Time Correlated Single Photon Counting - an Advancing Technique in a Plate Reader for Assay Development and High Throughput Screening

Dirk U. Näther*, Roger Fenske, Reynald Hurteaux, Sandra Majno, S. Desmond Smith
Edinburgh Instruments Ltd., 2 Bain Square, Livingston, EH54 7DQ, U.K.

ABSTRACT

A new plate reader (Nanotaurus) has been developed by Edinburgh Instruments that has the principle design features of a confocal microscope and utilises the technique of Time Correlated Single Photon Counting for data acquisition. The advantages of Fluorescence Lifetime Measurements in the nanosecond time scale and analysis methods to recover lifetime parameters are discussed based on experimental data. First working assays using changes of lifetime parameters are presented that clearly demonstrate the advantages of the new instrument for biochemical assays and show strong promise for cell-based assays, by utilising the independence of lifetime parameters from sample volume and concentration.

Keywords: Fluorescence Lifetime Plate Reader, Time Correlated Single Photon Counting, Biochemical Assays, Cell Based Assays, Confocal Detection, Fluorescence Lifetime Measurements

1 INTRODUCTION

Fluorescence detection techniques are now widely used in assay development and high throughput screening, and they are progressively replacing radiometric assay methods [1-3]. Fluorescence detection avoids the problem of working with and disposing of radioactive materials and has several other advantages, such as practically unlimited shelf life of labels, significantly increased stability of reagents and comparatively short read times. A major technical advantage is that fluorophores are highly sensitive to their environment, whereas radioactive isotopes provide little intrinsic information of their surroundings [4]. All this combines to make high throughput screening by fluorescence techniques more cost effective and more amenable to miniaturization and ultra high throughput screening.

Fluorescence intensity, FI, is currently the most popular readout parameter and is utilized in more than 30% of all screens [3]. Measurements of fluorescence intensity require inexpensive instrumentation, but they are often affected by background problems. The known high potential of unmatched sensitivity and high selectivity of fluorescence is often compromised by background that originates from sample carriers, sample reagents, solvents and test compounds [3, 4] and also components of the optical system [5].

Time-gated fluorescence detection, often referred to as time-resolved fluorescence, TRF, is the second most popular fluorescence readout method. Time-gated detection requires pulsed sample excitation and makes use of the decay process of long living (1-2ms) lanthanide chelates [7] and cryptates [8]. It remains intrinsically an intensity measurement. The advantage over a steady state fluorescence intensity measurement is that the short living (<10ns) background signals and residuals of the lamp excitation pulse are removed by delaying the active detection gate in respect to the exciting light flash.

By using multiple gates and ratioing the signals from different time windows, or by measurement and analysis of the entire decay process, TRF essentially measures a lifetime parameter. Some TRET (time resolved energy transfer) assays work on the basis of this detection principle. Although the robustness of these assays is improved, the long lifetime lanthanide complexes are relatively specialized and expensive and their molecular size imposes limitations in their use as biochemical labels [4].

*dnather@edinst.com; phone +44 1506 425 300; fax +44 1506 425 320; edinst.com

Advanced Photon Counting Techniques, edited by Wolfgang Becker, Proc. of SPIE
Vol. 6372, 637208, (2006) · 0277-786X/06/$15 · doi: 10.1117/12.688419

Direct measurement of the fluorescence lifetime, FLT [9,10], of organic fluorophores within an assay offers the cost advantage of FI assays while matching or indeed improving the robustness and sensitivity of TRF assays. Such fluorophores have excited state lifetimes in the nanosecond time regime which are an intrinsic property independent of volume or concentration.

Fluorescence lifetime measurements in the nanosecond and picosecond time scale using the technique of Time Correlated Single Photon Counting, TCSPC, are well established in the research market. In the past, such Time Correlated Single Photon Counting has been considered technically challenging with complex electronics, that required expensive specialized light sources (typically lasers), and with long data acquisition times [11,12]. Today, the availability of high speed data acquisition electronics, e.g. in this work the Edinburgh Instruments TCC900 plug-in card, high repetition rate picosecond diode lasers of suitable wavelength, and fast single photon counting detectors, makes this technique ideally suited for high throughput applications and assay development.

2 INSTRUMENT

2.1 Optical design considerations

For plate readers, a collinear optical configuration where a dichroic beam splitter is used to steer radiation into the sample and allowing longer wavelength emission to pass back through the filter to the detector is most common. It appears to be the logical approach, in particular for plates with 384, 1536, and even higher number of wells.

Spectral filtering is typically used to separate the fluorescence signal from unwanted scattered light (both Rayleigh and Raman) and from other background emission sources, such as optical components (dichroic, lenses, filters, beam dumps) and sample auto-fluorescence. In addition, spatial filtering can provide further signal / background discrimination. For collinear optical arrangements the confocal setup is by far the best to achieve separation between sample emission and other, spatially separable, sources of emission. The smaller the confocal volume, the better the separation. The dilemma is, that the spatial resolution collinear to the optical axes is always lower than that in the plane perpendicular to the optical axes; the resolution in the z-direction only becomes satisfactory for objectives with very high numerical aperture, when the volume is in the order of 1 μm^3 (1 femtoliter sample volume) or less.

Objectives with high numerical aperture (NA>1) are expensive and are not readily compatible with the aperture ratio of commercially available well plates. Furthermore, with sample concentrations of 1nM or less the conditions of fluctuation spectroscopy with only a limited number of molecules in the excited sample volume is reached, resulting in an unavoidable increase in measurement time per well.

The optical layout shown in figure 1 was found to be the best compromise. It has the design principles of a confocal setup, but with a much larger confocal volume than generally used in confocal microscopy. The effective sample volume ellipsoid is of 200μm diameter in the short x-y axis and has a length along the optical axis of approximately 2mm. With this set-up emission from components of the optical system is virtually eliminated and emission from sample carrier plates is kept at a minimum.

Even with the fully optimized optical system, residual Raman scattered light (predominantly water Raman) and sample auto-fluorescence can interfere FLT measurements of low fluorophore concentrations. In homogeneous assays these unwanted signals can not be spatially separated from the fluorescence signal. In cell based assays, where the local dye concentration is high, a reduction of the confocal volume may be beneficial.

Figure 2 shows typical measurements of a 4ns and a 14ns dye, the dyes being at a concentration of 1nM. The figures demonstrate the data quality that can be achieved due to the TCSPC technique, but they also show the presence of temporally dependent background originating from the material of the bottom of the well plate. It should be noted that the background curves in figure 2 are graphically enhanced, due to the demonstration in semi-logarithmic scale that is common for presentation of TCSPC data.

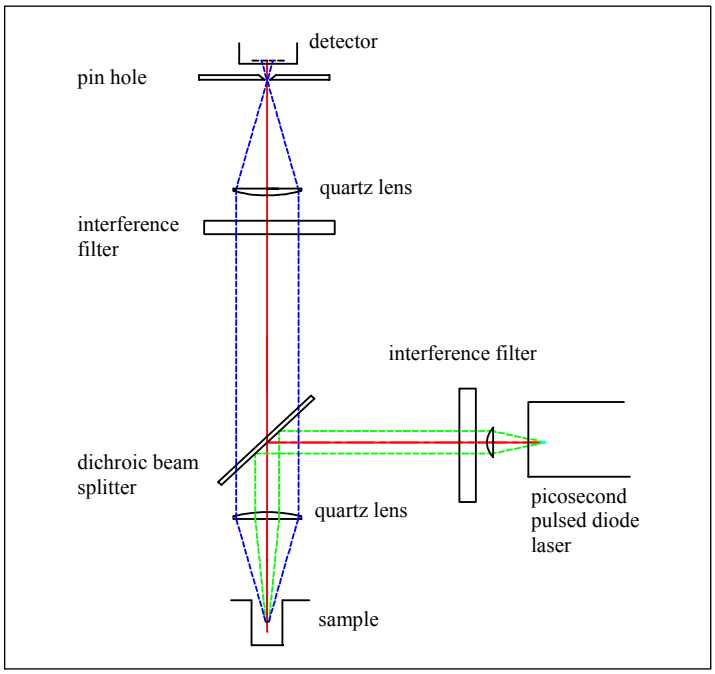

Fig. 1. Principle of the plate reader optics. Background minimization is obtained by using the confocal principle together with reduction in the number of optical elements, use of quartz optics, minimum material thickness and special selection of coating materials.

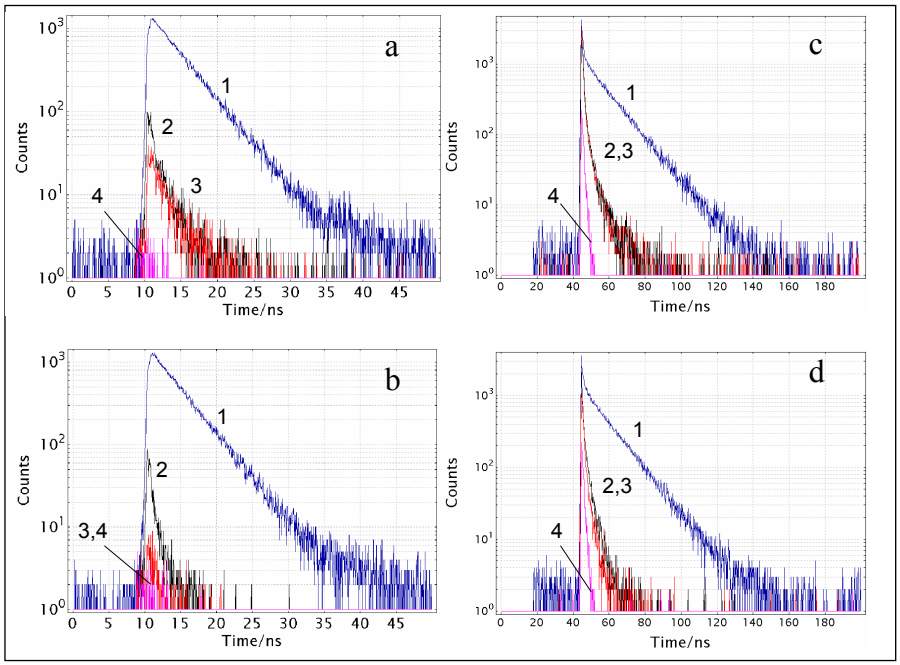

Fig. 2. a) and b): 1 nM fluorescein in PBS (1), PBS only (2), empty well (3), and no well plate (4), all excited at 473nm, detected at 520nm, laser attenuated. a) black Greiner plate, b) clear NUNC glass bottom plate.
c) and d): 1 nM PT14 (Assaymetrics) in PBS (1) , PBS only (2), empty well (3), and no well plate (4), all excited at 405nm, detected at 450nm, laser not attenuated. All data collected with 1s integration time.

Further reduction of background interference can be achieved by selection of specific dye properties, i.e. a) use of long wavelength fluorescing dyes with high extinction at laser wavelengths between 375nm and 475nm and b) use of fluorescent labels that have a fluorescence lifetime longer than 10ns.

2.2 TCSPC specific considerations

TCSPC has the well known advantages of being a highly repetitive technique while detecting signals at the quantum limit. The noise statistics are Poissonian, i.e. the standard deviation of each individual data point is the square root of the value of the data point. This means that small signals (data at the tail of a decay) contain – relatively to Gaussian distributed data – more information. (This is the reason why TCSPC data are typically shown in a semi-logarithmic scale and why TCSPC data allow more precise lifetime recovery and higher exponential fits than alternative techniques.) The beginning of the sample decay process, also, contains better information than alternative techniques, as the instrumental response function with TCSPC is narrower than in those of alternative techniques [11].

To fully exploit these advantages of TCSPC the plate reader uses picosecond diode lasers for sample excitation and photomultiplier detectors with low transit time spread for signal detection. The speed of acquisition is dictated by the pulse repetition rate, and the known phenomenon of pulse pile up. The following limitations apply:

1. The highest pulse repetition rate that can be used is set by the lifetime of the fluorophore. The time window that is required to fully cover the decay (see figure 2) is approximately 10 times the fluorescence lifetime of the dye. The maximum pulse repetition rate will therefore be between 5MHz (20ns dye in 200ns window) to 20MHz (5ns dye in 50ns window).

2. The highest rate of signal photon pulses that is allowed for recovering fluorescence lifetimes with better than 1% precision is 3% of the laser pulse repetition rate (figure 3). The maximum signal rate will therefore be between 150 000cps (20ns dye in 200ns window) to 600 000cps (5ns dye in 50ns window).

A decay curve histogram with a total number of 100 000 single photon counts provides sufficient resolution in both x-axes (500 time bins) and y-axes (>1000 counts at the peak) for high precision data analysis. This results in data acquisition times of ca 0.7s per well (20ns dye) to 0.2s (5ns dye).

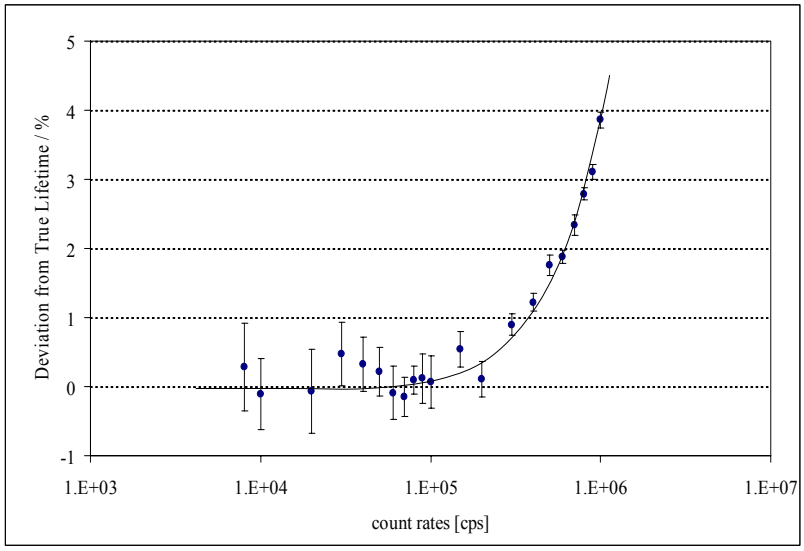

Fig. 3. Results of fluorescence lifetime measurements of a 13.7ns dye, measured at different signal count rates, with the fixed laser repetition rate of 10MHz. Data Acquisition time = 1s per well, 8 wells to calculate the standard deviation (error bars). At about 300 000 cps the fluorescence lifetime is 1% skewed towards shorter values due to the pulse pile-up.

2.3 Instrument control

User friendliness and ease of use are crucial for plate readers. The Nanotaurus plate reader comes as a compact, computer controlled module which can present lifetime parameters in full research quality or as pseudo colour lifetime dependent array pictures.

The instrument is fitted with up to two picosecond diode lasers of different wavelength (375nm, 405nm, 445nm, 475nm and 635nm are available) and has either a single photon counting detector that is sensitive to 670nm or to 850nm. The laser radiation can be optically attenuated by 4 orders of magnitude. Attenuation level, laser and dichroic selection, filter turret with up to 8 filters, laser pulse repetition frequency and focal height of the objective lens are all controlled via software.

Various modes of operation are designed for different types of assays, with or without background measurements, and with or without separate measurements of products and substrates.

2.4 Data analysis

The software offers nine different data analysis routines, some universal exponential fits, some specifically tailored for assay development (table 1). The four different methods of the routines 6-9 (table 1) use different ways to numerically treat the background, and they are different in respect to the number of exponentials of the substrate or product functions.

Fluorescence lifetime data are accurately fitted using a modified Marquardt-Levenberg algorithm that is 100% convergent.

The robustness of an assay is directly related to the repeatability of measured lifetime data. In contrast, the absolute accuracy of recovered lifetime data is of secondary importance. Considerable effort was put into obtaining correct readouts for those parameters that determine the robustness. The Poissonian statistic of the data allows representative errors to be calculated, even if there is only one data point (one well measurement) available. All recovered lifetime parameters and their errors have undergone scrutiny tests against standard deviations obtained by repeated single well measurements and measurements of multiple wells and plates.

		Background	Substrate	Product
1.	Force Fit Single Exponential			
2.	Force Fit Single Exponential with Background Emission	X		
3.	Force Fit Double Exponential			
4.	Force Fit Double Exponential with Background Emission	X		
5.	Fluorescent Marker with Optional Correction of Background	(X)		
6.	Conversion Efficiency from Substrate to Products – Method 1	(X)	(X)	(X)
7.	Conversion Efficiency from Substrate to Products – Method 2	(X)	(X)	(X)
8.	Conversion Efficiency from Substrate to Products – Method 3	(X)	(X)	(X)
9.	Conversion Efficiency from Substrate to Products – Method 4	(X)	(X)	(X)

Tab. 1. Analysis Routines. The last three columns indicate whether or not the assay requires additional reference measurements: X – measurement required, (X) – measurement may not be required, depending on whether or not specific fit parameters have been fixed.

It is not necessary to perform full numerical reconvolution, involving the sample decay and the instrumental response function, to obtain high repeatability and hence high assay robustness. The use of a simple tail fit analysis directly improves the ease of use and the speed of lifetime parameter recovery. Figure 4 shows that even without reconvolution short lifetimes can be recovered with high repeatability (relative error); but they are systematically calculated too large. If the knowledge of the accurate (absolute) lifetime values is required, they can be obtained by using an advanced data analysis package that contains reconvolution.

If a fluorescence background measurement shows non-zero background, it will be subtracted from the sample measurement with a freely floating scaling factor in the fitting procedure. This algorithm avoids fitting of the background with a complex model function [6]. This simple and robust method is possible, because only one (temporally dependent) background is observed. The instrument never shows a constant (temporally independent) background.

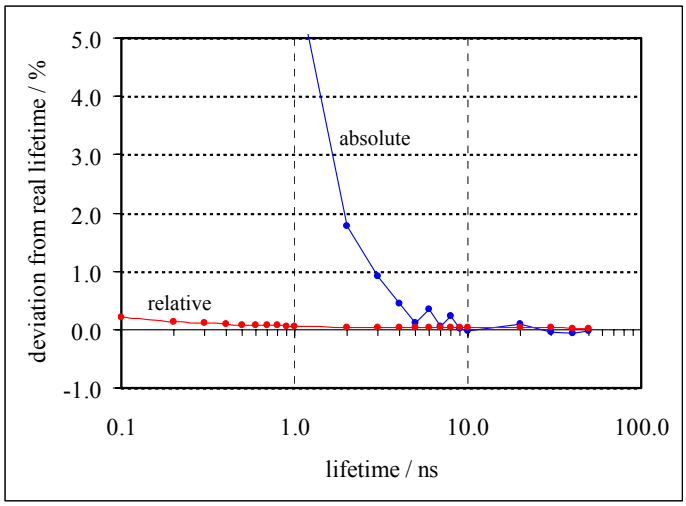

Fig. 4. Relative and absolute errors of fluorescence lifetimes obtained with tail fit analysis. Short lifetimes are systematically skewed, but measured with high repeatability. The graphs represent fit result from simulated data curves.

Data analysis is performed automatically in the process of a well plate measurement. The lifetime parameters of each sample well are numerically recovered directly after the data acquisition. The time for analyzing the data is minimal and has practically no influence on the overall plate acquisition time.

3 VALIDATION

3.1 Precision of fluorescence lifetimes

The advantage of fluorescence lifetime measurements in data precision is demonstrated on the example of fluorescein in PBS. Figure 5a shows that a lifetime of 4ns is accurately measured over the full concentration range to within 0.5%. A small systematic deviation towards shorter lifetimes is observed at concentrations less than 1nM where background emission becomes more important.

Figure 5b shows the repeatability of the lifetime measurements, expressed in the coefficient of variances. The figure clearly shows that the lifetime is measured with higher repeatability (<0.4%) than other parameters calculated from the single exponential decay. It can also be seen that fluorescence intensity and fluorescence amplitude related parameters have essentially the same larger error. Tests have also shown (not demonstrated here) that with prolonging the integration time the lifetime-CV decreases with the square root of the integration time, whereas the intensity error remains stable at around 1%. This is most likely the pipetting error that can not be improved with longer integration times of the fluorescence intensity. In contrast, FLT measurements are concentration and volume independent and therefore assays based on the fluorescence lifetime have the potential to be made more robust by longer signal integration.

The advantage of fluorescence lifetime assays over intensity assays (including TRF using gated detection) will be far more significant in inhomogeneous assays (e.g. cell based assays), where local dye concentrations vary dramatically. An example is given in section 4.2.

Even in those cases where the fluorescence decay can not be accurately described with a single exponential decay model, it is still advantageous to "force fit" the decay with a single exponential function. The decay constant obtained with those fits is representative for an average lifetime that will change depending on the conditions of the dye label within the assay. An example for this type of analysis is given in section 4.3.

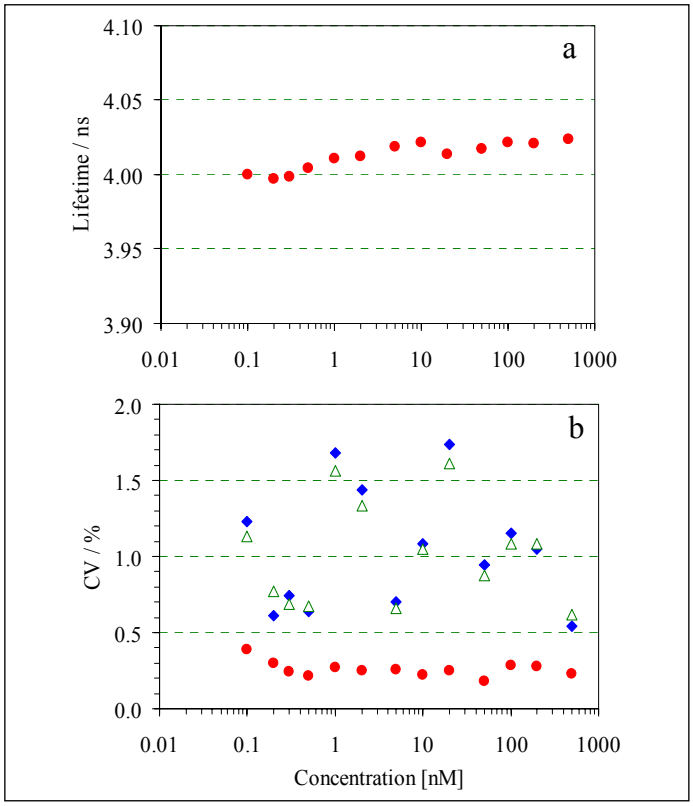

Fig. 5. Fluorescein in PBS pH7.2 at concentrations between 100pM and 500nM, measurement time per well = 2s, 16 wells were measured and analyzed. a) average of fluorescence lifetimes b) relative errors of fluorescence lifetime (●), fluorescence intensities (Δ), and pre-exponential factors (◆)

3.2 Precision of relative lifetime amplitudes – lifetime discriminated measurements

The previous paragraph showed that in multi-well applications fluorescence lifetimes can be more precisely measured than fluorescence intensities or the related amplitude of the decay. The following validation experiment demonstrates the strength of using the *amplitude ratios* of multi-exponential decays for accurate recovery of fractions of a mixture of fluorophores. Similarly to the fluorescence lifetimes, amplitude ratios are also intensity independent.

The spectra of two fluorophores used in this dye mixture experiment and the spectrum of the solvent, a cell culture medium that shows high auto-fluorescence, are shown in figure 6. The spectral overlap of the three components of the solution is significant. Separating these components by a single intensity measurement would be impossible, and even a two-color ratioing technique would have limited success.

Figure 7 shows FLT measurements of the two different dyes in the cell culture medium alone, and one example of a mixture of the two dyes. It can be seen that the background created by the cell culture medium is significant. Nevertheless, the fractional components of the two fluorophores can be accurately retrieved by a simple two step measurement (sample and background), due to the high quality of the lifetime data and the power of the numerical analysis. The lifetimes were 8.7ns and 2.9ns for TG404 and Cascade Yellow, respectively. The background would need to be fitted multi-exponential. It has an average lifetime of 3.9ns.

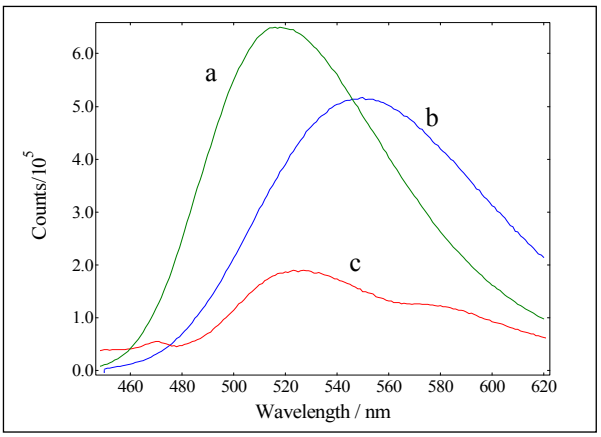

Fig. 6. Emission spectra of the two dyes TG404, SETA Biomedicals (a); Cascade Yellow, Molecular Probes (b); and the cell culture medium DMEM F12, Cambrex (c).

The measured dye concentration ratios are in good agreement to the calculated values (figure 8). There is a small recognizable systematic deviation from theoretical values at small Cascade Yellow concentrations. This is not due to limited recovery capability caused by the fluorescence background, it is due to the fact that the initial, absolute, amplitude of both dyes has to be determined once (through a separate measurements). The initial amplitudes are affected by the extinction coefficient of the individual dyes at the excitation wavelength and their fluorescence quantum yields.

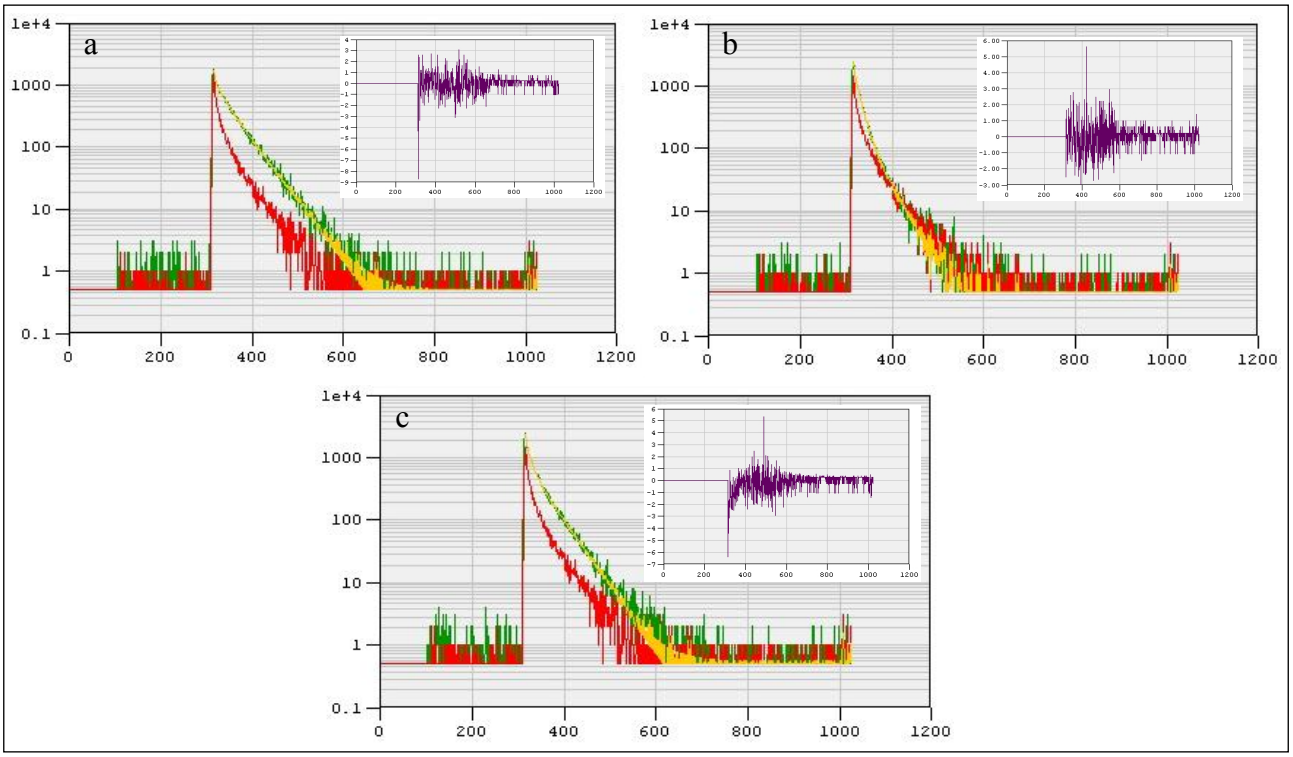

Fig. 7. Examples of lifetime measurements: a) TG404 in DMEM F12; b) Cascade Yellow in DMEM F12; c) 1:1 mixture of TG404 and Cascade Yellow. Upper dark curves represent sample decay measurements; lower curves show background measurements (DMEM F12 emission); light curves superimposing sample decay measurements are fitted decay curves; inserts: residuals. The emission filter used for the experiment was centered at 520nm with a band pass of 40nm. The x-axes are shown in channels, y-axes in counts.

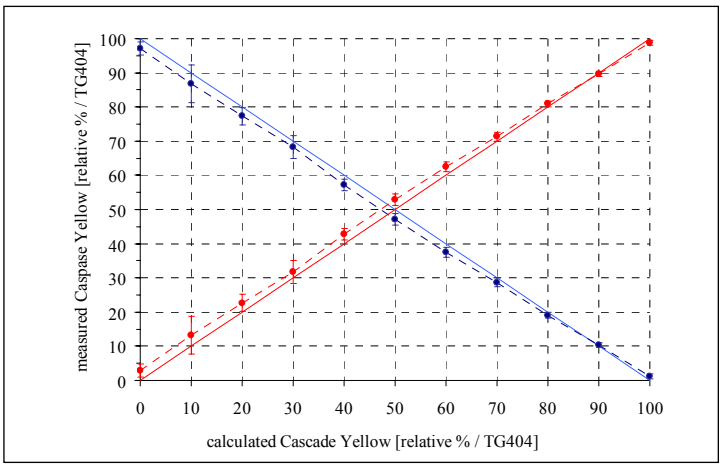

Fig. 8. Results of the dye mixture experiment of TG404 and Cascade Yellow. The dotted lines combine the measured data points, whereas the drawn lines symbolize the theoretical concentration ratios.

Many assays can be reduced to the analysis of a double exponential decay model and the extraction of the ratio of pre-exponential factors and fluorescence lifetimes. This includes FRET based assays (an application is shown in section 4.2) and fluorescence anisotropy measurements.

3.3 Anisotropy parameters – recovered by a single FLT measurement

This paragraph demonstrates the capability of the Nanotaurus plate reader to perform polarization measurements and to accurately recover fluorescence anisotropy parameters.

Fluorescence anisotropy reveals information on the rotational diffusion dynamics of fluorophores. In many cases the molecular rotation of fluorophores is in the time scale of their excited state lifetime, and single fluorescence photons will be emitted while the molecules randomly rotate from one polarization plane into the other.

In FI and TRF plate readers the steady state fluorescence anisotropy may be obtained by measuring two linearly polarized fluorescence intensities, I_{VV} and I_{VH}, and by use of a correction factor G that takes account the efficiency of the optical system in the two different polarization planes. This steady state anisotropy is a time integrated value that contains information about the average speed of rotation of the excited molecules and the orientation of initial dipoles, but it is also affected by the fluorescence lifetime of the rotating molecules [13].

By measuring the time courses of the linearly polarized fluorescence components, $I_{VV}(t)$ and $I_{VH}(t)$, instead of the of the integrated fluorescence intensities I_{VV} and I_{VH}, the two processes rotational diffusion and fluorescence (energy) relaxation can be clearly separated and the dynamic information of the fluorescence anisotropy, r(t), becomes available. Furthermore, it can be shown, that in many cases only one single polarized measurement is sufficient to retrieve the parameters of the fluorophore's rotational diffusion. Using only one of the two polarized decays, $I_{VV}(t)$ or $I_{VH}(t)$, not only has the advantage of saving the time for a second measurement, it also makes G-factor correction, that is unavoidable when two polarization planes are measured, obsolete.

For example, for a spherical fluorophore the rotational diffusion is

$$r(t) = r_0 \exp\left\{-\frac{t}{\varphi}\right\}, \tag{1}$$

with r_0 the initial anisotropy and ϕ the rotational diffusion time constant. If the fluorescence intensity decays single exponentially,

$$I(t) = I_0 \exp\left\{-\frac{t}{\tau}\right\}, \tag{2}$$

the two linearly polarized components decay with [13]:

$$I_{VV}(t) = I_0 \exp\left\{-\frac{t}{\tau}\right\} + 2\, I_0\, r_0 \exp\left\{-\, t\, \frac{\varphi + \tau}{\varphi\, \tau}\right\} \quad (3)$$

$$I_{VH}(t) = I_0 \exp\left\{-\frac{t}{\tau}\right\} - I_0\, r_0 \exp\left\{-\, t\, \frac{\varphi + \tau}{\varphi\, \tau}\right\}. \quad (4)$$

If, for example, only the parallel fluorescence component, $I_{VV}(t)$, is recorded, the whole task of obtaining reliable anisotropy parameters reduces to an accurate measurement of pre-exponential factors and lifetimes in a double exponential decay model. The ratio of the two pre-exponential factors provides the initial anisotropy r_0, and the rotational diffusion time, ϕ, can be calculated from the two exponential time constants.

Rotational diffusion processes can be more complex. However, the principle of using only one polarized time resolved fluorescence measurement is still applicable, in particular if other information of the system is already available, like initial and "infinity" anisotropies, fluorescence lifetimes, etc.

Figure 9 shows time resolved fluorescence anisotropy curves of mixtures of glycerol and PBS for the dye PT14 at a temperature of 15°C. These example anisotropy time courses, obtained using the Nanotaurus plate reader, where generated by the conventional way, i.e. by recording and processing $I_{VV}(t)$, $I_{VH}(t)$ and G. Figure 10 shows the results obtained for the same sample measurements, but processed using only the decay model function $I_{VV}(t)$, Eqn. 3. The results show that, by using a single polarized FLT measurement, anisotropy parameters can be recovered with high repeatability, paving the way for robust fluorescence anisotropy assays.

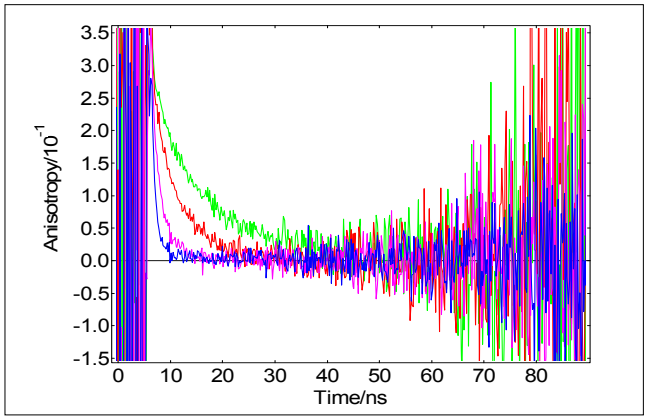

Fig. 9. Fluorescence anisotropy time courses of PT14 (1µM) in glycerol/PBS mixtures. Four example curves are shown for mixtures of 20/80 (lower curve), 40/60, 60/40, and 80/20 (upper curve). Measurements were made at 15°C.

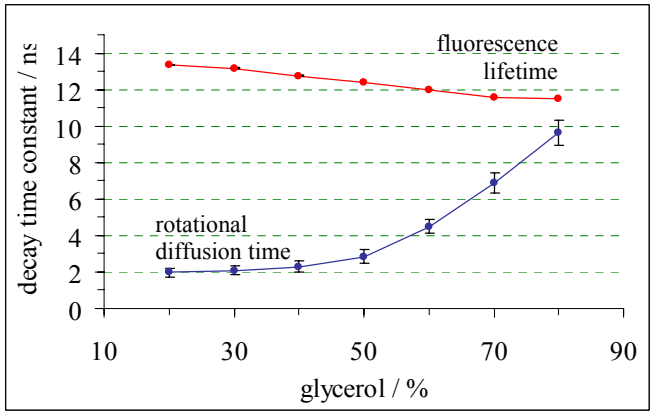

Fig. 10. Fluorescence anisotropy parameters recovered with only a single polarized FLT measurement for samples with different glycerol/PBS mixtures. By changing the solvent viscosity the rotational diffusion time changes significantly. A small but significant change is also observed for the fluorescence lifetime. Error bars represent the repeatability calculated from 4 independent measurements.

4 ASSAY APPLICATIONS

4.1 Caspase assay

A caspase-3 assay has been developed using the fluorescence lifetime technique. The assay uses a substrate that contains tryptophan. Tryptophan is known to shorten the lifetime of certain fluorophores. The presence of tryptophan in an aminoacid sequence shortens the lifetime of the covalently linked dye from 13.3ns to 8.0ns. The mechanism behind the quenching is believed to be electron transfer.

Figure 11 shows the change in the average fluorescence lifetime. The cleavage of the substrate peptide by the enzyme separates the fluorophore from the tryptophan, causing the increase in the fluorophore's lifetime.

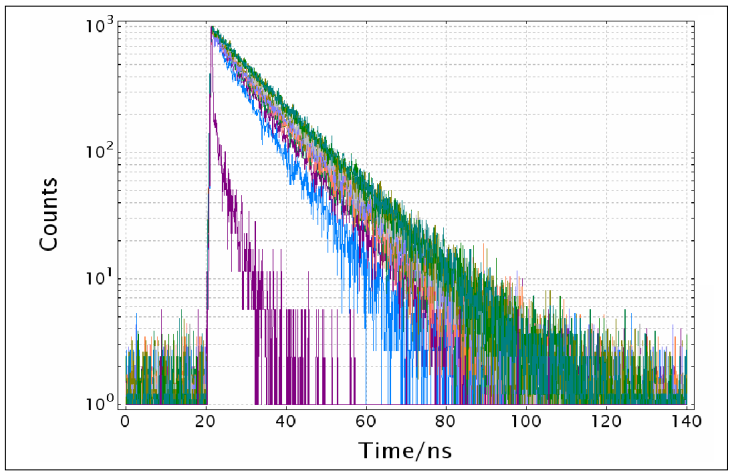

Fig. 11. FLT measurements of the caspase-3 assay for incubation times between zero and 180min. Curves are normalized to 1000 counts at the peak, artificially enhancing the background measurement. All fluorophore decays are precisely fitted with a single exponential decay model.

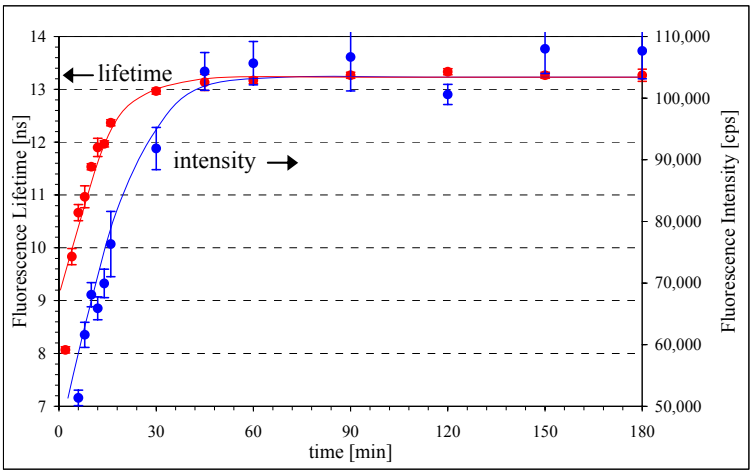

Fig. 12. Average fluorescence lifetime (τ) and total fluorescence intensity (T) of the caspase substrate in dependence of the time after beginning of the incubation of the caspase-3 enzyme. The no-enzyme control (not shown here) has a constant fluorescence lifetime of 7.5ns. Error bars obtained from 4 independent well measurements.

Figure 12 shows the course of the fluorescence lifetime and fluorescence intensity changes. The slopes of both parameters differ, indicating the cleavage may involve not only dynamic, but also static quenching processes. The differences between the two parameters lifetime and intensity have been found to be even more dramatic when other fluorescent probes were used. The change in the fluorescence lifetime is directly linked to dynamic quenching and therefore is the true representation of the cleavage process.

This example of a caspase-3 assay shows that the FLT technique is applicable for cleavage experiment, even if there is only one fluorophore present in the assay. The presence of other molecules, such as certain natural amino acids, can change the lifetime of the fluorophore. This may not only be used for cleavage, but also for conformational studies.

4.2 Protein-protein interaction and protein conformation assay

A cell based FRET assay that involves the dye pair Alexa Fluor 488 and Cy3 has been tested using the fluorescence lifetime technique. The dyes were coupled to antibodies (anti-tubilin and anti-IgG) used for immunohistochemistry in formalin fixed cells that were grown on the bottom of the well plate.

In cell based assays, local concentrations vary significantly. This enhances errors in intensity readings (fluorescence intensity and time gated readings) dramatically, practically yielding them ad absurdum. The measurement of the example presented here showed errors of the intensity readings of more than 40%, whereas intensity independent lifetime parameters gave consistently less than 5% error. (figure 14).

By systematic de-focusing, fluorescence of a larger assembly of cells fixed to the well bottom is collected and a bigger averaging effect is achieved. This slightly improves the statistics of the intensity readings, but they still do not come close to the precision the lifetime parameters offer.

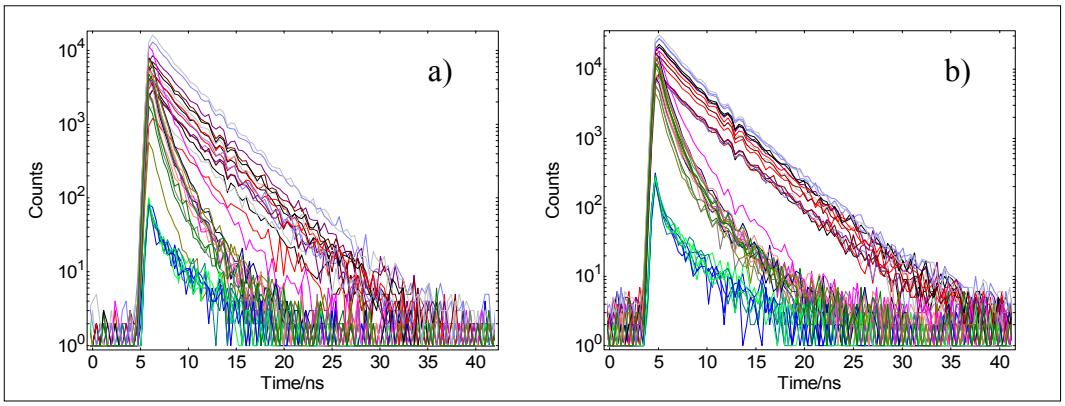

Fig. 13. Focused (a) and defocused by 3mm (b) measurement of a cell based FRET assay. Excitation 475nm, emission 520nm (Alexa Fluor 488), measurement time per well = 1s. Each plot contains 16 curves of cells without FRET action (upper curves), 16 curves where FRET shortens the lifetime of Alexa Fluor 488 (centre curves), and 16 background measurements. The curves showing FRET were analyzed using a double exponential fit with background elimination.

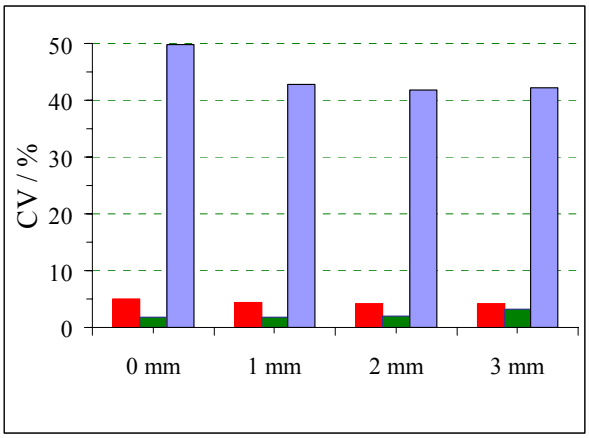

Fig. 14. Coefficient of variances for the ratio τ_1/τ_2 (average = 0.25) – left bars, for the relative pre-exponential factors $B_1/(B_1+B_2)$ (average = 93%) – middle bars, and for the total intensities – right bars, in dependence of the focal point: 0mm=focus on the cells, 1, 2, 3mm= focus above the cells.

In FRET based studies, FLT measurements not only produce better data repeatability, they also contain more information than available to FI and TRF measurements. Analyzed by double exponential decay model, the ratio of the short and the long lifetime contains information of the FRET efficiency (i.e. the average distance between donor and acceptor molecules), while the ratio of the pre-exponential factors gives information on the FRET strength (i.e. the ratio between donor molecules that are involved in FRET and those that do not participate) [6].

4.3 Kinase assay

A protein kinase-B (PKB) assay has been developed for use with the fluorescence lifetime technique. The peptide substrates are labeled with a fluorescent dye. The phosphorylation of the substrate changes the local polarity in the vicinity of the phosphor and makes it susceptible to iron and chromium chelates. The proximity of the chelate quenches the average fluorescence lifetime of the fluorescent label (figures 15, 16).

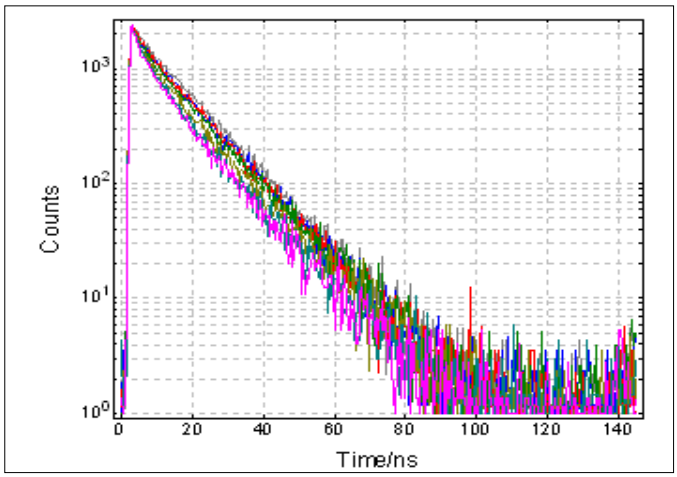

Fig. 15. Fluorescence lifetime measurements on the PKB substrate at subsequent times (0, 2, 5, 10, 20, 30, 40 mins after reaction start). Decay curves are multi-exponential. The average fluorescence lifetime systematically reduces from 12.5ns to 10.2ns.

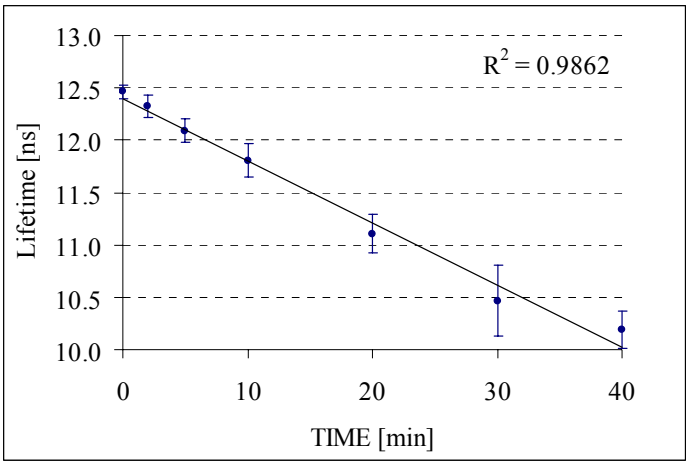

Fig. 16. Measured average lifetimes, errors calculated from four independent measurements, linear regression.

The fluorescence lifetime PKB assay demonstrated here has been directly compared to the radiometric counterpart with very encouraging results. Further optimization is underway to replace the radiometric method by the robust, safer, and more cost effective FLT technique.

5 CONCLUSIONS

The Nanotaurus plate reader from Edinburgh Instruments is a new FLT plate reader that is based on the data acquisition technique of time correlated single photon counting. The plate reader has fully optimized optics that have been specifically designed to minimize temporally dependent background for both homogeneous and inhomogeneous assays.

We have demonstrated that the fluorescence lifetime technology in general, and the technique of time correlated single photon counting in particular, has distinct advantages over fluorescence intensity and time gated fluorescence intensity measurements. The advantages are the use of organic fluorophores as probes (rather then lanthanide chelates), independence of sample concentrations and sample volume and therefore higher multi-well repeatability, higher information content, and higher robustness due to effective background elimination. These unique advantages, utilized in a commercial plate reader, improve assay robustness and reduce consumable cost.

We have demonstrated the advantage of this higher information content using example of both a FRET based assay and with fluorescence anisotropy validation data. In FRET studies, FRET efficiency and FRET strength can be separated by means of the FLT measurements. In fluorescence anisotropy studies, dynamic information of the rotational diffusion can clearly be separated from the energy relaxation of the fluorophore.

We have demonstrated that information on rotational diffusion can be obtained from a single polarized time resolved fluorescence measurement only. This reduces technical complexity, saves measurement time, is intensity independent and does not require G-factor correction. This is a clear advantage over all time integrating techniques.

It is our belief that a commercial FLT plate reader must use the TCSPC technique for data acquisition in order to fully exploit the capability the FLT technology can offer. Only with the uncompromised data precision of TCSPC reliable measurements down to picomolar concentrations are possible, background elimination can be performed with high accuracy, and the full information content intrinsic to FLT measurements can be recovered.

ACKNOWLEDGEMENTS

We wish to thank Dr. Brian Bacskai (Massachusetts General Hospital) and Dr. Alexander Gray (School of Life Sciences, University of Dundee) for their support in supplying data and information and for their work in assay development using the FLT technology. We also wish to thank Dr. Dmitry Gakamsky and Dr. Richard Dennis (Edinburgh Instruments) for helpful discussions.

REFERENCES

1. R. P Hertzberg and A. J Pope, *High-throughput screening: new technology for the 21st century,* Current Opinion in Chemical Biology, 4, 445–451, 2000
2. E. Trinquet, G. Mathis, *Fluorescence technologies for the investigation of chemical libraries*, Mol. BioSyst., 2, 380-387, 2006
3. J. Comley, *New HTS technologies serve pharmaceutical sector*, SP2, June 2004.
4. S. Turconi, R. P. Bingham, U. Haupts and A. J. Pope, *Developments in fluorescence lifetime-based analysis for ultra-HTS*, *DDT* Vol. 6, No. 12 (HTS Suppl.) 2001.
5. J. Owicky, *Fluorescence polarisation and anisotropy in high throughput screening; perspectives and primer*, J. Biomol Screening, 4, 67-73, 2000
6. P.B. Jones, L. Herl, O. Berezovska, A.T.N. Kumar, B. J. Bacskai, B. T. Hyman, *A time domain fluorescent plate reader for cell based protein-protein interaction and protein conformation assays,* in press
7. I. Hemmila, *LANCE; homogeneouse assay platform for HTS*, J. Biomol. Screening, 4, 303-307, 1999
8. G. Mathis, *HTRF Tecnology* , J. Biomol. Screening, 4, 309-313, 1999
9. J. Comley, *TR-FRET Based Assays – getting better with age*, Drug Discovery World Spring 2006.
10. J. Comley, *ASSAY Interference a limiting factor in HTS?*, Drug Discovery World Summer 2003.
11. D.V. O'Connor, D. Phillips, *Time Correlated Single Photon Counting,* Academic Press, London, 1984
12. W. Becker, *Advanced Time Correlated Single Photon Counting Techniques*, Springer, Heidelberg, 2005
13. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Second Edition, Plenum Publishers, New York, 1999