

# Fluorescence Lifetime and Nano-TRF Assay Technology for HTS

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## Introduction

In recent years, novel fluorescence assay technologies using the detection of nanosecond fluorescence lifetimes (FLT) and nanosecond time-resolved fluorescence signals (Nano-TRF) emerged. The aim is to overcome limitations of existing assay formats concerning false positive and false negative results caused by stray light due to sample turbidity, the refractive index jump at the boundary between air and the liquid, or sample autofluorescence. Assays based on the measurement of fluorescence intensity (FI) largely suffer from these effects.

Assays based on the direct FLT measurement enable the observation of the fluorophore molecules during their interaction with the micro-environment. Binding to other molecules, quenching, solvent polarity or pH may alter the measured lifetime values. Since the fluorescence lifetimes usually are on the nanosecond time scale advanced instrumentation is required.

Nano-TRF assays use long-lifetime fluorescence labels in combination with a time-delayed fluorescence signal detection. The time-scale for signal integration is somewhere between 100 ns and 5 μs depending on the used fluorophore in contrast to several hundred microseconds for common TRF.

In this work we present a comparison of different numerical methods for the analysis of multiple fluorescence decay curves routinely obtained in microplate analysis and their effect on achievable z'-factors. We present examples of FLT and Nano-TRF readout.

## Experimental

All experiments have been carried out on a LF 502 NanoScan FLT microplate reader (IOM, Germany). This instrument is equipped with a Nitrogen/dye laser combination and provides different excitation wavelengths between 337 and 785 nm. It is capable of performing time-resolved measurements of fluorescence decays within an adjustable time window of between 10 ns and 20,000 ns.



The laser repetition rate is adjustable from 10 to 30 Hz. Throughout all experiments a repetition rate of 30 Hz was used.

Data analysis is performed after completion of the measurement on the instrument controlling PC.

## FLT-based assays - Description of numerical methods

Basically two different numerical methods are employed [1]:

1. a serial deconvolution of the single fluorescence decay curves well per well with independent parameters describing each experiment (each well)
2. a "global" deconvolution of all decay curves combined together in a common matrix where a logic linkage of experimental parameters is possible. As an example may serve the linkage of the analysed fluorescence lifetimes over the entire plate. This may be justified because in each well there is the same fluorophore e.g. in a mixture of two different states.

The basic formula describing the fluorescence decay is:

$$I^k(t) = \sum_{i=1}^n \alpha_i^k \cdot \exp\left(-\frac{t}{\tau_i^k}\right) \quad (1)$$

In this formula I is the fluorescence intensity as a function of time t.  $\alpha$  and  $\tau$  are amplitude and fluorescence lifetime of a specific fluorescence relaxation process and n is the number of observed lifetime components (typically 1 ... 3). The index k stands for each experiment in a microplate read-out (the well number).

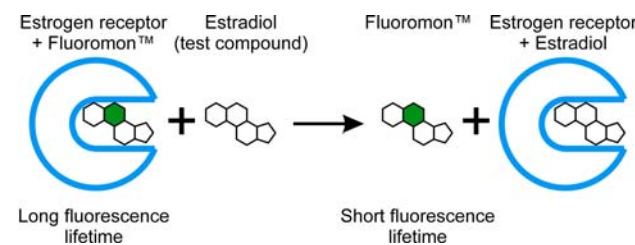
In the case that a measured decay curve optimally is described by a "mixture" of more than one fluorescence lifetime an average lifetime can be defined:

$$\bar{\tau} = \frac{\sum_{i=1}^n \alpha_i \cdot \tau_i^2}{\sum_{i=1}^n \alpha_i \cdot \tau_i} \quad (2)$$

Upon the comparison of different numerical analysis methods a single exponential and a double exponential deconvolution of each well serially is compared to a "Global" double exponential analysis where both lifetimes were linked.

## FLT-Assay example – Estrogen Receptor Assay:

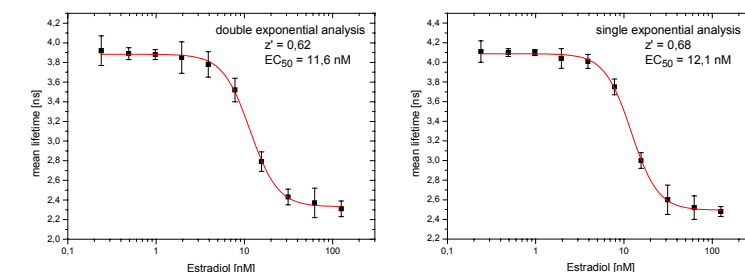
A first study was performed with a commercially available Estrogen Receptor- $\alpha$  Competitor Assay (P2698, Invitrogen, USA). Assay principle is as derived from the assay protocol:



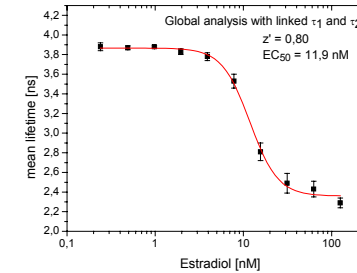
Experiments were performed in a black 384 well microplate (781076, Fluotrac 200, Greiner, Germany) with a total sample volume of 40 μl.  $\beta$ -Estradiol was obtained from Sigma (E-8875). Final concentrations of Fluoromon™ and receptor were 1 nM and 15 nM respectively.

Excitation was at 488 nm. Emission was detected at 520 nm within a time window of 50 ns. With this time window the complete decay curve was obtained. Averaging occurred over 32 laser pulses for each well.

## Data analysis



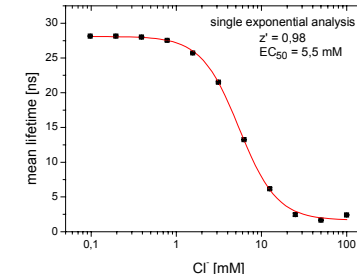
Using the same set of data from a standard curve with 4 replicates the improvement of data analysis by "Global" analysis compared to a serial curve analysis is demonstrated. Though the decay curves are best described by a double exponential fit, the single exponential fit gives a better statistics. In this respect the resolved lifetime is to be understood as a mean lifetime value approximating that of a correct double exponential fit.



## FLT-example with SPQ – Cl<sup>-</sup> sensitive dye:

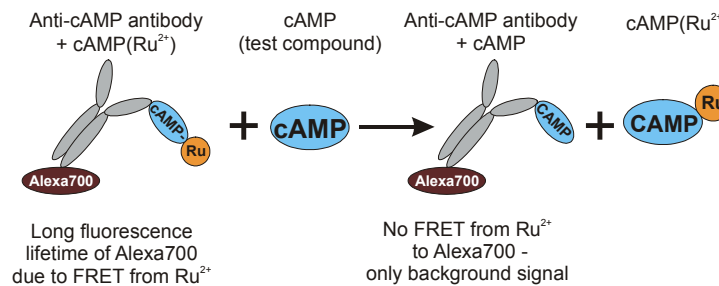
A second example for potential FLT based assay is based on the dye 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) which is sensitive to the samples Cl<sup>-</sup> concentration. Sensitivity is based on collisional quenching of the dye molecules with chloride ions. Thus, with increasing Cl<sup>-</sup> concentration as well fluorescence intensity as fluorescence lifetime will decrease.

Excitation for this experiment occurred at 365 nm and fluorescence was detected at 450 nm. The measurement of a standard curve occurred in a black 96 well microplate (655076, Fluotrac 200, Greiner, Germany) with 8 replicates. Averaging was done over 32 laser pulses.



Data analysis was performed using the single exponential fit law. Double exponential or "Global" fitting is not appropriate since the single exponential decay behavior of the unquenched dye is not altered due to collisional quenching. Only the resulting lifetime of the decay curve is varied.

## Nano-TRF assay - cAMP immunoassay

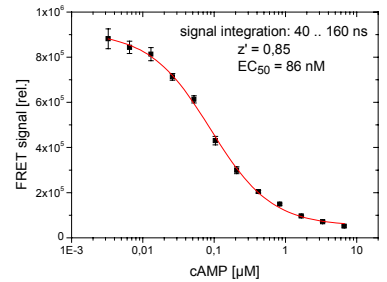


This experiment was performed with a homogeneous assay developed on the basis of a highly fluorescent Ruthenium complex [2]. This competitive assay is based on the energy transfer between Ru<sup>2+</sup>-marked cAMP and Alexa700. Free cAMP displaces Ru<sup>2+</sup>-cAMP and diminishes the rate of energy transfer.

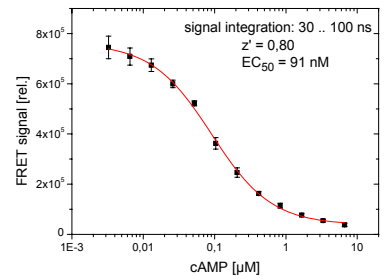
Excitation occurred at 456 nm. Fluorescence was detected simultaneously in two detection channels for the donor at 630 nm and for the acceptor at 700 nm. The measurement of the standard curve occurred in a black 384 well microplate (781076, Fluotrac 200, Greiner, Germany) with a final sample volume of 40 μl and 4 replicates. Averaging was done over 32 laser pulses.

Like for common TRF assays the FRET signal is to be detected in a time-gate after the pulsed fluorescence excitation.

Two different integration windows are compared:  
- 40 to 160 ns and  
- 30 to 100 ns  
after the laser pulse.



As indicated in the figures to the right this minor change in the integration range leads to altered z'-factor and EC<sub>50</sub> values.



Thus optimization of the measurement settings has to be done carefully. This optimization procedure may be more important for Nano-TRF assays than for TRF assays.

## Conclusion

FLT-based assays as well as Nano-TRF assays have the potential to serve as robust and reliable assay formats for routine application in HTS. A high importance for the evaluation of FLT-based assays has to be ascribed to the data analysis. The reduction of the number of free parameters by using "Global" curve analysis procedures has proven to increase the statistical security of revealed lifetime values.

For Nano-TRF assays the precise adjustment of the integration range according to the actual lifetimes of the fluorescence markers is important.

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## References

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