

Fluorescence Lifetime Read-Out of Quantum Dots in a Homogeneous Immunoassay



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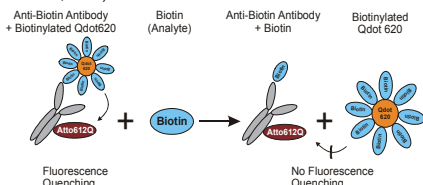
Introduction

Latest advances in fluorescence lifetime (FLT) measurement technique provide a promising basis for the development of very robust high throughput screening (HTS) assays for compound screening and profiling. Only recently, the successful application of ultra-violet absorbing fluorescent labels with lifetimes in the order of 10 to 20 nanoseconds for protease activity assays was reported underlining the potential of this technology (1). However, the huge majority of the red absorbing and emitting organic fluorophores has short lifetimes in the lower nanosecond region (2). This constrains the temporal discrimination of short-lived autofluorescence and limits the use-ful window of detection in this visible and especially in the otherwise attractive NIR region.

Here, we present an assay scheme based on quantum dots (QDots) and FLT read-out. QDots possess very interesting spectroscopic properties like a size-dependent absorption and emission as well as fluorescence lifetimes in the order of 25 to 30 nanoseconds. We report on an example immunoassay based on fluorescence quenching of the QDots by the organic quencher Atto612Q and with FLT read-out for the determination of Biotin concentrations.

Assay principle

The anti-biotin-IgG-antibody (B7653, Clone BN-34, Sigma Aldrich, St. Louis, MO, USA) was covalently labeled with the fluorescence quencher Atto612Q (Atto-Tec, Siegen, Germany). Biotinylated EviFluor@620 quantum dots were obtained from Evident Technologies (Troy, NY, USA). For standard curves biotin was obtained from Sigma (B4501, Sigma Aldrich, USA)



When the biotinylated QDot binds via a biotin group to the antibody the QDot comes into close proximity to the quencher molecules. This leads to an energy transfer from the excited QDot to the quencher and, due to the non-radiative de-excitation of the quencher, subsequently to a loss of fluorescence intensity.

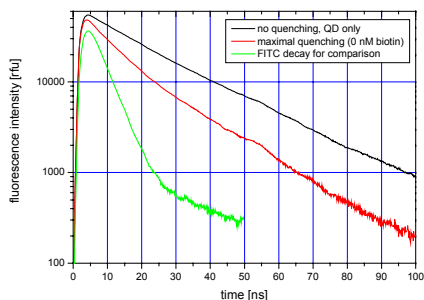
All dilutions were done in a pH 7.4 PBS buffer (P5368 Sigma) with 0.1% bovine serum albumin (BSA, A6003, Sigma). For obtaining a standard curve 15 μ l of a biotin dilution series with concentrations between 6 nM and 100 μ M were prepared in a Corning 384 well plate (#3712, Corning, NY, USA). 1 μ l of a 300 nM QDot stock solution was added. Finally 14 μ l of a 4 μ M stock solution of the anti-biotin-antibody was added. The final assay concentrations of the QDot and the antibody were 10 nM and 1.87 μ M respectively in 30 μ l sample volume.

As well-known for the FRET process the FLT of the fluorescing donor is expected to be reduced with increasing FRET efficiency. At the same time the donor fluorescence intensity is reduced. Both, the reduced fluorescence intensity and the FLT can be used as read-out parameter for this assay.

Instrumentation

Assay read-out was performed with a **LF502 NanoScan FLT** microplate reader (IOM, Berlin, Germany). The instrument is equipped with a nitrogen/dye laser combination and was used with the 488 nm laser module for fluorescence excitation. Fluorescence detection occurred at 620/10 nm.

The fluorescence decay curve was acquired on a 100 ns time scale with 0.125 ns temporal resolution. In order to reduce noise averaging over 32 laser pulses was applied. Together with the 30 Hz repetition rate of the laser this leads to a measurement time per well of approximately 1 s.



The decay curves of the QDot fluorescence as shown as black curve in the graph above did not exhibit a single exponential behavior. Instead all decay curves with (red curve) and without fluorescence quenching were fitted with a double-exponential function with good fit quality.

In contrast to this the fluorescence of a fluorescein sample (green curve) showed a 4 ns single exponential behavior in the time range 0.25 ns, apart from a tail of background signal later on. According to previous experiences (3) the mean lifetime of the decay was calculated using the following formula:

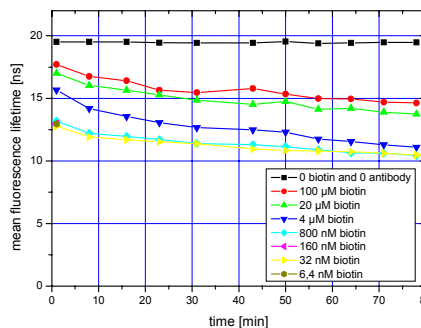
$$\bar{\tau} = \frac{A_1 * \tau_1^2 + A_2 * \tau_2^2}{A_1 * \tau_1 + A_2 * \tau_2} \quad (1)$$

A_1 and τ_1 are amplitudes and fluorescence lifetimes of the two recalculated lifetime values, respectively.

Results

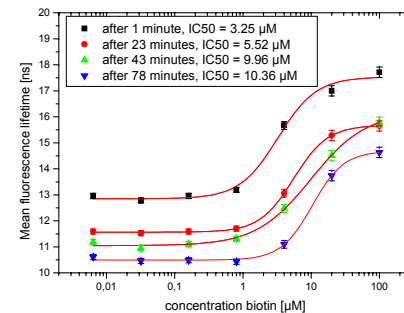
A standard curve was measured repeatedly roughly every 10 minutes. At the same time a reference sample without antibody addition was monitored.

For the reference sample a constant signal over a time period of 80 minutes was measured for the fluorescence intensity as well as for the fluorescence lifetime. The mean fluorescence lifetime value was constantly 19.47 ± 0.04 ns (black curve in next graph).



The kinetics of the QDot-antibody-interaction shows an overall declining lifetime and intensity signal. Already the initial fluorescence lifetime value of the sample with maximum biotin concentration (100 nM) was reduced in comparison to the unquenched QDot.

The total difference between the maximum and minimum QDot quenching is in the order of 4.5 ns and remains constant over time. There is a very good reproducibility of the lifetime measurement with a precision of less than 0.1 ns.



A comparing plot of the standard curves at different times shows additionally a shift of the IC50 towards bigger values. All IC50 values are in the range of 3 to 10 μ M i.e. in the order of the antibody concentration.

Conclusion

The presented assay has demonstrated that the red wavelength and long lifetime QDot fluorescence can be used to develop robust homogeneous assays for screening applications.

The use of the FLT read-out significantly improves signal quality and provides a tool for discovering and to eliminate false positive screening results. The long excitation and emission wavelengths further reduce the influence of compound interference.

The principle of this assay scheme can easily be adapted to other binding assays with HTS relevance.

Together with the LF502 NanoScan microplate reader there can additionally be used the Nano-TRF read-out mode further improving data quality.

Acknowledgement

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References

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