APPLICATION NOTE

Time Resolved Fluorescence Techniques to FRET Based Immunoassay

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Introduction

Förster resonance energy transfer (FRET) is a phenomenon of non-radiative transfer of the optical excitation from an excited electronic level of one molecule, called the donor, to a resonant electronic level of another molecule, called the acceptor.[1] If the electronic levels of donor (D) and acceptor (A) are in a strong resonance the optical excitation can be up to 10 nm. Efficiency of energy transfer exponentially decays as a sixth power of D-A separation distance. Hence, this phenomenon is used as a "ruler" to measure the separating distance between two molecules or the ascertainment of proximity or spatial coordination of two molecules in solution or on a cell surface. FRET has found massive applications in biology to study protein folding, protein-protein interactions or protein organisation in molecular complexes in solution and on the cellular membrane.[2-4]

Lanthanides, as for example Europium Cryptate (EuK), have been extensively used as luminescence donors because of a relatively long emission lifetime (1.2 ms) and high transfer efficiency to an acceptor, as for example Allophycocyanin (APC). Due to the energy transfer from millisecond EuK to nanosecond APC the latter emits in the hundreds of microseconds range.[5,6]

A commonly used format of FRET-based immunoassay (usually abbreviated as TR-FRET) relies on the ratio of donor (620nm) to acceptor (665 nm) intensities measured in the "gated"mode, i.e. the signal is integrated over a time-period starting after the end of the excitation pulse. The gating separates the emission of donor and acceptor from nanosecond background.[7]Since the intensities are measured in the "gated" mode, this method is misleadingly called Time-Resolved FRET or TR-FRET although it is actually not based on "real" time-resolved measurements.

A serious drawback of the TR-FRET format is that the emission spectrum of APC and the 642 nm emission line of EuK overlap. As a result, the directly excited emission of the donor bleeds through into the FRET channel thereby significantly limiting the method sensitivity. In addition the method in the intensity format requires two detectors. We show in this application note that one can increase the method sensitivity and reduce cost of the assay by applying a time-resolved fluorescence format to the FRET-based immunoassay.

The Assay Principle

The assay principle is illustrated in Fig. 1. The assay is based on the specific binding of a "matched" pair of antibodies, AB1 and AB2, to an antigen (Ag) which is the analyte molecule of interest. In the measurements reported here the antigen used was Alpha Fetoprotein (AFP). When both labelled antibodies bind to Ag they produce a ternary complex Ab1-Ag-Ab2. Upon certain experimental conditions when concentration of the donor-labelled antibody is high enough to allow binding of all Ag molecules, but still comparable with Ag concentration, one can resolve a short lifetime component in EuK time-response, the amplitude of which is proportional to the "sandwiches" concentration.

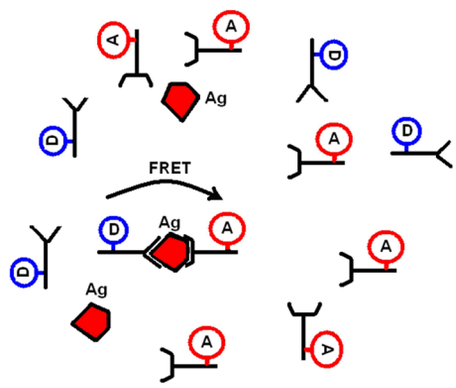

Figure 1: The principle of FRET-based immunoassay

Methods and Materials

An AFP KRYPTOR kit (B·R·A·H·M·S) was used in this study. The standard protocol for the kit was modified to reduce concentration of the donor-labelled antibody (Ab1-EuK). The reaction was started by mixing 21 µl of Ab1-EuK, 70 µl of Ab2-APC, 49 µl of buffer and 70 µl of Ag solution to give the total volume 210 µl. The final concentration of Ag in the sample varied from 0 to 833 ng/ml. The sample was incubated for 1 hour at 20°C to complete the complex formation reaction. The sample was transferred into a semi-micro fused silica cuvette and time-resolved EuK emission was measured using an FLS980 Fluorescence Spectrometer operating in the multi-channel scaling (MCS) mode. Excitation was provided by a 60 W Xenon microsecond flash lamp with the monochromator set at 310 nm with a 16 nm spectral bandwidth. EuK fluorescence was monitored through the emission monochromator set at 620 nm (16 nm spectral bandwidth). A cooled red sensitive photomultiplier (PMT-900) was used for the gated photon counting (80 ms delay and 10 ms gate width). Emission time-courses were acquired for 30 s.

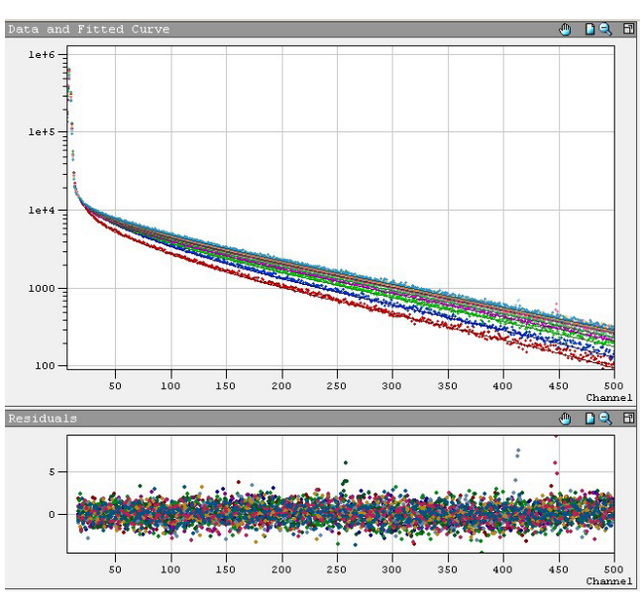

Figure 2: FAST analysis of a series of fluorescence time-responses of EuK at different concentrations of AFP

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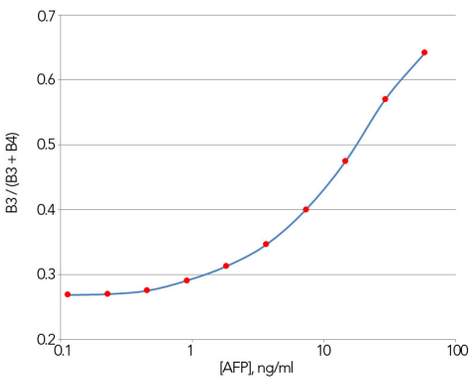

Figure 3: The relative concentration of bound EuK-labelled antibody at different concentrations of the antigen.

Results-Discussion

A family of emission time-courses were evaluated using the Exponential Component Analysis routine of the Fluorescence Analysis Software Technology (FAST, Edinburgh Instruments) and are shown in Fig. 2. A 4-exponential model with fixed lifetime constants was used:

$$I(t) = \sum_{i=1}^{3} B_i \exp(-t/\tau_i) \qquad (1)$$

The longest lifetime (τ_4=1.2 ms) corresponding to the natural emission of EuK was measured independently in a control sample and fixed in the analysis. The third lifetime component (τ_3=330 µs) was attributed to FRET quenching. The two fastest lifetime components (τ_1=40 µs and τ_2=150 µs) were attributed to microsecond background or stray light. By decomposing the fluorescence time-responses into individual lifetime components and taking for calculation only the component associated with FRET, we can separate microsecond background from the emission of EuK which is not possible in the intensity TR-FRET format. Ag concentration can be calculated as B3/(B3+B4), were B3 and B4 are pre-exponential coefficients.

A Standard Assay Curve was calculated from the evaluated data for samples containing different concentrations of the antigen (Fig. 3).

Conclusion

This work shows that the optimisation of concentration of the donor labelled antibody and implementation of MCS mode to measure the donor emission time-responses endows the method with a "real" temporal resolution. By dealing only with the donor emission (EuK), which can be measured in the spectral range free from the acceptor (APC) emission, we can circumvent the unavoidable spectral "cross-talk" problem of the TR-FRET format. In addition, the lifetime mode allows microsecond background to be discriminated, which also limits the sensitivity of the "gated" intensity format. Taking together the lifetime format makes the method less costly and more sensitive.

References

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