Optimisation of TR-FRET Assay Performance with the Varioskan[R] Spectral Scanning Time-Resolved Fluorometer

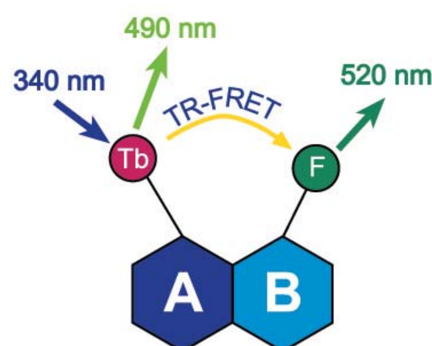
Marika Raitio, Hanna Granö, Veera Välisalo and Jorma Lampinen, Thermo Electron Oy, Vantaa, Finland

Abstract

Time-resolved fluorescence energy transfer (TR-FRET) is a commonly used variation of fluorescence energy transfer (FRET), and it uses a time-resolved fluorescence label as a FRET donor. Compared to fluorescence intensity based FRET, it has a benefit of lacking biological background fluorescence because of the time dependent phenomena. The most important factor affecting TR-FRET assay performance is the differentiation of the donor signal emitted by the free donor from the TR-FRET signal. In this work, measurement parameters for a common TR-FRET application were optimized using the Varioskan spectral scanning microtiter plate fluorometer. Excitation and emission spectra of free donor and TR-FRET combination were measured using time-resolved spectral scanning and the resulting spectra were analyzed to determine the best possible resolution between donor and TR-FRET emissions. Additionally, time-resolved fluorescence signal decay curves over time from the donor and TR-FRET complex were measured to determine the optimal measurement window for the TR-FRET assay. Optimization of the wavelengths and measurement window for a TR-FRET can easily be done using a spectral scanning reader. Remarkable improvements can be achieved with only small changes in the TR-FRET measurement parameters.

Introduction

In the process of fluorescence energy transfer (FRET) the donor is excited, and if the acceptor is in close proximity, the excited state energy will be transferred. This leads to a reduction in the donor fluorescence intensity and excited state lifetime, and an increase in the acceptors emission intensity. The basic principle of the FRET technology is shown in Picture 1. The efficiency of energy transfer strongly depends on the distance between the donor and acceptor molecules and on the overlap of the donor emission and acceptor excitation spectra. (Picture 2.). Time-resolved fluorescence resonance energy transfer (TR-FRET) combines the advantages of time-resolved fluorescence and FRET. Time resolution is used to separate the energy transfer signal from the acceptor prompt fluorescence and background. The purpose of this paper is to describe the optimization process of measurement parameters using the scanning features of Thermo's Varioskan[R] and SkanIt[R] Software. The effect of the optimization is demonstrated by comparing the assay results with the default and the optimized parameters. The type of the assay used in this study was a tyrosine kinase assay from Invitrogen Corp. utilizing terbium as donor and fluorescein as an acceptor.

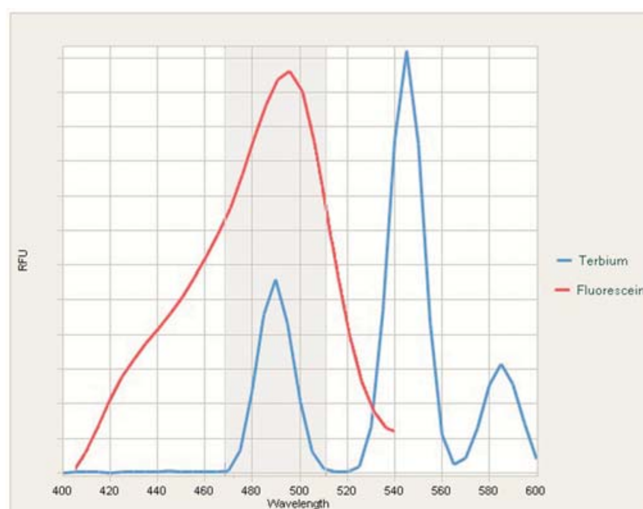


Picture 1. Principle of the FRET technology.

A fluorescent donor (D) is excited at its specific fluorescence excitation wavelength. By a long-range dipole-dipole coupling mechanism, this excited state is non-radiatively transferred to the acceptor (A). The donor returns to the electronic ground state and the fluorescence signal is emitted by the acceptor.

Materials and Methods

The LanthaScreen TR-FRET Tyrosine kinase assay was used as an example of TR-FRET optimization. This assay uses a terbium chelate labeled anti-phosphotyrosine peptide specific antibody as a TR-FRET donor and a phosphorylated fluorescein labelled substrate peptide as TR-FRET acceptor (LanthaScreen[TM] TR-FRET Tyrosine kinase assay kit, prod. no. PV3149 with Tb-PY72 terbium labeled antibody, prod. no. PV3554, and fluorescein-PTK substrate, positive control, prod. no. PV35249, all from Invitrogen Corp., Carlsbad, CA). All assays were made according to the kit insert with a white 110 µl 384 square well plate (Thermo Electron Corp., prod. no. 8225). The optimal measurement parameters for the Varioskan with this assay were determined from the fluorescence spectra and decay curves. These parameters were compared with the default measurement parameters from the kit instructions. First, fluorescence excitation spectra of terbium label was determined to ensure the most efficient excitation of the FRET measurement. Then, fluorescence emission spectra were determined from the terbium label alone, fluorescein label alone and their TR-FRET complex. Optimal measurement wavelengths were determined based on these spectra. TRF decay curves were measured from major terbium emission peaks at 490 and 540 nm using pure terbium label and the TR-FRET decay curve was determined using terbium-fluorescein FRET complex as a sample. These curves were used to determine the optimal timing of the FRET measurement. Terbium decay curves were also compared to the theoretical decay profiled based on the standard TRF decay mathematics. Optimized measurement parameters were then verified by determining the titration curve of the positive control peptide, fluorescein PTK substrate, with constant terbium antibody concentration. All measurements were made with Thermo Electron's Varioskan spectral scanning microplate fluorometer connected to SkanIt Software (version 2.2).



Picture 2. Excitation and emission spectra of the fluorochromes used for this study.

The overlap of the donor emission and acceptor excitation is marked with gray on the image. Terbium's minor emission peak at 490 nm is used for the fluorescein excitation in this TR-FRET reaction. Fluorescein emission is measured at 520 nm where the donor emission is minimal.

Legends:

Blue line = terbium emission spectra,

Red line = fluorescein excitation spectra.

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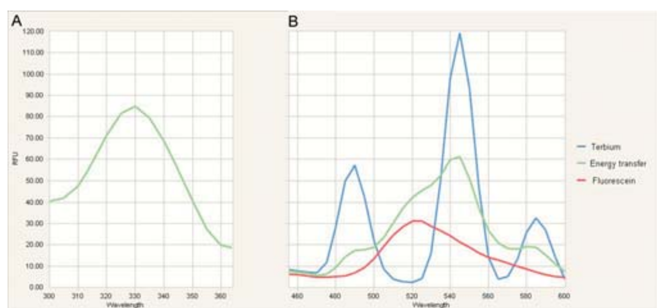
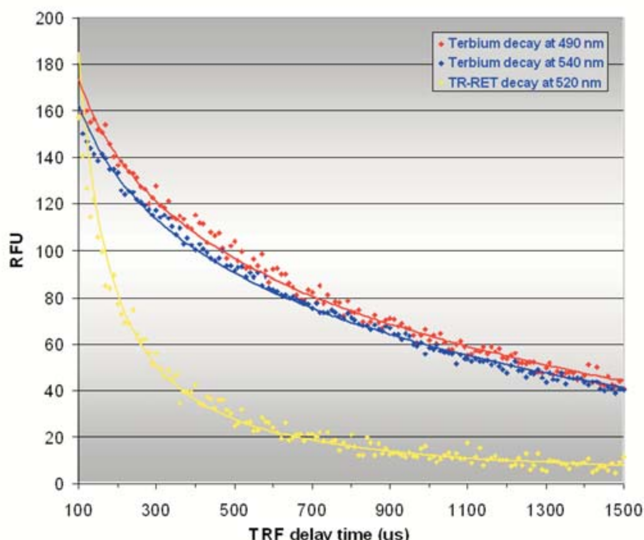
Results

Determined fluorescence spectra of both labels and their TR-FRET complex are shown in Picture 3. From these spectra, the optimal excitation and emission wavelengths were chosen for further measurements. As seen from Picture 3A., the terbium excitation spectrum has an excitation maximum at 330 nm and this wavelength was chosen as the test setting for all subsequent measurements. Emission wavelengths were selected from the spectra shown in Picture 3B. As expected, the terbium secondary peak was located at the emission maximum at 490 nm and this was chosen for donor emission measurements in the TR-FRET assay. Fluorescein emission maximum was achieved at 520 nm where terbium emission was minimum. This wavelength also gives the maximum difference between TR-FRET emission and donor emission and that wavelength was selected to be used for acceptor emission measurements. The TRF decay curves were determined for the terbium label and the terbium-fluorescein TR-FRET complex.Tthe decay curves are shown in Picture 4. By analyzing the difference between the decay curves and calculated data the optimal delay and integration times were chosen for the assay. Optimal assay parameters determined from the TRF spectra and decay curves are shown in Table I. Positive control peptide titration curves determined with both the optimal and manufacturer's recommended measurement parameters are shown in Picture 5.



Picture 3. Fluorescence spectra of the labels and their TR-FRET complex.

A) Excitation spectrum of terbium label in terbium-fluorescein TR-FRET complex. B) Emission spectra of all three label combinations.
Legends:
Blue line = terbium emission spectra,
Red line = fluorescein emission spectra,
Green line = terbium-fluorescein TR-FRET complex emission spectra.

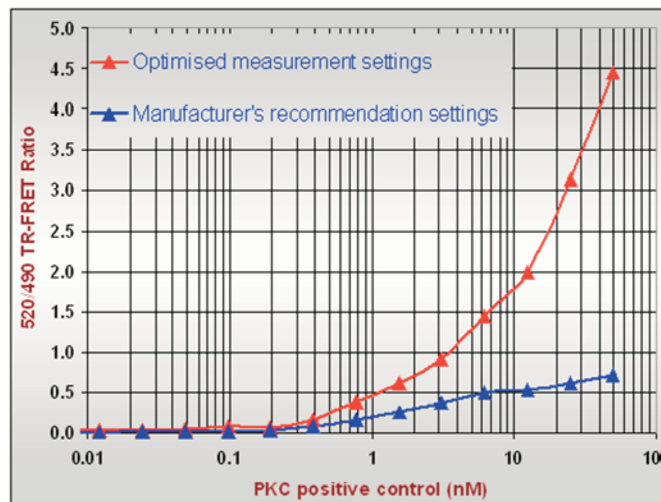


Picture 4. TRF decay curves of terbium and terbium-fluorescein TR-FRET complex.

TRF decay curves were determined from both major emission peaks of terbium label and from terbium-fluorescein TR-FRET complex.

	Recommended parameters	Optimized parameters
Donor excitation (nm)	340	330
Acceptor emission (nm)	520	520
Donor emission	490	490
Delay (us)	100	20
Integration time (us)	200	100
Measurement time (ms)	1000	1000

Table I. Optimized assay parameters for terbium-fluorescein TR-FRET measurements with Varioskan.



Picture 5. PKC positive control titration curves.

Conclusions

- The assay optimization process can easily and effectively be performed with a spectral scanning time-resolved fluorometer.
- Varioskan is an excellent instrument for assay optimization because it can be used for both TR-FRET assay optimization and actual measurement. Because of the possibility to visually examine the results, the optimization process is informative. The spectral data can easily be processed; e.g. the blank spectra reduced from the sample data. This makes the analysis of the matrix and its possible effect easier, which is important for a homogeneous assay type.
- The TR-FRET complex shows significantly shorter fluorescence lifetimes than the TRF label used in the complex. This has a very strong effect on the FRET ratio because 490 nm emission has a different lifetime than the 520 nmemission. Because of this, it is recommended to measure TR-FRET reactions with very short TRF integration times.
- Optimization has an effect on assay performance, specially changes in TRF delay time have a strong effect on the FRET ratio. As a general rule, one should choose as shorta delay time as the assay's prompt fluorescence background and instrument performance allows.

For more information contact:
Thermo Electron Corp.
PO. Box 100, FI-01621 Vantaa, Finland
Email: info.microplateinstruments@thermo.com
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