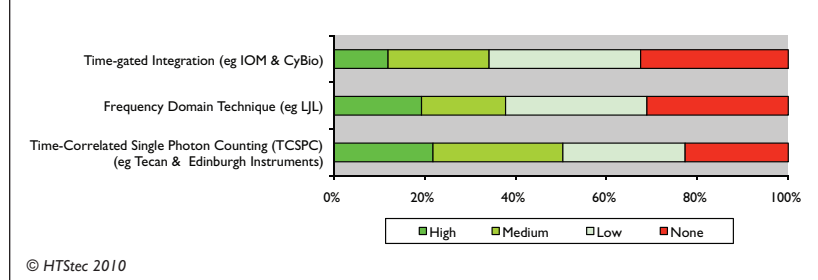
Figure 1: Perception of FLT as a screening technology



What is known about FLT?

Of the different approaches to FLT measurement survey respondents were most aware and had greatest understanding of time-correlated single photon counting (TCSPC), as enabled by Tecan and Edinburgh Instruments. Least awareness and understanding was shown for time-gated integration, as enabled by IOM-Berthold and CyBio (Figure 2). Overall, less than 1 in 5 of all survey respondents had a high understanding of the different approaches to FLT measurement, which is suggestive that more needs to be done to educate screeners about the principles and inherent advantages of FLT measurements (see box). More recently a third alternative method for measuring FLT has been developed by Fluorescence Innovations, based on microchip laser excitation sources and proprietary digitisers (see below).

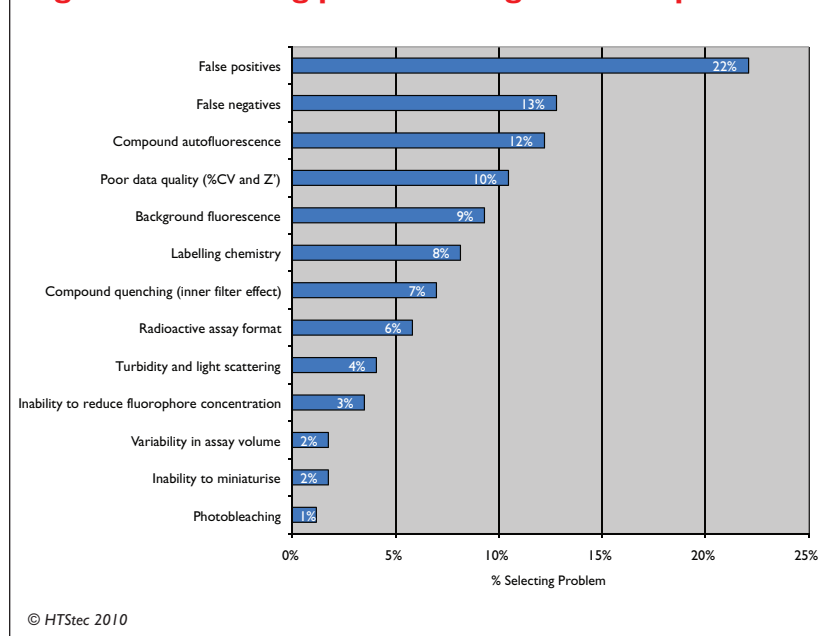
Figure 2: Awareness/understanding of approaches to FLT measurement



What are main problems encountered in screening?

The screening problems of greatest importance to survey respondents are shown in Figure 3. False positives were ranked by far the most problematic, this was followed by compound autofluorescence, poor data quality (%CV and Z') and then background fluorescence. Photobleaching was ranked of least importance. It should be pointed out here that FLT has the potential to impact nearly all of these screening problems, which probably explains the latent appetite within the screening community.

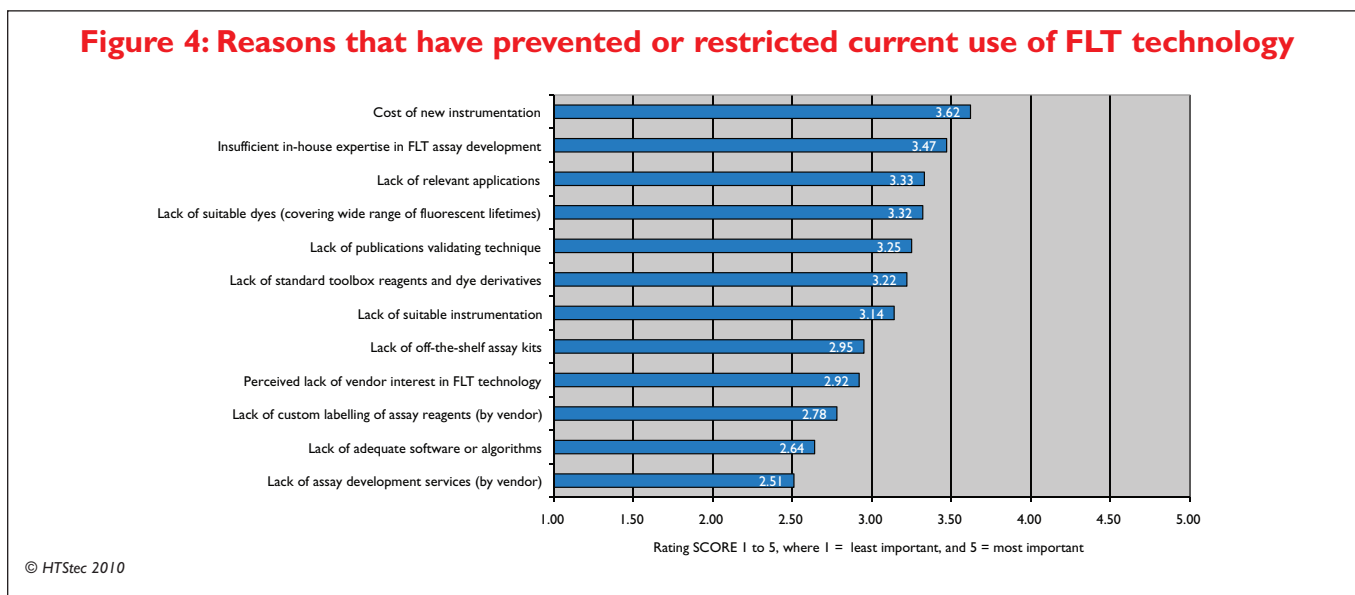
Figure 3: Screening problems of greatest importance



What has hitherto restricted the widespread use of FLT?

Feedback on the reasons that have prevented or restricted the use of FLT technology is given in Figure 4. Cost of new instrumentation was rated the main reason, and still poses a major barrier to the adoption of any new technology, particularly in the current economic climate with constraints on capex budgets. It calls for more inventive strategies (eg free loan, lease, lease-to-buy etc) by vendors regarding instrument placement to nurture initial FLT adoption. The next most important restriction was insufficient in-house expertise in FLT assay development. Again this calls for new efforts by vendors and early adopters to encourage new investigations. The next four reasons were all similarly rated (ie lack of relevant applications; lack of suitable dyes (eg covering wide range of fluorescent lifetimes); lack of publications validating technique; and lack of standard toolbox reagents and dye derivatives) all have been subject to much improvement over the past few years (see the updates below).

Figure 4: Reasons that have prevented or restricted current use of FLT technology



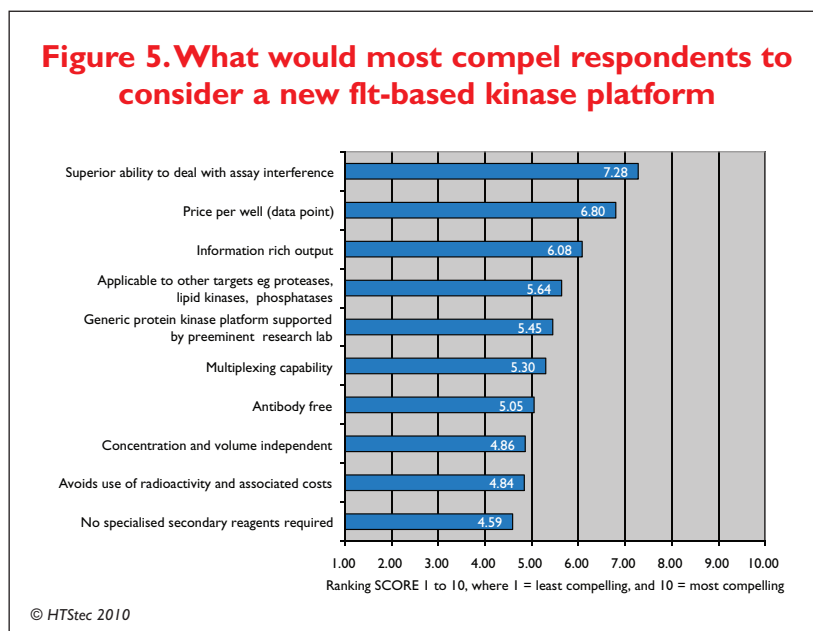
What would make an FLT-based kinase assay attractive?

When survey respondents were asked what would most compel them to consider an FLT-based kinase platform they ranked superior ability to deal with assay interference as the most compelling reason (Figure 5). What we think respondents are saying here is if FLT's ability to deal with assay interference in kinase assays resulted in better assay quality (ie better Z' values than other assay technologies) it would almost certainly trigger adoption by some screening groups. Other important criteria were price per well (data point) and then information rich output. All these three reasons match well with the strengths of the new FLT assay kit offerings described below (see Almac FLEXYTE®).

What might FLT displace?

If FLT were successful in capturing a proportion of the kinase screening market share, where will it impact current screening technologies (Figure 6)? Survey feedback suggests that most respondents (28%) think TR-FRET (ie HTRF, LANCE, TR-IMAP, LanthaScreen) is the kinase platform/technology most likely to be displaced overall by an FLT-based kinase platform. This was followed by glow luminescence and then FP with ligand, (ie NO antibody). This finding is perhaps a little surprising, as these happen to be the most used and most preferred kinase platforms today. This finding is probably suggestive of a degree of grudging acceptance of these technologies, ie in the absence of better alternatives and no doubt sweetened by highly competitive price incentives.

Figure 5. What would most compel respondents to consider a new flt-based kinase platform



What are the prospects for the wider adoption of FLT?

Opinion on the future adoption of FLT into survey respondents workplaces (surveyed in 2007) suggests that FLT will remain a largely unimportant (0% targets screened) or a low importance tool (<5% of targets screened) in the near term (next 1-2 years) (Figure 7). In the medium term (after 3 years, ie in 2010) survey respondents were evenly split between no or low importance and medium or high importance. In the long term (after 5 years, ie by 2012) respondents' expectations for FLT have risen considerably with

Fluorescence lifetime assays – the basics

When a light photon is absorbed by a molecule, the energy is used to lift an outer electron from its ground state into an excited state. Upon its return to the ground state, the energy can be dissipated through various channels; one possibility is to re-emit a photon, usually with a slightly reduced energy. This process is called fluorescence. The time interval between absorbing a photon and re-emitting one, is called the fluorescence lifetime. For each individual molecule the length of time spent in the excited state will vary. As an ensemble of many molecules, the decay of the proportion of molecules in the excited state is similar to a radioactive decay, and the mathematical description is an exponential function. The fluorescence lifetime is the time after which the exponential curve reaches a value of $1/e$, or about 37%.

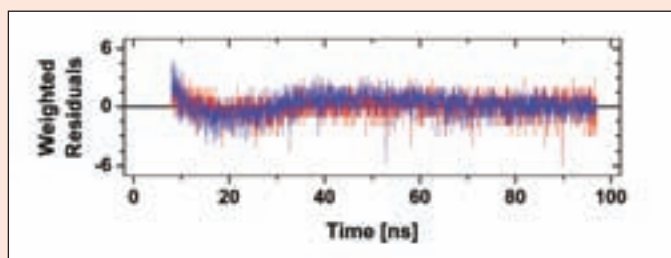
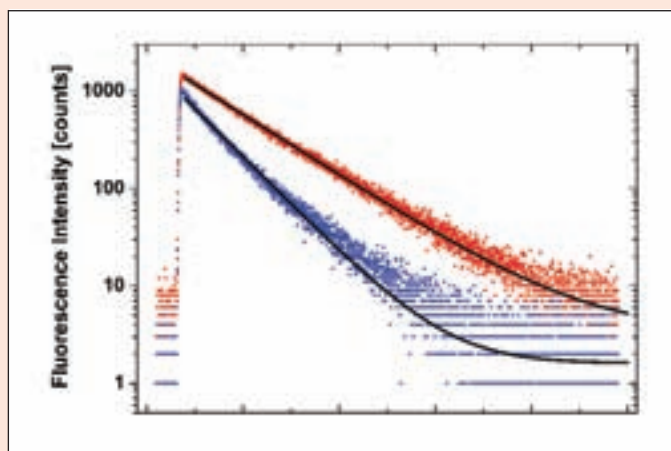
Most fluorescent samples do not convert 100% of the absorbed photons into fluorescence photons. The relative probabilities between the radiative process and those channels where no photon is emitted (thus: non-radiative), govern the fluorescence lifetime of the sample. The balance between the radiative and non-radiative channels can be altered, eg by bringing a quencher into the vicinity of the fluorescent molecule: the quencher adds a non-radiative channel, thereby reducing the fluorescence lifetime.

Typical values for the fluorescence lifetime are in the order of one to five nanoseconds (10^{-9} s). Fluorescein, rhodamine and similar dyes fall into this class. Some better known exceptions are rare earth metal ions in a chelate or kryptate complex; these have fluorescence lifetimes of up to one millisecond (10^{-3} s). Similarly, a complex with the transition metal ruthenium displays fluorescence with lifetimes of several hundreds of nanoseconds (10^{-5} to 10^{-6} s).

Measuring the fluorescence lifetime has been done in several ways. The most intuitive method is to excite the sample with a very short light pulse and record the fluorescence intensity decay with sub-nanosecond resolution. The resulting decay curve can then be analysed by a mathematical curve fit, assuming a mono-exponential function in the easiest case. The best known technique for recording these extremely fast time courses is time-correlated single photon counting (TCSPC). Alternatively, one can analyse the sample's response to excitation light modulated at a high-frequency (phase-modulation method). Lesser known methods employ, for instance, boxcar integration with varying delay and gate widths, and fast digitising of the entire decay curve.

Note that the term 'time-resolved fluorescence' is often used to describe measurements in which the fluorescence intensity is integrated after a fixed delay time. This technique can help suppress background fluorescence if a label with suitably long lifetime is used (see the above mentioned rare-earth and ruthenium complexes). No real time resolution is currently used in these assays but may provide further information and assay stability.

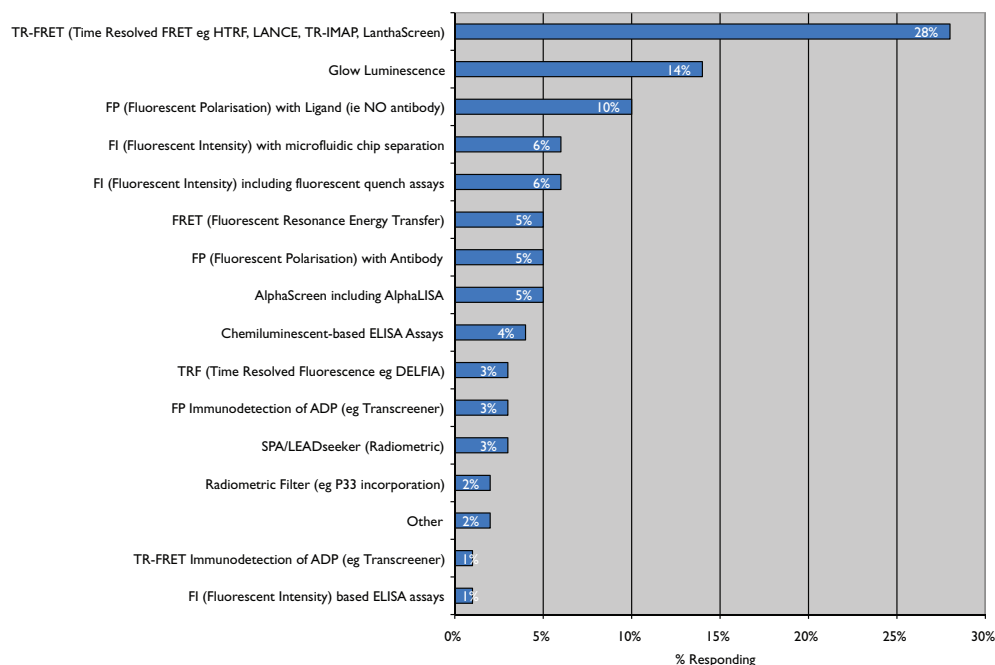
Utilising fluorescence lifetime as a readout signal in drug screening assays requires a sufficiently large change of the lifetime. With current measurement techniques a lifetime value can be recorded with a precision of a few tens of picoseconds, while keeping recording times at about one second per sample. This necessitates that the assay signal changes by several nanoseconds. Standard fluorophores with lifetimes between 1 ns and 5 ns are therefore relatively impractical starting points. More recent developments (see Almac, Assaymetrics below) have produced labels with lifetimes between 10 and 25 nanoseconds, making fluorescence lifetime available for assay development in drug discovery.



Top panel: Typical FLT data recorded by TCSPC. The fluorescence intensity is plotted semi-logarithmically versus a nanosecond timescale. Two sets of data are shown, corresponding to samples with 10 ns (blue) and 14 ns lifetimes (red). Each curve is made up of approximately 2,500 points, with a time resolution below 50 ps between points. A total of 200,000 photons were collected per curve. The continuous lines are the fitted mono-exponential curves, yielding the lifetime values, at a typical relative error of 0.5%.

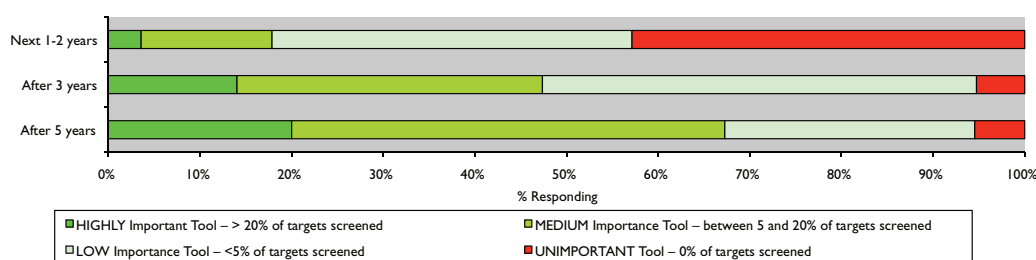
Lower panel: Displays the weighted residuals for the fits, which are the normalised deviations of the fit from the data. They are useful as a quality control tool during assay development.

Figure 6. Kinase platform/technology likely to be displaced by FLT-based kinase platform



© HTStec 2010

Figure 7: Opinion on adoption of FLT over the coming years



© HTStec 2010

20% viewing it as high importance (>20% of targets screened) and 47% as medium importance (between 5% and 20% of targets screened). Supporting this finding is the fact that there is already significant adoption of FLT in some companies, eg the Protease Platform at Novartis⁶.

Latest vendor offerings

The following snapshots provide details of some of the current status and recent progress vendors have made in the development of FLT-related instrumentation, dyes and assays.

Tecan (www.tecan.com) pioneered the field of developing multimode microplate readers that include a FLT detection module, when it marketed the ULTRA Evolution with FLT option in 2002. FLT measurements on the ULTRA Evolution are performed using the Time-Correlated Single Photon Counting (TCSPC) method, where a pulsed laser repeatedly excites the sample. The intensity of the excitation pulses is adjusted so that each individual pulse generates one photon counting event in the detector. The time between the laser pulse and the detector event is measured, and count numbers versus time

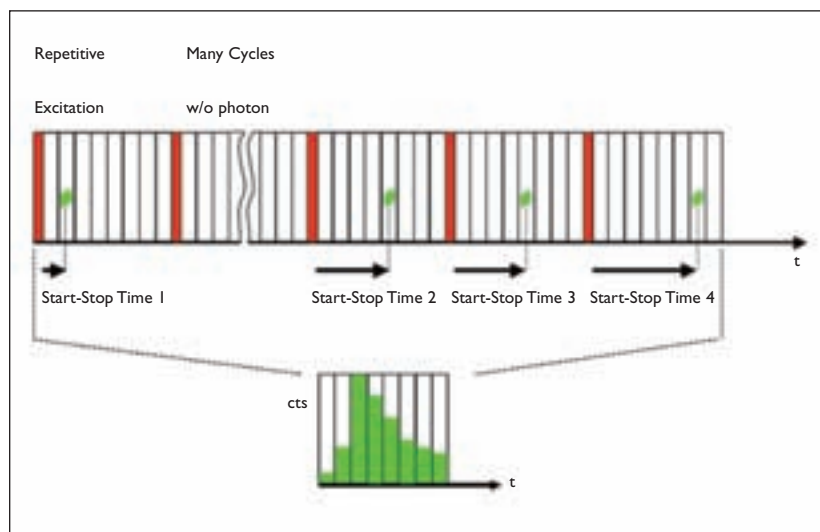


Figure 8: Principles of TCSPC measurements on Tecan's Ultra Evolution. A pulsed laser repetitively excites the sample. The intensity of the excitation pulses is adjusted such that for any one pulse no more than one photon counting event is generated on the detector. According to the time measured between the laser pulse and the detector event, this count is added to a histogram collecting count numbers. The channel numbers are sorted to reflect the arrival time of the single photons at the detector. One to ten million repetitions of this elementary experiment lead to smooth histogram data that represent the fluorescence decay curve

differences plotted on a histogram. Between one and ten million repetitions of these elementary events produces a smooth histogram representing the fluorescence decay curve. As fluorescence intensity-based readouts are background limited, they are susceptible to numerous assay interferences. Consequently the drug discovery community has investigated more

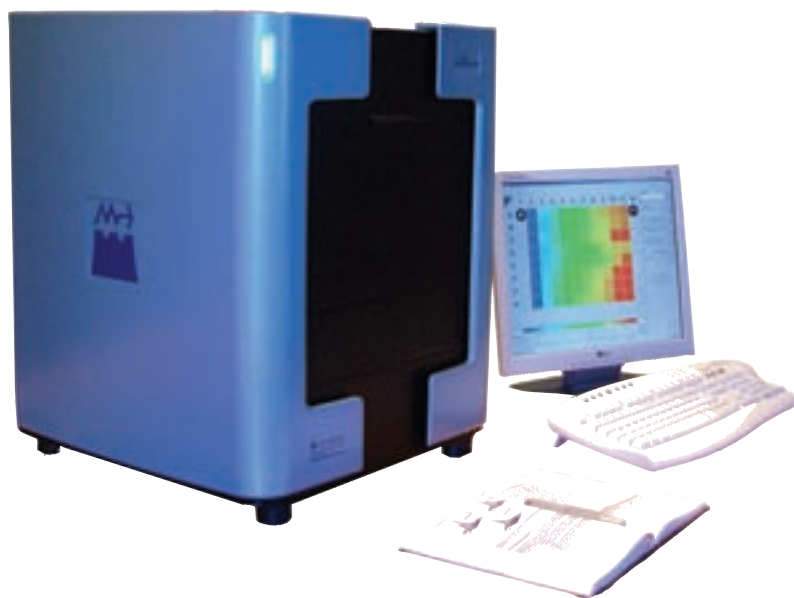


Figure 9: Edinburgh Instruments' NanoTaurus, the first dedicated FLT plate reader

robust assay principles, including fluorescence polarisation (FP) and, more recently, time-resolved fluorescence (TRF) and time-resolved fluorescence resonance energy transfer (TR-FRET) assays. The challenge is to reduce the number of short-lived autofluorescence signals from plates, buffers and compounds. When it comes to data robustness and reduction of fluorescence-based interferences, Tecan's customers find fluorescence lifetime methodology a powerful and robust alternative to TR-FRET-based measurements. FLT is highly susceptible to changes in the micro-environment surrounding the label, an aspect which could be exploited to allow the development of assay platforms targeting a variety of enzyme classes. In addition to a suitable microplate reader, researchers also need access to appropriate reagents which are compatible with FLT applications, and previously there has been a lack of reagent providers offering ready-made FLT kits suitable for specific drug discovery applications. In addition, the complexity of FLT readout demands that researchers become familiar with the principles and practice of FLT-based applications. These prerequisites currently limit the broad use of FLT. The latest drug discovery related conferences show interesting new activities and offerings around FLT, striving for robust, complementary and synergistic screening solutions. Tecan is working closely with its customers, exploring this promising technique and identifying opportunities where it can offer the technology to a broader industrial base (Figure 8).

Edinburgh Instruments (www.edinst.com), with more than 30 years' experience in pioneering developments in FLT, has developed the NanoTaurus, the first dedicated FLT plate reader. The NanoTaurus is based on the FLT method of choice, ie Time Correlated Single Photon Counting (TCSPC). The principle of TCSPC is analogous to using a fast stop watch to repetitively measure the time difference between each excitation pulse and the detection of the first fluorescence photon. Each measurement is added to form a histogram which depicts the time resolved sample emission. TCSPC outperforms all other methods for time resolved fluorescence in terms of sensitivity (detection at the quantum limit of single photons), dynamic range, accuracy and precision. Importantly, as a digital process, the data noise in TCSPC is Poissonian in nature (the noise of each data point is given by the square root of the number of counts) meaning that decays can be analysed further into the tail, helping precision. The measured fluorescence time response in an assay is made up by a linear combination of the emissions from the dye labelled substrate and its



Figure 10
Berthold Detection Systems' LF 502 NanoScan multimode fluorescence microplate reader, with lifetime capability

product. The complex data from each well measured in the NanoTaurus can be converted to a convenient, single parameter readout of average lifetime, $\langle\tau\rangle$, given by:

$$\langle\tau\rangle = (B_1\tau_1)^2 + (B_2\tau_2)^2 / (B_1\tau_1 + B_2\tau_2)$$

where B_1 and B_2 are the amplitudes and τ_1 and τ_2 are the lifetimes of the two components. The NanoTaurus uses proprietary, fast data harvesting acquisition electronics and fluorescence decay analysis software to obtain high quality measurements in a 384-well plate, with sample volumes less than 10 μ l, in less than five minutes. In Almac FLEXYTE[®] assays (see below), a typical variation in average lifetime between substrate and product of 5ns is obtained, compared to the measurement precision of the NanoTaurus of better than 0.1ns. Using a long lifetime fluorophore as reporter, a high level of separation from interfering compounds, typically with a lifetime of a few nanoseconds, can be routinely achieved. More sophisticated expert software can be used to indicate, and in some cases to automatically compensate for, compound interference, hence dramatically reducing the incidence of false hits (Figure 9).

The LF 502 NanoScan multimode fluorescence microplate reader from Berthold Detection Systems (www.berthold-ds.com) (formerly IOM GmbH) is probably the most versatile microplate

reader on the market among the instruments dedicated to fluorescence lifetime analysis. Technically, the basis is given by a nanosecond-pulsed laser with a low repetition rate and across the UV and visible range variable wavelength. These features lead directly to the unrivalled capability of seamlessly measuring fluorescence lifetimes between nanoseconds and milliseconds, ie over more than six orders of magnitude. Nearly every fluorophore can be analysed. Detection is accomplished by two ultrafast PMTs in two separate channels enabling the simultaneous measurement at two different fluorescence wavelengths. The NanoScan is capable of reading not only FLT assays over the whole span of lifetimes but also TRF and HTRF, Nano-TRF or fluorescence intensity assays. Due to its flexibility the NanoScan opens up a whole new world of applications. The availability of blue and green excitation wavelengths enables the use of novel quantum dot labels with outstanding long nanosecond lifetimes accompanied by large Stokes shifts while avoiding compound fluorescence due to UV excitation. Furthermore, quantum dots are considered as basis for the development of multiplexed assays. Short and intense UV laser pulses enable the efficient excitation of Terbium and Europium labels thus extending their use not only for TRF applications but rather in FLT mode within FRET or quenching assays. The microsecond-order lifetimes of transition metal complexes (eg Ruthenium) can easily be exploited by the NanoScan either in Nano-TRF assays (eg in a commercial cAMP assay) or directly in FLT mode. Recently progress was made to extend the span of detection methods to nanosecond time-resolved polarisation assays as recently demonstrated with a red fluorescing label in an enzymatic assay⁴. The LF502 NanoScan is ready to be integrated with stackers, dispensers and assay processors for applications in 96 to 1536 well microplates (Figure 10).

Fluorescence Innovations (FI2) (www.fluorescenceinnovations.com) and **Montana Molecular** (www.montanamolecular.com) have teamed up to develop new live cell assays for FI2's fluorescence lifetime microplate reader, which is based on microchip laser excitation sources and proprietary digitisers that record the complete fluorescence decay waveform on every excitation pulse. In addition to lifetime, the reader (see Figure 11) is compatible with fluorescence intensity, polarisation, and time-resolved polarisation formats in 96-, 384, and 1536 formats. The reading can be conducted for continuous scanning across the central 50% of the well or in stop-and-go mode. Read time for a

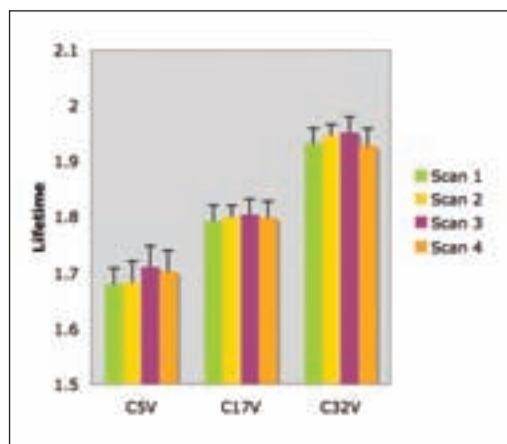


Figure 12: Fluorescence lifetimes of three FRET pairs obtained with direct waveform recording using FI2's lifetime reader. The samples consisted of Cerulean and Venus fluorescent proteins separated by 5, 17, and 32 amino acid linkers. See Koushik et al, *Biophysical Journal*, 91, L99-L101 (2006) for details on construct generation and comparison data obtained via two-photon excited time-correlated single photon counting. Each protein was expressed in a total of 22 wells on a 96-well plate and the entire plate was scanned four times. Scan time per plate was 29 seconds. Fluorescence decays for the Cerulean donor undergoing FRET were fit to a single exponential decay. Error bars represent one standard deviation and are substantially smaller than in the Koushik et al work

1536 plate in continuous scanning mode is less than two minutes. The FI2 direct recording approach is extremely fast, accurate and precise. The response of thousands of photoevents is recorded on every excitation pulse rather than one count for 100 excitation pulses as in time-correlated single photon counting (TCSPC). Signal-to-noise ratio (SNR) at the peak of the decay waveform can easily exceed 500:1 even at 10 wells per second read speed, equivalent to 250,000 counts in the peak channel by TCSPC. The excellent precision makes it possible to reliably monitor extremely small and subtle changes in the fluorescence decay characteristics even when operating in high throughput screening (HTS) mode. FI2 plans to bring to market a next-generation plate reader designed to accommodate a wider variety of laser excitation sources by the end of 2010. Fluorescence lifetime imaging microscopy (FLIM) is the usual form in which live cells expressing genetically encoded sensors are combined with fluorescence lifetime, but FLIM is unsuitable for HTS. FI2 and Montana Molecular have demonstrated the feasibility of simply averaging the fluorescence emitted by a collection of cells in the well. Illustrative data is shown in **Figure 12** for three Cerulean::Venus FRET pairs that have a short link-



Figure 11: Current version of the two-channel FI2 plate reader equipped with a microchip laser excitation source. Filters are used to isolate either the 532nm or 355nm laser wavelengths for excitation. Each channel has a six-position filter wheel for fluorescence wavelength selection

er consisting of 5, 17, or 32 amino acids between the chromophores. The FRET pairs were transiently expressed in HEK293 cells. As expected, the shorter the linker, the higher the FRET efficiency and the shorter the Cerulean donor lifetime. Twenty-two wells for each of the three pairs were studied in a 96-well plate. The figure shows that lifetime differences of just 0.1ns or 0.2ns are reliably distinguished.

AssayMetrics (www.assaymetrics.com) has developed a range of fluorescence labels specifically suitable for assays using fluorescence lifetime (FLT) as their readout signal. The first distinctive feature of these labels is their very long lifetime compared to, for example, fluorescein or rhodamine, providing a much enhanced signal window. The second is that their fluorescence can be dynamically quenched by two natural amino acids, tyrosine and tryptophan. Further, the labels are extremely photo stable. Together, these characteristics allow the researcher in drug discovery to rapidly develop cost-effective, customised, robust assays. The assay signal is produced by a single label attached to, for example, a peptidic enzyme substrate. No secondary components such as antibodies, beads, etc are necessary.

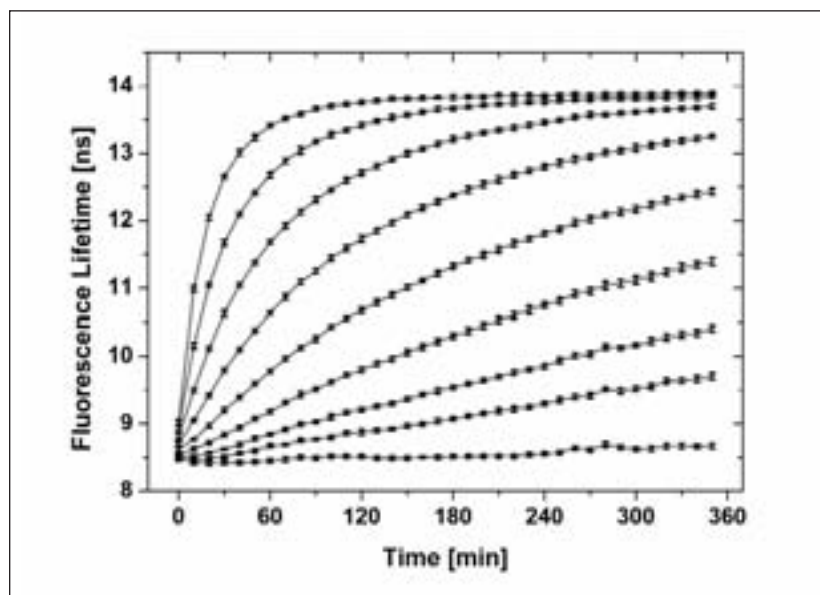


Figure 13: Proteolytic cleavage of Puretime14-labelled peptide. Various enzyme concentrations between 0nM and 25nM, substrate concentration 600nM. Each point depicts the mean value and standard deviation from 40 samples, sample volume 25uL in a 384-well microplate

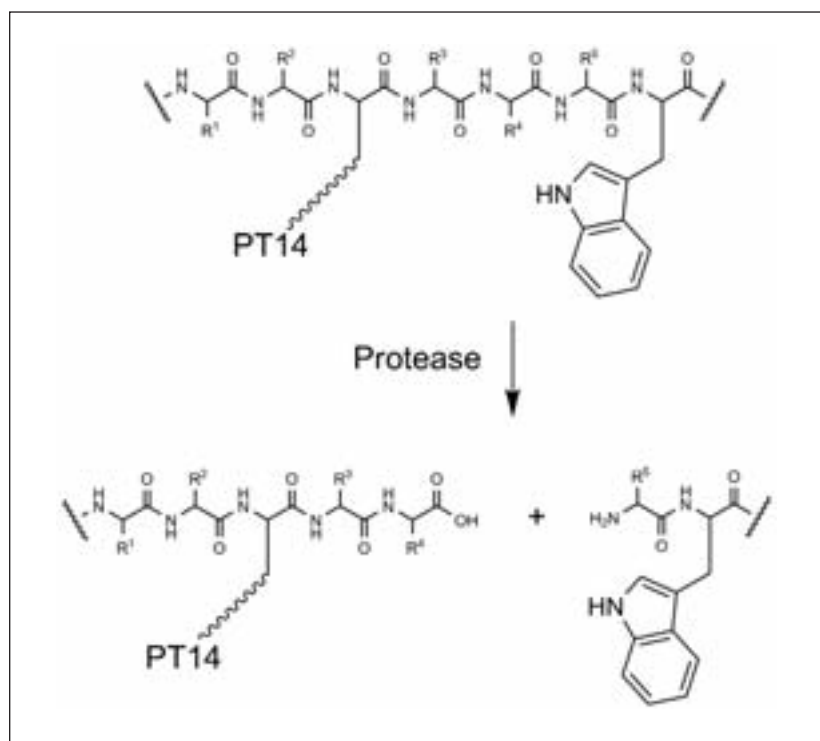


Figure 14: Protease assay principle. The peptidic substrate carries a Puretime14 label and a tryptophan either side of the scissile bond. While PT14's fluorescence is dynamically quenched in the parent structure, after cleavage label and quencher are separated, with a concurrent increase in the fluorescence lifetime

Thus the signal change develops in real-time together with the biochemical reaction under scrutiny. Determining kinetic parameters of enzymatic reactions becomes the norm without further assay development effort. In order to enable users of this technique to further optimise their protocols, AssayMetrics are developing their own dedicated fluorescence lifetime reader, focusing on short read times while keeping it highly flexible for a wide variety of applications. The Protease Platform at Novartis Pharma AG (Basel, Switzerland) uses this assay principle routinely for large scale inhibitor profiling and medium-throughput screening (MTS) campaigns on proteases. In comparison to assays employing standard protease substrates that are dual-labelled with UV-active fluorophore/quencher pairs, the FLT assay format led to an increase in the robustness against background fluorescence. Additionally, the work-flow was significantly simplified due to the universal assay format, allowing for running automated assay on broad panels of endo- and exopeptidases with just one common reader setup. The high flexibility in substrate design facilitated the in-house development of assays for approximately 80 different proteases from all four classes to date⁵⁻⁷ (Figures 13 and 14).

Almac (www.flexyte-assays.com) has drawn on its peptide sciences expertise in a collaboration with Dundee University and Edinburgh Instruments to develop an economical, homogeneous, antibody-free assay platform branded as FLEXYTE[®]. This new platform exploits the potential of FLT to offer robust approaches to screening and profiling that minimise background interference. Key to the success of the FLEXYTE[®] platform has been the development and application of 9-aminoacridine (9AA) as a long lifetime fluorescent reporter ($\tau = 17\text{ns}$)⁸. This photostable fluorophore has a high quantum yield and its lifetime can be modulated in a defined fashion through interaction with specific aromatic moieties. By exploiting the fluorescence properties of this dye, a series of 9AA labelled substrates have been developed, which produce a significant change in fluorescence lifetime upon modification by a variety of target enzymes. The initial focus was on Ser/Thr protein kinases. The FLEXYTE[®] protein kinase platform uses three generic peptide substrates, developed through the collaboration with the University of Dundee, to configure assays for a broad panel of kinases⁹. The assay uses a phosphoserine/phosphothreonine chelating agent to induce a change in the fluorescence lifetime of the 9AA label. Typical lifetimes

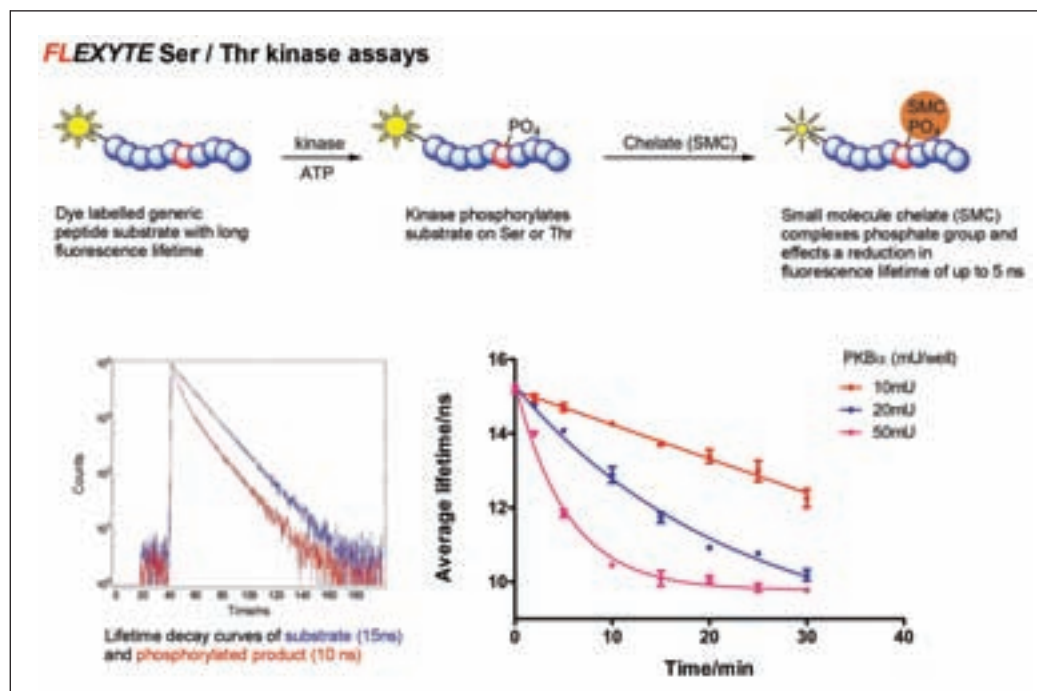


Figure 15 Almac FLEXYTE® Ser/Thr kinase assays. A small molecule chelate (SMC) complexes to the phosphate group of 9AA-labelled kinase product resulting in a reduction of fluorescence lifetime compared to the non-phosphorylated substrate. Phosphorylation of the substrate by the enzyme is directly reported through the corresponding decrease in the average fluorescence lifetime

are around 15ns for unmodified labelled substrate and 10ns for the phosphorylated product after reaction with the chelator (Figure 15). Since FLT measurements can be made accurately to within 0.05ns, the 5ns change provides a large assay window. The technology can be expanded to any protein kinase with appropriate peptide substrates. The platform is compatible with a wide range of ATP concentrations and performs very well in head

to head comparisons with radiometric and TR-FRET approaches. The second focus for FLEXYTE® was development of protease assays, opening up the potential of FLT to another therapeutically important class of targets. In this case 9AA-labelled substrates were designed so that cleavage by the protease causes a significant increase in fluorescence lifetime, typically from 11ns to 17ns (Figure 16), enabling enzyme activity

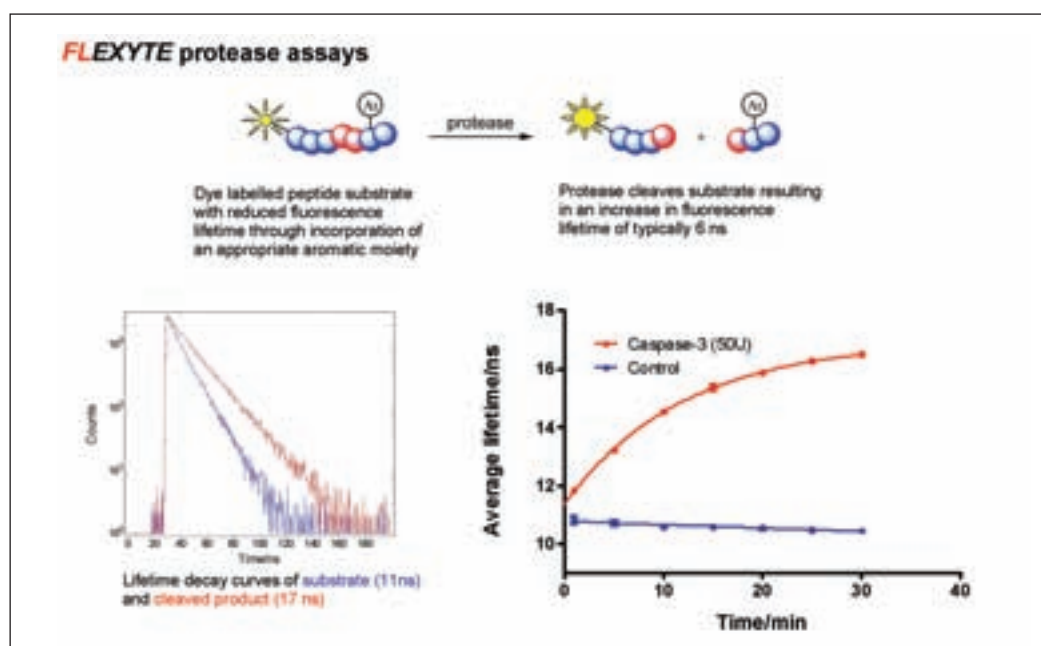


Figure 16 Almac FLEXYTE® protease assays. The protease substrate incorporates a 9AA dye and an aromatic moiety, upon enzymatic cleavage the aromatic moiety is released from the substrate resulting in an increase in fluorescence lifetime. Protease activity is monitored in real time through the concomitant increase in average fluorescence lifetime

References

- 1 Comley, J. Assay Interference a limiting factor in HTS? *Drug Discovery World* (2003): 4(3): 91-98.
- 2 Fluorescence Lifetime Trends 2007 Report, published by HTStec Limited, Cambridge, UK, March 2007.
- 3 FLT-Based Kinase Platform Trends 2009 Report, published by HTStec Limited, Cambridge UK, September 2009.
- 4 Riester, D et al. Non-isotopic dual parameter competition assay suitable for high-throughput screening of histone deacetylases. *Bioorg. Med. Chem. Lett.* (2009): 19:3651-3656.
- 5 Doering, K et al. A Fluorescence Lifetime-Based Assay for Inhibitor Profiling on Human Kallikrein 7. *J. Biomol. Screening* 2009: 14(1):1-9.
- 6 Hassiepen, U et al. Fluorescence Lifetime Assays – A Smart Solution for Inhibitor Profiling on Protease Panels. *Screening – Trends in Drug Discovery* 2009; 4:11-13.
- 7 Pritz, S et al. A Fluorescence Lifetime-Based Assay for Abelson Kinase. *J. Biomol. Screening* (2010): Submitted for publication.
- 8 Ramage, R et al. Novel fluorescent dyes and uses thereof. PCT/GB2006/004015 2006.
- 9 Paterson, MJ et al. A fluorescence lifetime-based assay for serine and threonine kinases that is suitable for high-throughput screening. *Anal. Biochem.* (2010): 402(1): 54-64.

to be directly monitored in real time through a change in the average lifetime. The ability to develop individual substrates for the significant majority of enzymes in this class indicates the potential of FLT for assay and screening of proteases. One of the clear advantages of employing FLT as a reading modality is its robustness. With Z' factors consistently above 0.9, the FLEXYTE® platform clearly lends itself to large scale screening and profiling applications. In the development of the platform, Almac has sought to engage major screening centres and following a successful evaluation of the FLEXYTE® platform, AstraZeneca's scientists plan to submit their work for publication. With the focus expanding further in the near future to lipid kinases, Tyr kinases, and ligand binding assays, Almac's FLEXYTE® assays offer to deliver on FLT's long promised potential to circumvent assay interference and provide an effective assay platform for screening and profiling groups alike.

Discussion

FLT largely failed to make a significant inroad as a screening technology when it was first introduced nearly a decade ago, despite the fact that intrinsically it has a lot going for it, particularly in dealing with assay interferences¹. Market studies undertaken by HTStec in the intervening period have consistently uncovered a latent appetite within drug discovery for FLT assays^{2,3}. Up until recently this appetite has for the most part gone unmet. However, recent published work at Novartis⁶⁻⁸ and unpublished work at other major pharmas have successfully addressed target class applicability (in the protease and kinase areas), assay development and validation, and assay reagent availability issues using FLT. The marketing of the Puretime dyes from AssayMetrics and the Almac's 9AA dye, plus the possibility of new dyes under development, has brought new life to this field and led to the development of a new generation of off-the-shelf assay kits based on lifetime (FLEXYTE®).

The FLT reader situation has also changed over the past eight years. Tecan no longer offers an FLT option on its current generation of Infinite microplate readers, yet it still has customers using FLT on the ULTRA Evolution. CyBio withdrew the CyBi@-NanoScan HT after a change of strategic direction within the company. Most Evotec Mk1 to Mk3 screening systems able to offer lifetime as a component of a multi-parameter fluorescence readout have largely ceased routine operation. The IOM MicroScan reader which originally offered Microsecond FLT has now evolved into the LF 502 NanoScan multimode fluorescence microplate

reader from Berthold Detection Systems. Edinburgh Instruments' NanoTaurus dedicated FLT plate reader is a main new addition that has already proven itself able to generate a large signal window with excellent precision in Almac FLEXYTE® assays. However, other yet to be commercialised FLT reading possibilities also exist, and may become available in the near future. Fluorescent Innovations developed a lifetime reader several years ago that so far has been mainly used internally. This reader does not utilise the better characterised TCSPC approach, but uses digitisers to enable direct recording methods resulting in extremely fast read times, in low assay volumes and in 1536 – all key requirements for some potential Pharma customers. AssayMetrics is also now developing its own dedicated fluorescence lifetime reader, focusing on short read times and better automation while keeping it highly flexible for a wide variety of applications. Furthermore, it is rumoured that one additional instrumentation company will also enter the market with a new FLT reader later this year. It is not clear whether this additional investment in new instrumentation was triggered by the availability of new lifetime dyes and kits, but there is an undoubted sense that the momentum around FLT is picking up. Clearly the advances in FLT highlighted in this article when viewed collectively make FLT a much more attractive option today than in 2002. The question is will this translate into sufficient probe, assay kit and instrumentation sales to fund future investment in those new and emerging areas where FLT has even greater potential to impact, for example, epigenetic enzyme assays and some cell-based targets.

Acknowledgement

The assistance of Klaus Döring in the preparation of 'Fluorescence lifetime assays – the basics' info box is gratefully acknowledged. **DDW**

Dr John Comley is Managing Director of HTStec Limited, an independent market research consultancy whose focus is on assisting clients delivering novel enabling platform technologies (liquid handling, laboratory automation, detection instrumentation and assay reagent technologies) to drug discovery and the life sciences. Since its formation seven years ago, HTStec has published more than 50 market reports on drug discovery technologies and Dr Comley has authored more than 30 review articles in Drug Discovery World. Please contact info@htstec.com for more information.