HTRF® application note

Cisbio Bioassays

Homogeneous Time-Resolved Fluorescence Part 1: Methodological aspects

Abstract

Rapid, homogeneous, easy to use and to automate, allowing extreme miniaturization and easy assay development, Cisbio's HTRF® technology has been one of the leading tools in drug discovery. The technology is now used for over 10 years by scientists in top pharmaceutical, biotechnology, and academic institutions.

Methodological aspects of the technology are described in this document.

Fluorescence and homogeneous assays

Fluorescence is, in theory, the most sensitive analytical technique. As many intra and intermolecular processes are able to modulate the label emission, it was therefore under consideration very early on as a technique of choice for designing homogeneous assays. Polarization, quenching, time correlation, lifetime variation as well as fluorescence resonance energy transfer (FRET) have been applied to probing molecular interactions. Among these techniques, FRET is of particular interest. Many domains of biology were explored with FRET, such as enzymatic activity, protein-DNA interactions, and cell surface lectin-revealed receptor-ligand interactions. DNA hybridization has attracted much attention and is now becoming a field of investigation for FRET experiments, be this in structural investigations, oligonucleotide hybridizations or the detection of gene translocation. Because the processes involved in FRET occur within distances characteristic of antigen-antibody interactions, homogeneous fluoroimmunoassays have also been developed. FRET was used to monitor receptor oligomerisation, a key process in the cell signaling pathway.

HTRF is a homogeneous method which combines standard FRET technology with the time-resolved measurement of fluorescence.

FRET (Fluorescence Resonance Energy Transfer) is based on the transfer of energy between two fluorophores, a donor and an acceptor, when in close proximity. Molecular interactions between biomolecules can be assessed by coupling each partner with a fluorescent label and detecting the level of energy transfer.

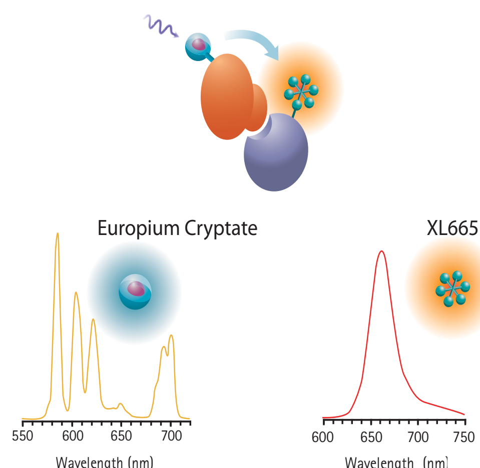


Fig.1 : HTRF principle with Eu^{3+} cryptate and XL665 as respectively donor and acceptor. When the two entities come into close proximity and upon excitation, FRET occurs and XL665 re-emits a specific long-lived fluorescence at 665 nm.

HTRF®: the reference for time-resolved FRET

Through time-resolved measurement of fluorescence, HTRF allows the elimination of short-lived background fluorescence. Introducing a time delay (50-150 µseconds) between the system excitation and fluorescence measurement allows the signal to be cleared of all non-specific short lived emissions. In contrast, HTRF fluorophores emit long-lived fluorescence when engaged in the

TR-FRET process. Therefore, long-lived emissions signify energy transfer through proximity of the labeled biomolecules (fig. 2).

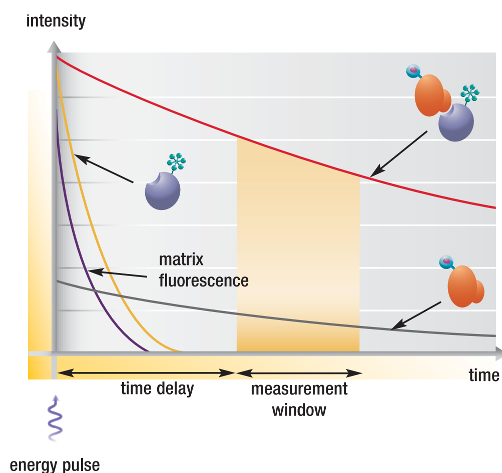


Fig. 2: The energy pulse from the excitation source (flash lamp, laser) is followed by a time delay, allowing interfering short lived fluorescence (compounds, proteins, medium etc.) to decay.

Time-Resolved Fluorescence and Rare Earth Complexes

Many compounds and proteins present in biological fluids or serum are naturally fluorescent, and the use of conventional fluorophores may lead to serious limitations of sensitivity. Most of these background signals being short-lived, the use of long-lived labels combined with detection on a time-resolved fluorescence basis allow the minimization of prompt fluorescence interferences.

HTRF® fluorescent partners

HTRF involves several carefully selected fluorophores. Obviously, FRET partners must fulfill multiple compatibility criteria. First, their emission spectra must show non-overlapping regions in order to be able to measure each partner's fluorescence individually. Second, the FRET quantum yield – i.e. its efficacy – must be as high as possible. Third, fluorescence emission must occur within a region of the spectrum remote from that naturally produced by proteins; in other words, a red-shifted emission is better for avoiding medium-intrinsic fluorescence.

The selection of new fluorescent tracers and the subsequent generation of rare earth cryptates were driven by the specific constraints of homogeneous assays.

HTRF uses four specific fluorophores to form different TR-FRET systems.

The central element, the energy donor, is either Europium cryptate (Eu^{3+}) or Lumi4-Tb™, a Terbium cryptate (Tb^{2+}), the fruit of a recent collaboration with Lumiphore Inc..

The maximum of the excitation wavelength of Eu^{3+} and Tb^{2+} cryptate fits with most energy sources (e.g. nitrogen laser, flash lamp) and compatible with all the HTRF certified readers. The long-lived emission obtained upon excitation (300 µsec to 2.2 msec) is characteristic of Eu^{3+} and Tb^{2+} cryptate fluorescence emission between 480 to 720 nm (fig. 3)

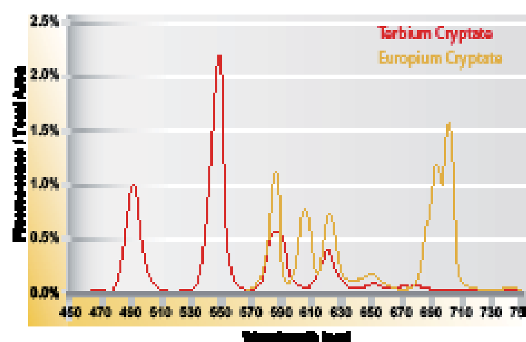


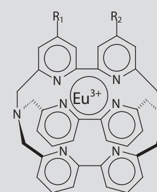
Fig. 3: Emission spectra for Europium and Terbium cryptates

The first acceptor developed for HTRF was XL665, a phycobilliprotein pigment purified from red algae. XL665 is a large heterohexameric edifice of 105 kDa, cross-linked after isolation for better stability and preservation of its photophysical properties in HTRF assays.

Awarded Cryptate Chemistry

Prof. J.M. Lehn's was awarded a Nobel Prize for Chemistry in 1987, for his work on rare earth complexes.

These complexes consist of a macrocycle within which a Eu^{3+} ion is tightly embedded. This cage allows both energy collection and transfer to the Eu^{3+} ion, which ultimately releases this energy with a specific fluorescent pattern. Moreover, this type of structure confers long-lived fluorescence, one of Eu^{3+} cryptate's fundamental properties, and has proven its stability. In particular, cryptate is not subject to the photobleaching that effects a number of more conventional fluorophores, and the ion is almost inseparable from its macrocycle.



The second generation of acceptors is characterized by organic structures 100 times smaller, displaying a series of photophysical properties very close to those of XL665. The

comparison of d2 to XL665 was achieved by screening 14,700 compounds on an assay for quantifying a phosphorylated peptide (fig.4). As a much smaller entity, d2 limits the steric hindrance problems sometimes suspected in XL665 based systems. The acceptor contributed to significantly greater stability of immuno-competitive assays, and in some cases to better assay sensitivity.

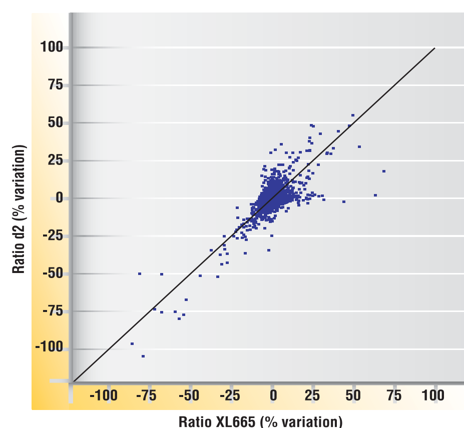


Fig. 4: XL665 and d2 were compared in an HTRF assay involving an anti-phosphotyrosine antibody conjugated to Eu^{3+} cryptate (PT66), a phosphorylated biotinylated substrate, and streptavidin alternatively labeled with XL665 and d2. Each system was tested with a library of 14,700 compounds (Schering AG). Comparison shows that the two acceptors behave very similarly and that d2 represents a promising alternative to XL665

Dual wavelength detection and signal ratio

HTRF emissions are measured at two different wavelengths, 620nm (donor) and 665nm (acceptor). This feature of HTRF is extremely advantageous, particularly for reducing well-to-well variations that may arise in homogeneous assay formats. Homogeneous formats do not require washing or separation steps, so all the assay components are present at the time of assay readout. Because wells contain a range of compounds and/or medium additives, each well will have different photophysical properties and cause varying degrees of signal interference and therefore altered signal intensities. This well-to-well signal variation is not due to true differences in light transition, but it can lead to misleading results if only a single emission wavelength is measured.

Cisbio has developed a unique ratiometric measurement of two emission wavelengths (patent US 5,527,684 and foreign equivalents) that corrects for well-to-well variability and signal quenching from assay components and medium variability. Emissions at 620nm (donor fluor) are used as an internal reference while emissions at 665nm (acceptor fluor) are used as an indicator of the biological reaction being assessed. Because both the 620nm and 665nm emissions are decreased by sample interferences, the ratio remains unchanged.

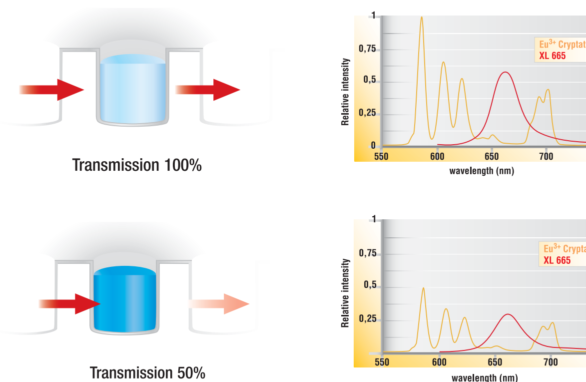


Fig. 5: Signal ratio. The specific signal at 665 nm may be affected by light transmission. The ratio (i.e. 665nm/620 nm) will normalize the signal measured and generate a variable that is independent of the optical properties of the medium in which the interaction is studied. In the case shown above, the 665 nm fluorescence decreases proportionally to the transmission, whereas the ratio corrects this interference and is equivalent in both situations.

A large range of compatible readers

The HTRF technology requires simple but specific detection conditions not necessarily available on all fluorometers.

The readout must be performed at two different wavelengths (typically 620 nm and 665 nm), for the calculation of the fluorescence ratio, and with a time-resolved detection module which enables the introduction of a delay between the excitation pulse and the time-gated measurement window. The wavelength and the energy delivered by the excitation source must also be compatible with the spectral characteristics of Europium and Terbium cryptates.

In order to broaden the HTRF readout capabilities for the Life Sciences domain, Cisbio has initiated collaborations with different detection system manufacturers.

An HTRF sticker clearly identifies the instruments that have been certified. This label guarantees that the performances of the reader meet the specifications for an optimal HTRF readout. A comprehensive and updated list of compatible instruments can be found at www.htrf.com.

Related articles

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Document reference: 68AN2ZZZ MAR08 rev00

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