

# Review: Increasing the Luminescence of Lanthanide Complexes

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## ABSTRACT

This review compares the chemical and physical properties of lanthanide ion complexes and of other narrow-emitting species that can be used as labels for cytometry. A series of luminescent lanthanide ion macrocyclic complexes, Quantum Dyes®, which do not release or exchange their central lanthanide ion, do accept energy transfer from ligands, and are capable of covalent binding to macromolecules, including proteins and nucleic acids, is described and their properties are discussed.

Two methods are described for increasing the luminescence intensity of lanthanide ion complexes, which intrinsically is not as high as that of standard fluorophores or quantum dots. One method consists of adding a complex of a second lanthanide ion in a micellar solution (columinescence); the other method produces dry preparations by evaporation of a homogeneous solution containing an added complex of a second lanthanide ion and/or an excess of an unbound antenna ligand. Both methods involve the Resonance Energy Transfer Enhanced Luminescence, RETEL, effect as the mechanism for the luminescence enhancement.

**Key terms:** Quantum Dyes, RETEL, lanthanide, macrocycle, luminescence, columinescence, time-delayed, europium, terbium

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## 1. INTRODUCTION

Only one article published in *Cytometry*, by Seveus et al. (1), has described in any detail the use of a lanthanide ion complex as a luminescent label for an antibody and this article, published in 1992, dealt primarily with the instrumentation for time-gated microscopy. Thus, it is appropriate for a special issue of *Cytometry* to include a focused review of this class of luminescent labels. Extensive reviews of the clinical and other uses of lanthanide complexes have previously been published by Hemmilä and coworkers (2,3,4).

This review presents a comparison of the spectral properties, relative sizes, and essential chemical features of lanthanide complexes and other narrow emitting labels. It also provides a critical description of two related approaches that can be used to overcome the comparatively low molar extinction coefficients (molar absorptivities) of lanthanide ion complexes: 1) the columinescence effect, where the luminescence of a lanthanide complex is increased in a micellar solution by energy transfer from a complex of a non-emitting lanthanide to a complex of an emitting lanthanide, and 2) the Resonance Energy Transfer Enhanced Luminescence, RETEL, effect (5) where the energy transfer occurs in the solid state.

A companion Technical Note (6) describes the experimental aspects of the RETEL effect (5), which resulted in a major increase of the luminescence intensity of a specific type of lanthanide macrocycles, the Quantum Dyes®. This increase in luminescence facilitates the use of the Quantum Dyes as labels, either with fluorescent microscopes conventionally illuminated by a Mercury-Xenon (Hg-Xe) arc or--when it is helpful to eliminate contamination from the emissions of conventional fluorophores--with new cost-effective time-gating instrumentation.

The emissions of lanthanide complexes can be separated easily from those of conventional fluorophores because lanthanide complexes have both very narrow emissions and very long lifetimes. As shown in Figure 1 and Table 1 of Section 2.1, the emission band-width of the europium macrocyclic complex at half maximum is 5.2 nm, compared to 37.5 nm for the organic fluorophore fluorescein, and to 27.3 nm for a quantum dot. Also, the emission lifetime of the europium macrocyclic complex is approximately 2 ms; thus, the background emissions from conventional synthetic or natural fluorophores, which have lifetimes in the order of nanoseconds, can be eliminated by the use of simple and relatively inexpensive time-gating equipment (5). Accordingly, the multispectral imaging of lanthanide ion complexes, in combination with a limited number of organic fluorophores, can be performed rapidly and with inexpensive UV LED based instrumentation. Alternatively, if a large number of organic fluorophores is measured by sophisticated multispectral instrumentation in an experimental or diagnostic procedure, lanthanide complexes can be added to increase the number of detectable labels (7).

The emission spectra of lanthanide ions and their complexes consist of very narrow bands because they arise from inner-shell electronic transitions that are unaffected by the environment and occur at well defined wavelengths. Because of these features, they have been used to calibrate spectral instruments, such as spectrophotometers, and are potentially useful for spectroscopic microscopes including confocal spectroscopic systems (8).

## 2. REVIEW OF THE LITERATURE

Three complementary approaches for maximizing the signal-to-noise ratio of fluorescence and/or luminescence measurements are: 1) to employ labels with narrow emission band(s), thus allowing the use of narrow band-pass filters to eliminate the background; 2) to increase the number of labels attached to the molecule to be detected, thus increasing the signal intensity; and 3) to use time-delayed measurements in order to reduce or eliminate contamination by the emissions of other labels having different emission life-times. The first two approaches are achieved by the use of

narrow band-width emitters with significant Stokes' shifts (separation between the excitation and emission). For such emitters, the concentration quenching that results from the use of closely spaced multiple fluorophores or lumiphores (compounds that luminesce) is minimized because the narrow emission band has minimal overlap with the excitation band (9). The third approach, time-delayed measurements, requires special instrumentation that records only photons emitted by long-lived lumiphores after illumination has stopped and the emission of short-lived fluorophores has decayed (1,2,3,6).

Most luminescent labels based on inorganic elements have large Stokes' shifts as well as narrow emissions, and thus fulfill the first two of the above requirements. Three narrow emitting species of importance as labels are: coated semiconductor nanocrystals, generally referred to as quantum dots (10), inorganic phosphor particles (11-14), and complexes of trivalent lanthanide ions ( $\text{Ln}^{3+}$ ) (7,15-18). All these labels have one feature in common--their light-emitting electrons are essentially unaffected by the vibrations of the atomic framework of the surrounding molecular or crystalline structure.

### **2.1. Comparison of Quantum Dots, Nanocrystals and Lanthanide Complexes**

Figure 1 shows the excitation and emission spectra of a conventional organic fluorophore, fluorescein, of a quantum dot with an emission peak at a 605 nm (Qdot 605), and of a  $\text{Eu}^{3+}$  macrocyclic complex, EuMac.

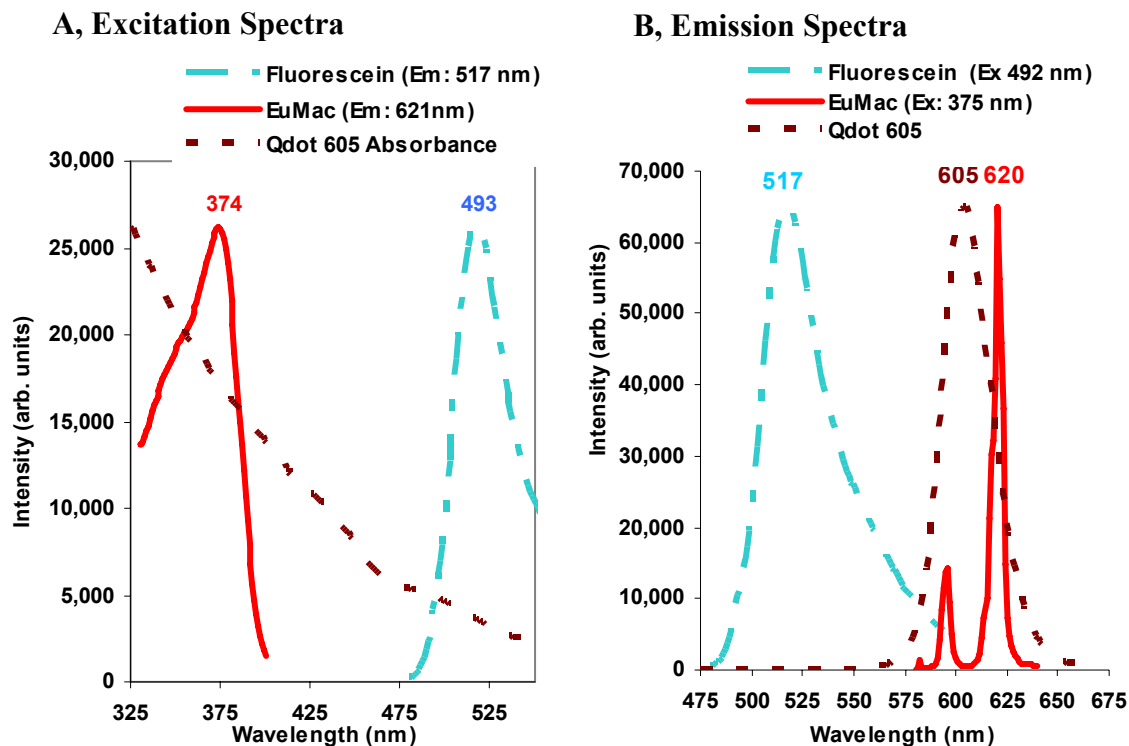


Figure 1. Excitation (A) and Emission (B) spectra of fluorescein, the EuMac (Eu Quantum Dye), and a quantum dot that emits in the red (Qdot 605). The spectra of fluorescein and EuMac were obtained with a SLM spectrofluorometer and have been redrawn from data previously published in reference 19. The spectrum of the Qdot 605 was redrawn from a spreadsheet obtained from [www.qdots.com](http://www.qdots.com). Fluorescein ( $1.0 \times 10^{-5}$  M) was in a hexamethylenetetramine buffer (1.0 M, pH 8.03); the EuMac ( $1.0 \times 10^{-5}$  M) was in an emulsion containing  $\text{Gd}(\text{TtFA})_3$ . (TtFA is the mono-negative anion of thenoyltrifluoroacetone.) For the excitation measurements (A), the excitation and emission slits (resolution) were set to 4 and 8 nm, respectively. The shape of the EuMac excitation spectrum is similar to that of fluorescein or other fluorophores; whereas, the quantum dot excitation efficiency continuously increases as the wavelength decreases. For the emission measurements (B), the excitation and emission slits were set to 8 and 1 nm, respectively. The emission band-width of the EuMac is much narrower than that of either fluorescein or the quantum dot. The spectra of both fluorescein and the quantum dot were scaled to be of the same maximum height as that of the EuMac.

**Table 1. Comparison of the Emission Spectra of Fluorescein, the EuMac in a  $\text{Gd}^{3+}$  Containing Micellar Solution, and a Quantum Dot.**

Emitting Compound	Emission Width at Half-Maximum (nm)	Emission Maximum (nm)	Excitation-Emission Separation (nm)
Fluorescein	37.5	517	24
EuMac	5.2	620	246
Quantum Dot	27.3	605	N.A.

The data in Figure 1 and Table 1 show that the emission band-width of the EuMac is very narrow, 5.2 nm at half-maximum, compared to that of an organic emitter, such as fluorescein, or of the Qdot 605 (Figure 1). Similar data have been obtained for EuMac--labeled beads by Zucker et al (8). Since the emission band-width at half-maximum determines the minimum number of nanometers that should be allocated to any one label to avoid spectral overlap with neighboring emitters, the number of labels that can be measured in the same sample is to a first approximation inversely proportional to the sum of the individual band-widths.

The emission of the EuMac is narrow because, as discussed in Section 2.1.3, it arises from an electronic transition between the non-bonding orbitals of the  $\text{Eu}^{3+}$  ion, and these orbitals are only minimally affected by vibrational coupling with the rest of the molecule. In contrast, the emitting electronic states of fluorescein are coupled to molecular vibrations, and the emission band-width of quantum dots depends on their size distribution, which in turn is related to how they were manufactured.

Table 1 also shows that the excitation-emission separation (Stoke's shift) is about 10 times greater for the EuMac than for organic fluorophores, e.g. fluorescein (19). This simplifies the instrumentation and software required to separate the exciting from the emitted light. It also completely eliminates the overlap between excitation and emission bands, responsible for the concentration quenching usually observed for organic emitters, and leads to the high luminescence intensity observed with multi-EuMac-labeled polymers (20,21) or dendrimers (22). The excitation bands of the EuMac complex and fluorescein are both broad because they closely mirror the respective absorption bands, and these are broad because the electrons involved in the photon uptake are affected by the vibrations of the surrounding structure--the organic ligands bound to the EuMac or the molecule of fluorescein. No specific value can be given for the Stoke's shift of quantum dots because their absorption intensity, and hence their excitation intensity, continually increases as the wavelength of the exciting light decreases (Figure 1A). According to the manufacturer's data, the absorbance of the Qdot 605 at its emission peak is about 12.6% of the absorbance at the 365 nm excitation, and thus there is overlap, even though its significance is reduced by the narrow emission band-width of the quantum dots. For lanthanide-doped inorganic phosphor particles, the narrow emission band-width and Stokes' shifts are similar to those generally observed for lanthanide complexes (12).

The physical and chemical properties of a label, and especially the size, mass and surface properties, can affect the behavior of its conjugates in different ways. The addition of a large, high molecular weight label to a macromolecule can significantly decrease its diffusion coefficient as well as its permeability across cellular membranes and into tissue sections. Also, the minimum distance between two large labels may approach the Förster distance, and thus decrease the efficiency of energy transfer between their energy accepting/energy emitting sites. The Förster distance is the space separation between donor and acceptor, where the energy transfer efficiency is one-half of the maximum value (9). Thus, large size may decrease the desirability of labels for cytological studies; however, it decreases the possibility of concentration quenching.

Figure 2 illustrates the size difference between a EuMac and a quantum dot. Table 2 lists the sizes and masses of three narrow emitters--the EuMac isothiocyanate, fluorescein isothiocyanate, and a quantum dot.

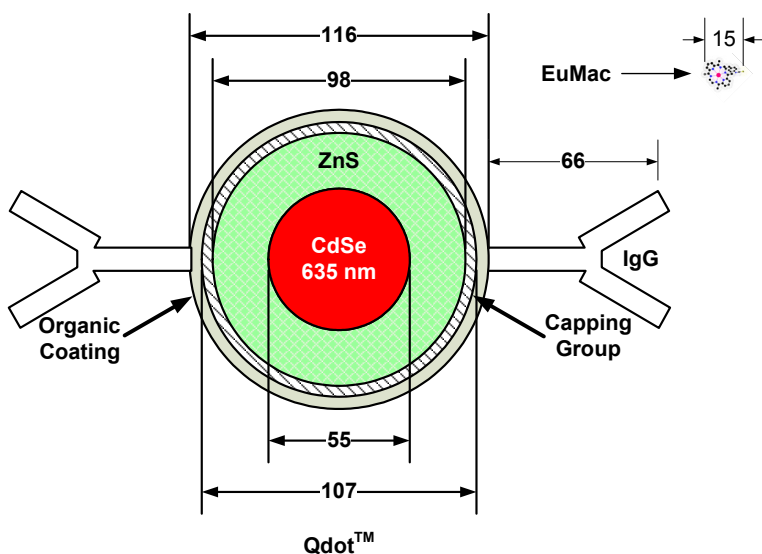


Figure 2. Comparison between the sizes of a quantum dot and the EuMac. Because the thickness of the zinc sulfide, ZnS, and organic layers are considered proprietary information by the manufacturer, the dimensions (in Angstroms) of the multiple layers of the quantum dot were inferred from patents listed on the Quantum Dot Corp's web page (23,24) and other literature. The dimensions of the EuMac were determined from computer models prepared with Chem 3D (<http://www.cambridgesoft.com>). The drawing of the red-emitting quantum dot assumed that 7 layers of ZnS were present to passivate the cadmium selenide, CdSe, surface.

**Table 2. Comparison of a Quantum Dot, a Quantum Dye (EuMac), and an Organic Dye (FITC)**

Label	Diameter, Å	Volume, Å <sup>3</sup>	Mass, Atomic units
Qdot 605	116	825,800	1,500,000
EuMac <sup>1</sup>	15 Max	*504	674
Qdot-to-EuMac ratio	7.8	990	2,223
Small Phosphor Particles (11)	1,000-3,000		
FITC <sup>2</sup>			389

<sup>1</sup>EuMac-mono-NCS. <sup>2</sup>FITC is fluorescein isothiocyanate. \*Connolly solvent-excluded volume from Chem 3D.

The data in Table 2 are based on the dimensions given in Figure 2. Since the luminescence of lanthanide macrocycles (LnMacs) does not quench with increasing concentration, many LnMac units can fit into the volume that would be occupied by a single quantum dot or a small phosphor particle, with a concomitant increase in luminescence intensity. Also, the total volume and mass of 10 to 50 LnMacs are approximately one-fifth relative to those of a single immunoglobulin G molecule; therefore, the addition of multiple LnMacs will have only a minimal effect on the capacity of a diagnostic macromolecule to diffuse or to cross cell membranes. In contrast, the high mass of the quantum dot will slow the diffusion of a diagnostic macromolecule and consequently its binding reactions.

### 2.1.1. Coated Semiconductor Nanocrystals (Quantum Dots)

Semiconductor nanocrystals (10,25), quantum dots, are marketed by Evident Technologies, Inc. (EviDots™) (www.evidenttech.com) and Invitrogen (26) (Formerly Quantum Dot Corp. (Qdot®)) (www.qdots.com) for use as light-emitting labels. Quantum dots have been successfully employed for microscopy (27), flow cytometry (28), and diagnostics (29,30). Individual nanocrystals have been reported (10) to have very narrow emission spectra, for example 12 nm width at half maximum for ZnS capped CdSe; however, because of the size heterogeneity, the collective emissions from a population of these and other nanocrystals are significantly broader. For emissions with maxima at 561–569, 582–588, and 604–612 nm, the widths at half-maximum were reported by a manufacturer to be “less than” 34, 34, and 27 nm, respectively (31). Many of these nanocrystals have broad absorptions that tail into the ultraviolet; thus, maximizing the emission intensity and number of separate emissions of a population of nanocrystals requires excitation of the shortest wavelength emitters with either blue or UV light. Irradiation at ca. 365 nm is usually sufficient to excite most quantum dots simultaneously. Recently, there has been a preliminary report (32) of a narrow emitting quantum dot which contained terbium. However, because of the high background fluorescence, the Tb<sup>3+</sup> luminescence could be detected only after time-gating.

As stated by Chan and Nie (10), “The properties of quantum dots result from quantum-size confinement, which occurs when metal and semiconductor particles are smaller than their excitation Bohr radii (about 1 to 5 nm).” Since the wavelength of the emission maximum is proportional to a physical property (25), namely the size of the nanocrystal, the size heterogeneity of quantum dots results in a broadening of the emission band to 27.3 nm width at half maximum. By comparison, the emission of the europium macrocycle in the lanthanide enhanced luminescence solution (33), when observed at high resolution, had a 5.2 nm width at half maximum (19).

The higher emission intensity of quantum dots compared to conventional fluorophores has been explained by Gao et al. (34) who stated, “In practice, however, fluorescence imaging usually operates under absorption-limited conditions, in which the rate of absorption is the main limiting factor of fluorescence emission. Because the molar extinction coefficients ( $0.5\text{--}2 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ ) of QDs (quantum dots) are about 10–50 times larger than those of organic dyes ( $5\text{--}10 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ), the QD absorption rates will be 10–50 times faster than those of organic dyes at the same excitation photon flux.” In order to minimize the losses from nonradiative recombination, the cores of the quantum dots must be coated with a shell, usually consisting of zinc sulfide, ZnS, which increases the probability of the conduction band electrons relaxing directly to the valence band (35).

Intermittent dark states of individual nanocrystals can be a significant problem (36). According to Lacoste et al. (36), “They exhibit dark states that can span any duration from microseconds to seconds, sometimes accompanied by intermittent spectral jumps. This photophysical behavior can result in a strong nonlinear relationship between the laser excitation power and the fluorescence emission.” Although the previous quotation concerns single particles, it indicates that for systems involving only a small number of particles the precision of measurements could be compromised by one or more particles entering a dark state.

Quantum dots and the doped metal oxide nanocrystals considered above have one feature in common. All have multiple protein (antibody) binding sites on their surfaces. Thus, the conjugates of these particles could cross-link their antigens and, unless special care is taken (27), could form aggregates by two particles being bridged through a protein molecule. Another possible problem has recently been reported by Bocsi et al. (37) “QDs (quantum dots) lost their fluorescence by

mounting [in] anti-fading medium.”

### 2.1.2. Inorganic Phosphor Particles

For flow cytometry and other applications that can employ a focused laser, two-photon absorption of infrared laser light (14,38) is a potentially very useful technique for the excitation of inorganic phosphor particles. The use of these particles is often limited by the background emission resulting from their nonspecific binding (11,12,13). However, the elegant coatings (34) developed for quantum dots to inhibit both nonspecific binding and aggregation might be applicable also to phosphor particles.

### 2.1.3. Lanthanide Ion Complexes

**Electronic transitions:** The 14 elements that comprise the lanthanide series are present as tripositive ions,  $\text{Ln}^{3+}$ , in almost all their compounds. The  $\text{Ln}^{3+}$  ions differ markedly in their spectral properties that arise from their (inner-shell) 4f orbitals configurations. (In the 4f notation, the number 4 identifies the principal energy level--the principal quantum number--and the letter f identifies the type of sublevel--the orbital--to which the electrons belong.) However, the  $\text{Ln}^{3+}$  ions are all very similar in their chemical properties. Specifically, they bind preferentially to highly electronegative elements, present either as simple ions or as ligating atoms of polyatomic molecules or ions (“ligands”).

The complexes of several trivalent lanthanide ions, especially those with organic ligands that include aromatic or other  $\pi$  bonded groups exhibit a unique kind of luminescence. When irradiated with ultraviolet light, the complexes of the  $\text{Sm}^{3+}$ ,  $\text{Eu}^{3+}$ ,  $\text{Tb}^{3+}$ , and  $\text{Dy}^{3+}$  ions emit visible light and, while the absorption and excitation spectra are broad and fairly similar for all complexes of the same ligand, the emission spectra consist of several very narrow bands that are characteristic of each  $\text{Ln}^{3+}$ . As an example, Figure 1 shows the emission and excitation spectra of a  $\text{Eu}^{3+}$  complex of an organic macrocyclic ligand that includes pyridine head-units. Figure 3 illustrates the mechanism for the  $\text{Eu}^{3+}$  luminescence in complexes of organic ligands.

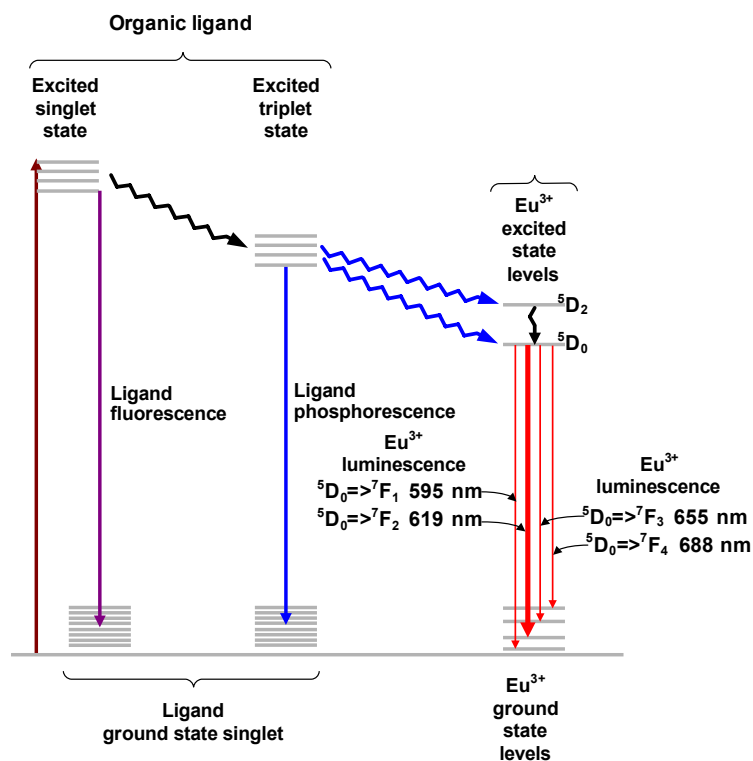


Figure 3. Energy level (Jablonski, Tailbones) diagram of the energy transfer process involved in the luminescence of Eu<sup>3+</sup> complexes of organic ligands. The up and down arrows indicate excitation and emission, respectively. The squiggly arrows indicate radiationless energy transfer. The electronic transitions of the 4f electrons of the Eu<sup>3+</sup> ion, from the excited <sup>5</sup>D<sub>2</sub> and <sup>5</sup>D<sub>0</sub> configurations of the <sup>5</sup>D excited state to various components of the <sup>7</sup>F ground state, are shown on the right. In these symbols, the capital letter represents symbolically (39) the total orbital angular momentum of the electronic configuration, while the superscript and subscript represent respectively the spin multiplicity (number of unpaired electrons + 1) and the value of the J quantum number (J values vary by increments of 1/2) (39). The emissions resulting from the (weak) 595 nm transition and the (strong) 619 nm transition are shown in Figure 1.

As shown in Figure 3, absorption of a UV photon causes one of the electrons of the organic ligand to be promoted from the ground singlet state to the first excited singlet state. The term “singlet” means that the spins of each pair of electrons are opposite. In the first excited singlet state the promoted electron has the same spin as in the original ground state--that is, its spin is still opposite to that of the other member of the pair in the ground state. The organic ligand may return from the first excited singlet state to the ground singlet state by radiative emission--the phenomenon of fluorescence. Alternatively, intersystem crossing may occur to an excited triplet state, if one with a suitable energy level is available. The term “triplet” means that the excited electron has changed its spin orientation to be the same as that of its unexcited original partner. In this process, the electron changes its spin orientation and the organic ligand passes from the excited singlet to the excited triplet state with radiationless emission of a small amount of energy. The organic ligand may then decay from the excited triplet state to the original ground singlet state by radiative emission--the phenomenon of phosphorescence, which again involves a change in electron spin orientation. However, when an organic ligand is bound to a lanthanide ion, the deactivation of the excited electron may follow a different path. In this case, radiationless energy transfer occurs from the excited triplet state of the ligand to the lanthanide ion, resulting in the promotion of its 4f electrons to either the <sup>5</sup>D<sub>0</sub> or the <sup>5</sup>D<sub>2</sub> electronic excited state. Finally, the lanthanide ion returns to one of its <sup>7</sup>F electronic ground states by radiative emission and, because the <sup>7</sup>F state consists of a number of energy sub-levels corresponding to different configurations of the 4f electrons, several distinct emissions are observed.

This generally accepted mechanism for the luminescence of  $\text{Ln}^{3+}$  complexes of organic ligands accounts for both the broadness of the absorption and the narrowness of the emissions in the spectra of the complexes. The absorption is broad because both the ground and the excited electronic states of the polyatomic organic ligand consist of many closely spaced vibrational levels. The emissions are sharp because they involve the decay of electrons from higher to lower energy levels of the 4f electrons, and these constitute an “inner shell” that is shielded from vibrational interaction with the environment by the surrounding shell of 5d electrons.

The energy transfer pathway illustrated in Figure 3 shows that both the phosphorescence of the organic ligand and the luminescence of the  $\text{Ln}^{3+}$  complexes involve excited singlet-to-excited triplet intersystem crossing steps. This provides a simple rationalization for the very long lifetimes, up to 2 milliseconds, observed (40) for the luminescence of  $\text{Ln}^{3+}$  complexes.

Recently, complexes of the neodymium ion,  $\text{Nd}^{3+}$ , with the anions of polycarboxylic acids have been reported by Xiao et al. (41) to upconvert in solution, emitting at wavelengths shorter than those employed for excitation. This is a standard example of two-photon excitation, because the emission intensity depended quadratically on the incident laser power and the wavelengths of the exciting radiations (592-599 nm) were longer than the wavelengths of the strongly emitted radiations, located near 360 nm, 387 nm, and 417 nm. More efficient upconversion was observed with the anion of ethylenediaminetetraacetic acid (EDTA) than with the anion of dipicolinic acid (DPA), also known as 2,6-pyridinedicarboxylic acid ( $\text{H}_2\text{PDCA}$ ). These authors also studied the use of excitation by two lasers, one of which emitted near 587 nm and the other emitted between 791 and 799 nm. The lifetime of the first excited state ( $^4\text{F}_{3/2}$ ) of  $\text{Nd}^{3+}$ , produced by excitation near 800 nm, was much longer (55-684 ns) than the lifetime (less than 20 ns) of the emitting excited state ( $^4\text{D}_{3/2}$ ) produced by excitation near 590 nm. The long lifetime of the first excited state should permit two-photon excitation with much simpler lasers than those presently used to produce picosecond pulses for the excitation of conventional fluorophores.

**Past Use of Lanthanide Complexes as Labels:** Leif and Vallarino were the first to propose the use of lanthanide complexes as luminescence labels (7). In 1977, Wieder patented (42) an instrument for the time-gated detection of their luminescence. In order to be broadly useful as a label for cytology and histology, a lanthanide complex should meet several simultaneous requirements. Specifically, the complex should: 1) be sufficiently stable or inert to prevent the release of the lanthanide ion into the surrounding solution, or the exchange of lanthanide ions between two or more complexes, 2) absorb a significant fraction of the exciting radiation, 3) efficiently convert the absorbed energy to intense light emission, 4) include a reactive functionality capable of covalent binding to a macromolecule, and 5) be sufficiently soluble in aqueous systems to permit coupling and avoid precipitation of the macromolecule after coupling.

The first of the above requirements--the need for high stability or inertness--is related to two features that are common to the complexes of the lanthanide ions: 1) Their stability increases with the number of bonds that the lanthanide ion forms with each ligand. Thus, the greatest stability is achieved for complexes of poly-chelating ligands, which form three or more bonds with the same lanthanide ion. 2) The complexes of the lanthanide ions, even when stable, are usually substitution-labile. Thus, a lanthanide ion may release its original ligand and bind to different ligands present in the solution; also, rapid exchange may occur in solution between the lanthanide ions of different complexes (43).

For solid phase clinical assays, Soini and Lonvgren (44) developed a protocol that circumvented the above requirements by removing the first from the other four. These authors started by

attaching a non-luminescent lanthanide complex to a reporter molecule, bound the reporter molecule to an analyte, removed by washing any excess of labeled reporter molecules, dissociated the lanthanide ion from the original non-luminescent complex, and used it to form a new soluble luminescent complex with a ligand present in the assay medium. This complex was finally assayed by time-delayed luminescence. Since this protocol removes the lanthanide from the analyte, it is not applicable to cytology or histology.

Many lanthanide complexes have been synthesized in an effort to obtain species that would satisfy all the above requirements for general use. Especially difficult is the simultaneous achievement of the first three requirements: chemical stability and inertness, high absorbance, and intense luminescence. Because lanthanide complexes, unlike conventional fluorophores, do not undergo concentration quenching, the problem of their relatively low luminescence intensity can be overcome to some extent by employing very high levels of ligation to the targeting species. An example are the complexes of 4,7-bis(chlorosulfonylphenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), for which the optimum labeling level was approximately 100 complex units per unit of substrate (45). However, for direct assays the binding of such a large number of lanthanide complexes to a targeting species can impair its functionality and decrease its solubility.

Lehn et al. (46,47) approached the requirements of high stability and high luminescence, as well as the problem of solvent quenching of luminescence, by developing luminescent lanthanide complexes of cryptands that included bipyridine side-units. These are polycyclic organic ligands that surround and essentially sequester the lanthanide ion into a three-dimensional cavity, with the bipyridine units serving as light absorbers (antenna groups). More recently, Rodrigues-Ubis et al. have described (48) lanthanide complexes of a new class of cryptands in which the light absorbing species, the antenna group, is a substituted coumarin (1,2-benzopyrone) attached to a single benzene ring that is part of the cryptand cage. The absorption and excitation spectra of these lanthanide complexes could be shifted systematically to longer wavelengths by substituting the hydrogens on the coumarin moiety by one or two methoxy groups, or one diethylamino group. The absorption maxima of these substituted species were 338, 353, and 421 nm, respectively. Only the lanthanide complexes with the methoxy substituents were found to be appreciably luminescent.

Two new types of luminescent lanthanide complexes have been reported recently by Raymond et al. (49, 50, 51). The first type (49, 50) includes complexes of bidentate, tetradentate and higher polydentate ligands related to 2-hydroxyisophthalamidylamide. Excitation between 350 to 360 nm produced strong emission from the europium and terbium complexes. Also, luminescence was observed (50) when  $10^{-4}$  M aqueous solutions of each of the macrobicyclic complexes of  $Tb^{3+}$ ,  $Eu^{3+}$ ,  $Sm^{3+}$ , and  $Dy^{3+}$  were excited at 354 nm. The quantum efficiencies of these complexes were reported to be 0.61, 0.06, 0.01, and 0.03, respectively. The second type (51) includes complexes of ligands containing the salicylamidyl moiety. The patent states (51) that there is "one type of complex in solution" but "the stability of this complex is low." To comply with the first of the requirements listed above, the spectra were obtained in a nonaqueous solvent, acetonitrile.

An excellent review of the literature on lanthanide complexes has been written by Parker and Williams (40). These authors state that, in order to overcome the low molar absorption coefficient of the lanthanide ion, "the ligand should incorporate a chromophore (sometimes referred to as an antenna) which absorbs strongly at a suitable wavelength and transfers its excitation energy to the metal which, in accepting this energy, becomes excited to the emissive state." This review describes extensively the chemistry of macrocyclic ligands based on cyclen (1,4,7,10-tetraazacyclododecane), the most important examples of which are the derivatives of DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid). These ligands include an organic moiety that serves as a light-absorbing antenna, the absorbed energy being subsequently

transferred to a lanthanide ion bound in the macrocycle.

A similar approach by Ge and Selvin (52) involved the attachment of derivatives of Carbostyryl (7-amino-4-methyl-2(1H)-quinolinone, cs124,) to diethylenetriamine-pentaacetic acid (DTPA). A derivative with good water solubility, DTPA-cs124-6-SO<sub>3</sub>H, had an absorbance peak at ca. 340 nm, and the complexes formed by its anion with Tb<sup>3+</sup> and Eu<sup>3+</sup> had emission intensities with an approximate 10 to 1 ratio.

Vallarino and Leif solved the problem of the lability of lanthanide complexes by employing functionalized six-nitrogen-donor monomacrocyclic ligands (15,53-55). These are poly-chelating ligands with a cyclic structure (56) (Figure 4A), capable of sequestering the lanthanide ion into their cavity. Unlike all the complexes described above, these monomacrocycles are formed by template synthesis around the desired lanthanide ion, rather than by replacement of another cation by a lanthanide ion within a preformed macrocyclic ligand, and are especially useful when high resistance to the release or exchange of the lanthanide ion is essential for a particular application. These monomacrocyclic complexes also circumvent the requirement for incorporation of a photo-trapping antenna, because light of appropriate energy can be absorbed by exchangeable ligands (Figure 4B) that are not part of the macrocycle structure and bind to the Ln<sup>3+</sup> on opposite sides of the two-dimensional macrocycle. These exocyclic ligands can then transfer the energy of the absorbed light to the lanthanide ion, thus acting as antennas and luminescence enhancers. Both symmetrically di-isothiocyanate-functionalized and mono-isothiocyanate-functionalized monomacrocyclic complexes of Ln<sup>3+</sup> ions have been synthesized. The isothiocyanate functionalities allow covalent coupling of the Ln<sup>3+</sup> macrocycles to biosubstrates that contain primary amine functionalities.

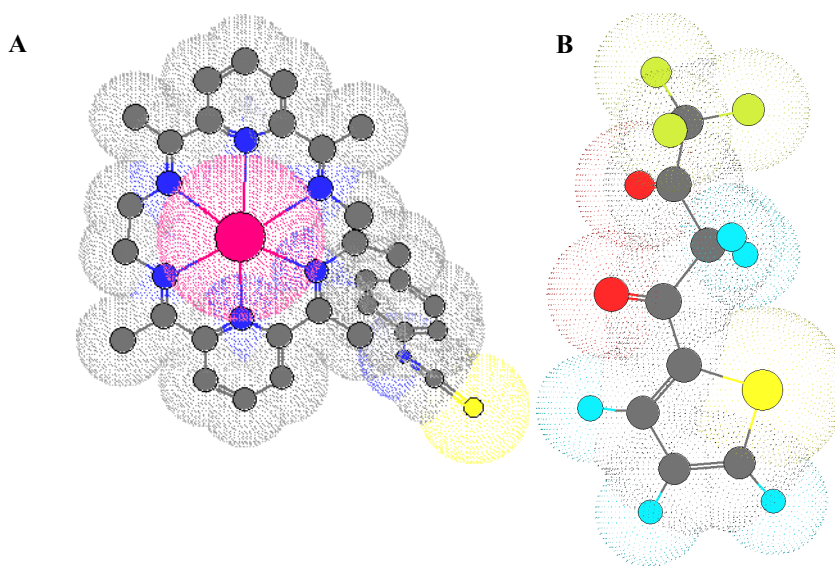


Figure 4. A, 3D Image of the europium macrocycle mono-isothiocyanate (55) (Quantum Dye®). The central europium ion is shown in red, the six surrounding nitrogens in blue, and the sulfur of the mono-isothiocyanate in yellow. The use of a single isothiocyanate coupling functional group (lower right) prevents cross-linking. B, 3D Image of the luminescence enhancer. The anion of thenoyltrifluoroacetone (TTFA) is excited by UV light (365 nm), and the energy of the absorbed photons is transferred to the europium and samarium ions of the macrocycles (57).

The macrocyclic complexes of the  $\text{Eu}^{3+}$ ,  $\text{Sm}^{3+}$  and  $\text{Tb}^{3+}$  ions (LnMacs), together with their antenna ligands, possess a set of properties--water solubility, inertness to release or exchange of the lanthanide ion over a wide pH range, ligand-sensitized narrow-band luminescence, large Stoke's shift, and long excited-state lifetime--that provide ease of detection as well as an emission signal that has minimum interference from background autofluorescence. Since the antenna ligands are not part of the macrocycle, a wide variety of ligands including some of those mentioned above could be used as antennas for these macrocyclic complexes.

The luminescence of these macrocyclic complexes, however, is highly sensitive to their environment. Leif, Vallarino, and coworkers stated (15), "The results with the  $^5\text{D}_0 \Rightarrow ^7\text{F}_2$  (610-625 nm)  $\text{Eu}^{3+}$  transition, which is the major signal source (Figure 3), show that the luminescence of the EuMac-enhancer system is highly dependent upon the choice of both buffer and solvent. The emission intensity increases dramatically in the absence of those buffers that contain anions, such as carbonate, capable of competing with the  $\beta$ -diketonate enhancers as ligands for  $\text{Eu}^{3+}$ . The emission intensity also increases greatly in the less hydroxylic solvents."

At present, the low emission intensity of the EuMac in a homogeneous aqueous medium impedes its use as an optical-label for the observation and measurements of live cells (54). The long lifetime (20) of the EuMac luminescence, of the order of 1 to 2 milliseconds, renders marginal its use for conventional flow cytometry with conventional optics and flow rates (58), and minimizes the signal that can be obtained from conventional, rapid laser-scanning microscopes including confocal microscopes (59). The time needed to acquire a significant signal would be too long -- for example, it would take 131 seconds to scan a 256 by 256 area with a dwell time of 2 ms. However, as described below, the EuMac complex could be used effectively in the dry state for clinical diagnostics and conventional fluorescence (luminescence) microscopy, providing the cells are initially mounted in an appropriate nonaqueous medium, which is then allowed to dry.

## **2.2. Increasing the Luminescence of Lanthanide Complexes by Energy Transfer**

The optimization of lanthanide ion complexes as labels for macromolecules required the development of new means to increase their luminescence, as well as the development of new instrumentation and special software.

### **2.2.1. Columinescence**

Compared to quantum dots and conventional fluorophores, lanthanide complexes have the advantage of narrower emission bands; however, their emission intensities are generally weaker (1). The primary cause of this reduced emission intensity is that the molar extinction coefficients (absorptivities) of the ligands of lanthanide complexes are much lower than those of either quantum dots or highly absorbing organic fluorophores, such as cyanine dyes. For example, the molar extinction coefficients of the sodium salt and  $\text{Gd}^{3+}$  complex of the thenoyltrifluoroacetate (TTFA) ligand in ethanol solution are 15,280 and 47,040, respectively, when measured at the absorption maximum (340 nm), and are further reduced to 7,120 and 25,960 at the wavelength presently used for their excitation, 365 nm. For comparison, the molar extinction coefficient of Cy3 has been reported (60) to be 150,000 at 550 nm. Thus, at the same light flux, the TTFA ligands absorb far fewer photons than either quantum dots or highly absorbing organic fluorophores.

The problem of ligands with low extinction coefficients was first circumvented by Xu and Hemmilä (61), who increased the luminescence of lanthanide complexes by taking advantage of energy transfer from a complex of a non-emitting  $\text{Ln}^{3+}$  to a complex of an emitting  $\text{Ln}^{3+}$ . This

process, here referred to as “columinescence”, was originally called “cofluorescence”. These authors (61) described a luminescence enhancement system containing an emitting lanthanide ion ( $\text{Eu}^{3+}$ ,  $\text{Sm}^{3+}$ ,  $\text{Tb}^{3+}$  or  $\text{Dy}^{3+}$ ), present as a complex with the anions of pivaloyltrifluoroacetone (PTA) and with 1,10-phenanthroline (Phen), as well as a non-emitting ion ( $\text{Y}^{3+}$ ,  $\text{Lu}^{3+}$ ,  $\text{Gd}^{3+}$ , or  $\text{La}^{3+}$ ) as energy transfer donor. These authors stated (61), “The optimum PTA concentration for  $\text{Tb}^{3+}$  detection was narrow and the fluorescence diminished rapidly at PTA concentrations above 70  $\mu\text{M}$ , whereas for  $\text{Eu}^{3+}$  detection the optimum PTA concentration was wider.” The optimum concentration of Phen was 50  $\mu\text{M}$  and Triton X-100 was employed as the surfactant. The greatest luminescence enhancement was produced by  $\text{Y}^{3+}$  at 75  $\mu\text{M}$ , followed by  $\text{Lu}^{3+}$  and  $\text{Gd}^{3+}$ . The optimum pH of the columinescence solution was in the 7.2-7.3 range.

Subsequently, Tong et al. reported (62) that the luminescence of the complex of  $\text{Dy}^{3+}$  with the anion of 1,6-bis(1'-phenyl-3'-methyl-5'-pyrazol-4'-one)hexanedione (BPMPHD) was enhanced about seven-fold by the addition of the cationic surfactant cetyltrimethyl-ammonium bromide (CTMAB), and that the addition of  $\text{Gd}^{3+}$  resulted in a further eightfold enhancement. These Authors also demonstrated the necessity of a micellar system for the columinescence effect, and stated: “...that the fluorescence intensity of the system changes greatest when CTMAB is at its apparent CMC (critical micellar concentration) indicates that the formation of micelles has a great effect on the increase in the fluorescence intensity in the system.” A micellar system was also shown (62) to be required for maximum luminescence because “The columinescence effect disappeared if the organic solvent concentrations were more than 70% for ethanol, 50% for acetone and 80% for dimethyl sulfoxide, when the turbid system became transparent.” Tong et al. concluded: “From the effects of surfactants and solvents on the fluorescence intensity, we conclude that the co-luminescence effect only occurs in the surfactant micellar system or turbid coprecipitated system.”

On the basis of the above observations, Tong et al. (62) described the mechanism of columinescence as follows: “Because  $\text{Gd}^{3+}$  possesses a relatively stable half-filled 4f shell and the luminescence level of  $\text{Gd}^{3+}$   ${}^6\text{P}_{7/2}$  is higher than the triplet state of BPMPHD in the complex  $[\text{Gd}(\text{BPMPHD})_2] \cdot \text{CTMAB}^+$ , the energy of the latter cannot be transferred to  $\text{Gd}^{3+}$ , but can be transferred to the luminescence  ${}^4\text{F}_{9/2}$  level of  $\text{Dy}^{3+}$  in the  $[\text{Dy}(\text{BPMPHD})_2] \cdot \text{CTMAB}^+$  complex by intermolecular energy transfer owing to the short distance between the two complexes in the micelle.”

The above columinescence studies, however important, are not directly relevant to cytometry because the emitting lanthanide ions of the original complexes, either with the PTA and Phen ligands (61) or with BPMPHD (62), undergo exchange with the non-emitting ions present in the system, thus delocalizing the luminescence signal. However, these studies partially explained the mechanism of the energy transfer from the antenna ligands to the emitting lanthanide ions.

Leif, Vallarino, and coworkers found (19,33,63,64) that the luminescence of the EuMac, in the presence of the thenoyltrifluoroacetate (TTFA) ligand or other suitable ligands, was significantly enhanced by addition of a second trivalent ion ( $\text{Gd}^{3+}$  or  $\text{Y}^{3+}$ ) in a micellar solution. Under these conditions localized luminescence could be observed, proving that the light-emitting  $\text{Eu}^{3+}$  ion of the EuMac had not been exchanged by the non-emitting  $\text{Gd}^{3+}$  or  $\text{Y}^{3+}$  ions. Fixed apoptotic (65) and S phase (6,66) cells, labeled with the EuMac conjugate of anti-5BrdU, could be imaged with a standard epifluorescence microscope under illumination at 365 nm with a standard Hg-Xe 100 watt arc lamp. Thus, effective imaging could be obtained without the

previously needed expensive and complicated time-gated or laser-based systems, and/or without heavy labeling of the anti-5BrdU.

The importance of micellar organization for the enhanced luminescence composition was demonstrated by the observation (33) that a water-miscible polar solvent such as ethanol, when added to the characteristically cloudy and luminous micellar composition, completely eliminated the luminescence enhancement while simultaneously turning the cloudy micellar liquid to a clear solution. A simple explanation for this effect is that only in the micelles the concentration of  $\text{Gd}(\text{TTFa})_3$  complexes present within the Förster distance of the EuMac units was sufficiently high to permit energy transfer, presumably by the mechanism suggested by Tong et al. (62).

It was also observed that the photo-decomposition of some component of the columinescence solution (presumably, the TTFa anion) resulted in a loss of luminescence. This loss could be reversed by the addition of fresh columinescence solution, and it was noted that the rate of luminescence loss was much slower for a plastic embedded sample than for the aqueous columinescence solution (65). Thus the LnMacs, similarly to quantum dots, are protected from fading by encapsulation in plastic, which eliminates the presence of water and lowers the concentration and diffusion rate of oxygen from air.

### **2.2.2. Resonance Energy Transfer Enhanced Luminescence (RETEL)**

The enhancement of lanthanide luminescence by cofluorescence in micellar solutions involves a major problem, because such solutions are not sufficiently stable for storage and shipping. Also, the surfactants required for the formation of micelles can have a deleterious effect on cellular morphology. This problem led to the search for, and development of, a new method for providing energy transfer ligands and/or complexes within the Förster distance of the LnMacs. A EuMac labeled analyte was bound to a solid support, and a thin layer of a solution containing the enhancer ligands and/or a gadolinium complex was applied to it (5,6,67); ethanol, methanol, or other volatile organic liquids could be used as solvents. Spontaneous evaporation of the solution resulted in a thin dry film which showed EuMac enhanced luminescence. Figure 5 is a diagrammatic representation of the luminescence enhancement process in such a dry system. Since the energy-transfer mechanism leading to enhanced luminescence is not completely understood, the previously used (5) term FRETTEL, which implied fluorescence as an intrinsic feature, has been changed to the more general term RETEL (Resonance Energy Transfer Enhanced Luminescence). The increase of the EuMac luminescence intensity that results from the RETEL effect upon drying is described in the accompanying Technical Note (6).

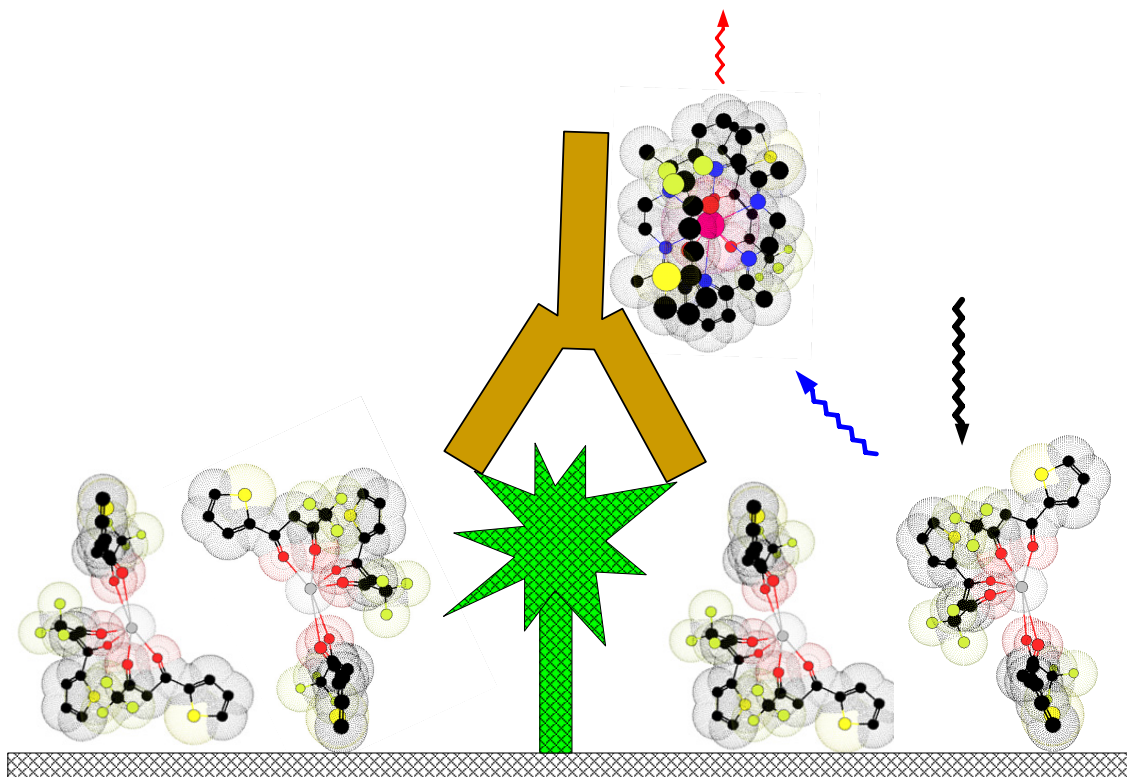


Figure 5. Diagrammatic representation of the resonance energy transfer enhanced luminescence, RETEL, effect for a solid sample. The drawing shows an antibody (brown) labeled with an EuMac (top) and bound to a cell surface antigen (bottom, green). This antigen-antibody complex is embedded in a thin layer of  $\text{Gd}(\text{TTFA})_3$  (shown as four complexes at the bottom) that remains after evaporation of the solvent (ethanol). The light-emitting  $\text{Eu}^{3+}$  of the EuMac label is shown in red and the non-emitting  $\text{Gd}^{3+}$  of the  $\text{Gd}(\text{TTFA})_3$  complexes are shown in gray. An ultraviolet photon, large black arrow, excites one of the  $\text{Gd}(\text{TTFA})_3$  complexes, which then releases energy, medium sized blue arrow. This energy can be transferred to a neighboring  $\text{Gd}(\text{TTFA})_3$  complex or can excite the europium ion of the EuMac, which in turn releases a red photon, small red arrow. Since the system may contain over one million  $\text{Gd}(\text{TTFA})_3$  complexes for each EuMac, and the energy absorbed by many of these complexes can be transferred to the EuMac, the luminescence enhancement is significant.

In Figure 5, the RETEL effect is illustrated using the  $\text{Gd}(\text{TTFA})_3$  complexes as the energy transfer donors. However, anionic ligands other than those bound to the central lanthanide ion of the EuMac or other Quantum Dye, as well as the protonated form of these ligands, can also absorb a photon from incident light and transfer energy to the central lanthanide ion (5), thus enhancing its luminescence. Formerly, the presence of a second lanthanide ion such as  $\text{Gd}^{3+}$ , or of another metal ion such as  $\text{Y}^{3+}$ , was considered necessary for the luminescence enhancement. However, it has been observed (5) that an increasing concentration of the non-ionic, protonated form of the TTFA ligand (HTTFA) can result in a parallel increase in the luminescence of the EuMac; in fact the addition of either HTTFA or NaTTFA to  $\text{Gd}(\text{TTFA})_3$  has provided an approximately 70% increase in luminescence, compared to the use of  $\text{Gd}(\text{TTFA})_3$  alone (5).

The RETEL effect illustrated in Figure 5 is not limited to europium complexes. Simultaneous enhancement of the luminescence of both the TbMac and EuMac has been achieved using the anion of pyridinedicarboxylic acid,  $\text{PDCA}^{2-}$ , as the ligand and its sodium salt,  $\text{Na}_2\text{PDCA}$ , and

gadolinium complex,  $\text{Na}_3\text{Gd}(\text{PDCA})_3$ , as the energy transfer donors. It was also observed that an increase in the concentration of either  $\text{Na}_2\text{PDCA}$  or  $\text{Na}_3\text{Gd}(\text{PDCA})_3$  resulted in an increase of the emission intensities of the TbMac and EuMac only until a specific maximum concentration was reached, which was different for  $\text{Na}_2\text{PDCA}$  and  $\text{Na}_3\text{Gd}(\text{PDCA})_3$ . After these emission maxima were reached, the emission intensities decreased as the concentration of the enhancers increased (5). Since the above results are inconsistent with a simple model of luminescence, additional studies will be required to provide a better understanding of these phenomena and suggest other means to further increase the luminescence of the LnMacs.

At present, two strongly luminescent labels are available, the TbMac and EuMac. Weaker luminescence has also been detected with the Samarium Quantum Dye (57), which has not yet been studied in the dry state. Other lanthanides have also been shown to luminesce in the solid state, some emitting in the near infrared. Identification of their optimum enhancers may result in the development of other useful LnMacs labels.

An important feature of the emission spectra of the TbMac and EuMac is that they do not overlap each other and are well separated from the emission spectrum of DAPI. Since conditions have been found where the TbMac and EuMac have similar emission intensities, it is now possible to use these LnMacs as background-free ratiometric labels for fluorescence in situ hybridization, FISH, and comparative genomic hybridization (68). The problem of selective dye binding should be eliminated because the TbMac and EuMac have virtually identical structure and chemical properties. The only significant obstacle to the simultaneous use of the TbMac and EuMac is that their excitation, at 280 nm, requires the use of expensive fused silica optics or reflecting objectives. Since four different lanthanide ions, when present in the same multidentate 2-hydroxyiso-phthalamide complex, have been reported to luminesce upon excitation at 354 nm (50), further studies may identify other enhancers suitable for the simultaneous excitation of the  $\text{Tb}^{3+}$ ,  $\text{Eu}^{3+}$ , and other LnMacs at wavelengths that can be transmitted by conventional fluorescence objectives.

The increase in luminescence brought about by the use of the RETEL effect described in this paper can be associated with another method for increasing the luminescence of the LnMacs (20,21). This previously reported method was based on the fact that lanthanide luminescence, in contrast to the fluorescence of organic dyes (9,69), does not undergo concentration quenching (9,70,71). Commercial peptide synthesis technology (20,21,72,73) was employed to produce bead-bound peptide carriers labeled with multiple LnMacs and containing a reactive functionality that could bind to a protein or a polynucleotide (20,21). The luminescence of these labeled peptide carriers increased linearly with the number of LnMacs bound to each peptide (20) and the peptide carriers could be cleaved from the beads, by the use of Proteinase K (74), in a manner that had no noticeable effect on fluorescein labeled anti-5BrdU and without loss of LnMacs luminescence (20).

Finally, the use of relatively inexpensive instrumentation for time-delayed measurements, which eliminates the background fluorescence from the DAPI-DNA complex in the cell nucleus, permits the visualization and segmentation of lanthanide-labeled probes (6).

### 3. CONCLUSIONS

The problem of the relatively low intrinsic intensity of lanthanide luminescence, which was a major impediment to their use as labels for cytometry (1), has been solved by the development of the RETEL technology. Also, since the lanthanide macrocycles, LnMacs, do not undergo concentration quenching, the increase in emission intensity resulting from the RETEL effect can be multiplied by the number of LnMacs bound to a peptide produced by solid-phase

nanochemistry technology (20,21), or by the use of multiply-labeled dendrimers (22). In some cases, this might eliminate the need for PCR.

The application of the RETEL effect to the measurement of the luminescence of LnMacs in the solid state is appropriate for their main projected uses: microscopic examination and analysis of samples on slides, and quantitation in micro-wells or dip-sticks. The RETEL effect has facilitated the ratiometric measurement of the simultaneous emissions of the TbMac and EuMac in the same sample. In order to maximize the emission signals, both LnMacs were excited at 280 nm; their individual emission intensities could be measured separately because the emission bands of the TbMac and EuMac are very narrow and do not overlap. Also, owing to their very long lifetimes, the emissions of the LnMacs could be separated from the DAPI emission by time-gating (6).

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#### 5. REFERENCES

1. Seveus L, Väisälä M, Syrjänen S, Sandberg M, Kuusisto A, Harju R, Salo J, Hemmilä I, Kojola H, Soini E. Time-resolved fluorescence imaging of europium chelate label in immunohistochemistry and in situ hybridization. *Cytometry* 1992;13:329-338.
2. Hemmilä I. Applications of Fluorescence in Immunoassays. New York: Wiley Interscience, 1990.
3. Hemmilä I, Mukkala V-M. Time-resolution in fluorometry technologies, labels and applications in bioanalytical assays. *Crit. Rev. Clin. Lab. Sci.* 2001;38:441-519.
4. Hemmilä I, Laitala V. Progress in lanthanides as luminescent probes. *J Fluoresc.* 2005;15:529-42.
5. Leif RC, Becker MC, Bromm Jr. AJ, Vallarino LM, Yang S. Fluorescence resonance energy transfer enhanced luminescence (FRETTEL) of Quantum Dyes®. In: Enderlein GJ, Gryczynski ZK, editors. *Ultrasensitive and Single-Molecule Detection Technologies: Proc. SPIE 2006;6092:29-41.*
6. Leif RC, Vallarino LM, Becker MC, Yang S. Technical Note: Increasing the Luminescence of Lanthanide Complexes for Cytometry by use of the RETEL Effect. *Cytometry* 2006; **{to be added by publisher}**;
7. Leif RC, Clay SP, Gratzner HG, Haines HG, Rao KV, Vallarino LM. Markers for Instrumental Evaluation of Cells of the Female Reproductive Tract: Existing and New Markers. In: Wied GL, Bahr GF, Bartels PH, editors; *Chicago: Tutorials of Cytology* 1976; 313-344.
8. Zucker RM, Chua M, Salmon W, Rigby P, Clements I. *Cytometry* 2006; **{to be added by publisher}**;
9. Lakowicz JR. Principles of fluorescence spectroscopy, Chapter 13, Energy Transfer. New York: Kluwer Academic/Plenum Press, 1999.
10. Chan WC, Nie S. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. *Science* 1998;281(5385):2016-2018.
11. Beverloo HB, van Schadewijk A, Bonnet J, van der Geest R, Runia R, Verwoerd NP, Vrolijk J, Ploem JS, Tanke HJ. Preparation and microscopic visualization of multicolor luminescent immunophosphors. *Cytometry* 1992;13(6):561-570.

12. Beverloo HB, van Schadewijk A, van Gelderen-Boele S, Tanke HJ. Inorganic phosphors as new luminescent labels for immunocytochemistry and time-resolved microscopy. *Cytometry* 1990;11(7):784-792.
13. Beverloo HB, van Schadewijk A, Zijlmans HJ, Tanke HJ. Immunochemical detection of proteins and nucleic acids on filters using small luminescent inorganic crystals as markers. *Anal Biochem* 1992;203(2):326-334.
14. Zarling A, Rossi MJ, Peppers NA, Kane J, Faris GW, Dyer MJ, Ng SY, Schneider LV. Up-converting reporters for biological and other assays using laser excitation techniques. US Patent 5,736,410. 1998.
15. Leif RC, Harlow PM, Vallarino LM. Production, fixation, and staining of cells on slides for maximum photometric sensitivity. In: Bonner RF, Cohn GE, Laue TM, Priezzhev AV, editors. *Proceedings of Biochemical Diagnostic Instrumentation: Proc. SPIE* 1994;2136:255-262.
16. Leif RC, Thomas RA, Yopp TA, Watson BD, Guarino VR, Hindman DHK, Lefkove N, Vallarino LM. Development of instrumentation and fluorochromes for automated multiparameter analysis of cells. *Clin. Chem.* 1977;23:1492-1498.
17. Takalo H, Mikkala VM, Mikola H, Litti P, Hemmilä I. Synthesis of europium(III) chelates suitable for labeling of bioactive molecules. *Bioconjugate Chemistry* 1994;5:278-282.
18. Vallarino LM, Watson BD, Hindman DHK, Jagodic V, Leif RC. Quantum Dyes, a new tool for cytology automation. In: Pressman HJ, Wied GL, editors. *Chicago: Tutorials of Cytology*; 1979. p. 53-62.
19. Leif RC, Becker MC, Bromm Jr. AJ, Vallarino LM, Williams JW, Williams SA, Yang S. Optimizing the luminescence of lanthanide(III) macrocyclic complexes for the detection of Anti5BrdU. In: Farkas DL, Leif RC, editors. *Optical Diagnostics of Living Cells V: Proc. SPIE* 2002;4622:250-261.
20. Leif RC, Becker MC, Bromm Jr. AJ, Vallarino LM, Williams SA, Yang S. Increasing the luminescence of lanthanide(III) macrocyclic complexes by the use of polymers and lanthanide enhanced luminescence. In: Farkas DL, Leif RC, editors. *Optical Diagnostics of Living Cells IV: Proc. SPIE* 2001;4260:184-197.
21. Leif RC, Vallarino LM. Conjugated polymer tag complexes. PCT WO 01/27625 A1. 2001.
22. Cross JP, Lauz M, Badger PD, Petoud S. Polymetallic lanthanide complexes with PAMAM-naphthalimide dendritic ligands: luminescent lanthanide complexes formed in solution. *J Am Chem Soc* 2004;126:16278-16279.
23. Bawendi M, Jensen KF, Dabbousi BO, Rodriguez-Viejo X, Mikulec FV. Highly luminescent color-selective nano-crystalline materials. US Patent 6,322,901. 2001.
24. Treadway JA, Zehnder DA, Schrier MD. Luminescent nanoparticles and methods for their preparation. US Patent 6,815,064. 2004.
25. Bruchez M, Jr., Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor nanocrystals as fluorescent biological labels. *Science* 1998;281(5385):2013-2016.
26. Invitrogen, <http://probes.invitrogen.com/handbook/sections/0105.html>.
27. Lidke DS, Nagy P, Heintzmann R, Arndt-Jovin DJ, Post JN, Grecco HE, Jares-Erijman EA, Jovin TM. Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. *Nat Biotechnol* 2004;22(2):198-203.
28. Pearl JP, Parris J, Hale DA, Hoffmann SC, Bernstein WB, McCoy KL, Swanson SJ, Mannon RB, Roederer M, Kirk AD. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transplant* 2005;5(3):465-474.
29. Nikiiforov T. The use of Qdot™ Streptavidin Conjugate in solid-phase immunosorbent assays: a useful alternative to enzyme amplified assays. *Quantum Dot Vision* 2003;1:7-9.
30. Ornberg RL, Harper TF, Hongjian L. Western blot analysis with Quantum Dot fluorescence technology: A sensitive and quantitative method for multiplexed proteomics. *Nature Methods* 2005;2:79-81.
31. Anonymous. Qdot™ Streptavidin Conjugates a new class of fluorescent bio-labels. *Quantum Dot Vision* 2003;1:2-4.

32. Chengelis DA, Yingling AM, Badger PD, Shade CM, and Petoud S. Incorporating lanthanide cations with cadmium selenide nanocrystals: A strategy to sensitize and protect Tb(III). *J Am Chem Soc* 2005;127:16752 -16753.
33. Bromm Jr. AJ, Leif RC, Quagliano JR, Vallarino LM. Addition of a second lanthanide ion to increase the luminescence of europium(III) macrocyclic complexes. In: Farkas DL, Leif RC, Tromberg BJ, editors. *Optical Diagnostics of Living Cells II: Proc. SPIE* 1999;3604:263-272.
34. Gao X, Cui Y, Levenson RM, Chung LW, Nie S. In vivo cancer targeting and imaging with semiconductor quantum dots. *Nat Biotechnol* 2004;22(8):969-976.
35. Anonymous. Quantum dots explained: Features, adding shells to Evidots. <http://www.evidenttech.com/qdot-definition/quantum-dot-features.php>.
36. Lacoste TD, Michalet X, Pinaud F, Chemla DS, Alivisatos AP, Weiss S. Ultrahigh-resolution multicolor colocalization of single fluorescent probes. *Proc Natl Acad Sci U S A* 2000;97(17):9461-9466.
37. Bocsi J, Lenz D, Mittag A, Varga VS, Molnar B, Tulassay Z, Sack U, Tárnok A. Automated four-color analysis of leukocytes by scanning fluorescence microscopy using quantum dots. *Cytometry* 2006;69A:131-134.
38. Balda R, Fernandez JR, Mendioroz A, Voda M, Al-Saleh M. Spectroscopy and frequency upconversion in  $\text{KPb}_2\text{Cl}_5:\text{Pr}^{3+}$  crystal. In: Jiang S, Keys RW. editors. *Rare-Earth-Doped Materials and Devices VI: Proc. SPIE* 2002;4645: 97-104.
39. Cotton S. Lanthanides and actinide chemistry, Chapter 5, Electronic and magnetic properties of the lanthanides. Wiley, 2006. p.61-74.
40. Parker D, Williams JAG. Getting excited about lanthanide complexation chemistry. *J Chem Soc, Dalton Trans* 1996;18:3613-3628.
41. Xiao X, Herring ME, Haushalter J, Lee S, Kalogerakis KS, Faris GW. Optical property measurements of a novel type of upconverting reporter. In: Savitsky AP, Bornhop DJ, Raghavachari R, Achilefu S, editors. *Genetically Engineered and Optical Probes for Biomedical Applications. SPIE Proc.* 2003;4967:172-178.
42. Wieder I. Method and apparatus for improved analytical fluorescent spectroscopy. US Patent 4,058,732. 1977.
43. Basolo F, Pearson RG. Mechanisms of inorganic reactions. New York: J. Wiley & Sons; 1967: p.155.
44. Soini E, Lovgren T. Time-resolved fluorescence of lanthanide probes and applications in biotechnology. *CRC Crit Rev Anal Chem* 1987;18:105-154.
45. Scorilas A, Magklara A, Hoffman BR, Bromberg RM, Bjartell A, Diamandis EP. Highly sensitive array analysis using time resolved fluorescence and a novel Streptavidin-based reagent. *Analytical Sciences, Supplement* 2001;17:547-550.
46. Lehn JM, Mathis G, Alpha B, Deschenaux R, Jolu E. Rare earth cryptates, processes for their preparation, synthesis intermediates and application as fluorescent tracers. US Patent 5,162,508. 1992.
47. Lehn JM, Mathis G, Alpha B, Deschenaux R, Jolu E. Rare earth cryptates, processes for their preparation, synthesis intermediates and application as fluorescent tracers. US Patent 5,534,622. 1996.
48. Rodriguez-Ubis JC, Alonso MT, Juanes O, Brunet E. Luminescent cryptands. 3-Aroylcoumarin macrobicyclic complexes of europium(III) and terbium(III): the effect of coumarin substitution. *Luminescence* 2000;15:331-340.
49. Raymond KN, Petoud S, Cohen SM, Xu J. Phthalamide lanthanide complexes for use as luminescent markers. US Patent 6,515,113. 2003.
50. Petoud S, Cohen SM, Bünzli J-CG, and Raymond KN, Stable lanthanide luminescence agents highly emissive in aqueous solution: Multidentate 2-hydroxyisophthalamide complexes of  $\text{Sm}^{3+}$ ,  $\text{Eu}^{3+}$ ,  $\text{Tb}^{3+}$ ,  $\text{Dy}^{3+}$ . *J Am Chem Soc* 2003;125:13324-13325

51. Raymond KN, Petoud S, Cohen SM, Xu J. Salicylamide-lanthanide complexes for use as luminescent markers. US Patent 6,406,297. 2002.
52. Ge P, Selvin PR. Carbostyryl derivatives as antenna molecules for luminescent lanthanide chelates. *Bioconjugate Chem* 2004;15:1088-1094.
53. Leif RC, Vallarino LM. Rare-Earth chelates as fluorescent markers in cell separation and analysis. In: Kompala DS, Todd PW, editors; 1991. ACS: 41-58.
54. Vallarino LM, Leif RC. Macrocyclic complexes of yttrium, the lanthanides and the actinides having peripheral coupling functionalities. US Patent 5,373,093. 1994.
55. Vallarino LM, Leif RC. Macrocyclic complexes of yttrium, the lanthanides and the actinides having peripheral coupling functionalities. Continuation-In-Part, US Patent 5,696,240. 1997.
56. Benetollo F, Bombieri G, Vallarino LM. Synthesis, characterization, and crystal structure of a lanthanum(III)-isothiocyanate complex of a macrocyclic ligand with aromatic side-chains. *Polyhedron* 1994;13:573-578.
57. Quagliano JR, Leif RC, Vallarino LM, Williams SA. Methods to increase the luminescence of lanthanide(III) macrocyclic complexes. In: Farkas DL, Leif RC, editors. *Optical Diagnostics of Living Cells III: Proc. SPIE* 2000;3921:124-133.
58. Condrau MA, Schwendener RA, Zimmermann M, Muser MH, Graf U, Niederer P, Anliker M. Time-Resolved Flow Cytometry for the Measurement of Lanthanide Chelate Fluorescence: II. Instrument Design and Experimental Results. *Cytometry* 1994;16:195-205.
59. Leif RC, Becker MC, Bromm Jr. AJ, Chen N, Cowan AE, Vallarino LM, Yang S, Zucker RM. Lanthanide-enhanced luminescence (LEL) with one- and two-photon excitation of Quantum Dyes lanthanide(III)-macrocyclics. In: Nicolau DV, Enderlein J, Leif RC, Farkas D, editors. *Manipulation and Analysis of Biomolecules, Cells, and Tissues: Proc. SPIE* 2004;5322:187-199.
60. Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS. Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. *Bioconjug Chem* 1993;4:105-111.
61. Xu YY, Hemmilä IA. Co-fluorescence enhancement system based on pivaloyltrifluoroacetone and yttrium for the simultaneous detection of europium, terbium, samarium and dysprosium. *Analytica Chimica Acta* 1992;256:9-16.
62. Tong C, Zhub Y, Liua W. Study on the co-luminescence system of Dy–Gd–1,6-bis(1'-phenyl-3'-methyl-5'-pyrazol-4'-one)hexanedione–cetyltrimethylammonium bromide and its analytical application. *Analyst* 2001;126:1168–1171.
63. Leif RC, Vallarino LM. A reagent system and method for increasing the luminescence of lanthanide(III) macrocyclic complexes. US Patent 6,340,744. 2002.
64. Leif RC, Vallarino LM; A reagent system and method for increasing the luminescence of lanthanide(III) macrocyclic complexes. US Patent 6,750,005. 2004.
65. Leif RC, Becker MC, Vallarino LM, Williams JW, Yang S. Progress in the use of Quantum Dye Eu(III)-macrocyclics. In: Nicolau DV, Enderlein J, and RCL, editors. *Manipulation and Analysis of Biomolecules, Cells and Tissues: Proc. SPIE* 2003;4962:341-353.
66. Leif RC. CytometryML binary data standards. In: Nicolau DV, Enderlein J, Leif RC, and DF, editors. *Manipulation and Analysis of Biomolecules, Cells, and Tissues II: Proc. SPIE* 2005; 5699:325-333.
67. Leif RC, Yang S, Vallarino LM. A reagent system and method for increasing the luminescence of lanthanide(III) macrocyclic complexes. PCT/US2004/037314.
68. Kallioniemi A, Kallioniemi O-P, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM. Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci USA*. 1994;91:2156-2160.
69. Haralambidis J, Angus K, Pownall S, Duncan L, Chai M, Tregear GW. The preparation of polyamide-oligonucleotide probes containing multiple non-radioactive labels. *Nucleic Acids Research* 1990;18:501-505.

70. Lamture JB, Wensel TG. Intensely luminescent immunoreactive conjugates of proteins and dipicolinate-based polymeric Tb(III) chelates. *Bioconjug Chem* 1995;6:88-92.
71. Kwiatkowski M, Samiotaki M, Lamminmaki U, Mikkala VM, Landegren U. Solid-phase synthesis of chelate-labeled oligonucleotides: Application in triple-color ligase-mediated gene analysis. *Nucleic Acids Research* 1994;22:2604-2611.
72. Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J Am Chem Soc* 1963;85:2149-2154.
73. Peterson JJ, Meares CF. Enzymatic cleavage of peptide-linked radiolabels from immunoconjugates. *Bioconjugate Chemistry* 1999;10:553-557.
74. Bromme K, Peters K, Fink S, Fittau S. Enzyme-substrate interactions in the hydrolysis of peptide substrates by Thermitase, Subtilisin BPN, and Proteinase K. *Archives of Biochemistry and Biophysics* 1986;244:439-446.