

Suitability of Long-nanosecond Lifetime Probes for Nanosecond Time-resolved Fluorescence Assays



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Introduction

In high-throughput screening (HTS) fluorescence based assays play an important role due to the method's inherent high sensitivity and versatility.

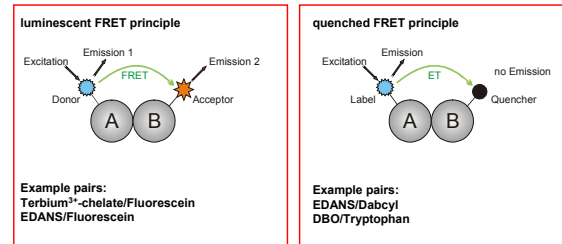
In recent years, novel fluorescence detection technologies measuring directly nanosecond fluorescence lifetimes (FLT) and nanosecond time-resolved fluorescence signals (Nano-TRF) emerged. The aim is to overcome limitations of existing assay formats concerning false positive and false negative results caused by colored compounds, sample auto-fluorescence or stray light. Assays based on the measurement of fluorescence intensity (FI) are largely suffering from these effects.

We describe the investigation of five different fluorescent labels in relation to their potential use as donor in time-resolved fluorescence resonance energy transfer (TR-FRET) assays with Nano-TRF read-out. Spectroscopic properties like fluorescence lifetimes and detection sensitivities have been determined. Based on the spectroscopic properties the profitable use of the labels in Nano-TRF assays is discussed.

General conclusions for the appropriate selection of long-nanosecond lifetime probes for Nano-TRF assays are drawn.

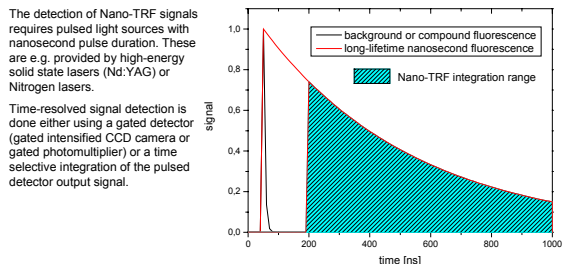
Nano-TRF Assay principle

Time-resolved fluorescence detection on the nanosecond time scale can favorably be used in energy transfer assay schemes. Energy transfer of the Förster type from a first probe to a second probe may result in (i) the emission of a wavelength-shifted luminescence originating from the second probe (acceptor) thus increasing the acceptor's luminescence or in (ii) quenching of the transferred energy.



Detection scheme in Nano-TRF assays

Like in common time-resolved fluorescence detection methods the signal recognition occurs within a specified time window. The position and duration of this window is chosen in order to exclude disturbing signals. In the figure below a synthetic fluorescence signal with a long lifetime of 500 ns is drawn together with a disturbing fluorescence signal of 5 ns decay time. Integration of the signal lasts 150 ns after the exciting light pulse when short-lifetime background signals completely disappeared.



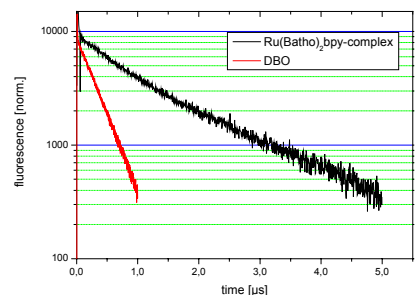
The detection of Nano-TRF signals requires pulsed light sources with nanosecond pulse duration. These are e.g. provided by high-energy solid state lasers (Nd:YAG) or Nitrogen lasers.

Time-resolved signal detection is done either using a gated detector (gated intensified CCD camera or gated photomultiplier) or a time selective integration of the pulsed detector output signal.

Experimental

The fluorescence decay behavior and the detection limits of five different probes suitable for Nano-TRF assays have been determined using a microplate reader (LF501 NanoScan FLT, IOM, Germany) which is able to temporally resolve fluorescence signals. The light source of the instrument is a dye laser pumped by a sealed Nitrogen laser with a repetition rate of up to 30 Hz, 2.5 ns pulse width and 130 μJ pulse energy. The signal from a photomultiplier is digitized by means of a fast transient recorder. The instrument is capable of resolving fluorescence lifetimes in the range of 0,5 ns up to more than 20 μs.

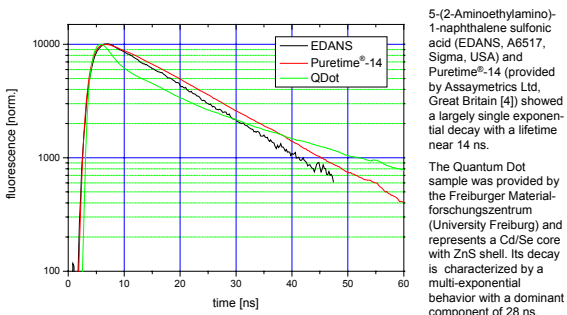
The samples have been prepared in black 384 well microplates (Greiner, 781076) with a sample volume of 50 μl per well. Estimation of the detection limit was performed using a three-fold 1:2 dilution series. Detection limit is set as 3 times standard deviation of the blank sample (solvent). Samples have been excited near their absorption maxima. Fluorescence lifetimes were determined at sample concentrations well within the linear range of instrumental response. Two time ranges had to be distinguished: first a range of 100 ns and secondly a range of 5 μs. The following graphs show fluorescence decay curves of the investigated fluorophores.



2,3-diazabicyclo[2.2.2] oct-2-ene (DBO) [1] was supplied by Prof. W. Nau, Jacobs University Bremen, and measured in aqueous solution.

A bipyridyl Ruthenium complex (for details see [2]) was measured in PBS buffer.

DBO and the Ru²⁺-complex showed essentially a single exponential decay on a microsecond scale.



5-(2-Aminoethylamino)-1-naphthalene sulfonic acid (EDANS, A6517, Sigma, USA) and Puretime®-14 (provided by Assaymetrics Ltd, Great Britain [4]) showed a largely single exponential decay with a lifetime near 14 ns.

The Quantum Dot sample was provided by the Freiburger Materialforschungszentrum (University Freiburg) and represents a Cd/Se core with ZnS shell. Its decay is characterized by a multi-exponential behavior with a dominant component of 28 ns.

Long nanosecond lifetime probe	solvent	absorption λ _{max} [nm]	emission λ _{max} [nm]	fluorescence lifetime [ns]	detection limit [M]
Ru ²⁺ -complex	PBS	450	620	1.474 +/- 12	1 * 10 ⁻¹¹
DBO	water	365	430	323 +/- 4	6 * 10 ⁻⁹
EDANS	water	335	493	13,6	5 * 10 ⁻¹⁰
Puretime®-14	water	394	426	15,8 +/- 0,5	NA
Qdot (CdSe/ZnS)	cyclohexane	< 500	582	28,4 +/- 0,4	2 * 10 ⁻¹²

Large differences for the lowest detectable concentration limit were found. For Puretime®-14 a detection limit was not determined. The reason for the rather poor detection limit of DBO is its low extinction of only 100 M⁻¹cm⁻¹. [1]

Conclusions:

The main property indicating the usefulness of a probe for Nano-TRF assays is given by its fluorescence lifetime. Three probes, EDANS, Puretime®-14 and a CdSe-Quantum Dot, have characteristic lifetimes between 14 and 30 ns. Since in most cases compound auto-fluorescence is of short nanosecond duration [3] in general these probes are suitable as donor for TR-FRET assays with Nano-TRF read-out although discrimination of auto-fluorescence will not be complete in the minor number of cases where auto-fluorescence has a long lifetime. In the case of EDANS and Puretime®-14 a further disadvantage is the required ultraviolet excitation which leads to efficient excitation of compound auto-fluorescence.

With this respect the quantum dot is more attractive. The rather long excitation wavelength of nearly 500 nm, the very low detection limit and the rather long lifetime of nearly 30 ns make it a promising candidate as donor for Nano-TRF based assays.

The probes DBO and Ru²⁺-complex exhibit lifetimes of several hundred nanoseconds up to 1,5 microseconds making them ideal candidates for Nano-TRF read-out. However, in the case of DBO the extinction and hence the detection limit is rather poor. This limits the use of DBO to quenched FRET assays where the analyte concentration is allowed to be in the micromolar range.

For the Ru²⁺-complex as well excitation wavelength, lifetime as detection limit predestine this label for luminescent FRET which already successfully has been demonstrated. [2] The potential use for quenched FRET assays is yet outstanding.

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probe	time delay/gate	applicable for lum. FRET	applicable for quen. FRET
Ru ²⁺ -complex	> 100 ns / 1,5 μs	++	o
DBO	> 100 ns / 0,5 - 2 μs	-	++
EDANS	~ 20 ns / ~100 ns	+	+
Puretime®-14	~ 20 ns / ~100 ns	++	+
Qdot (CdSe/ZnS)	~ 30...50ns / ~100 ns	++	o

legend: ++ well suited; + suited; - not suited; o not known

Literature:

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