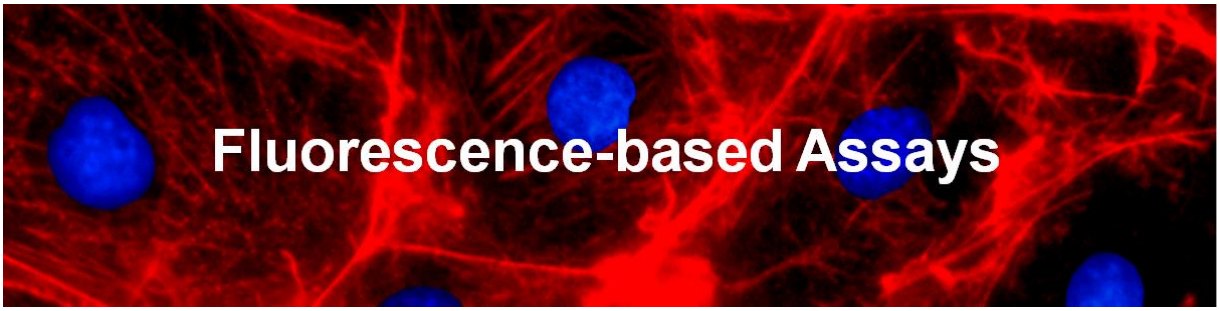

Fluorescence-based Assays

Fluorescent probes are molecules that absorb light of one wavelength and emit light of another, usually a longer wavelength (a process known as fluorescence). Fluorescent molecules are widely used in biological research, and their popularity is growing due to their variety, sensitivity, and quantitative capabilities. Fluorescent probes are used to detect protein localization and activation, identify protein complex formation and conformational changes, and monitor biological processes in vivo, among other things. This representative image was created using immunofluorescence techniques.

The Fluorescence Mechanism

Fluorescent compounds, commonly known as fluorophores or simply fluors, respond differently to light than other molecules. A photon of excitation light is absorbed by an electron of a fluorescent particle, raising the electron's energy level to an excited state. During this brief excitation phase, some energy is wasted by molecular collisions or transferred to a nearby molecule, and the remainder is emitted as a photon to return the electron to its ground state. Because the released photon typically carries less energy and has a longer wavelength than the excitation photon, the emitted fluorescence can be separated from the excitation light. A fluorophore's excitation and photon emission are cyclical, and it can be excited repeatedly until irreparably damaged. Fluorophores can thus emit multiple photons through this cycle of Excitation and emission, and fluorescent molecules are consequently used for a wide range of research purposes.

Fluorophores and their classifications

Fluorescence has been employed in biological study for over a century, but advances in fluorescence chemistry, as well as technological breakthroughs, have fuelled the development of a wide range of fluorophores. Today's broad array of fluorophores offers greater flexibility, variation, and fluorophore performance than ever before for research purposes. Fluorophores are classified into three broad categories:

Organic Fluorescent Dyes

Fluorescent dyes, also known as fluorophores/reactive dyes, are non-protein molecules typically containing numerous aromatic groups or plane or cyclic moieties. Fluorescent dyes are widely used in life science due to their higher photostability, sensitivity, and selectivity in target detection when compared to fluorescent proteins; however, they still suffer from the limitations of small Stokes shifts, insufficient fluorescent efficiency, low photostability, and autofluorescence interference for precise quantification.

Fluorescent Proteins

Fluorescent proteins are genetically encoded protein sequences containing fluorescent amino acids (tryptophan, tyrosine, and phenylalanine), which absorb excitation light and then reemit emission light. Fluorescent proteins are widely used as Biomarkers and Biosensors for monitoring protein-to-protein interaction, visualizing protein localization, and detecting transgenic expression *in vivo***.**

Quantum Dots

Quantum dots are nanometer-sized semiconducting materials with diameters of 2 to 50 nm. Quantum dots, with their unique optical properties, emit light of specific wavelengths based on their shape, material composition, and size if energy is applied to them. Quantum dots

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are widely used for immunolabeling, multiplexed biological detection, and molecular imaging, both in vitro and in vivo assays. One of the main disadvantages of quantum dots is their intermittent luminescence (blinking), which may interfere with some molecular identification methods.

Although those conventional fluorophores have been widely used over the past decades, they are still suffering from either one or *several* limitations in terms of applicability and efficiency*:*

1. Narrow excitation bands cause higher background signals.
2. Smaller Stokes shift often produces self-quenching.
3. Fluorescence is sensitive to environmental factors such as metallic ion concentration, pH, temperature, and solvent polarity.
4. Fluorescence intensity is not high enough for detecting a single biomolecular.
5. Fluorescence intermittency (blinking) affects some processes of molecule detection.
6. Only a few bioconjugatable forms are commercially available.
7. It is easily aggregated because of hydrophobicity.

More improved fluorescence techniques have been developed to address the shortcomings of today's fluorophores. TR-FRET is one of them, and it has proven to be a highly versatile assay technique, allowing researchers to explore a broad spectrum of biological interactions with low to high affinity, employing both small and large compounds.

Time-resolved fluorescence (TRF)

Fluorescence measurement can be divided into two categories: steady-state fluorescence and time-resolved fluorescence. TRF is quite similar to ordinary fluorometric detection. The primary distinction between the two measures is the timing of the excitation/emission process. Excitation and emission are simultaneous during typical fluorometric detection; the light emitted by the sample is measured while Excitation is occurring. On the other hand, TRF relies on the employment of very particular fluorescent molecules known as lanthanide chelate labels, which allow detection of the produced light to occur after Excitation. The europium ion (Eu3+) is the most frequent lanthanide chelate label.

Förster resonance energy transfer (FRET)

Förster resonance energy transfer (FRET) is based on the transfer of energy between two fluorophores in close proximity, a donor (long-lived fluorescence) and an acceptor (short-lived fluorescence) (Fig.1). The level of energy transfer between biomolecules can be measured by labeling each partner with a fluorescent marker and measuring the level of interaction. Organic luminous chemicals such as fluorescein and rhodamine were once commonly utilized in fluorescence assays. However, these bioassays have significant disadvantages in that fluorescent detection is significantly inhibited by noise in the background derived from scattered excitation light and interfered with by fluorescence from coexisting material in the sample (fluorescent compounds and dust/line), making obtaining a highly sensitive measurement difficult.

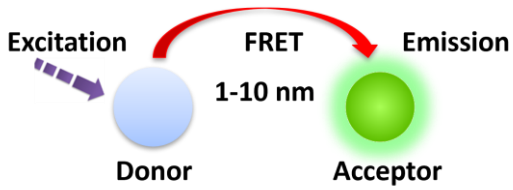

Fig.1 Förster resonance energy transfer (FRET)

BcMag™ TR-FRET Assay

BcMag™ TR-FRET Assay, in contrast to typical FRET assays, uses time-resolved fluorescent magnetic beads (BcMag™ TR-Magnetic Beads) as the donor fluorophore. The donor and acceptor can be two proteins, two DNA strands, an antigen, an antibody, or a ligand and its receptor. After a reasonable time delay (usually 50 to 100 s), a signal is generated by fluorescence resonance energy transfer between a donor and an acceptor molecule when they are close and monitored in a time-resolved way. In BcMag™ TR-FRET Assay, a trace amount of analytes can be easily enriched from the complex by TR-Magnetic Beads, resulting in higher sensitivity. This assay practically eliminates all

fluorescence backgrounds caused by the sample and plastic microplate, as well as by direct acceptor excitation. As a result, the signal-to-noise ratios of the BcMag™ TR-FRET Assay are very high, and the background is quite low. Furthermore, the assay does not need washing steps. BcMag™ TR-FRET Assay offers substantial advantages to bioassays in high throughput screening, such as assay flexibility, dependability, increased assay sensitivity, higher throughput, and fewer false positive/false negative results.

Magnetic bead separation is a fast, effective, and clean method used by scientists to replace filtering, centrifugation, and separation processes. Time-resolved Fluorescence Magnetic Beads can be used for immunoassays and other applications. They have high surface-to-volume ratios, small sizes (0.1-10μm), various functional groups attached to the surfaces (e.g., antibodies, DNA, and chemical groups), and the ability to manipulate the particles via an applied magnetic field easily. Combined with automated liquid handling and robust detection instrumentation, these characteristics enable a wide range of high-throughput applications.

BcMag™ Time-resolved Fluorescence Magnetic Beads

Bioclone offers three unique Time-resolved Fluorescence Magnetic Beads: BcMag™ Europium Fluorescent Magnetic Beads, BcMag™ Terbium Fluorescent Magnetic Beads, and BcMag™ Ruthenium Fluorescent Magnetic Beads (Fig.2). They are *uniform* and *monodisperse* magnetic beads available in nominal *diameters* of 2.5μm and 5μm. The beads are manufactured using nanometer-scale superparamagnetic iron oxide and Europium (Eu^{3+} cryptate) or Terbium (Tb^{3+} cryptate), or Ruthenium (Ru^{2+} cryptate) metal as core and entirely encapsulated by a high purity silica shell, ensuring no leaching problems with the iron oxide and the metal. Their fluorescence properties are summarized in table 1.

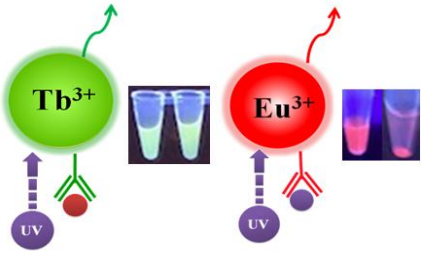

Fig.2 BcMag TRF Magnetic Beads

Fluorophore	Fluorescent color	Excitation (nm)	Emission (nm)	Fluorescence lifetime (τ) (μsec)	Stokes shifts (nm)
Europium ion (Eu^{3+})	Red	340	615	730	275
Terbium (Tb^{3+})	Green	320	545	1050	220
Ruthenium (Ru^{2+})	Far-Red	470	710	354.36	175

Table1. Fluorescent properties of TRF magnetic beads.

Advantages and benefits:

1. Perform a double function simultaneously on the same beads: The magnetic beads combine separation/preconcentration and detect analytes, allowing quick, simple, robust, and high-throughput analytes of trace amounts from complex biological samples on the same beads.
2. Ultra sensitive. Lower detection limits of 10 pg/mL versus typical fluorometric detection limits of 100 pg/mL
3. Extremely photostable and highly resistant to photobleaching. All the lanthanide chelate or cryptate molecules and iron oxide are entirely encapsulated inside each bead instead of merely on the bead's surface. The protective environment prevents iron oxide and dye from leaching into aqueous media, which makes the beads less sensitive to external conditions such as solvent, temperature, pH, etc.
4. Very high fluorescent intensity. Because a single bead has a large concentration of lanthanide chelate with a high quantum yield ranging from 40 to 90%, the beads show excellent fluorescence intensity, which increases test sensitivity without signal amplification. Such bright beads are also perfect for donors' use in time-resolved FRET assays.
5. Lanthanide chelate or cryptate has large Stokes shifts (>250 nm), narrow emission bands (~10 nm bandwidth), and long fluorescence lifetime (μs), which dramatically reduces background and increases the signal-to-noise ratio.
6. Most bioprocess ELISA assays can be converted to an HTRF assay.
7. No washing step is involved in the assays.

8. Have a hydrophilic silica surface grafted by different functional groups with linkers of variable lengths, allowing efficient conjugation of various ligands such as peptides, proteins, antibodies, small molecules, carbohydrates, aptamers, *DNA/RNA, etc.*
9. Due to the microsphere's magnetic properties, the fluorescent magnetic beads are suitable for high-throughput automation.

Workflow of TR-FRET Magnetic Beads Assay (Fig.3)

The TRF beads assay is straightforward. 1. Mix the antibody-conjugated donor beads with the cell lysates and incubate them with continuous rotation for a sufficient time. The beads remain suspended in the sample solution during mixing, allowing the target analytes to bind to the donor beads. 2. After incubation, the beads are collected and separated from the sample using a magnet rack. 3. Add the antibody-conjugated acceptor and incubate them with continuous rotation for a sufficient time. 4. Analysis of numerous microplate readers supports TR-FRET measurements.

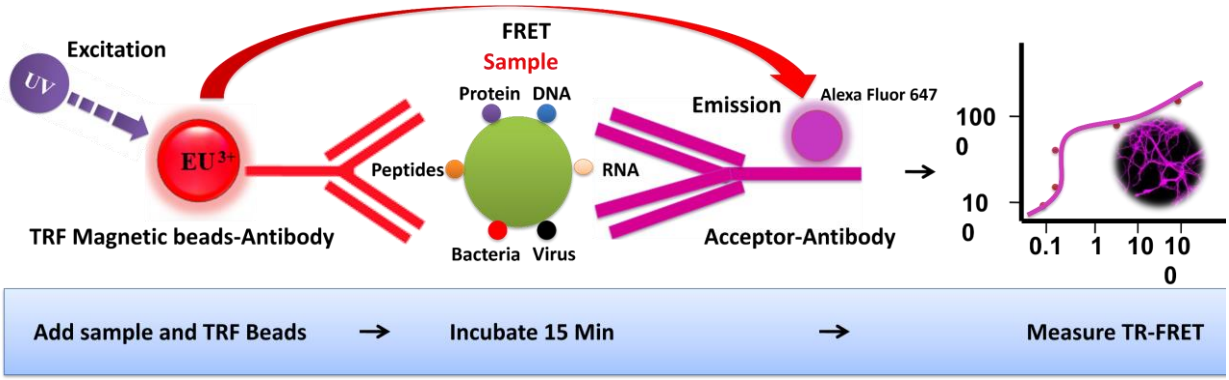

Fig.3 Workflow of TR-FRET Magnetic Beads Assay

Selection of TRF magnetic beads and acceptor

TRF-Magnetic Beas	Excitation	Emission	Acceptor
BcMag™ Europium Fluorescent Magnetic Beads	340 nm	615 nm	Surelight® APC
BcMag™ Europium Fluorescent Magnetic Beads	340 nm	615 nm	AlexaFluor 647
BcMag™ Europium Fluorescent Magnetic Beads	340 nm	615 nm	XL665/d2
BcMag™ Terbium Fluorescent Magnetic Beads	320 nm	545 nm	Fluorescein/GFP
BcMag™ Ruthenium Fluorescent Magnetic Beads	470 nm	710 nm	Far-red dye

Table.2 Selection of TRF magnetic beads and acceptor

Learn more

- Fluorescence Based Assays
- Covalent Conjugation of Fluorophore
- Time Resolved Fluorescence Magnetic Beads

Fluorescence Detection and Measurement

Fluorescence Detection and measurement can be used using the following instruments:

1. Molecular Devices, LLC.
   - The SpectraMax® iD3
   - The SpectraMax® iD5 Multimode Microplate Readers
2. Thermo Fisher Scientific
   - Varioskan LUX Multimode Microplate Reader
3. Medisensor, Inc
   - Qcare™ TRF-S reader

4. BioTek
   - Cytation 1 cell imaging multimode reader
5. PerkinElmer
   - EnVision 2104-0020 Multilabel Microplate Reader Pred 2105
   - EnSpire 2300 MultiMode Microplate Reader
6. BMG LABTECH
   - The PHERAstar® FSX

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