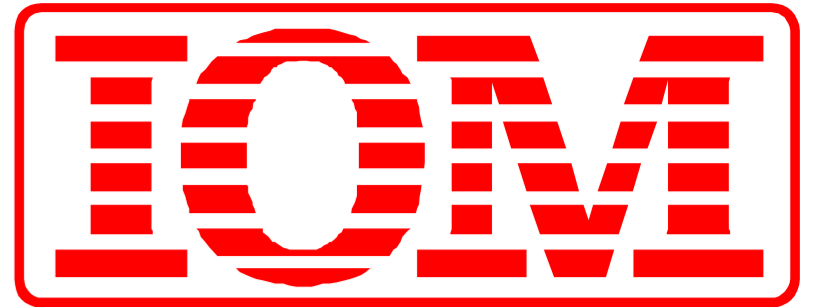


# Duplex HTS assay measured simultaneously with Nano-TRF and fluorescence lifetime method



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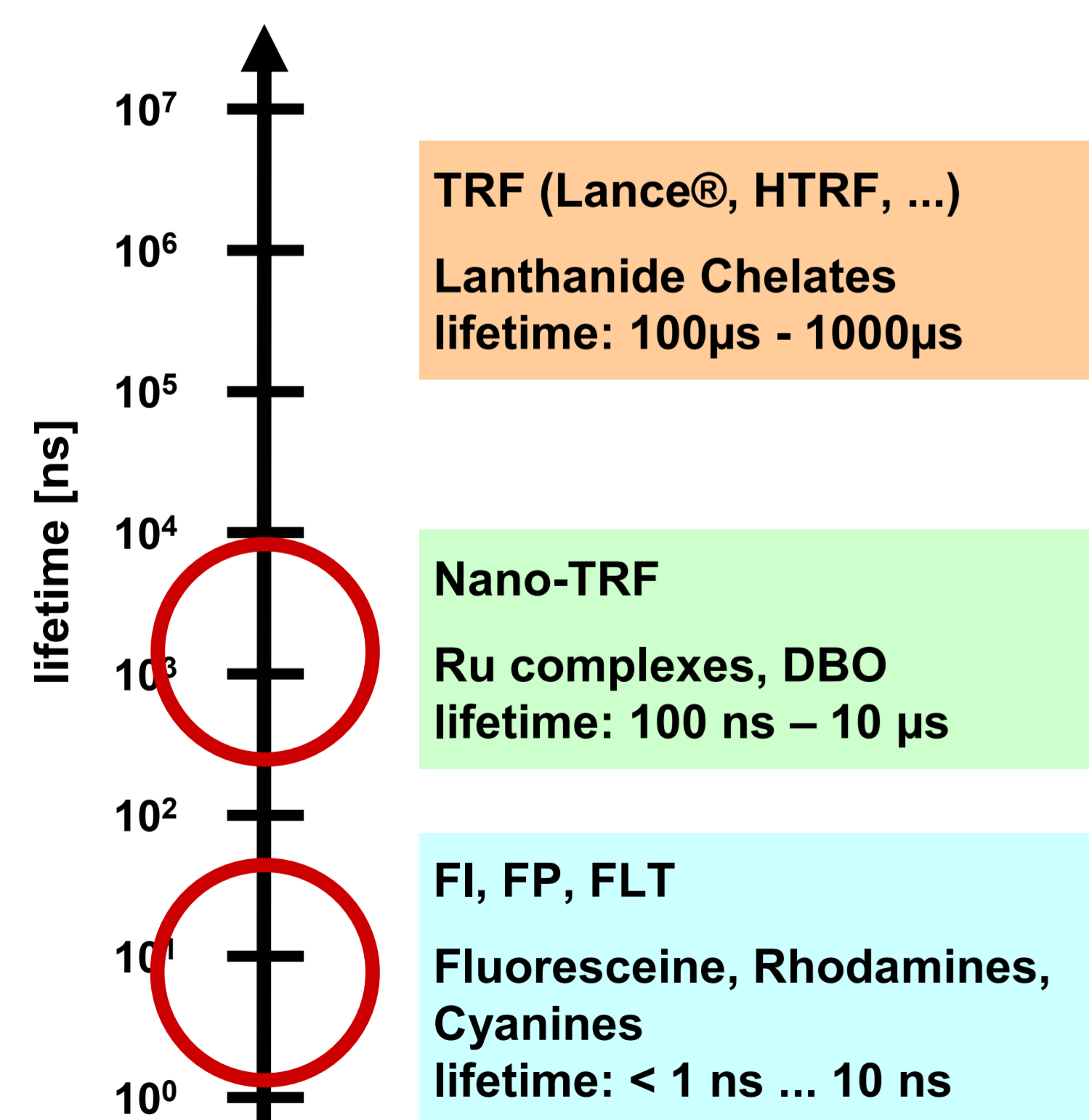
## Introduction

Multiparameter high-throughput screening (HTS) assays are interesting not only for economizing time and cost, but also to better illustrate the highly complex biochemical signal chains in cells and organisms that often influence each other.

The current state-of-the-art in HTS assays detects single analytes. Specifically this is the case for modern GPCR assays when detecting cAMP, Ca<sup>2+</sup> or IP1. In Nuclear receptor assays the activities of single receptor types like Estrogen, Androgen, or Cortisol receptors are analyzed. With other words, in today's HTS campaigns hundreds of thousands of compounds are screened for a potential drug activity but the complexity of the signal pathways is lost largely. As a consequence one result of big HTS campaigns is a high number of false positives, resulting in high costs for the subsequent confirmation and optimization steps. [1], [2]

In this presentation, we report preliminary experiments for establishing a novel duplex assay format exploiting different fluorescence lifetimes of labels with overlapping fluorescence spectra. We demonstrate the possibility of a nearly complete signal separation by means of time-resolved and lifetime resolved signal detection using the microplate reader LF502 NanoScan FLT.

## Duplex assay method

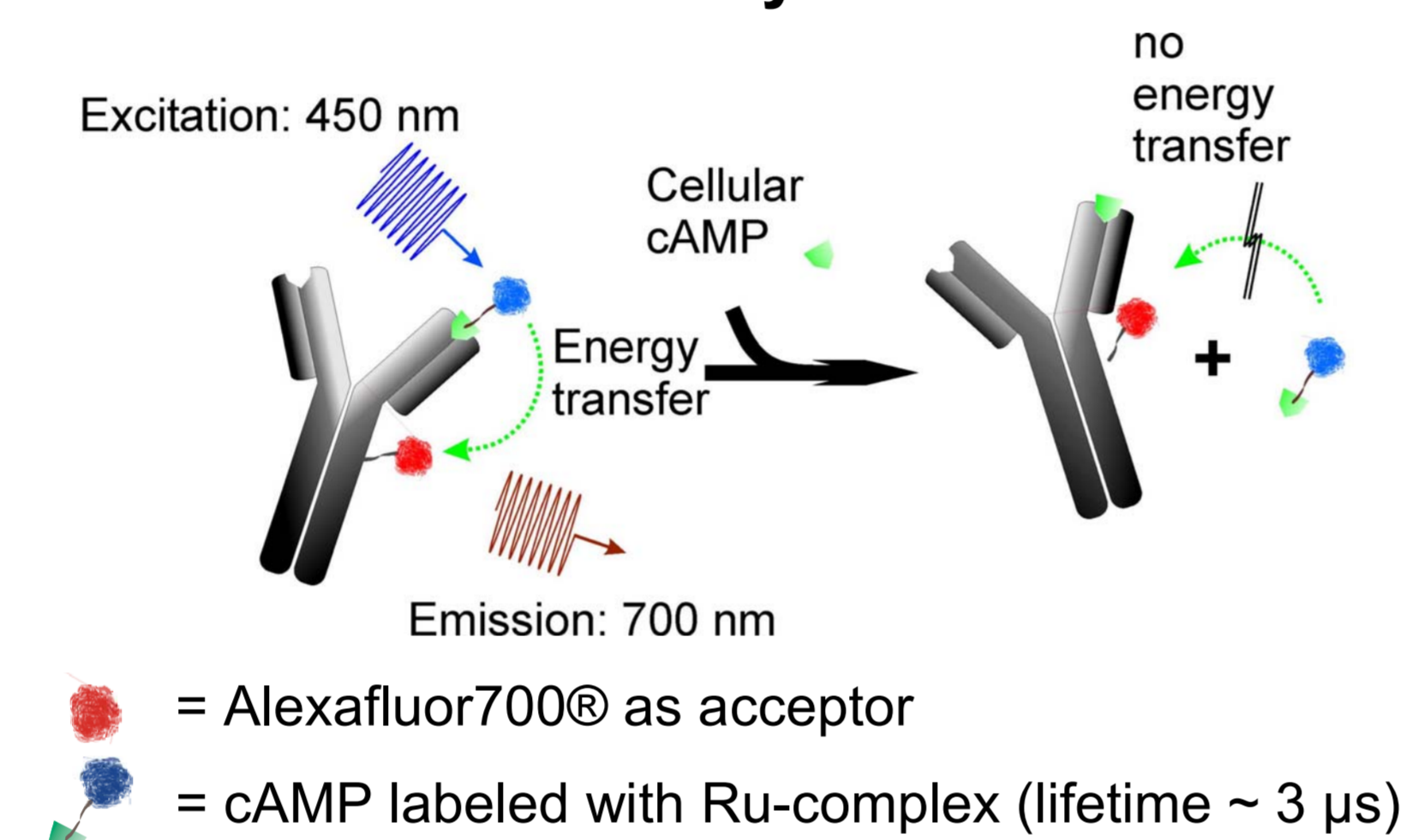


Our model is to use two labels with different fluorescence lifetimes measured simultaneously, rather than using fluorescent labels with differing fluorescence spectra measured repeatedly at different wavelengths.

The fluorescence lifetime scale spans approximately 7 decades ranging from about 1 ns to 10 ms. Fluorescence technologies involving organic dyes, which generally have shorter lifetimes, use detection methods such as fluorescence intensity and fluorescence polarization. Fluorophores with longer lifetimes, such as Lanthanide Chelates and Cryptates (Eu<sup>3+</sup>, Sm<sup>3+</sup>, Tb<sup>3+</sup>), are analyzed using assay formats such as HTRF or Lance@ (Cis Biointernational, France/ Perkin Elmer, USA).

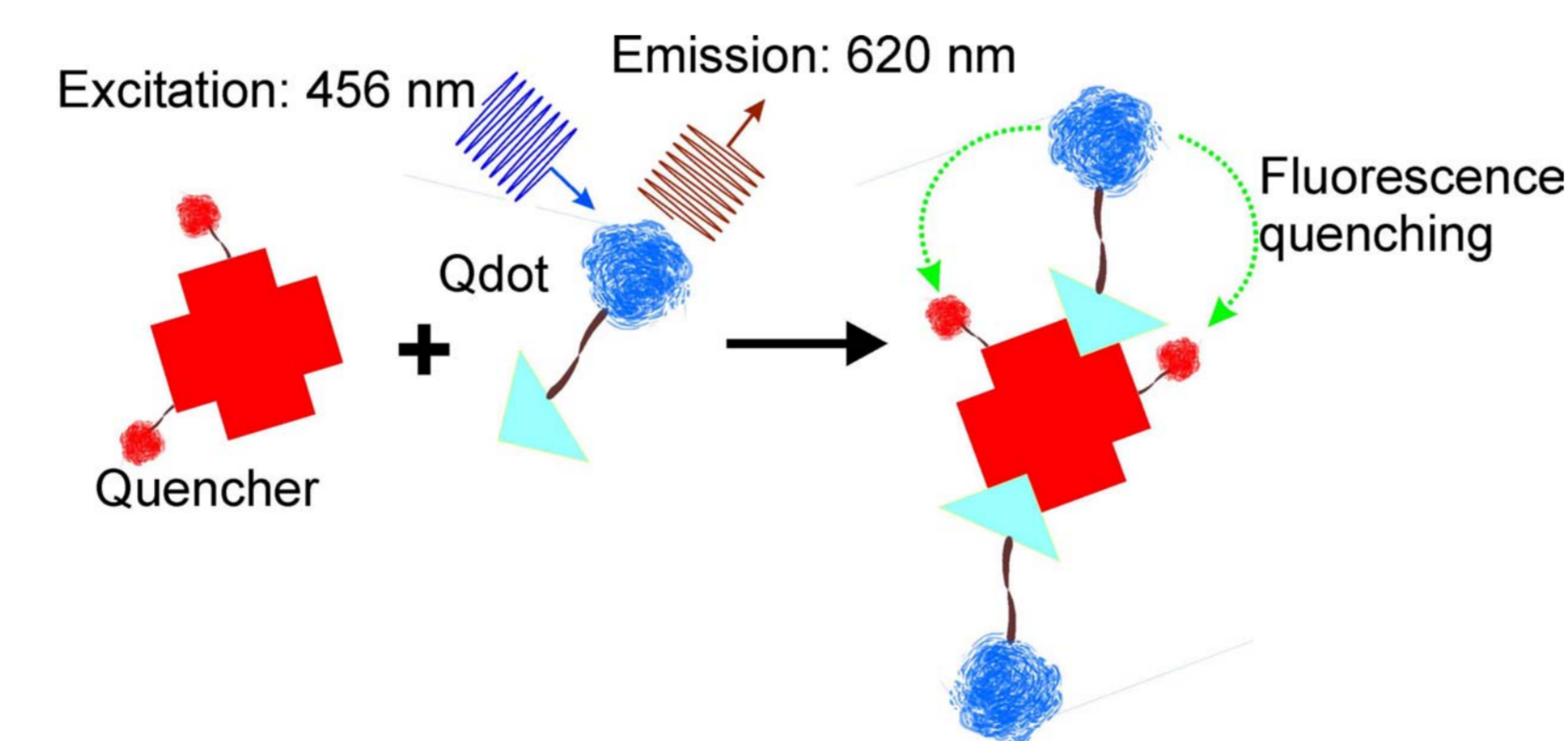
For the duplex assay a Ruthenium complex and a quantum dot as distinctive marker were used.

### 1 – Ru-based cAMP assay



The Ruthenium complex (Roche Diagnostics, Mannheim, Germany) as free acid in aqueous solution has a luminescence lifetime of ~3 µs. The energy transfer (FRET) principle of this assay prolongs the acceptor's lifetime (AlexaFluor@700, Invitrogen, Carlsbad, CA, USA) to ~100 ns. [3]

### 2 – Quantum dot based assay principle



Biotinylated EviFluor@620 quantum dots were from Evident Technologies (Troy, NY, USA). Streptavidin conjugated Atto612Q (Attotec, Siegen Germany) was used as a fluorescence quencher.

Quantum dots usually exhibit excited state lifetimes in the order of 20 ... 30 ns. Upon quenching fluorescence intensity and possibly lifetime are diminished.

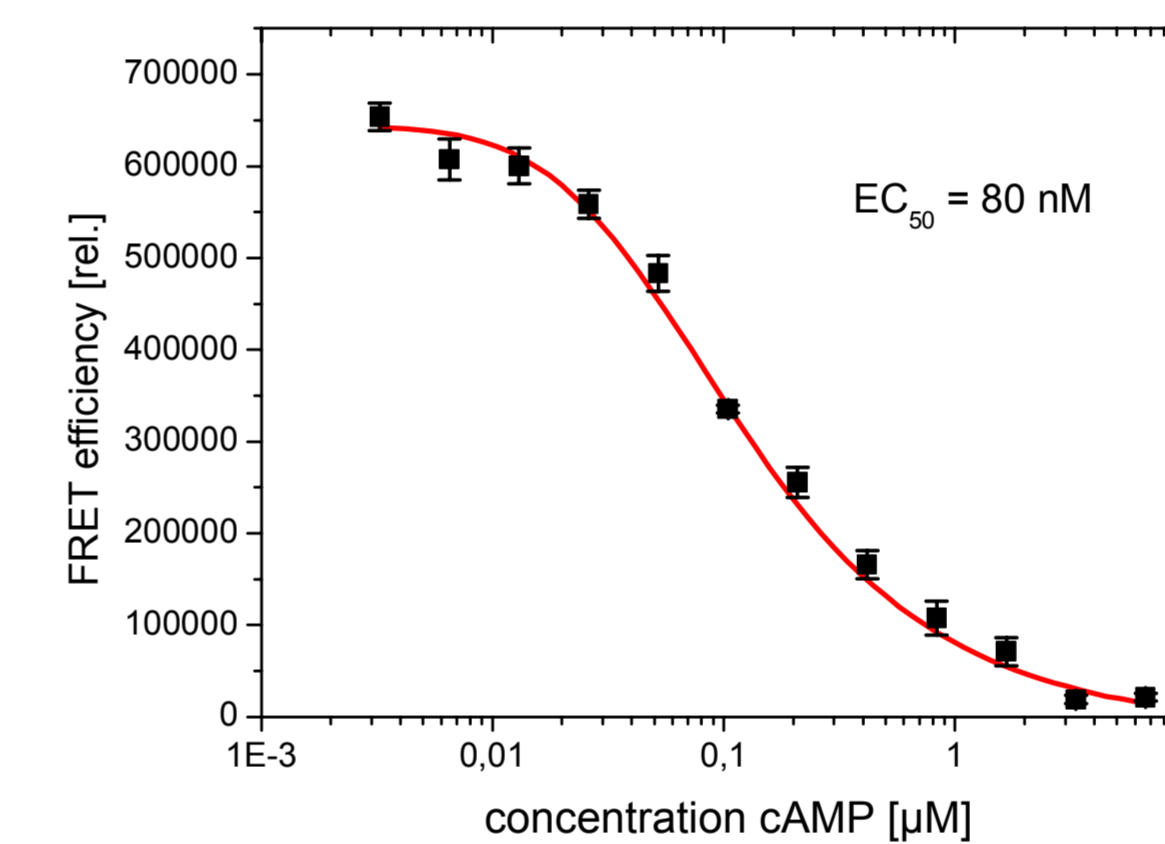
## Duplex assay read-out

Assay read-out was performed with LF502 NanoScan FLT microplate reader (IOM, Berlin, Germany). The instrument is equipped with a Nitrogen/dye laser combination and provides excitation wavelengths between 337 and 785 nm. It is capable of performing time-resolved fluorescence measurements within a time range between 1 nanosecond and 10 milliseconds.

The following read-out parameters were used for the cAMP-Nano-TRF assay :

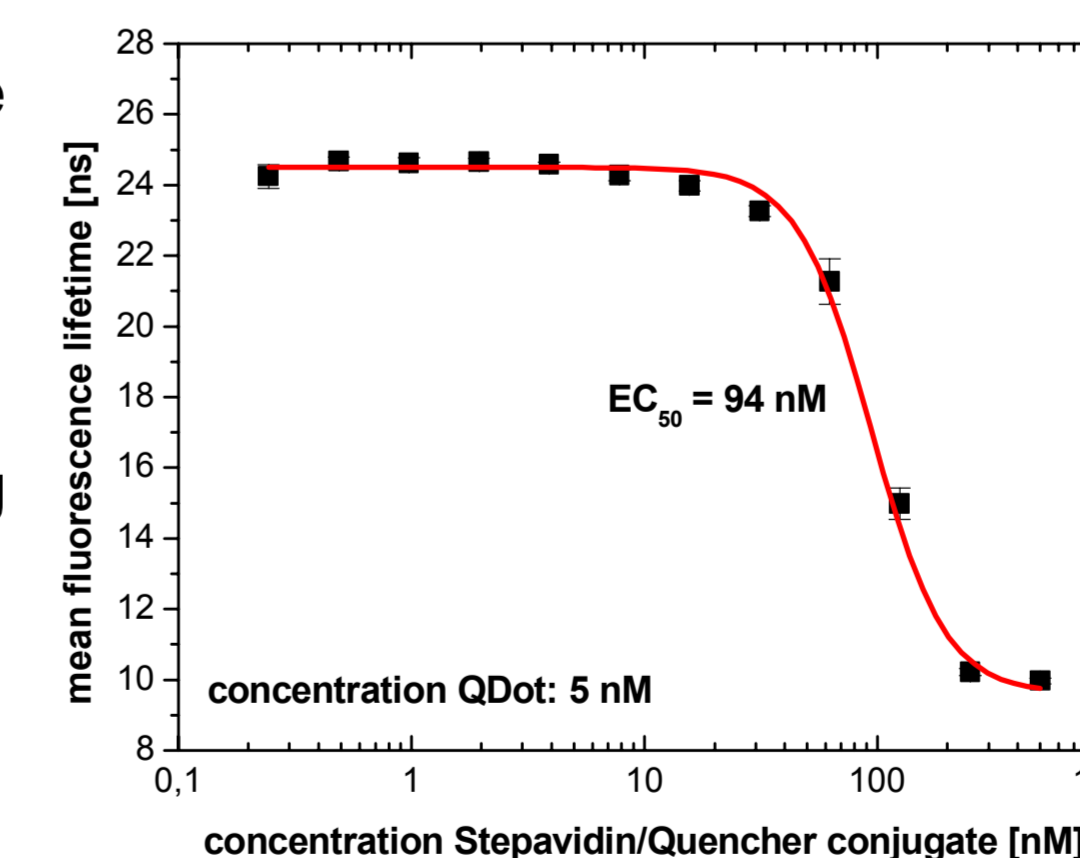
**excitation** 456 nm  
**emission donor Channel:** 630/50 nm  
 delay 500 ns, integration 1.5 µs  
**emission acceptor Channel:** 700/30 nm  
 delay: 40 ns, integration: 160 µs

Standard curve for the cAMP assay. Data acquisition was done with a high-speed digitizer with a maximum temporal resolution of 0.125 ns.



The quantum dot quenching was read-out using the same excitation and emission filters. Unlike for the Nano-TRF signal the highly resolved fluorescence decay curve was numerically analyzed in the range of 0 ... 100 ns in order to reveal the fluorescence lifetime.

For the standard curve the Qdot concentration was kept constant at 5 nM whereas the Atto621Q-Streptavidin conjugate was added with increasing concentration. The Qdot fluorescence was analyzed with a double-exponential fit.



The average fluorescence lifetime was calculated according to the following formula:

$$\tau = \frac{A_1 \cdot \tau_1^2 + A_2 \cdot \tau_2^2}{A_1 \cdot \tau_1 + A_2 \cdot \tau_2} \quad (1)$$

A<sub>1</sub> and τ<sub>1</sub> are amplitude and fluorescence lifetime of the two recalculated lifetime values, respectively.

Mixtures of high and low assay signals were measured in the same well to demonstrate the nearly zero overlap of both assays despite identical emission wavelengths.

	cAMP-Assay	Streptavidin-Biotin-Bindung [EviFluor@620] = 2.5 nM
1	cAMP-High [cAMP] = 2.5 µM	Streptavidin-Quencher-High [Streptavidin] = 250 nM
2	cAMP-High [cAMP] = 2.5 µM	Streptavidin-Quencher-Low [Streptavidin] = 0.5 nM
3	cAMP-Low [cAMP] = 2.5 nM	Streptavidin-Quencher-High [Streptavidin] = 250 nM
4	cAMP-Low [cAMP] = 2.5 nM	Streptavidin-Quencher-Low [Streptavidin] = 0.5 nM

Result of the simultaneous read-out:

	Streptavidin-Quencher-High	Streptavidin-Quencher-Low
cAMP-High	14.95 ± 0.25 ns / 240,573 ± 28,207	21.40 ± 0.23 ns / 190,946 ± 14,715
cAMP-Low	14.13 ± 0.24 ns / 846,961 ± 16,007	21.65 ± 0.10 ns / 737,999 ± 26,683

## Summary

Herein we demonstrate :

- A duplex assay on the basis of different lifetimes
- Negligible interference despite strong spectral overlap
- Statistically significant improvement in results from simultaneous read-out emission
- Excitation at visible wavelengths reduces possible compound fluorescence
- a potential for triplex assays on the 7 decade lifetime scale

### Acknowledgement

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### References

- [1] Methods Of Screening Compositions For G Protein-Coupled Receptors Against Agonists. US Patent Application US020070196865A1
- [2] Cassutt KJ, Orsini MJ, Abousleiman M, Colone D, Tang W. Identifying nonselective hits from a homogeneous calcium assay screen. J Biomol Screen. 2007;12(2):285-287.
- [3] Roth D, Matile H, Josel H-P, Enderle T. Fast TRF: Novel Time-Resolved Assays for Drug Discovery. Poster SBS 2005, Geneva, Switzerland