Fluorescence Lifetime ASSAYS

The benefits of fluorescence lifetime (FLT)-based technologies have been highlighted for more than a decade but thus far they have failed to gain widespread acceptance. Significant improvements in assay reagents and availability of HTS-compatible readers, however, have now delivered a cost-effective, robust technology applicable to a broad range of therapeutic targets. This article charts FLT development to be a highly attractive tool for drug discovery.

By Dr Graham Cotton, Dr Wayne Bowen, Dr Robert Grundy and Dr Ulrich Hassiepen

High-throughput screening (HTS) continues to evolve in response to the changing landscape of therapeutic targets and the needs of lead discovery teams. Having become a mature discipline in terms of screening capacity, recent years have seen a steady focus toward higher content technologies and data quality for the biological assays. The trend towards profiling of smaller, more chemically-focused compound libraries has placed increasing pressure on the relevance of the biological data generated and quality of the list of validated compounds against the target for optimisation. The presence of so-called false positives in the output from a HTS screen is therefore an increasingly unwanted occurrence, since primary hit validation and secondary profiling in follow-up assays have become significant time-consuming and costly efforts.

Luminescence and fluorescence-based assay methods prevail in modern HTS. Although invaluable as technologies, the problem of compound-related assay interferences such as quenching, auto-fluorescence and light scattering becomes important. The degree of interference experienced may depend on variables such as assay reagents, composition of the chemical library and reader technology. Where the amount of interference is unacceptably high, the only option is to delete the data and thus lose all information pertaining to compound activity. False positives occur when such interferences go undetected.

The use of fluorescence lifetime (FLT) as the readout parameter for *in vitro* assays increases biochemical assay robustness against artefacts such as well-to-well volume variations, turbidity by precipitating particles and compound-related interferences. This makes the readout superior to other optical readout formats and capable of providing high quality primary screening data[1]. In addition, while providing high data quality, FLT-based assays are homogenous and scalable, and therefore compatible with medium- and high-throughput experiments. With a predictable assay window size based on rationally-derived rules for substrate design and the ease of substrate synthesis, this assay format offers a convenient and cost-efficient alternative to other established formats[2]. Enzyme activity assays, whereby the fluorescence lifetime of a reporter-labelled peptide substrate is directly affected by enzyme modification, have been successfully introduced for routine inhibitor profiling in automated processes within the hit-to-lead and lead optimisation phases for enzyme classes such as proteases, kinases and phosphatases[3-7]. In addition, FLT assays have now been configured for deubiquitinases, protein-protein interactions and a number of different epi-enzyme targets.

FLT assay principles

The fluorescence lifetime is the average time between excitation of a fluorophore and its return to ground energy state. In simple systems,

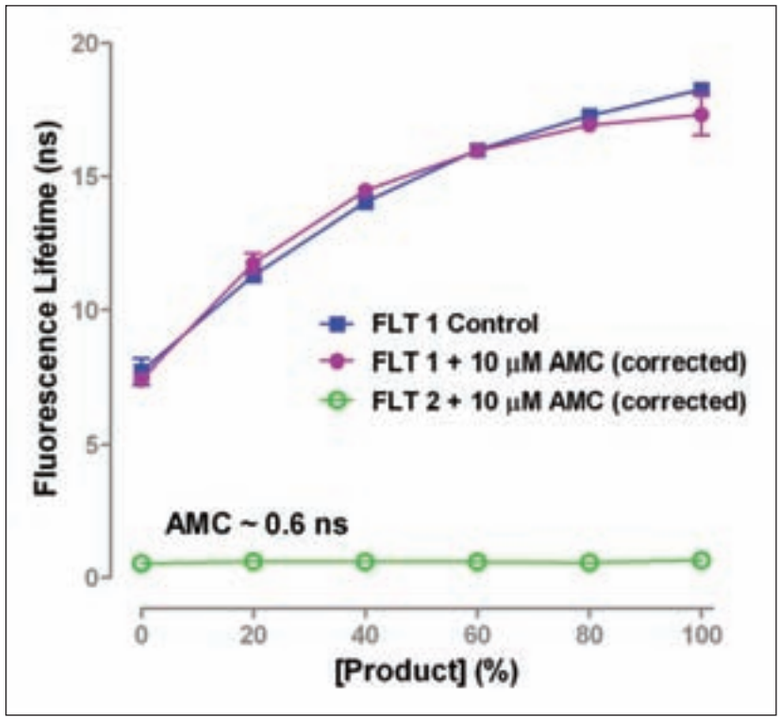


Figure 1
Addition of fluorophore 7-amino-4-methylcoumarin (AMC) to a FLEXYTE caspase-3 assay has a dramatic effect on the fluorescence decay curves. Unlike other fluorescence-based assay techniques, the information-rich RT-DCA output permits resolution of enzyme activity and AMC to restore the dataset

fluorescence intensity decays as a single exponential thus defining FLT as the time in which the fluorescence intensity decays to 1/e (~37%) of that immediately following excitation[8]. In drug screening applications fluorescence artefacts and compound interference typically have fluorescence lifetimes of less than 5ns. In addition, the background auto-fluorescence from cellular components is typically less than a few nanoseconds. However, many fluorophores currently used in assay applications have short fluorescence lifetimes in the range 1-5ns, and while these have been used in fluorescence lifetime applications they do not exploit the true potential of the technology. To this end, a number of fluorophores have been developed with fluorescence lifetimes in the 10-25ns range to facilitate clear discrimination of the reporter signal from compound interference. Thereby minimising compound interference and realising the benefits of FLT for the end-user.

In the last few years the two reporters that have been predominantly utilised in FLT assay applications are 9AA and PT14. PT14 (GE Healthcare) is an acridone-based fluorophore typically excited at 405nm and has a fluorescence lifetime of 14ns[3]. While 9AA (Almac Group, FLEXYTE™) is a bright 9-aminoacridine based reporter, similarly excited at 405nm and with a fluorescence lifetime of circa 17ns in physiological buffers[5]. PT22 (GE Healthcare) is a larger quinacridone fluorophore that is routinely excited at 473nm and has a lifetime of 22ns. It can also be excited at longer wavelengths, which has been utilised in fluorescence polarisation applications but it holds promise for FLT assay applications. Importantly, these fluorophores have now been incorporated into all manner of peptides and proteins, and off-the shelf and bespoke reagents are freely available to the end-user, as well as fully configured assays for a variety of different target classes. The latter being facilitated by an increased understanding on how the FLT of these fluorophores can be modulated in response to a variety of molecular, structural and environmental changes.

Changes in FLT can only occur upon dynamic quenching events such as collisional quenching or fluorescence resonance energy transfer (FRET). Both mechanisms have been used in assays employing FLT as the readout parameter and have enabled different configurations of FLT assays to be developed for a variety of different target classes.

A detailed analysis for the quenching mechanism in peptides accommodating acridine, acridone and quinacridone fluorophores and known quenching by side-chains of natural and non-natural amino-acids such as tryptophan, tyrosine, 4-amino-phenylalanine and napthalene is still missing. In such peptides, the effect of the fluorophore-quencher distance and relative positioning of the two moieties when compared to well described fluorophore-quencher systems suggests that the lifetime reduction is due to a collisional quenching mechanism, most likely photon-induced electron transfer (PET)[2].

Small-molecule lifetime modulators have been developed, which afford dynamic quenching and a lifetime reduction of the 9AA reporter over larger distance through space. While the exact mechanism of lifetime modulation has not been elucidated, this has enabled FLT protein-protein/protein-ligand assays to be configured by labelling of the two different binding partners with the fluorophore and the modulating agent respectively.

The knowledge of the underlying mechanism results in guidelines that allow for prediction of the assay window and enables FLT assay for new targets to be designed *a priori* with confidence[2,5]. Given the current availability of spectrally-distinct FLT dyes, the ability to development multiplex assays is a realistic possibility. This is an aspect that cannot be addressed by many HTRF and TR-FRET approaches.

Instrumentation

Two approaches are currently employed for the measurement of fluorescence lifetimes in microplates; time-correlated single photon counting (TCSPC) and real-time decay curve analysis (RT-DCA)[9]. To enable such measurements, the optics in readers comprise a pulsed laser as an excitation source, emission filters and sensitive photomultipliers tube (PMT) detectors. The approaches differ in the method used to quantify fluorescence emissions in the time domain. TCSPC involves measuring the time for the first emitted photon to reach the PMT detector following a brief excitation pulse. Repeating this measurement many times produces a histogram of detected 'first photons'. The electronics used in the first-generation plate readers limited the count rate to about 1%, ie, on average only one photon must be counted per 100 laser pulses for gaining a faithful representation of the true fluorescence decay. The latest developments are based on improved TCSPC boards with multi-stop capability and multiple channels enabling counting at higher rates with parallel detection. This increase in the count rate translates into a decrease in read time per microplate well from typically one second to below 0.2 seconds.

In real-time decay curve analysis (RT-DCA), the entire emission curve is recorded after a single excitation pulse, enabling high-throughput time-resolved data to be acquired. The Ameon™ microplate reader (TTP Labtech, Melbourn, UK) contains a proprietary digitiser that records a complete fluorescence decay curve for each laser pulse. Since RT-DCA is not limited to counting single photons, more powerful lasers can be utilised generating more emitted photons and improved statistics, enabling read times of ~2 mins for a 1536-well microplate. These fast reading times make it possible to implement FLT measurements in the challenging environment of high-throughput screening.

Compound interference and data correction

A constant challenge during compound profiling is the successful differentiation of compound-related activity from off-target interferences. The level of interference during HTS is dependent on multiple assay variables, but levels of 3-5% are commonly experienced. A major driver for re-initiating the implementation of FLT assays in drug-discovery was the development of dyes with remarkably long fluorescence lifetimes (9AA, PT14 and PT22). The fluorescent compounds in the screening decks used in the pharmaceutical companies are characterised by fluorescence lifetimes that are significantly shorter than those of such dyes. Therefore employing these long lifetime dyes in FLT-based assays enables automated correction of assay data by utilising the difference in the FLTs of the compounds and the dyes.

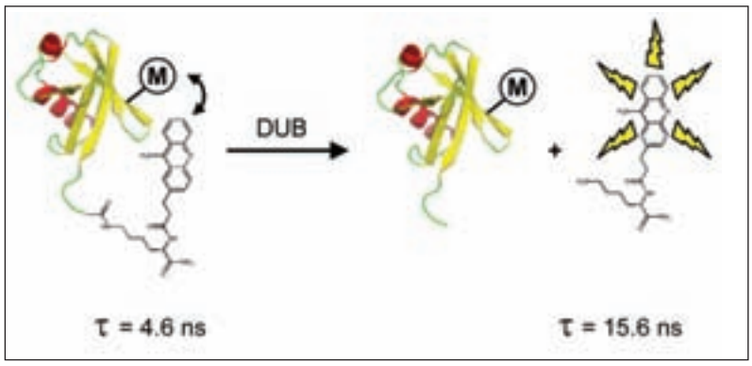
With the currently used acridone and quinacridone dyes, data distortion (distortion of the fluorescence intensity (FI) decay curve of the reporter dye) can only be caused by fluorescent compounds with spectroscopic properties similar to those of the reporter dye or by collisional quenching. The latter one can only occur at compound concentrations of 500µM and above, because the spatial distance of the fluorophore and the quencher (the quenching compounds) has to be in the range that the compound can move by lateral diffusion within the lifetime of the exited dye.

For fluorescent compounds basically two cases can be distinguished. First, the FLT of the compound is shorter than that of the reporter dye – this can be corrected in TCSPC and RT-DCA (**Figure 1**). Second, the FLT of the reporter dye is in the range or even longer than that of the reporter dye. Here, data recovery is generally more difficult but such situations are rare given the relatively long lifetime of the FLT dyes.

The information-rich FLT output allows determination of multiple fluorescence characteristics including amplitude and intensity. These characteristics can be interrogated to distinguish between fluorescence from interfering compounds with similar emission profiles to FLT probes. In recent years, detection and filter algorithms have been developed to provide rapid identification and where possible restore data anomalies.

However, the software provided with the first generation FLT readers did not offer the opportunity for any sophisticated, automated data processing. FLT data as provided by these instruments was used for detecting anomalies in the single wells such as a significant variation in the total intensity compared to control wells or a significant distortion of the FI decay curve. Wells with detected strong anomalies were disregarded and rejected from data evaluation.

With the second-generation instruments more advanced algorithms are provided and can be used automatically. AssayMetrics's PureCheck algorithm in combination with a bi-exponential fitting routine of the FI decay curve delivers excellent results[8]. Now, only data from wells containing fluorescent compounds with spectroscopic characteristics similar to those of the reporter dye cannot be corrected. However, such compounds are rare and



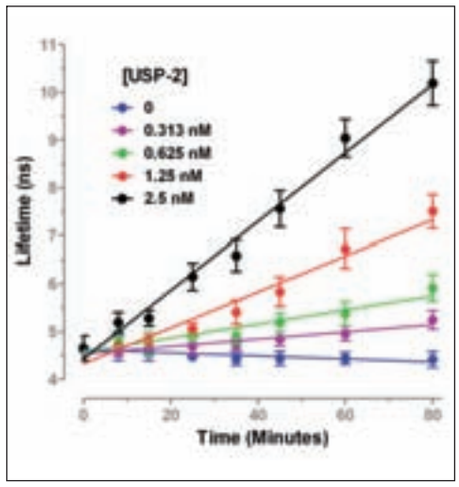


Figure 2: A: FLEXYTE isopeptide-DUB assay principle. 9AA is attached to the C-terminus via a natural isopeptide-linkage and a small-molecule modulator is incorporated into ubiquitin such that it decreases the FLT. Cleavage of the isopeptide-bond by DUB enzymes, removes modulation, causing an increase in the measured lifetime. B: Activity of the DUB enzyme USP2 is reported in real time through a change in FLT

their structural similarity to the reporter dye allows easily for identification and makes them unattractive as pharmacophores.

TTP Labtech has recently developed Validator, an algorithm which integrates the tasks of data acquisition, anomaly identification and lifetime determination. Hence, FLT technologies have the capacity to not only flag assay interferences but to also correct for their effect, leading to a reduction in the number of false positives and an expanded dataset.

Established assay principles – fields of application

Enzyme activity assays

A host of different FLT protease, kinase and phosphatase activity assays have been developed based on cleavage, phosphorylation or dephosphorylation respectively, of a short, artificial substrate peptide used as a surrogate for the endogenous protein substrate.

For proteases assays, peptide substrates are generated that are mono-labelled with 9AA, PT14 or PT22 and contain a quenching amino-acid side chain of tryptophan, tyrosine 4-amino-phenylalanine, or naphthalene in proximity to the fluorophore but on the opposite 'side' of the scissile bond. The peptide is designed such that the fluorescence lifetime of the reporter is significantly reduced in the substrate. Peptide cleavage by a protease causes separation of fluorophore and quencher enabling protease activity to be measured in real-time through an increased in the measured fluorescence lifetime[3,4,5]. Using this approach, assays for more than 100 different proteases have now been developed and productively used for inhibitor selectivity profiling[4]. Interestingly, when using these long-lifetime dyes, only small changes in fluorescence lifetime are required to configure workable assays for screening applications. The accuracy of the FLT measurements translates into respectable Z'-factors with assay windows of less than 1ns. This is clearly described in a worked example by Pritz et al[2]. For enzyme activity assays, the authors consider a situation where the lifetime changes between substrate and product is 4ns (the change in lifetime between substrate and product is often larger than this), and illustrate how a final assay with 20% substrate conversion results in Z'-factors of 0.7. Appreciating that larger lifetime changes are not a pre-requisite for successful assay development, maximises the potential applications of the technology.

The mechanisms employed in the protease assays have been further exploited to develop an FLT assay platform for screening the broader epi-enzyme target class. Utilising 9AA-labelled histone peptide substrates, a protease protection approach has been developed, whereby modification of the substrate by the epi-enzyme protects the substrate from proteolytic cleavage by an established protease. The histone substrates are designed in such a fashion that the proteolytic cleavage induces a change in FLT. Hence protease protection, which is reported by a change in fluorescence lifetime, is a direct measure of substrate to product turnover. Using this approach FLT assays for lysine methyl transferases, lysine demethylases, protein arginine

methyltransferases, histone deacetylases, histone acetyl transferases and deiminase assays have all been reported.

Recently, a generic FLT approach has been developed for assaying the deubiquitylating (DUB) family of enzymes. Ubiquitylation is the attachment of the C-terminal glycine of the 76 amino acid protein ubiquitin (Ub) to the amino group of a lysine side-chain in the target protein via an isopeptide bond. With removal of Ub, via cleavage of the isopeptide bond, being catalysed by the DUB enzymes. Given the broad role of ubiquitylation and deubiquitlyation in regulating normal cellular processes, and its increasing implication in a variety of different diseases, there is a growing interest in targeting the DUB family of enzymes.

The FLEXYTE FLT DUB assay platform employs full-length 'isopeptide-linked' ubiquitin substrates to determine enzyme activity. In this simple homogenous assay format, the ubiquitin substrates are generated with the 9AA FLT reporter site-specifically attached to the C-terminus of the protein via a lysine isopeptide bond. Enzymatic cleavage of this scissile isopeptide bond is directly reported, in real time, through a change in FLT of the system (**Figure 2**).

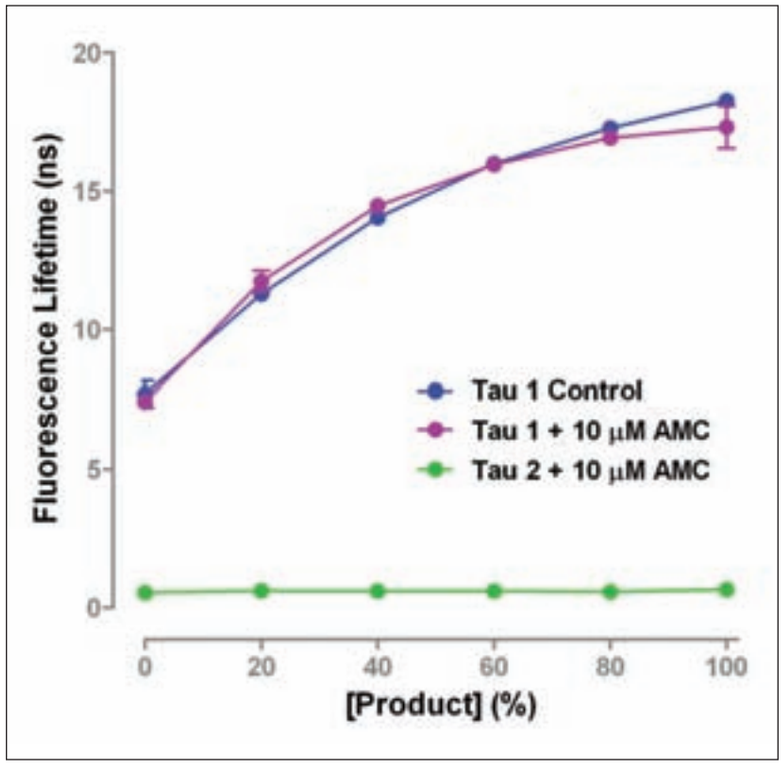
In a similar fashion to the protease assays, real time tyrosine kinase assays have been configured by exploiting the finding that tyrosine can dynamically quench the fluorescence of 9AA and PT14 labelled substrates, while phosphotyrosine cannot. By judicious positioning of the FLT label, protein tyrosine kinase substrates can be generated whereby phosphorylation of the tyrosine induces significant increases in the fluorescence lifetime of the system, enabling kinase activity to be monitored in real time through a change in lifetime[7].

For serine and threonine kinases a different approach has been developed. In these assays, selective co-ordination of phosphoserine and phosphothreonine with an iron(III) (FeIII) chelate is used to induce changes in the fluorescence lifetime[6]. 9AA-labelled peptides are utilised in these assays. After treatment with the kinase, the reaction is stopped by addition of an FeIII chelate under acidic conditions. The FeIII complex co-ordinates selectively with the phosphorylated product and not the unphosphorylated substrate, and in doing so reduces the lifetime of the 9AA reporter. The level of substrate phosphorylation, ie kinase activity, is simply reported through a change in the measured fluorescence lifetime. Using this approach a number of different serine and threonine kinase assays have been developed using generic and tailored substrate sequences.

Competitive binding assays

The assay principle and its application for drug-discovery has been demonstrated with proteases using a known inhibitor fluorescently-labelled with the lifetime dye as the probe[10]. The synthesis of the probe was driven by rational design. Previous work on FLT-based enzyme activity assays showed that nitrogen-containing aromatic heterocycles are potential dynamic quenchers of PT14's fluorescence[2]. Active-site directed, low-molecular weight inhibitors of S1 serine proteases comprising such heterocyclic, putatively quenching molecular moieties such as S1 or S1' pocket binders, respectively, were selected. The PT14 dye was conjugated to a site of the inhibitor in a way that the dye is most likely solvent exposed when the probe is bound to the proteases. In the bound state the inhibitor's quenching moieties will be completely or partially buried in the S1 or S1' pockets, respectively; thus the spatial separation of dye and quencher will result in a long observable fluorescence lifetime. In contrast, the probe's unbound states will be characterised by a short fluorescence lifetime because the probe's structural flexibility will allow for collisions of the PT14 and the quenching heterocycle. Data obtained with this novel assay format is in excellent agreement with those from the similar TR-FRET-based assay (**Figure 3**).

Figure 3
Comparative analysis of TR-FRET- and FLT-based displacement assay data for a serine protease. The two similar assays were developed for the same target. The TR-FRET-based assay required labelling of both the displacement probe and the receptor protein with a corresponding donor-acceptor pair. In contrast, labelling of just the displacement probe with PT14 was sufficient for developing the FLT assay. EC values ± error of duplicate measurements were plotted. The line illustrating the excellent correlation of the data from the two assays results from fitting a linear equation to the data



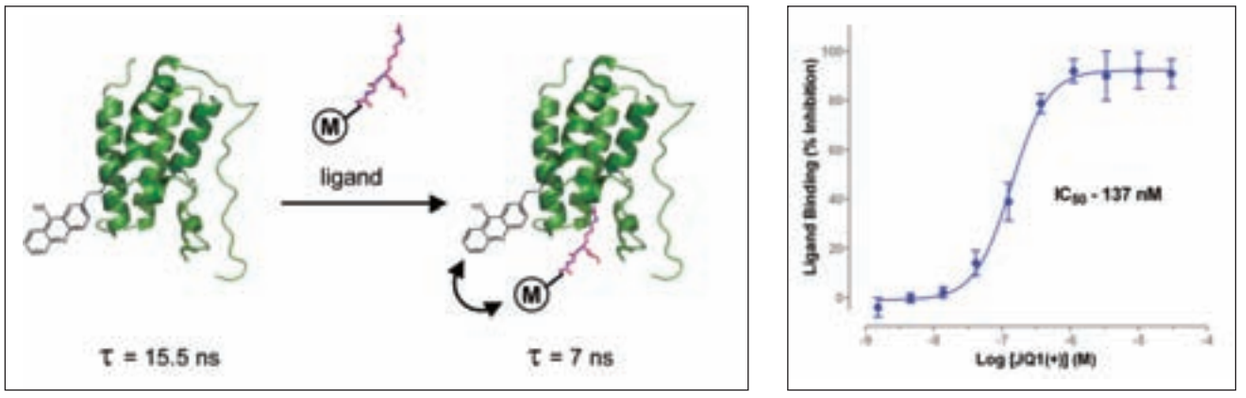


Figure 4: A: BRD4(1) FLT assay principle. Binding of an acetylated lysine histone H4 peptide to the 9AA labelled BRD4(1) domain induces significant changes in fluorescence lifetime. B: Inhibition of the BRD4(1) – H4 interaction is directly reported though a change in fluorescence lifetime of the system, data for the known small molecule inhibitor JQ1(+) is shown

FLT-kinase binding assays have also been developed using fluorophore-labelled derivatives of staurosporine, with binding to the kinase directly inducing small but measurable changes in FLT[11]. The fluorophore used in this study had an intrinsically short half-life. The application of long-lifetime reporters may open up new opportunities in this area.

Protein-protein interactions

In an alternative approach to enable FLT protein binding assays, a small molecule modulator has been specifically developed which changes the FLT of the 9AA reporter when the two are brought into proximity. Labelling two binding partners with the 9AA-modulator pair at appropriate positions enables protein-ligand (or protein-protein) interaction assays to be successfully configured (Z'-factor > 0.8) and used for assaying protein-protein interactions.

While a relatively recent development, the approach has been successfully applied to the epi-reader target class, in particular the interaction between the bromodomain, BRD4(1), and acetylated histone peptides. Inhibiting such interactions has been shown to elicit anti-tumour and anti-inflammatory effects in a number of disease models. The FLT assay employs BRD4(1) site-specifically labelled with 9AA and an acetylated histone peptide incorporating a FLT modulator that produces a significant change in FLT upon protein-ligand binding (**Figure 4**). The assay was validated for inhibitor screening using the reported small molecule inhibitor JQ1(+) with protein concentrations as low as 50nM. The IC_{50} of 137nM for JQ1(+) in this FLT assay is in-line with values reported in the literature using other assay formats. Given the nature of the assay configuration it should be widely applicable for screening other protein interaction assays.

Comparison of FLT with other assay formats

The FLT assay platforms that have been developed are straightforward in design. The assays are homogenous and antibody-free with the FLT measurements generally providing a direct readout of activity. Given the simplicity of the approach and the robust nature of the technology, target assays have proved relatively easy to configure and the technology as a whole easy to transfer into a laboratory and set-up from scratch. Consequently, there is no real barrier for adopting the technology. In addition, FLT assay platforms such as those for the protease, tyrosine kinases and protein-protein interactions enable real time measurements to be performed, facilitating kinetic analysis in screening.

The merits of FLT technology in comparison to other routinely-employed assay formats is nicely illustrated for the kinase area (**Table 1**). For this target class HTRF and Alphascreen are routinely employed technologies[12]. By assessing the three different approaches against key end-user criteria, FLT stacks-up as a very attractive option for the screening community. In terms of head-to-head data comparisons between FLT and other assay formats, although the number of disclosed data sets is limited, they do highlight the benefits and potential of the FLT technologies. A couple of examples are highlighted below.

In one interesting study, the FLT isopeptide-DUB assay described above was used to perform a focused fragment screen against a designated DUB family member. This same fragment library was also screened against the same DUB target but using an established fluorescence intensity-based DUB assay which employs Ub-Rh110 as the substrate. Interestingly, for the intensity-based assays, significant compound interference was observed for 20 out of the 47 fragments at concentrations of 500µM. While with FLT assay interference was only observed for one out of the 47 compounds at concentrations of 1mM.

In another comparator study, again using a serine/threonine kinase target, Astra Zeneca compared the ability of an FLT assay and a TR-FRET assay to determine IC_{50} values for a number of problem fluorescent compounds. The results, generated independently by each of these assay formats, were then directly compared to the internal AZ data set generated using mobility shift technology (Caliper). The compound set was biased to contain a number of known inhibitors for the kinase target with a range of IC_{50} values. Comparison of the IC_{50} values obtained by the TR-FRET assay with the mobility shift data gave a correlation of 0.57, while the IC_{50} values obtained by the FLT assay format gave a correlation of 0.95 with the mobility-shift assay data (**Figure 5**), demonstrating the utility of the FLT technology in this instance.

Discussion/concluding remarks

The exploitation of FLT in the context of drug discovery has evolved significantly over the last decade to adapt to the needs of drug discovery groups. Primary and secondary screening groups need to have at their disposal simple, robust, cost-efficient and widely applicable technologies. These needs have driven the development of FLT-based assays to the extent that there now exists highly effective instrumentation and reagents lines designed to focus the unique aspects of FLT towards extracting maximum value from compound screening libraries. Recent advances in FLT dye chemistry, substrate design and assay configuration have been combined effectively with transformative instrumentation and analysis software to provide a drug screening platform that is simple yet robust. Moreover, by harnessing the particular characteristics of FLT the amount of information one is able to extract from a screen allows a high degree of analysis, enabling the management of interfering compounds which would ordinarily compromise the success of a small molecule library screen. In summary, after following a path of continuous improvement, FLT is now poised to take its place amongst the most valued HTS technologies. DDW

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Table 1: Comparison of time-resolved technologies for protein kinase assay

	FLT	HTRF	ALPHASCREEN
Homogeneous format	✓	✓	✓
1536 compatible	✓	✓	✓
Antibodies-free	✓	✗	✗
Bead-free	✓	✓	✗
Intensity independent	✓	✗	✗
Kinetic measurements	Easy	Difficult	Difficult
Reagent cost	$	$$$	$$$

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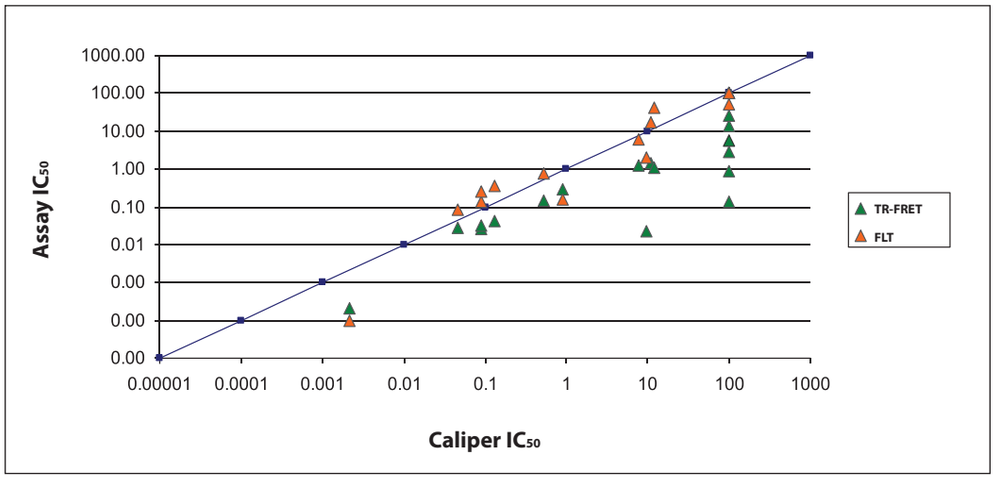

Figure 5: Correlation of IC_{50} values for a set of 'problem' compound against a designated kinase target using FLT, TR-FRET and mobility shift (Caliper) assay formats. The FLT results show a much greater correlation with the mobility shift technology than the TR-FRET data with the mobility-shift results

and within cells. In 2002 he moved to Almac (formerly Albachem), where as Senior Group Leader he is responsible for the R&D portfolio in protein-based technologies and their application to protein therapeutics and drug discovery, including the development of the Flexyte FLT assay platform.

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