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ULTRASENSITIVE BIOANALYTICAL ASSAYS USING TIME-RESOLVED FLUORESCENCE DETECTION

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Abstract—This article reviews the use of time-resolved fluorimetric detection of lanthanide chelate luminescence as a detection method for ultrasensitive bioanalytical assays. Assay formats and detection methods, and the principle of time-resolved fluorimetric detection, are described. Detection systems, assay formats, reagents, and instrumentation for time-resolved fluorimetric detection are outlined. A review of published and commercially available immunoassays and DNA hybridization assays using time-resolved fluorimetric detection of lanthanide chelate luminescence is given.

Keywords—Immunoassay, DNA hybridization, DNA probing, detection technique, time-resolved luminescence, lanthanide chelate.

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Abbreviations—ALP, alkaline phosphatase; APC, allophycocyanin; BCPDA, 4,7-bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid; DELFIA®, Dissociation-Enhanced Lanthanide Fluoroimmunoassay; DTPA, diethylenetriaminepentaacetic acid; EALL, enzyme-amplified lanthanide luminescence; HRP, horseradish peroxidase; pAS, *p*-aminosalicylic acid; PCR, polymerase chain reaction; SA, salicylic acid; SBR, specific binding reagent; TBP, trisbipyridine cryptate; TSH, thyrotropin.

1. INTRODUCTION: ULTRASENSITIVE BIOANALYTICAL ASSAYS

1.1. Background

Ultrasensitive bioanalytical assays are used routinely in the medical and biological sciences for such applications as cytology, disease diagnostics and monitoring of therapeutic drugs and drugs of abuse. Such assays can determine the concentration or presence of one or more analytes, in cells, serum, urine and other biological fluids, or tissues, at concentrations typically ranging from micrograms to picograms/millilitre. The analyte may be endogenous or exogenous, and can vary in size from a small organic molecule to a macromolecule. A labeled specific binding reagent (SBR), such as an antibody or nucleic acid fragment, is used to permit selective detection of analytes in highly complex samples in the presence of a host of possible interfering species.

The ideal bioanalytical assay would be reliable, accurate, rapid, inexpensive and simple. A critical factor in assay performance is the detection method, that is, the nature of the specific signal generated by the labeled SBR and how it is detected. A variety of highly sensitive detection systems have been developed, most of which rely upon the absorption or emission of electromagnetic radiation by the label itself or by molecules upon which it acts. These include radioisotopic, colorimetric, fluorimetric and chemiluminometric methods. This review covers the current status of bioanalytical assays that use time-resolved fluorescence (luminescence) detection of lanthanide, or other, metal chelates as an ultrasensitive detection method.

1.2. Types of Assays

1.2.1. *Immunoassays*

Immunoassays (Gosling, 1990; Diamandis, 1990) are based on the specific binding reaction of an antibody to a particular antigen (the analyte); they are used for detection and quantitation of any analyte to which antibodies can be raised, in the size range of small molecules to macromolecules, typically in the concentration range of 10^{-7}–10^{-13} mol/L. Immunoassays can be quantitative, semi-quantitative or qualitative. Immunoassays are used routinely in the medical sciences for monitoring levels of hormones, toxins, infectious agents, drugs, tumor markers and other proteins, or metabolites, in serum and other biological fluids, as well as in the field of cytology, hematology, pathology, immunology, veterinary medicine and environmental monitoring.

1.2.2. *Nucleic Acid Hybridization Assays*

Nucleic acid hybridization assays (Diamandis, 1990; Syvanen, 1986; Matthews and Kricka, 1988) are based on the specific binding reaction between a labeled DNA or RNA fragment and its complementary nucleic acid sequence (the analyte). The fragment may be either a short, synthetic oligonucleotide or a long nucleic acid sequence. Nucleic acid hybridization assays are usually semi-quantitative. They are typically used for diagnosis of genetic, malignant or infectious disease, to study disease predisposition or for forensic identification.

1.2.3. *Enzyme Assays*

Enzyme assays (Tijssen, 1985) measure a specific enzymatic reaction between an enzyme and its substrate. The analyte can be the enzyme, whose enzymatic activity is determined, or the substrate or enzyme cofactors. Such assays are used commonly in clinical biochemistry, hematology and cytology.

1.3. Assay Formats

1.3.1. *Homogeneous, or Nonseparation*

This is procedurally the simplest and the most desirable assay format, in which detection reagents are added to a known volume of the sample in solution, and a signal is generated with known relationship to the concentration of analyte. Enzyme assays are typically homogeneous. However, specific binding reactions of antibodies or nucleic acids do not easily permit distinguishing between bound and unbound detection reagent without a separation step. Thus, the homogeneous format has limited applicability in binding assays (immunoassays and nucleic acid hybridization assays); detection systems that do work usually do not permit sensitivities below 10^{-9} mol/L, and in addition, there are a host of possible interferences in biological samples that may alter the assay performance.

1.3.2. *Heterogeneous, or Separation*

This is the most common format for binding assays. The analyte is separated in some way from the remainder of the sample; this is performed by immobilization of the analyte on a solid phase either by a specific binding process, which separates only the analyte, or by adsorption or by chemical reaction, which also separates chemically similar species. Labelled detection reagents which bind specifically to the analyte can be included or subsequently added. At the end, excess unbound detection reagent is washed away. Reliability and sensitivity are usually improved compared with a homogeneous format due to removal of possible interferents, reduced backgrounds due to nonspecific binding and simpler detection reagents.

1.3.3. *Noncompetitive*

The detection SBR is labeled and binds to the analyte either directly or indirectly through other binding reagents. (a) In a heterogeneous format, the analyte is immobilized on a solid surface either directly or in a sandwich format by binding to a different immobilized SBR; excess labeled detection reagent is washed away and the signal is proportional to the concentration of detection reagent and, hence, analyte. Sandwich formats require that the analyte has at least two different sites capable of specific binding with two separate reagents. (b) In a homogeneous format, the bound detection reagent may generate either more or less signal than unbound detection reagent; the signal is then proportional or inversely proportional, respectively, to the concentration of bound detection reagent and, hence, analyte.

1.3.4. *Competitive*

These are performed as either homogeneous or heterogeneous immunoassays for small molecules that do not have two different potential specific binding sites. A known concentration of labeled analyte is added to the sample, which competes with unlabeled analyte for binding to a specific antibody. (a) In a heterogeneous format, excess reagents are washed away, and the signal is proportional to the concentration of immobilized labeled analyte and, hence, inversely proportional to concentration of analyte in sample. (b) In a homogeneous format, signal from the labeled analyte can be quenched or enhanced by binding. These are all generally less sensitive formats, as a low concentration of analyte corresponds to a small change in a large ‘blank’ signal.

1.3.5. *Nucleic Acid Amplification*

The natural function of nucleic acids permits their replication and thus, an assay format can be devised that permits an exponential amplification of their original concentration. This amplification step can be performed specifically for a particular nucleic acid sequence before applying any of the usual assay formats. The most common type of nucleic acid amplification assay uses the polymerase chain reaction (PCR). Such assays are highly sensitive and specific. A number of methods have been developed recently that permit quantitation of the number of copies of the original sequence, for example, via the amplification of an internal standard sequence, or an added competitive template sequence, either of which is present in known concentration in the starting sample.

1.3.6. *Common Assay Formats*

A typical heterogeneous format for a microwell-based binding assay is the ‘sandwich’ format in which one SBR for the analyte is bound to the microwell surface; a different labeled SBR subsequently binds to the bound analyte. Alternately, in the ‘dot blot’ format, the analyte is bound directly to a nylon or nitrocellulose membrane and detected on the surface with one labeled SBR. The ‘Western’, ‘Southern’ and ‘Northern’ blot formats for proteins, DNA and RNA, respectively, are an extension in which the analyte is first spatially separated from other similar species by gel electrophoresis, producing a variety of bands of protein or nucleic acid; then the bands are blotted onto a membrane for subsequent detection of the analyte with labeled SBR.

1.4. Detection Methods

Detection limits for the various labels used in ultrasensitive assays range from 10^{-14} to 10^{-20} mol (6×10^9 to 6×10^3 molecules), with enzyme labels generally having the best detection limits (Beck and Koster, 1990; Carlson *et al.*, 1990; Diamandis and Christopoulos, 1992a). The types of labels and detection methods associated with them are outlined below.

1.4.1. *Radioisotopic Labels*

These are radioactive isotopes, typically β and γ particle emitters, such as ^{125}I, ^{32}P or ^{3}H, which can replace nonradioactive atoms in the SBR with little or no loss of binding affinity or specificity. This detection method has been the workhorse of ultrasensitive bioassays and the benchmark against which reliability and sensitivity are now measured for replacement nonisotopic methods. Low backgrounds, high labeling efficiencies and SBR specificity result in excellent detection limits of analyte (down to 10^{-18} mol). However, the replacement of isotopic methods is inevitable for several reasons. Isotopic labels are environmentally unfriendly, difficult and potentially dangerous to handle, and labeled reagents can have short shelf-life due to radioactive decay. In addition, the method does not support the highest possible sensitivity because an isotopic label generates only one detectable signal event (decay) within its lifetime; as a result, in order to achieve the best detection limits, the measurement times must be prolonged to minutes and in some cases, hours or days.

1.4.2. *Fluorescent Labels* (*Hemmilä, 1989, 1991*)

In fluorescence, a single photon of light is absorbed by a molecule (excitation) and re-emitted at a slightly lower energy, or longer wavelength. In theory, this should be the most sensitive detection method, compared with absorption, chemiluminescence or radioisotopic decay. A single label can be measured many times during a relatively short measurement interval, thus increasing its detectibility. Sufficiently sensitive detection of fluorescence emission permits even single molecule detection. Fluorescently labeled reagents usually have excellent stability. However, in addition to the label, many species in a sample can generate fluorescence emission, and in some cases, nonspecific background signals can overwhelm specific signals.

Typical fluorescent labels, such as fluorescein, are small aromatic organic molecules. High labeling efficiencies are not usually possible due to the excessive chemical modification of SBR, resulting in loss of binding affinity; also, self-quenching of the fluorescence from the label will usually occur. Background fluorescence problems tend to be severe.

Metal chelates, e.g. lanthanide chelates, consist of an organic molecule bound to a metal ion. They have advantages over organic labels; their different emission characteristics make possible selective detection and excellent background rejection. Disadvantages include potentially unstable metal–chelator binding and low fluorescence yields relative to organic labels. These advantages and disadvantages are discussed in more detail in Section 2.2.

1.4.3. *Chemiluminescent Labels* (*Beck and Koster, 1990*)

Such a label consists of an organic molecule, which, when triggered by a chemical reaction, decomposes into an excited fluorescent molecule; a single detectable event per label is given as the original chemical state cannot be regenerated. Low backgrounds are usually achievable and reagents are usually stable on storage.

1.4.4. *Enzymes* (*Tijssen, 1985*)

Enzymes, such as alkaline phosphatase (ALP), horseradish peroxidase (HRP) and β-galactosidase, are widely used as labels. A single enzyme molecule can act upon its substrate (present in large excess) to generate many product molecules in a short time (conversion rates of 100–1000 molecules per enzyme molecule per second). Product molecules are detected by their characteristic fluorescence, chemiluminescence or light absorption. The result is many detectable events per single enzyme label. This signal amplification yields greatly improved detection limits. Backgrounds are generally associated with the method of signal detection and nonspecific binding of the SBR. Enzyme reagents vary in stability.

1.4.5. *Directly and Indirectly Labeled Detection Reagents*

Labels may be attached directly to the SBR, e.g. labeled antibody or nucleic acid fragment, or to the analyte (for a competitive format). Alternately, two SBRs may be utilized, e.g. biotin-labeled antibody and enzyme-labeled streptavidin, using the additional specific binding reaction between biotin and streptavidin (Diamandis and Christopoulos, 1991b).

2. TIME-RESOLVED FLUORESCENCE

2.1. Fluorescence

Luminescence is the most general term for light emission from a chemical species. Fluorescence is a specific type of luminescence, namely light absorption (excitation of an electron from its ground state to an excited state) followed by rapid light emission, from the lowest singlet excited state (generally, but not necessarily, the same as the absorbing state) to the ground state. The emitted light is at a longer wavelength (lower energy) than the absorbed light, as some energy is usually lost to vibrations; usually the emitted energy is spread over a broad band (for organics) in the ultraviolet to visible range of the electromagnetic spectrum. The timescale of the emission is on the order of 10^{-12} to 10^{-6} sec (psec to μsec); the yield is up to 1 photon emitted per photon absorbed.

Also possible, but generally much less probable, is phosphorescence emission from the lowest excited triplet state to the ground state. The wavelength of this emitted light is in the same approximate range as fluorescence, but, on average, of lower energy; the timescale is 10^{-6} to 1 sec; and the yield is usually very low in the liquid phase, as the slower speed of emission makes for a lower probability of occurrence (other competing deactivation processes will be faster).

Processes involving light-emitting transitions between other types of states are usually termed 'luminescence', which covers all such transitions; the term fluorescence is sometimes used loosely to include other such transitions.

2.2. Time-Resolved Fluorescence or Time-Resolved Luminescence

2.2.1. *Fundamental Principles (Gudgin Dickson et al., 1994; Diamandis, 1988; Soini and Lövgren, 1987; Wieder, 1978)*

All processes involving light emission or luminescence do not occur on the same timescale, as discussed in Section 2.1. If excitation is performed in a repetitive timed fashion (using pulsed light), the subsequent emission by different species may have different decay rates dependent on the lifetime of each species. Luminescence from any long-lived species can then be detected selectively by delaying measurement until such time after the excitation pulse that all short-lived species have decayed. Such 'time-gated' or 'time-resolved' measurements can discriminate selectively for long-lived species.

If a sufficiently long-lived luminescent molecule is used as label, the time-resolved fluorometric principle can be utilized in bioassays. Typical background emission in biological samples is due to organic fluorophores and is short-lived. An appropriate long-lived label would have an emission lifetime in the microsecond to millisecond range, with a reasonable fluorescence yield in an environment suitable for the detection step of the assay. In addition, favorable emission wavelength characteristics, such as a large wavelength gap between excitation and emission energies (Stokes' shift) or narrow emission bands, permits additional discrimination against normal fluorescence backgrounds or scattered excitation light, through the use of appropriate wavelength filtering. Application of this principle to detection of lanthanide chelate luminescence is outlined in more detail in the next section.

2.2.2. *Lanthanide Chelates and Other Long-Lived Fluorophores*

Various metal ion chelates can satisfy some or all of the aforementioned criteria. Such chelates consist of a metal ion bound to a chelator, which is an organic aromatic chromophore bearing several metal-binding groups, such as nitrogens and carboxyls. One or more additional chelators or metal-binding species may also be bound to the metal ion to compose the entire fluorescent chelate.

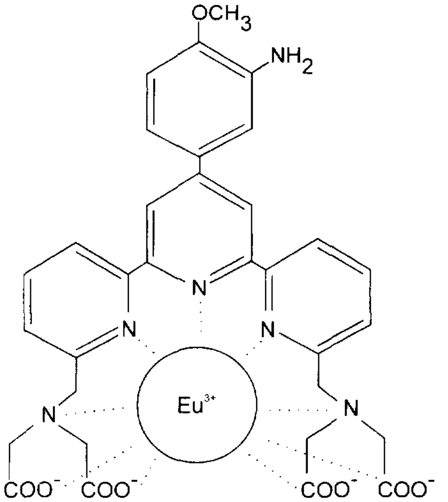


Fig. 1. The structure of a luminescent Eu(III) chelate, Eu(III) 4′-(3-amino-4-methoxyphenyl)-6,6″-bis(*N*,*N*-bis(carboxymethyl)aminomethyl)-2,2′:6′,2″-terpyridine.

In particular, the chelates of the luminescent lanthanides Eu(III), Tb(III) and Sm(III), and of Ru(II), have been used. The structure of a luminescent Eu(III) chelate used as a label is shown in Fig. 1. The light-absorbing chromophore consists of the substituted terpyridine portion, which transfers the excitation energy to the bound Eu(III) ion; chelation is performed by the nine nitrogen and carboxyl groups at the metal binding site.

Figure 2 outlines the principle of utilization of time-resolved luminescence to detect lanthanide chelate luminescence selectively. The particularly favorable emission characteristics of lanthanide chelates are due to the combination of the organic chromophore portion of the chelate, which absorbs the excitation light, with the lanthanide ion, which accepts the excitation energy and emits it at much longer wavelengths. The lanthanide ion luminescence (the transitions do not strictly correspond to fluorescence or phosphorescence) is mainly from a single excited state to a variety of lower states of the ion, and the width of the bands due to the individual transitions is generally in the range of 1–20 nm. The large Stokes' shift permits excellent wavelength filtering against nonspecific background signals arising from scattered excitation light and other luminescent species. The lanthanide chelate lifetime is typically in the range of microsecond to millisecond, and the chelate can have a reasonable luminescence yield because there are unusually few competing deactivation processes. One noteworthy competing or quenching process that does exist is radiationless deactivation of the lanthanide ion by bound water molecules; thus, design of a lanthanide chelate that luminesces with significant yield in water is difficult. The yield of luminescence is also significantly dependent on the efficiency of the energy transfer step, a parameter that is difficult to predict

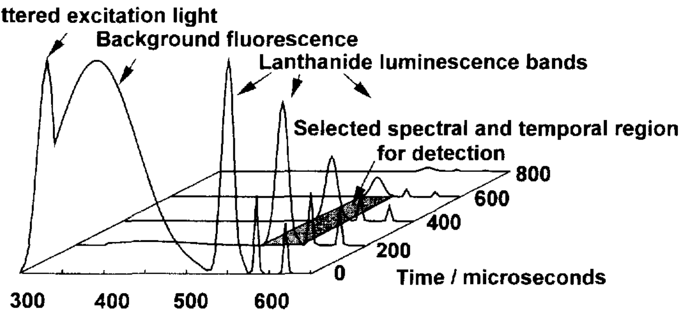


Fig. 2. Principles of time-resolved luminescence measurement. Reproduced from Gudgin Dickson, 1993, *J. Photochem. Photobiol. B* **17**: 293–297, with permission of the copyright holder, Elsevier Sequoia S.A., Lausanne.

Table 1. *Advantages and Disadvantages of Organic and Lanthanide Chelate Fluorescent Labels*

Fluorescent species	Advantages	Disadvantages
Typical organic labels such as fluorescein	Chemically stable, high fluorescence yield Can be used with conventional fluorescence instrumentation	Small Stokes' shift and short fluorescence lifetime; results in interference from background signals Fluorescence yield temperature and environment sensitive
Lanthanide chelates	Large Stokes' shift, narrow-band emission and long luminescence lifetime These properties permit selective discrimination against usual background signals by wavelength and temporal filtering Luminescence relatively insensitive to environment, temperature	Metal-complex stability may be low; requires special handling to ensure chelate binding Relatively low fluorescence yield compared with the best organic fluorophores Specifically tailored time-gated instrumentation required for selective detection

theoretically. The spectra, lifetimes and yields are relatively insensitive to fluctuations in environmentor temperature compared with many conventional organic fluorophores. The major advantages and disadvantages of time-resolved detection of lanthanide chelate labels compared with typical organic labels are outlined in Table 1.

2.2.3. *Instrumentation*

As outlined in Fig. 3, for a time-resolved fluorimetric measurement of a typical lanthanide chelate, the instrumentation must consist of: (i) a pulsed or time-gated light source, such as a flashlamp or laser; (ii) suitable wavelength filtering of the excitation light, if necessary, to provide ultraviolet excitation of the chelator; (iii) a holder to position the sample container or solid support for right angle or surface detection of luminescence, as appropriate; (iv) suitable wavelength filtering of the emitted light (narrow-band or long-pass filters in the visible region, appropriate to the metal ion emission); and (v) a time-gated detector, such that detection is only performed during the period, appropriate to the luminescence lifetime of the metal ion, when the light source is off and short-lived luminescence in the sample has decayed away.

There are a variety of instruments that have been specifically designed to meet these criteria, which are discussed in Section 4. The principles of time-resolved fluorimetric detection with respect to instrumentation components and practical problems in design have been reviewed thoroughly (Soini and Lövgren, 1987).

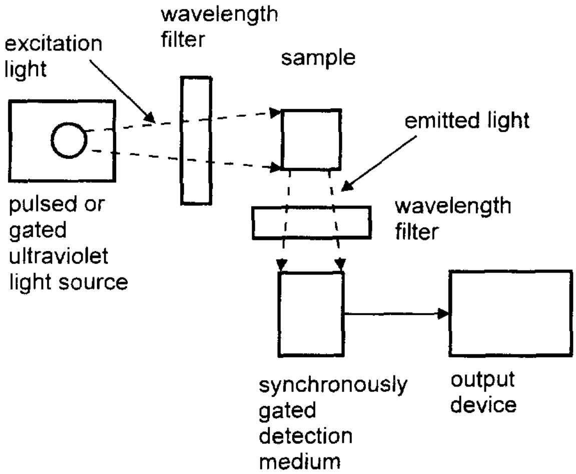


Fig. 3. General layout of time-resolved luminescence instrumentation.

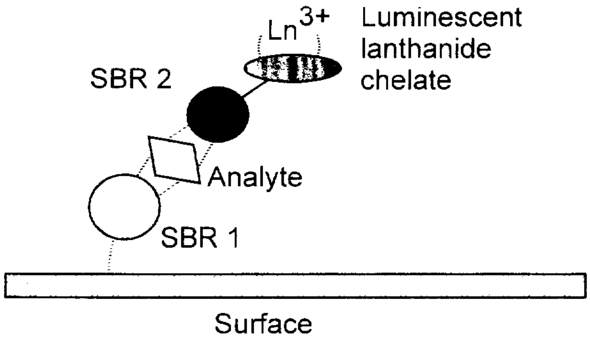


Fig. 4. Heterogeneous assay format using a directly luminescent lanthanide chelate-labeled specific binding reagent.

3. DETECTION SYSTEMS UTILIZING LANTHANIDE CHELATES AND OTHER LONG-LIVED FLUOROPHORES

Desired design characteristics for a time-resolved luminescence-based label and detection system suitable for ultrasensitive bioassays include: high luminescence yield of the chelate; high chelate binding stability; simple chemical synthesis of the chelate reagents; simple labeling methods; solubility of reagents forming the chelate in an aqueous or nonaqueous medium; good reproducibility of the method; low nonspecific binding of the labeled reagents to the solid phase; good homogeneity of the solid phase, if measurements are carried out directly on the solid phase; freedom from interferences caused by constituents of the sample or contamination by the environment; reliable, inexpensive instrumentation; the fewest and shortest possible required assay steps; and stable reagents, including resistance to chemical- or light-induced degradation of the chelate.

These requirements have been difficult to meet in a single system. Each system devised has had its strengths and weaknesses; research continues in an effort to achieve the ideal candidate for the task. This section outlines the lanthanide and ruthenium chelates that have been used in detection systems for bioassays. Section 4 describes the types of instrumentation that have been used for time-resolved luminescence detection, with emphasis on specialized and commercially available instrumentation.

3.1. Labelling with Luminescent Lanthanide Chelates for Heterogeneous Assay Formats

Figure 4 illustrates the principle of using a directly luminescent lanthanide chelate-labeled SBR in a heterogeneous sandwich format. This type of labeled reagent is the most desirable for use in heterogeneous and homogeneous assays because of the potential simplicity, stability and minimal susceptibility to contamination of the detection reagents. If the binding constant of lanthanide ion to the chelate is sufficiently high (e.g. $\geqslant 10^{10}\,\mathrm{M}^{-1}$), the lanthanide ion can be carried quantitatively through the assay in the bound form, and there is no need for addition of the lanthanide ion at the end of the assay. There is no problem of contamination by exogenous lanthanide ions because signal is proportional to the quantity of the chelator present, and excess lanthanide ion will not generate more signal. Signal remains spatially localized at all times in heterogeneous assays. *In situ* cellular detection should be possible with a suitable detection reagent. So far, however, no one luminescent lanthanide chelate label that can be used in all types of potential heterogeneous assay formats has been demonstrated. Common problems are: relatively low luminescence yield, which can result in poor detection limits, or require heavy labeling of the SBR, with potential resulting modification of its specific and nonspecific binding properties; unsuitability for aqueous detection; and insufficiently high chelate stability, requiring excess chelate components to be added in the final step to promote formation of the luminescent chelate. Thus most reagents have been optimized for one specific assay format.

The possible assay formats and labels that have been used are outlined below, and a selection of representative direct labels and chelate reagents from this list and their properties are given in Table 2.

Table 2. *Luminescence Characteristics and Detection Limits of Some of the Labels and Chelates that have been used in Time-Resolved Fluorescence-Based Assays*

Label or detectable species	Emission maximum of strongest band/nm	Lifetime of metal complex/μsec	Detection limit of single label	Reference
Eu(III) TBP	618	600	NR*	Prat *et al.*, 1991; Mathis, 1993
Tb(III) 1-(*p*-benzene-diazonium)-EDTA	NR	1000	NR	Kuo *et al.*, 1985
Tb(III) DTPA-pAS	545	NR	10^{-10} M	Siepak, 1989
Eu(III) terpyridine-bis-(methyleneamine)tetra-acetic acid	615	1400	3×10^{-17} mol	Saha *et al.*, 1993
Eu(III) BCPDA	613	400–700	10^{-11} M	Evangelista *et al.*, 1988
Tb(III) phosphor particles	546	500	NR	Beverloo *et al.*, 1992
Eu(III) phosphor particles	612–616	700–2000	NR	Beverloo *et al.*, 1992
Ru(II) *tris*-(bathophen-anthroline)	616 (broad)	1–8	10^{-14} M	Bannwarth *et al.*, 1988
Eu(III), detected in enhancement solution	613	700–900	3.5×10^{-14} M	Xu *et al.*, 1992b; Hemmilä, 1988b
Tb(III), detected in enhancement solution	543	500	3.4×10^{-13} M	Xu *et al.*, 1992b; Hemmilä, 1988b
Sm(III), detected in enhancement solution	643	60	7.9×10^{-12} M	Xu *et al.*, 1992b; Hemmilä, 1988b
Dy(III), detected in enhancement solution	573	approximately 1	4.6×10^{-11} M	Xu *et al.*, 1992b; Hemmilä, 1988b
ALP; product fluorosalicylic acid-Eu(III)-EDTA	— 546	— 1200	2×10^{-15} M; 1×10^{-9} M	Evangelista *et al.*, 1991; Christopoulos and Diamandis, 1992

*Not reported.

3.1.1. *Direct Solid Phase Detection*

The commercially available FIAgen™ detection system uses Eu-BCPDA (4,7-bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid) as label. In this system, Eu-BCPDA luminescence is typically detected from the labeled SBR immobilized and dry on an opaque solid phase such as a white microtiter well. Synthesis and characterization of BCPDA (Evangelista *et al.*, 1988) and general labeling procedures (Diamandis and Morton, 1988) have been reported. A variety of Eu-BCPDA–protein labels have been used in the FIAgen™ detection system, including directly multiple labeled antibodies, multiple labeled protein-conjugated antibodies, multiple labeled streptavidin, multiple labeled thyroglobulin–streptavidin and macromolecular complexes of the latter reagent (Diamandis *et al.*, 1989b; Morton and Diamandis, 1990; Diamandis, 1991; Reichstein *et al.*, 1988). Detection limits improve 5–50 times by using the thyroglobulin-containing reagents or macromolecular complexes compared with directly BCPDA-labeled streptavidin reagent (Diamandis, 1991).

A europium chelator of unspecified structure, W1014, has been reported for use in immunocytochemistry (Soini *et al.*, 1988b). It is recommended for dehydrated histological sections, as water quenches the signal. It has a lower signal and lower signal stability than fluorescein for comparable staining protocol, when viewed in a steady-state UV microscope.

Europium trisbipyridine cryptate, TBP(Eu(III)), (Prat *et al.*, 1991; Lopez *et al.*, 1993), has been used to label an anti-DNA-hybrid antibody for use in DNA hybridization assays.

A chelate that combines most of the desirable properties of a direct label, Eu(III) terpyridine-bis-(methyleneamine)tetraacetic acid (Saha *et al.*, 1993), has been synthesized recently and used to label DNA and antibodies. It has a good luminescence yield and long lifetime, as well as a high Eu(III) binding constant. It has shown excellent detection limits in the preliminary report.

An immunoassay for human choriogonadotropin has been demonstrated using the Eu(III) cryptate-type complex of 7,10-bis(chlorosulfophenyl)-2,15-diaza[3.3](2,9)-1,10-phenanthrolinenophane-N^2,N^{15}-diacetic acid as label (Sasamoto *et al.*, 1992).

The ternary complex Tb(III)-*p*-aminosalicylic acid–diethylenetriaminepentaacetic acid (Tb-pAS–DTPA) and related complexes have been used as labeling reagents (Bailey *et al.*, 1984; Siepak, 1989), and immunoassays performed (Canfi *et al.*, 1989). The same reagents have been used for labeling DNA for gel electrophoresis detection (Saavedra and Picozza, 1989) with detection by extraction of the labeled DNA from the gel. A similar Tb chelate-labeled psoralen has been used for photolabeling of oligonucleotides (Oser *et al.*, 1990). RNA detection has been performed with a similar label (Bush *et al.*, 1991).

Tb(III) aminophenylpyridine labels have been used in immunoassays (Dakubu *et al.*, 1988) and compared with the dissociative enhancement method.

Tb(III) 2-alkoxy-4,6-bis-(*N*,*N*,*N*′,*N*′-tetraalkyl)-aminotriazines have been used for labeling oligonucleotides (Sherman *et al.*, 1992).

Tb(III) (1-*p*-benzene-diazonium)-ethylenediaminetetraacetic acid has been used as a label for competitive immunoassay (Kuo *et al.*, 1985); a competition step was performed between labeled antigen and unlabeled antigen for immobilized antibody, followed by solution detection of the remaining unbound labeled antigen.

In addition, a large variety of chelators forming luminescent complexes with Eu(III) or Tb(III) have been reported by various groups and patented for use in binding assays or as labeling reagents; these are generally based on substituted phenols, phenylpyridines, phenylterpyridines and phenanthrolines (examples include Hinshaw *et al.*, 1983; Hale and Solas, 1986; Kankare *et al.*, 1986; Wieder and Hale, 1987; Toner, 1989; Sherman *et al.*, 1992).

3.1.2. *Immunocomplex Dissociation Assays*

In such assays, the immunocomplex is dissociated from the solid phase and the luminescence of the lanthanide chelate-labeled SBR is detected in solution. Such assays have the advantage of a homogeneous solution phase distribution of detected signal compared with the distribution on the dry solid phase, which may vary, depending on experimental conditions; this improves the assay precision.

Immunocomplex dissociation assays have been performed using BCPDA as label with biotinylated antibody and Eu-BCPDA–thyroglobulin-labeled streptavidin or Eu-BCPDA-labeled streptavidin detection reagents (Kropf *et al.*, 1991; Papanastasiou-Diamandis *et al.*, 1992b).

A patent for a similar type of immunocomplex dissociation-based assay has been reported using another Eu(III) chelate. The chelator is a derivative of 4-aminophenylethynyl-2,6-bis-[*N*,*N*-bis-(carboxymethyl)-aminomethyl]-pyridine isothiocyanate disulfide, which has been reacted to bind with a monoclonal anti-thyrotropin (TSH) antibody (Kankare, 1992).

3.2. Labelling with Nonluminescent Lanthanide Chelates and Dissociative Enhancement

This has been the most popular of time-resolved fluorometric detection systems, and is commercially available as DELFIA® (Dissociation-Enhanced Lanthanide Fluoroimmunoassay; Wallac Oy, Turku, Finland). The principle of the method for heterogeneous assay formats is outlined in Fig. 5. In general (Hemmilä, 1988b), the SBR is labeled with a nonluminescent lanthanide chelate, for example, diazophenyl-ethylenediaminetetraacetic acid-Eu(III). The lanthanide ion is carried quantitatively through the assay bound to the chelator. As the final step of the assay, the lanthanide ion is dissociated from the nonluminescent complex by reducing the pH to $\leqslant 3$, which reduces the chelator binding affinity for the lanthanide. The lanthanide ion is solubilized into a micelle containing additional reagents that form a luminescent chelate with the lanthanide ion. These reagents include energy transfer reagents, such as fluorinated aromatic β-diketones, to promote absorption and energy transfer to the lanthanide ion, and synergistic reagents, to increase the luminescence yield. The DELFIA® assay (Hemmilä *et al.*, 1984) is characterized by excellent reproducibility and sensitivity. The main disadvantages of the method are the necessity for solution phase detection, which results in the loss of spatial localization of signal, and susceptibility to exogenous Eu(III) contamination.

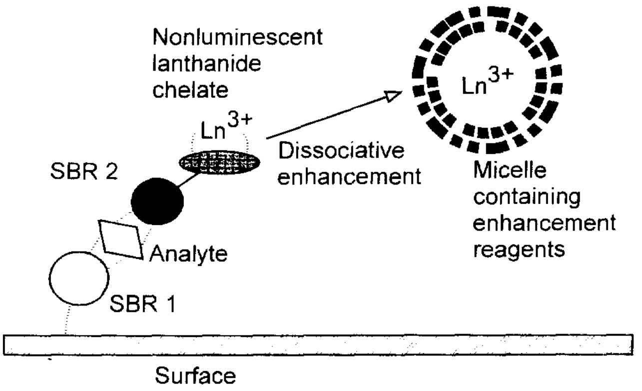


Fig. 5. Heterogeneous assay format using an SBR labeled with a nonluminescent lanthanide chelate, with detection of lanthanide luminescence by dissociative enhancement.

Subsequent improvements on the DELFIA^{R} and other dissociative enhancement assays include the use of stronger chelating reagents, more than one lanthanide ion in multianalyte assay formats, and modified enhancement solutions, as outlined below.

Labelling reagents originally employed the chelator ethylenediaminetetraacetic acid to bind the lanthanide ion (Hemmilä, 1988b); this chelating group has been replaced with DTPA, which binds the lanthanide ion more strongly, ensuring that it will be carried quantitatively through the assay procedure (Mukkala *et al.*, 1989).

The use of Sm(III) ion as label has been reported (Bador *et al.*, 1987), but the sensitivity is at least 10-fold inferior in comparison with Eu(III). Dual labeling has been performed with Eu(III) and Sm(III) (Saarma *et al.*, 1989; Ståhlberg, 1993) or Eu(III) and Tb(III) (Siitari, 1990; Hemmilä, 1988b; Hemmilä *et al.*, 1987) to permit simultaneous detection of two different antigens in immunoassay. Quadruple labeling has been performed with Eu(III), Tb(III), Sm(III) and Dy(III) (Xu *et al.*, 1992b); the different lanthanides were measured simultaneously using different filters and gating times, appropriate to each. The detection limits of the various luminescent lanthanide ions in this format are given in Table 2.

The original enhancement reagent for Eu(III) consisted of 15 μM β-napthoyltrifluoroacetone, 50 μM trioctylphosphine oxide and 0.1% Triton X-100 detergent in 0.1 M phthalate buffer, pH 3.2 (Hemmilä *et al.*, 1984). Alternate fluorescence enhancement reagents have been developed, for example, using a different synergistic reagent (Degan *et al.*, 1990) or energy transfer reagent (Keelan *et al.*, 1987) or using co-fluorescence enhancement, in which the enhancement solution contains an alternate synergistic reagent and, in addition, an enhancing lanthanide ion such as Y(III), which forms additional chelates acting as energy donors (Xu *et al.*, 1991, 1992a; Xu, 1992; Xu and Hemmilä, 1992a,b).

3.3. Homogeneous Assays

A variety of approaches has been used to design homogeneous assay formats with time-resolved fluorimetric detection. All involve some form of luminescence signal enhancement or quenching as a result of a binding reaction between the analyte and at least one SBR. To the sample containing the analyte may be added two labeled SBRs, in two-site binding format, or alternately, in a competition format, labeled analyte and an SBR are added.

3.3.1. *Energy Transfer to Lanthanide Ion Cryptates*

This method utilizes energy transfer from a lanthanide ion cryptate (the donor), which absorbs the excitation light, but has a low (but non-zero) luminescence yield itself, to an organic chromophore (the acceptor), which also has low luminescence yield in the absence of the donor. This energy transfer can only occur when the donor and acceptor are reasonably close to each other.

For homogeneous immunoassays, this requirement is satisfied in a two-site binding assay when donor and acceptor are used to label different SBRs to the same analyte and the ternary SBR complex with the analyte is formed. Thus, signal enhancement from the acceptor occurs only in the presence of the analyte.

This principle has been demonstrated with the donor–acceptor pair (TBP-Eu(III)) (the donor) and allphycocyanin (APC, the acceptor) (Mathis, 1993) (see Fig. 6). APC has an absorption band overlapping the TBP-Eu(III) emission. One monoclonal antibody against the analyte is labeled with TBP-Eu(III), another with APC; in the presence of analyte, the donor and acceptor are close enough for energy transfer to occur. In this detection system, the emission has the spectral characteristics of the acceptor (broad-band organic-type emission monitored at 665 nm); however, because of the energy transfer process, the APC emission timescale is delayed to match the lifetime of TBP-Eu(III) (lifetime ca. milliseconds). Thus, time-resolved fluorimetric detection at two different wavelengths can be used to monitor the donor and bound acceptor emission; the free acceptor emission is not measured as its lifetime is short.

This format overcomes many of the problems associated with interference due to light absorption by serum components. This is a feature of the simultaneous dual-wavelength monitoring of donor and acceptor emission (the instrumentation required is described in Section 4.1). Because the amount of free donor is always far in excess of that bound in the analyte complex, the free donor concentration can be taken as a constant and the luminescence contribution from the bound donor is minor. The luminescence from the free donor, at 620 nm, thus can be monitored as a reference intensity. This reference intensity may vary due to absorption of the excitation light by interfering serum components, as will the bound acceptor emission. However, the ratio of the acceptor to donor signal is unaffected by interfering absorption, and depends only on the concentration of the analyte. Detection limits in this immunoassay are reported to be comparable to heterogeneous immunoassay with radioisotopic detection, thus this detection system seems to be very promising.

3.3.2. *Ternary Chelate Formation*

In this format, signal is generated by a luminescent ternary lanthanide ion complex, which is formed only when the ion is near the two other components of the ternary complex. Two different SBRs must react with the analyte: one is labeled with a strongly bound, nonluminescent lanthanide ion

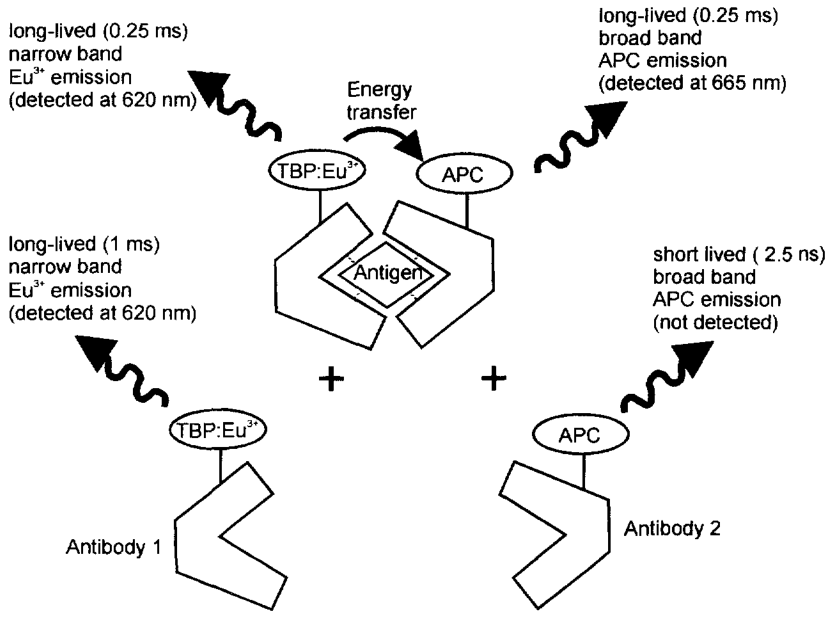


Fig. 6. Emission characteristics of bound and unbound reagents present in a homogeneous two-site binding assay, using detection by energy transfer from a lanthanide ion cryptate.

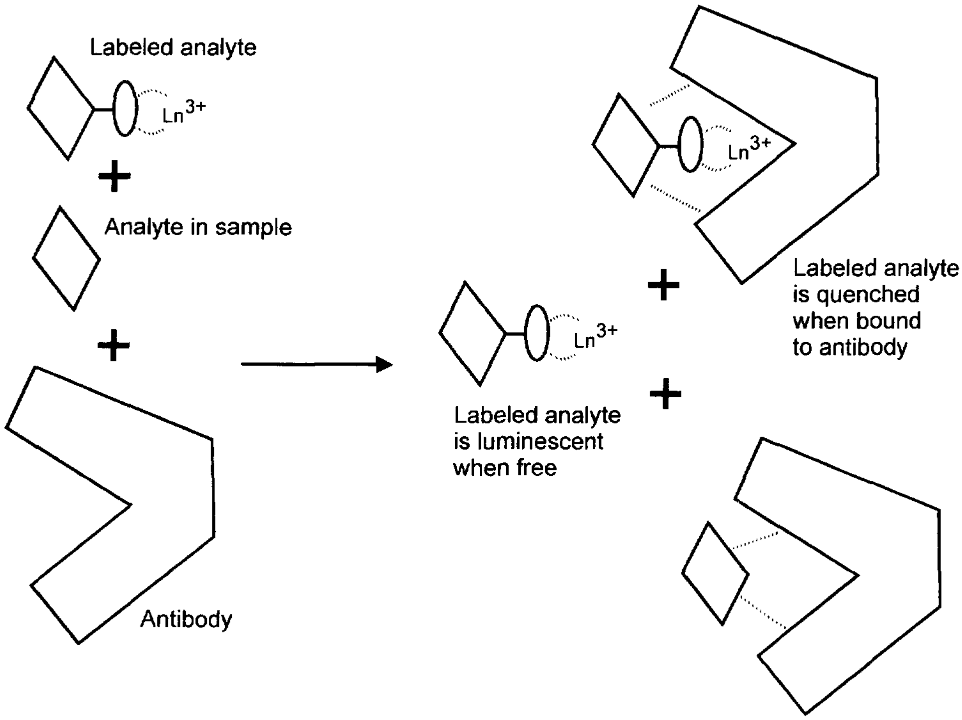


Fig. 7. A homogeneous competitive quenching assay.

chelate (just as in a dissociation enhancement-based assay); the other is labeled with the additional chelating agent responsible for light absorption and energy transfer. With the proper assay design, the ternary luminescent complex is formed only when both SBRs are bound to the analyte. A ternary Tb(III) complex has been used to detect nucleic acid hybridization, where two oligonucleotides, one labeled with *p*-AS and one with Tb(III)-DTPA, are bound to the analyte, a single template complementary strand (Oser and Valet, 1990). The two oligonucleotide-label pairs are oriented such that if the oligonucleotides are both bound, the ternary Tb(III) complex is formed and luminescence can occur. The detection limit of template DNA is 1 nM.

3.3.3. *Competitive Quenching Assay*

This assay format, illustrated in Fig. 7, has been reported using W1174, a fluorescent europium chelate, as label (structure not given) (Hemmilä *et al.*, 1988); the chelate luminescence is quenched by the binding of the antibody, permitting homogeneous assay detection. The method was deemed to be appropriate for assays that would benefit from rapidity, but do not require high sensitivity.

3.4. Enzyme-Amplified Lanthanide Luminescence

The principle of this method (Gudgin Dickson, 1993) is outlined in Fig. 8. An enzyme is used as label. In the usual configuration, the enzyme substrate is a species that is incapable of forming a luminescent lanthanide chelate; upon conversion by the enzyme, the resulting product species forms a luminescent chelate when a lanthanide ion and possible co-chelators are added. The requirement of this system is that the chemical alteration sufficiently changes the chelating or spectroscopic characteristics of the substrate/product pair to permit efficient distinction between the two by time-resolved luminescence detection (Evangelista *et al.*, 1991). This method has potentially the highest sensitivity of all existing lanthanide-based detection systems, because of the very high amplification factors possible using enzyme labels; this method has demonstrated the best single label detection limit compared with other reported time-resolved fluorimetric detection methods (see

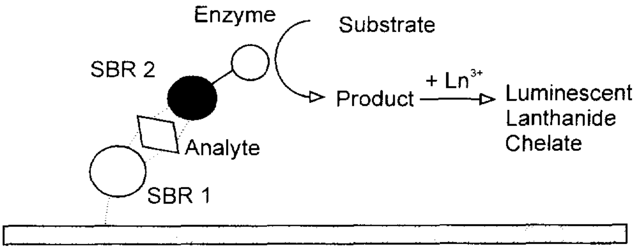


Fig. 8. Heterogeneous assay format using enzyme amplified lanthanide luminescence.

Table 2). Enzyme-amplified lanthanide luminescence (EALL) is potentially flexible, and has been demonstrated for assays in which signal localization is desirable. Substrates potentially can be designed for a variety of enzyme assays in which the enzyme is the analyte or the label. One disadvantage is the requirement for an extra signal development step during which substrate to product conversion takes place, before the addition of lanthanide ion to form the luminescent chelate.

Assays that use an ALP label and various substrates based on phosphates of salicylic acid (SA) derivatives have been reported (Gudgin Templeton *et al.*, 1991, 1992; Yu and Diamandis, 1993). Commercially available substrate reagents include; fluorosalicylphosphate or diflunisal phosphate, for solution phase detection, or *tert*-octylsalicylphosphate for assays requiring spatial localization of signal, e.g. membrane-based detection. The product of the enzymatic reaction, i.e. fluorosalicylic acid, diflunisal or *tert*-octylsalicylic acid, is detected subsequently as the luminescent ternary complex with Tb(III) ion and EDTA, at basic pH; the detection step also terminates the enzymatic reaction. This reaction can also be followed by monitoring the blue fluorescence of the dephosphorylated organic product, but sensitivity is inferior compared with time-resolved detection (Evangelista *et al.*, 1992). 4-Methylumbelliferone phosphate has been used as an ALP substrate in a TSH assay, in a reverse manner from the usual EALL format. In this application, the substrate forms a luminescent Eu(III) complex and the product does not (Diamandis, 1992).

A variety of detection reagents for other enzymes have been reported (Evangelista *et al.*, 1991). These include the glucose oxidase substrate 1,10-phenanthroline-2,9-dicarboxylic acid dihydrazide, giving the detectable chelate Eu(III)-phenanthroline–decarboxylic acid, and the xanthine oxidase and β-galactosidase substrates salicylaldehyde and salicyl-β-galactoside, respectively, both giving the detectable chelate Tb(III)-EDTA–SA.

3.5. Less Commonly Used Labels or Assay Formats

3.5.1. *Enzyme Amplified Covalent Attachment Assay*

In this assay, the immunoreactants are labeled with HRP. A typical solid-phase sandwich or competitive assay is performed and the HRP–SBR immobilized on the solid phase. The HRP is then provided with substrate, a biotin–tyramine conjugate, catalyzing the covalent attachment of biotin to the solid-phase immobilized proteins; the biotin is quantified with BCPDA-labeled streptavidin macromolecular complex reagent (Diamandis *et al.*, 1991).

3.5.2. *Liposome- or Target-Cell Encapsulated Lanthanide*

Eu-DTPA has been encapsulated in liposomes, and conjugated to antibodies for immunoassays (Vonk and Wagner, 1991); Eu(III) is released and detected by a typical dissociative enhancement step. Numerous patents have been filed on signal amplification with the use of latex beads or liposomes loaded with Eu(III) chelates (Frank and Sundberg, 1981; Burdick and Danielson, 1986; Schaeffer *et al.*, 1986; Wagner and Baffi, 1987a,b). Target cells have been labeled by incubation with Eu(III) ions, and used in cytotoxicity assays (Maley and Simon, 1990; Granberg *et al.*, 1988; Volgmann *et al.*, 1989) in which Eu(III) ion is released and measured in enhancement solution.

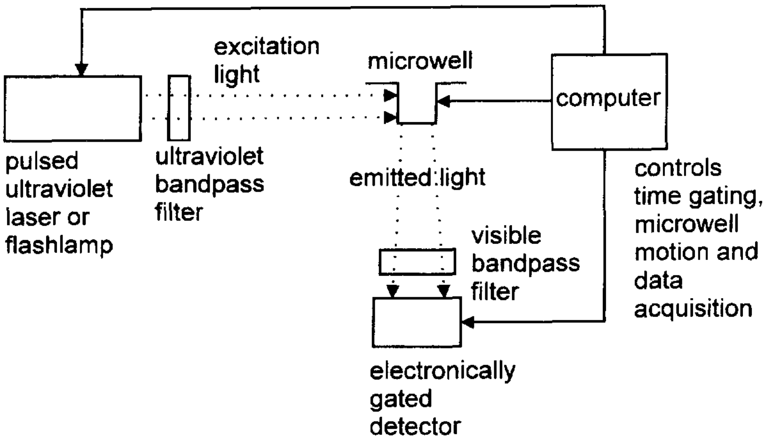


Fig. 9. Typical layout of a time-resolved luminescence microwell reader.

3.5.3. *Ruthenium Labels*

Ru(II) tris-(bathophenanthroline) complexes (Bannwarth *et al.*, 1988) have been used as labels for time-resolved fluorimetric detection, for example linked to DNA strands that have been modified with an aminoalkyl arm. The properties of a typical ruthenium-based label are outlined in Table 2. A ruthenium-tris-(phenanthroline)$^{2+}$-based reagent has also been used for protein labeling (Thompson and Vallarino, 1988) and immunoassays (Mueller and Schmidt, 1986).

A ruthenium dipyridophenazine complex has been used to label oligonucleotides and detect sequence-specific sections of single-stranded DNA, as it is only luminescent when intercalated in double-stranded nucleic acid after hybridization (Jenkins and Barton, 1992).

3.5.4. *Lanthanide Phosphors*

These consist of charge-stabilized suspensions of 0.1–0.3 μm phosphor particles (e.g. yttrium oxysulphide activated with europium or terbium, along with others doped with other metals) (Beverloo *et al.*, 1990, 1992); the suspensions are stabilized with polycarboxylic acids. Proteins are conjugated to the surface of the particles by physical adsorption. Properties of some of the phosphor labels are outlined in Table 2.

Immunocytochemistry has been performed on lymphocytes or erythrocytes by indirect labeling using a primary antibody, and a secondary antibody to it labeled with phosphor particles. Multiple labeling was also performed using different SBRs (avidin and antidigoxigenin antibodies) conjugated to different color phosphors. The progress of immunoreaction could be monitored directly under a time-resolved microscope. This method is not suitable for *in situ* hybridization inside cells, because the phosphor particle exceeds 1 nm in size and hence, will not penetrate the cell.

4. TIME-RESOLVED LUMINESCENCE INSTRUMENTATION

4.1. Tube, Cuvette or Microwell Readers

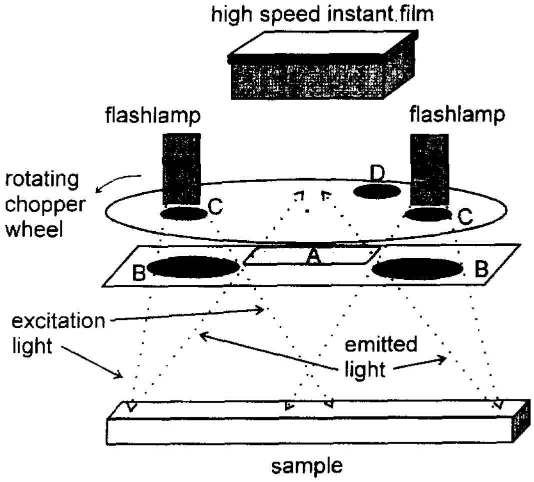
A number of commercially available multi-purpose spectrofluorometers are capable of time-resolved detection in the microsecond to millisecond regime using single-sample cuvettes. In addition, a number of instruments have been modified or designed for time-resolved detection of lanthanide chelate luminescence. The typical layout of such readers is illustrated in Fig. 9. Typically, the excitation conditions are fixed, with potentially varying emission conditions, i.e. one or more emission filters or time gates may be possible. The majority are designed to observe luminescence at right angles to the excitation beam through a transparent sample holder. The original designs were test tube readers for solution-phase fluorescence; variations included a tube reader using a nitrogen laser for excitation (Dechaud *et al.*, 1986) and the use of a flashlamp for ultraviolet excitation in the original commercial tube reader (Soini and Kojola, 1983) for DELFIA®. A conventional photon-counting fluorimeter that accepted cuvettes was modified with a chopped He–Cd laser at 325 nm, and delayed chopped detection at 550 nm, for quantitation of Tb(III)-labeled materials (Kuo *et al.*, 1985).

The DELFIA® tube reader subsequently was modified to automatically read transparent microwell strips (Soini and Lövgren, 1987). A commercial microwell plate reader (Diamandis *et al.*, 1989a) for the FIAgen™ system (CyberFluor Inc., Toronto, Canada) was developed for surface fluorescence measurement using pulsed nitrogen laser UV excitation at 337 nm and electronically gated detection. In this case, the labeled SBR is immobilized on an opaque microwell bottom. This instrument has also been modified to scan membranes (Christopoulos *et al.*, 1991) and agarose gels (Chan *et al.*, 1993). An instrument that also measures surface fluorescence in microwells, using a flashlamp for excitation, has been reported (Prat *et al.*, 1991). A double wavelength emission detection plate reader has been developed recently (Mathis, 1993) for the homogeneous energy transfer assays with TBP-Eu(III) as donor and APC as receptor; this reader uses a nitrogen laser for excitation and two filtered, gated detectors at right angles; detection of the emission of both Eu(III) and APC allows for correction for light filtering effects of the biological sample in homogeneous assays. A fluorescence particle concentration time-resolved microwell reader for use with polystyrene capture beads has been reported (Bush *et al.*, 1991).

Most recently, the commercial AutoDELFIA™ system (Wallac Oy, Finland) has been developed, with assays fully automated for batch processing and random access of up to 423 sample tubes, with up to 8 tests performed per tube. This represents an advance in the utility of time-resolved fluorescence-based detection for routine applications in both large and small clinical laboratories.

4.2. Photographic Detection

A commercial unit for time-resolved photography (Gudgin Templeton *et al.*, 1992) has been reported (see Fig. 10), with pulsed UV excitation from two flashlamps and gated emission detection in the visible, selectable by filter; two different time-gating settings are available. The excitation and emission light are gated by a rotating chopper; in the chopper position shown in the figure, the excitation light is passed by the apertures, while the emitted light is blocked by the chopper wheel.



A: visible wavelength emission filter; B: ultraviolet excitation filters; C: excitation apertures; D: emission aperture

Fig. 10. Time-resolved photography unit. In the position of the rotating chopper wheel shown, the lamps irradiate the sample through apertures C while the camera is blocked. As the wheel rotates, the lamps are blocked while aperture D moves under the camera to permit the time-delayed luminescence to be detected.

Subsequently, when the emission aperture rotates to permit the emitted light to strike the film, the flashlamps are gated off. This sequence is repeated as needed to sufficiently expose the film. The instrument records time-resolved lanthanide emission using fast instant film in order to obtain a qualitative or semi-quantitative picture of the distribution of signal on a membrane or gel surface or in a microwell plate. It is suitable for use with long-lived chelates such as those of Tb(III) and Eu(III). Its use has been reported for membrane and microwell DNA hybridization assays (Gudgin Templeton *et al.*, 1991) and microwell enzyme and immunoassays (Evangelista *et al.*, 1991).

4.3. Microscope

The time-resolved luminescence microscope (Beverloo *et al.*, 1990, 1992) is similar in concept to the time-resolved photography unit, and has been used for observing phosphor labels *in situ*. It is an epifluorescence microscope, modified with pulsed excitation light and emitted light gated by a rotating chopper wheel at the eyepiece. It possesses broad-band UV excitation and detects all species emitting at >430 nm, within a specified gating interval; typically this interval consists of a 300 μsec delay (available range 100–2000 μsec), and approximately 4.5 msec observation window. A cooled CCD camera has been used to record images. A non-time-resolved far-UV microscope has also been used to observe multiple phosphor labels.

4.4. Other Detection Instrumentation

Quantitation of tetracycline has been performed of a Eu-tetracycline micellar stabilized complex by using a HPLC with a time-resolved fluorescence detection attachment (Duggan, 1991).

5. REVIEW OF PUBLISHED AND COMMERCIALLY AVAILABLE ASSAYS

5.1. Immunoassays

5.1.1. *Background*

A text on fluorescence immunoassay principles has been published by Hemmilä containing an exhaustive review of published immunoassays that have used lanthanide-based detection up to 1990 (Hemmilä, 1991). The principles of time-resolved fluorescence-based immunoassays and the DELFIA® and FIAgen™ immunoassay detection systems have been extensively reviewed (Diamandis, 1988; Soini, 1984; Hemmilä, 1985, 1988a,b; Ekins and Dakubu, 1985; Soini and Lövgren, 1990).

A wide variety of immunoassays has been developed and evaluated, with the majority using the dissociative enhancement principle for detection. The types of antigens detected cover a broad spectrum, including infectious agents, proteins and enzymes, hormones, drugs and toxins. There has been comparatively little work in the field of cytochemistry and *in situ* hybridization, due to the dearth of suitable labeling systems; the optimum labeling system for this application would require a direct labeling reagent, containing multiple luminescent lanthanide chelates with extremely high binding constants, exhibiting low nonspecific binding and good cellular penetration, and luminescence in aqueous solution around neutral pH. One possible new candidate Eu(III) label has been reported recently (Saha *et al.*, 1993).

5.1.2. *Practical Applications to Heterogeneous and Homogeneous Assay Formats*

References to representative methods for development and evaluation of immunoassays in standard heterogeneous or homogeneous formats utilizing time-resolved fluorimetric detection, in particular covering 1990 and subsequently, are tabulated in the following sections (see Tables 3, 4 and 5). The type of lanthanide-based detection system is noted for each reference, as follows: D (DELFIA® or dissociative enhancement based assay, using Eu unless otherwise stated); F (FIAgen™ or BCPDA-based); EALL; or other, as described.

Table 3. *Representative Reported Immunoassays using Time-Resolved Fluorimetric Detection: Infectious Agent Antigens and Antibodies*

Analyte	Detection method	References
Simultaneous adenovirus and rotavirus	D, dual-label Eu(III) and Tb(III)	Siitari, 1990
Enteric adenoviruses	F	Brown *et al.*, 1990
Human immunodeficiency virus antibodies	D	Lombardi *et al.*, 1992; Siitari *et al.*, 1990; Roberts *et al.*, 1991
Rotavirus, adenovirus, respiratory viruses	D	Halonen *et al.*, 1983
Influenza A and B	D	Nikkari *et al.*, 1989
Hepatitis B	D	Siitari *et al.*, 1983
Potato viruses	D	Sinijarv *et al.*, 1988;
	D, dual labeling Eu(III) and Sm(III)	Saarma *et al.*, 1989
Tetanus antitoxin	D	Schroeder and Kuhlmann, 1991
Rubella virus antibodies	F	Shankaren *et al.*, 1990
	D	Meurman *et al.*, 1982

5.1.3. *Immunocytochemistry*

Antigens in peripheral blood lymphocytes and erythrocytes have been visualized (Beverloo *et al.*, 1992) using time-resolved microscopy. Immunological reagents labeled with Eu(III)-W1014 have been used to visualize human smooth muscle myosin, in a fluorescence microscope without time-resolved detection (Soini *et al.*, 1988b).

5.1.4. *Western Blot and Related Assays*

A Western blot assay has been performed using FIAgen™ reagents in assays for mouse immunoglobulin G, α-fetoprotein, carcinomebryonic antigen and ferritin (Diamandis *et al.*, 1992). These reagents have also been applied successfully in detecting the same antigens immobilized in microwells subsequent to HPLC separation (Diamandis and Christopoulos, 1991a).

5.2. DNA Hybridization Assays

A review on this subject has appeared recently (Diamandis, 1993). A number of papers covering general labeling methods and protocols for use in hybridization assays have also been published.

A variety of labeling methods for oligonucleotides and DNA with luminescent and non-luminescent lanthanide or ruthenium chelates have been reported. These include: a Tb(III) reagent for labeling of oligonucleotides (Sherman *et al.*, 1992); a Tb(III)-*p*AS DNA-labeling reagent for gel electrophoresis detection (Saavedra and Picozza, 1989), with extraction of the labeled band from the gel and measurement in solution in a time-resolved fluorometer; a similar Tb(III)-chelate substituted psoralen for photolabeling of oligonucleotides (Oser *et al.*, 1990); FIAgen™-based (F) labeling methods for PCR-based detection in gels (Chan *et al.*, 1993); a Ru(II) bathophenanthroline reagent for labeling of oligonucleotides and hybridization (Bannwarth *et al.*, 1988); and Eu(III) labeling methods for DELFIA™-type (D) detection (Hurskainen *et al.*, 1991; Sund *et al.*, 1988).

The following general protocols for a number of detection systems have also been published, with the detection method indicated in brackets (see Section 5.1 for abbreviations of detection methods). A treatise on nonisotopic DNA probe protocols includes chapters on microwell hybridizations, PCR and labeling of long probes and oligonucleotides (D) (Lövgren *et al.*, 1992); membrane hybridizations (F) (Diamandis and Christopoulos, 1992b); and microwell and membrane dot blot and Southern hybridizations (EALL) (Gudgin Templeton *et al.*, 1992). Methods and assays for typical DNA dot blots or microwell assays and Southern blots include detection of: adenovirus in mucus by sandwich hybridization in microwells (D) (Syvanen *et al.*, 1986); adenovirus in a model assay in microwells (D) (Dahlèn, 1987); adenovirus by dot blot on nitrocellulose filters (D) (Dahlèn

Table 4. *Representative Reported Immunoassays using Time-Resolved Fluorimetric Detection: Hormones*

Analyte	Detection method	References
Progesterone, estradiol	D	Lövgren, 1987; Sjoeblom *et al.*, 1990
Progesterone	D	Ius *et al.*, 1989, 1993
	D	Bacigalupo *et al.*, 1990
	F	Kakabakos and Khosravi, 1992; Dechaud *et al.*, 1988
17-α-Hydroxyprogesterone	D	Gonzalez *et al.*, 1990
Estrone-3-glucuronide	Homogeneous W1174 label	Barnard *et al.*, 1989b,c
Estrogen	D	Thomas *et al.*, 1990
Estriol	D	Ius *et al.*, 1991, 1993
Testosterone	D	Canfi *et al.*, 1989; Lövgren *et al.*, 1984
Cortisol	F	Diamandis *et al.*, 1988
	D	Lövgren *et al.*, 1984; Eskola *et al.*, 1985
Triiodothyronine and	F	Tan *et al.*, 1990
uptake	F + immunocomplex dissociation	Eskola *et al.*, 1985
Thyroxin	D	Nuutila *et al.*, 1990
	Homogeneous W1174	Hemmilä *et al.*, 1988
TSH	EALL	Papanastasiou-Diamandi *et al.*, 1992a
	F	Reichstein *et al.*, 1989
	D	Lawson *et al.*, 1986; Paterson *et al.*, 1985; Arends and Nørgaard-Pedersen, 1986; Kaihola *et al.*, 1985; Lövgren *et al.*, 1984; Suonpää *et al.*, 1992
Gonadotropin	D	Alfthan, 1986; Pettersson *et al.*, 1983; Alfthan *et al.*, 1988; Bieglmayer and Fischl, 1987; Iwasawa *et al.*, 1992
	F	Khosravi and Diamandis, 1987
Follitropin	D	Bador *et al.*, 1987; Madersbacher *et al.*, 1993; Suonpää *et al.*, 1992
Lutropin	F	Khosravi *et al.*, 1988b
	D	Lövgren *et al.*, 1984
Simultaneous lutropin and follitropin	D, dual label Eu(III) and Tb(III)	Hemmilä *et al.*, 1987
Somatotropin	F	Kahan *et al.*, 1990
	D	Albertsson-Wikland *et al.*, 1993; Strasburger *et al.*, 1989
Corticotropin	D	Dobson *et al.*, 1987
Prolactin	D	Mathis, 1993; Dechaud *et al.*, 1986
Insulin	D	Toivonen *et al.*, 1986
Simultaneous lutropin, follitropin, gonadotropin and prolactin	F, stick method, solid-phase fluorimetric reading	Kakabakos *et al.*, 1992
Simultaneous TSH 17α-hydroxy-progesterone, trypsin and creatine kinase MM isoenzyme	D, quadruple label Eu(III), Tb(III), Sm(III), Dy(III)	Xu *et al.*, 1992b

et al., 1988); and β-lactamase gene in *Escherichia coli* in a microwell sandwich assay (D) (Dahlèn *et al.*, 1987). Model assays for plasmid DNA include: a nitrocellulose filter dot blot (Oser *et al.*, 1988) (using the ternary Tb(III)-*p*AS–DTPA label); a microwell assay (D) (Oser and Valet, 1988); on nitrocellulose membranes or in microwells (using Eu(III) cryptate label) (Prat *et al.*, 1991); in a Southern blot on membrane (using Ru(II) bathophenanthroline as label) (Bannwarth *et al.*, 1988); in microwells and on membranes for Southern and dot blots (EALL) (Gudgin Templeton *et al.*, 1991); and on nitrocellulose in dot and Southern blots (F) (Christopoulos *et al.*, 1991).

A homogeneous oligonucleotide sandwich-type assay has been demonstrated using energy transfer-based detection with a ternary Tb(III) chelate (Oser and Valet, 1990).

Nucleic acid amplification assays have been performed typically using PCR, with sandwich hybridization in solution, followed by capture onto solid support using an SBR. The general methodology for use of Eu(III)-labeled PCR probes (D) has been reported (Dàhlen *et al.*, 1991a). Published assays include: *E. coli* rRNA (using ternary Tb(III)-EDTA-chelate label, on beads) (Bush

Table 5. *Representative Reported Immunoassays using Time-Resolved Fluorimetric Detection: Other, Including Proteins, Enzymes, Toxins, Peptides, Antigens, Metabolites, Drugs*

Analyte	Detection method	References
α-Fetoprotein	EALL	Christopoulos and Diamandis, 1992
	F	Chan *et al.*, 1987; Christopoulos *et al.*, 1990
	D	Suonpää *et al.*, 1985
α-Fetoprotein and free β-subunit of choriogonadotropin	D, dual label using Eu(III) and Sm(III)	Pettersson *et al.*, 1993
Ferritin	F	Khosravi *et al.*, 1988a
Fibronectin	F + immunocomplex dissociation	Kropf *et al.*, 1991
p53 Tumor suppressor gene protein	EALL	Angelopoulou *et al.*, 1994; Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1994
p53 Tumor suppressor gene product	EALL	Hassapoglidou and Diamandis, 1992; Angelopoulo and Diamandis, 1993
Myoglobin and carbonic anhydrase	D, dual label Eu(III) and Sm(III)	Vuori *et al.*, 1991
Human pancreatic isoamylase	F	Diamandis *et al.*, 1989c
Human pancreatic phospholipase A_2	D	Eskola *et al.*, 1983; Nevaleinen and Eskola, 1989
17β-Hydroxysteroid dehydrogenase	D	Maentausta *et al.*, 1991
Creatine kinase MB isoenzyme	F	Lianidou *et al.*, 1990
Trypsinogen	D	Itkonen *et al.*, 1990
Somatomedin binding protein	D	Kolstinen *et al.*, 1987
Sex hormone binding globulin	D	Soini *et al.*, 1988a; Tomas *et al.*, 1987
Placental protein 5	D	Butzow *et al.*, 1988
β2-Microglobulin	D	Tienhaara *et al.*, 1990
Human albumin	F	Diamandis and Ogilvie, 1990; Canfi *et al.*, 1989
	D	Nesbet *et al.*, 1993
Prostaglandin $F_{2\alpha}$	D	Lueke and Schlegel, 1990
Prostate-specific antigen	EALL	Yu and Diamandis, 1993; Yu *et al.*, 1994
	D	Vihko *et al.*, 1990
Prostate-specific antigen and its complex with α_1-antichymotrypsin	D, dual label Eu(III) and Sm(III)	Leinonen *et al.*, 1993
Nerve growth factor	D	Arumae *et al.*, 1989
Vasular permeability factor	D	Yeo *et al.*, 1992
Immunoglobulin M-rheumatoid factor	D	Van der Sluijs Veer and Soons, 1992
Aflatoxin	D	Degan *et al.*, 1989
Carcinoembryonic antigen	F	Bhayana and Diamandis, 1989
Tumor necrosis factor α and interleukin-6	D	Ogata *et al.*, 1992
Cysteine proteinase inhibitors	D	Joronen *et al.*, 1986
Ovarian carcinoma-associated antigen CA-125	D	Boerman *et al.*, 1987
Urinary metabolites	D	Barnard *et al.*, 1989a
Advanced glycosylation end products	EALL	Papanastasiou *et al.*, 1994
5-Methyl-2′-deoxycytidine and methylated DNA	D	Vilpo *et al.*, 1986; Rasi *et al.*, 1989
Digoxin	F	Papanastasiou-Diamandi *et al.*, 1989
	D	Helsingius *et al.*, 1986; Paterson and Stewart, 1987
Zidovudine	D	Tadepalli and Quinn, 1990

et al., 1991); human immunodeficiency virus Type 1 suitable for automation (D in microwells) (Dahlèn *et al.*, 1991b); detection of point mutations demonstrated with detection of the Z mutation leading to α_1-antitrypsin deficiency (D in microwells) (Dahlèn *et al.*, 1993); papilloma virus in smears (TBP(Eu(III)) label on microwells) (Lopez *et al.*, 1993); direct labeling of PCR products followed by gel electrophoretic separation and quantitation in the gel or after blotting (F) (Chan *et al.*, 1993).

5.3. Other

Cytolytic and cytotoxic activity of, for example, killer cells and cytotoxic agents has been determined using Eu(III)-labeled cells as targets (Maley and Simon, 1990; Volgmann *et al.*, 1989; Granberg *et al.*, 1988).

6. FUTURE DIRECTIONS

Because of its unique features and the variety of labeling and detection systems, lanthanide chelate luminescence detection has the potential for adaptation to many different assay formats and diverse applications.

In the area of improvement of the labeling methods, more stable and luminescent direct labeling reagents, such as the recently reported Eu(III) chelate (Saha *et al.*, 1993), are needed. In particular, application to *in situ* analysis would be facilitated.

The development of a variety of EALL-type reagents could yield improved detection limits and flexibility in selecting the enzyme label; increased application of ALP-based EALL detection should be possible given the availability of ALP-labeled reagents.

Most clinical assays will require automation, and reagents easily adaptable to automated formats will likely dominate. Multiple analyte analysis is particularly well-suited to lanthanide-based detection because of the variety of lanthanide ions that can be detected simultaneously. The use of the four luminescent lanthanides for nucleic acid sequencing applications is an interesting possibility.

The homogeneous assay format is the method of choice in many cases because of its simplicity. Homogeneous time-resolved fluorimetric assays should be successful if high sensitivities can be demonstrated, as for example, using energy transfer to lanthanide ion cryptates (Mathis, 1993).

Tb(III) and Eu(III) chelates have been used as luminescent stains or labels for nucleic acids (Topal and Fresco, 1980) and proteins (Banville *et al.*, 1993); the potential exists for lanthanide-based stains with improved sensitivities or background rejection compared with traditional fluorescent staining methods. Such reagents might be particularly appropriate to *in situ* formats.

We conclude that lanthanide chelate luminescence is a rapidly growing field with diverse applications, which will further evolve in the near future as the technique is reaching maturation and acceptance. In the future, lanthanide-based reagents should have a secure place amongst nonisotopic detection methods for bioanalytical application, due to their versatility and robustness compared with many other conventional methods.

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