

# Methods to Increase the Luminescence of Lanthanide(III) Macrocylic Complexes

John R. Quagliano<sup>a</sup>, Robert. C. Leif<sup>\*b</sup>, Lidia. M. Vallarino<sup>c</sup>, and Steven A. Williams<sup>c</sup>

<sup>a</sup>Los Alamos National Laboratory, Los Alamos, N.M, 87545, USA

<sup>b</sup>Newport Instruments, San Diego, CA 92115-1022, USA

<sup>c</sup>Department of Chemistry, Virginia Commonwealth University, Richmond, VA 23284-2006, USA

## ABSTRACT

Simultaneous detection of both a Eu(III) and a Sm(III) Quantum Dye<sup>TM</sup> is now possible because the enhanced luminescence (cofluorescence) of the Eu(III) and Sm(III) macrocycles occurs in the same solution and with excitation at the same wavelengths between 350 to 370 nm. Since DAPI is also excited between 350 to 370 nm, it is possible to use common excitation optics and a single dichroic mirror for measuring two molecular species and DNA. The narrow emissions of these macrocycles can be detected with negligible overlap between themselves or with DAPI-stained DNA. This will permit precise pixel by pixel ratio measurements of the Eu(III) macrocycle to Sm(III) macrocycle, and of each macrocycle to DNA. This technology should be applicable to antibodies, FISH, comparative genomic hybridization, and chromosome painting. Cofluorescence of the Tb(III)-macrocycle has also been obtained under different conditions. The luminescence of these lanthanide macrocycles can be observed with conventional fluorescence instrumentation at previously unattainable low levels. Thus, it will be possible to employ narrow bandwidth lanthanide luminescent tags to identify three molecular species with a conventional microscope.

**Keywords:** Luminescence, macrocycle, europium, samarium, terbium, gadolinium, cofluorescence, CGH, FISH, digital microscopy.

## 1. INTRODUCTION

### 1.1 Cytogenetic Imaging with Multiple Dyes

The full utilization of both flow cytometry and digital microscopy requires the ability to detect and measure an increased number of different molecular species<sup>1,2</sup>. Both digital microscopy and flow cytometry share the problem of maximizing the number of molecular species that can be measured while at the same time minimizing the lack of precision that can result from the cross-talk of multiple fluorescent markers. In contrast to most commercial flow cytometers, digital microscopes have the very significant advantage of employing mercury arc lamps that provide a very bright emission at 366 nm, suitable for the excitation of DAPI--the most specific DNA dye available. DAPI staining of DNA has been combined with standard fluorochromes for many types of analysis; three very significant examples are: fluorescence in situ hybridization, FISH, comparative genomic hybridization, CGH, and chromosome painting.

A combination of five dyes has been described<sup>3,4</sup> for chromosome painting. A set of six fluorophores and corresponding optical filters spaced across the 350-770 nm spectral region have been identified that allow excellent discrimination between all possible fluorophores including DNA. The fluorophores employed were: 4'-6-diamidino 2-phenyl indole (DAPI), fluorescein (FITC), Cy3, Cy3.5, Cy5 and Cy7. The excitation and emission maxima for these fluorophores were published in an excellent review article by Waggoner<sup>5</sup> and are collected in Table 1. For comparison, the Table also includes similar data for the prototype europium and samarium macrocycles (SmMac and EuMac) under the conditions described below.

\*Correspondence: Email: rleif@rleif.com; Telephone: 619 582 0437; Fax: 619 582 0437

**Table 1 Speicher et al. Dye Combination**

| Dye   | Excitation Maximum | Emission Maximum | Difference    | Optimum Light Source |
|-------|--------------------|------------------|---------------|----------------------|
| DAPI  | 350                | 456              | 106           | Hg @366              |
| FITC  | 490                | 520              | 30            | Ar+ @488             |
| Cy3   | 554                | 568              | 14            | Hg @ 546             |
| Cy3.5 | 581                | 588              | 7             | ?                    |
| Cy5   | 652                | 672              | 20            | Laser Diode @ 650    |
| Cy7   | 755                | 778              | 23            | Laser Diode @ 750    |
| EuMac | 370                | 619              | 249           | Hg @366              |
| SmMac | 367                | 599, 645-652     | 232, 278, 285 | Hg @366              |

Speicher et al.<sup>3</sup> were able to employ a 75 W Xenon arc lamp to excite all six dyes shown in Table 1. They state: “To attain the required selectivity, filters with band-widths in the range of 5-15nm (compared to approximately 50 nm or more for “standard” filter sets) were required.” In fact, quantitative imaging with the small quantities of light passed by these narrow spectral bandwidths provides a significant challenge even with the use of a cooled CCD. Multispectral imaging<sup>6</sup> provides a reasonable, although expensive, solution, as it permits the use of excitation filters with a much larger bandwidth. The use of an interferometer-based Fourier-transform spectrometer (Applied Spectral Imaging) permits obtaining a spectrum from each pixel; this system requires a cooled CCD and a xenon lamp.

The use of dyes with narrow emission bandwidths would facilitate chromosome painting as well as

other measurements of nucleic acid probes. One year ago, we reported<sup>7</sup> on the use of a second lanthanide ion to significantly increase the emission intensity of a europium macrocycle<sup>8,9,10</sup>, Quantum Dye<sup>TM</sup>, by the effect commonly termed “cofluorescence”. Now we wish to report that it has been possible to obtain significant emission from a samarium macrocycle. Conditions have been found that permit the emissions of both europium and samarium macrocycles to be simultaneously increased by cofluorescence. These same conditions also permit fluorescence to be simultaneously obtained from DAPI-stained DNA, as the two lanthanide macrocycles and DAPI are all excited by the 366 nm mercury line and can share the same excitation filter and dichroic mirror. The three fluorophores do, of course, require separate emission filters. Under the conditions described below, the emission intensities should be sufficiently high to allow measurement with a conventional digital microscope instead of a complex, expensive time-gated instrument<sup>11,12</sup>. Thus a very simple system (DAPI and the two lanthanide macrocycles) can be employed to provide three of the dyes listed in Table 1. It should be noted that this group of three dyes is sufficient for comparative genomic hybridization studies<sup>13</sup>.

## 1.2 Multiple Lanthanide-Containing Luminescent Markers.

The luminescence enhancement, or cofluorescence, caused by certain lanthanide(III) and lanthanide-like salts on aqueous solutions of europium(III) containing chelating  $\beta$ -diketonates as well as synergistic additives was first reported by Melentjeva<sup>14</sup>. This effect has since been the object of several investigations<sup>15,16</sup> and has been shown to apply not only to europium but also to other luminescent lanthanides<sup>17,18,19,20,21</sup>. Xu reported<sup>22</sup> in a patent that the presence of yttrium(III) and other additives can greatly enhance the emission intensity of aqueous micellar solutions of certain  $\beta$ -diketonate complexes of samarium(III), europium(III), terbium(III) and dysprosium(III).

We have extended these studies to a class of functionalized lanthanide macrocyclic complexes that have potential applications as luminescent biomarkers for cytology and immunology. One year ago we reported<sup>7</sup> that the luminescence of europium(III) complexes of six-nitrogen-donor macrocyclic ligands (Figure 1) is dramatically enhanced--by a factor of one-hundred or more in the presence of Gd(III) ions, and to a lesser extent also of La(III) and Y(III) ions, in an aqueous micellar system. This enhancement occurs both with the unfunctionalized prototype of Figure 1 and with the functionalized analog coupled to avidin, either in an aqueous micellar solution or immobilized on a solid substrate. These results showed that gadolinium-induced cofluorescence permits the utilization of europium(III) macrocycles as luminescent markers without the need for time-gated luminescence microscopy, which at present is costly, not widely available, and often involves loss of signal or precision. The presence of gadolinium(III), besides increasing the Eu-luminescence, offers the advantage of competing with charged lanthanide macrocycles for non-specific binding to anionic species.

## 2. EXPERIMENTAL METHODS

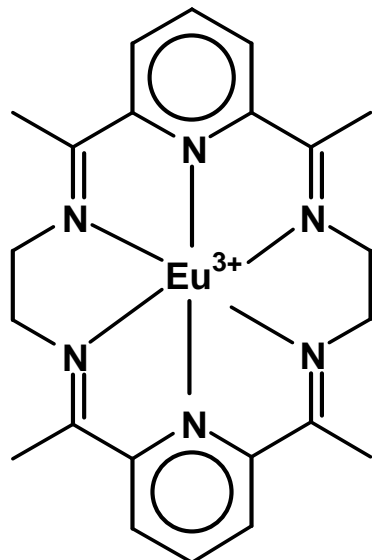


Figure 1. Schematic formula of the cationic entity of a six-nitrogen-donor macrocyclic complex. The structure shown is the unfunctionalized prototype of the Quantum Dye™.

### 2.1. Materials.

Cetyltrimethylammonium bromide (CTAB), hexamethylenetetramine, ACS Reagent (HMTA), 1,10-phenanthroline (PHEN), trioctylphosphine oxide (TOPO), 4,4,4-trifluoro-1(2-naphthyl)-1,3-butanedione (naphthoyltrifluoroacetone, HTFNA), all from Aldrich Chem. Co., and 5,5-dimethyl-1,1,1-trifluoro-2,4-hexanedione (pivaloyltrifluoroacetone, HPTFA, from Lancaster Synthesis Inc.) were checked for purity by IR and/or proton HNMN spectra and were used as received. The diketone 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione (thenoyltrifluoroacetone, HTTFA, from Aldrich) was purified by recrystallization from ethanol(charcoal)/hexane and stored at 4°C in a dark glass container. The complexes [Sm-macrocycle(acetate)<sub>2</sub>](acetate), SmMac, [Eu-macrocycle(acetate)<sub>2</sub>](acetate), EuMac, and [Tb-macrocycle(acetate)<sub>2</sub>](acetate), TbMac, were synthesized as previously described<sup>23</sup>. A high-purity sample of [Sm(macrocycle)(acetate)<sub>2</sub>](acetate) was similarly synthesized using as starting material a Sm(III) acetate prepared from the high-purity oxide (Sm<sub>2</sub>O<sub>3</sub>, 99.999%, REO, from Alfa Aesar); the oxide was dissolved in 50% acetic acid with mild heating and the resulting solution was evaporated to dryness under reduced pressure to give Sm(III) acetate trihydrate as a white crystalline solid. High purity Gd(III) chloride, was obtained from the oxide (Gd<sub>2</sub>O<sub>3</sub>, 99.999%, REO, from Alfa Aesar) by a procedure similar to that described for Sm(III) acetate, with the substitution of 15% hydrochloric for acetic acid. All common reagents and solvents were of reagent grade and were used as received. Only high purity deionized and Micropore-filtered water was used to prepare solutions and for the final rinsing of glassware.

### 2.2. Equipment and Instruments.

All glassware was cleaned with a methanol/conc.hydrochloric acid mixture (90/10 v/v), rinsed with deionized water and methanol, and dried at 60°C. The emission and excitation spectra shown in figures 2, 3, 4 and 9 were obtained with a SPEX 1692T spectrofluorometer (at LANL) and those in figures 5, 6, 7 and 8 were obtained with the SLM-8000 instrument (at VCU), equipped with a Hamamatsu R3896 PMT for emission measurements. Samples were examined in stoppered triangular quartz cuvettes, so oriented that the excitation beam entered the diagonal face at a 45 degree angle and the emitted light was collected through the bulk of the sample at 90 degrees relative to excitation. Slits (both excitation and emission) were varied as required. All experiments and measurements were performed at ambient temperature unless stated otherwise.

### 2.3. Stock Solutions.

(1) *Surfactant*: (a) Cetyltrimethylammonium bromide (CTAB), 1.00x10<sup>-3</sup> M in water. (2) *Buffer*: (a) Hexamethylenetetramine, 10% m/v, 0.71 M in water, adjusted to pH 6.0 with HCl (HMTA buffer), (b) Hexamethylenetetramine, 10% m/v, 0.71 M in water (HMTA base). *Synergistic Ligands*: (a) 1,10-phenanthroline (phen), 5.50 x 10<sup>-3</sup> M in ethanol, (b) trioctylphosphine oxide (TOPO), 5.00x10<sup>-2</sup> M in ethanol, (4) *Diketones*: (a) 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione (HTTFA), (b) 4,4,4-trifluoro-1(2-naphthyl)-1,3-butanedione (naphthoyltrifluoroacetone, HTFNA), (c) 5,5-dimethyl-1,1,1-trifluoro-2,4-hexanedione (pivaloyltrifluoroacetone (HPTFA), all 1.00x10<sup>-2</sup> M in ethanol, (5) *Light-emitting Complexes*: (a) SmMac(macrocycle)(acetate)<sub>2</sub>(acetate), SmMac, (b) [Eu(macrocycle)(acetate)<sub>2</sub>](acetate), EuMac, (c) and [Tb(macrocycle)(acetate)<sub>2</sub>](acetate), TbMac. Ethanol solutions of the macrocycles (1.0x10<sup>-3</sup> M) were used as primary stocks from which more dilute stock solutions were made as necessary. *Luminescence Enhancer*: Gd(III) chloride, 1.0x10<sup>-3</sup> M water.

### 2.4. Preparation of Solutions for Cofluorescence Studies.

A series of experiments was performed to determine the conditions of optimized cofluorescence for the SmMac and TbMac complexes, and for the SmMac/Eu-Mac mixtures. In these experiments, the concentration of the emitting complex was kept constant at a value chosen to provide a suitable range of emission intensities for screening tests. Each micellar solution also contained HMTA as the buffer, CTAB as the surfactant, either PHEN or TOPO, or both, as the synergistic ligands, HTTFA or HTFNA (for SmMac and EuMac), and HPTFA (for TbMac) as the diketone, and Gd(III) chloride as the energy transfer donor.

Various final concentrations of each of these components were tested and the pH of the final solution was kept in the 5.9-6.4 range.

The detailed protocol used for the preparation of a 5-mL sample of an optimized-cofluorescence micellar solution containing SmMac ( $1.00 \times 10^{-5}$  M) as the emitter is given here as example. All components were used as the stock solutions listed in Section 2.3; volumes were measured with calibrated micropipets. In a glass vial, the following are mixed: (a) 0.080 mL of PHEN, (b) 0.050 mL of CTAB, (c) 0.800 mL of HMTA buffer (10% m/v in water, adjusted to pH 6.0 with hydrochloric acid), (d) 0.400 mL of HMTA base (10% m/v in water), (e) 0.600 mL of  $\text{GdCl}_3$  ( $1 \times 10^{-3}$  M in water), (f) mL of SmMac ( $1.0 \times 10^{-3}$  M), and (g) the volume of water required to bring to total volume of the mixture to 5.00 mL after all components are added. The HTTFA (0.400 mL of a  $1.00 \times 10^{-2}$  M solution in ethanol.) is then added with gentle shaking and the previously clear solution becomes slightly cloudy owing to the formation of micelles. The micellar solution is allowed to stand at room temperature for 15-30 min, after which time 0.080 mL of TOPO ( $5.00 \times 10^{-3}$  M in ethanol) are added and the cloudiness of the solution becomes more pronounced. The mixture is incubated for an additional 5 min at room temperature; it is then placed in a quartz cell and its luminescence is obtained without further delay under the instrumental condition indicated in Section 2.2. The concentrations of the components in this optimized cofluorescence solution are listed in Table 2; minor variations ( $\pm 5\%$ ) in the concentration of any component except SmMac do not affect the luminescence intensity of the solution.

Table 2: Concentrations of Components in Optimized Cofluorescence Solutions Containing Gd(III) as the Energy Transfer Donor.

| Component   | Moles/L               |
|---|-----------------------|
| 1,10-Phenanthroline, PHEN   | $8.80 \times 10^{-5}$ |
| Cetyltrimethylammonium bromide, CTAB  | $1.00 \times 10^{-5}$ |
| Hexamethylenetetramine buffer, HMTA buffer  | $1.14 \times 10^{-1}$ |
| Hexamethylenetetramine base, HMTA base  | $5.68 \times 10^{-2}$ |
| 1,1,1-trifluoro-4(2-Thienyl)-2,4-butanedione<br>(Thenoyltrifluoro-acetone, HTTFA) | $8.00 \times 10^{-4}$ |
| Trioctylphosphine oxide, TOPO   | $8.00 \times 10^{-5}$ |
| Gd(III) chloride  | $1.20 \times 10^{-4}$ |
| SmMac, EuMac, and TbMac   | varied<br>(see text)  |

### 3. RESULTS AND DISCUSSION

#### 3.1. Enhancement of the Luminescence of the SmMac Complex by Gadolinium(III) in Aqueous Micellar Solutions.

A set of Gd-containing cofluorescence solutions having decreasing SmMac concentrations ( $1.0 \times 10^{-5}$  M,  $1.0 \times 10^{-6}$  M,  $1.0 \times 10^{-7}$  M,  $1.0 \times 10^{-8}$  M,  $5.0 \times 10^{-9}$  M) were prepared according to the protocol described in Section 2.4. For comparison, a cofluorescence solution of the SmMac complex ( $1.0 \times 10^{-5}$  M) containing all components at the concentrations of Table 2, but no gadolinium, was also prepared. The emission spectra of all solutions, obtained on a SPEX 1692T spectrofluorometer, are illustrated in . It should be pointed out that the problem of Eu(III) contamination in the gadolinium used as energy transfer donor, discussed previously<sup>7</sup> for EuMac, also affects the SmMac spectra in the lowest concentration range.

### 3.2. Comparison of the Luminescence Intensity of the SmMac Complex in a Gd-Containing Optimized Cofluorescence Solution and in Ethanol Solutions with Diketone Enhancers

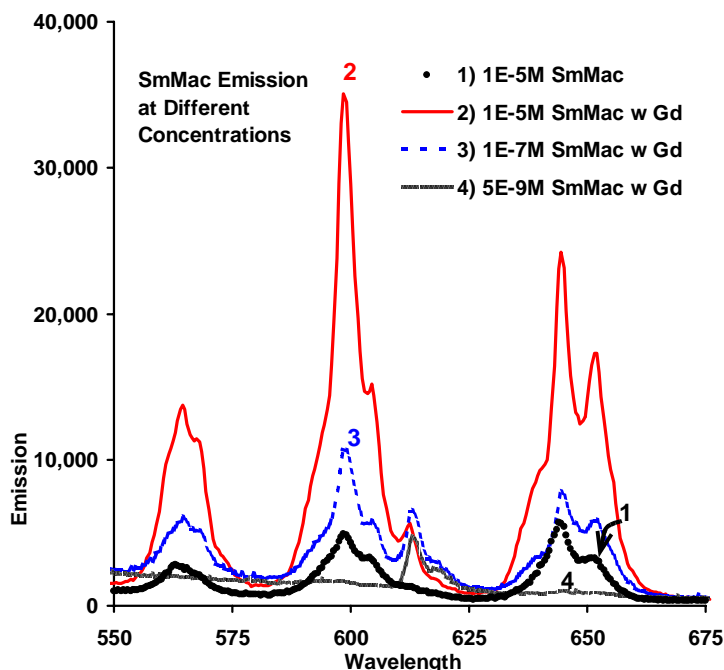


Figure 2. Emission spectra (excitation, 367 nm) of  $[\text{Sm-macrocycle}(\text{acetate})_2](\text{acetate})$  at three different concentrations,  $1 \times 10^{-5} \text{ M}$ ,  $1 \times 10^{-7} \text{ M}$ ,  $1 \times 10^{-9} \text{ M}$ , in cofluorescence-optimized solutions with and without Gd(III). The solutions that contain gadolinium also contain “free” Eu(III) (approximately  $4 \times 10^{-11} \text{ M}$ ) as contaminant. The SmMac spectrum shows three emissions at {563}, {599}, and {644} nm, arising from the  $^4\text{G}_{5/2} \rightarrow ^6\text{H}_{5/2}$ ,  $^4\text{G}_{5/2} \rightarrow ^6\text{H}_{7/2}$ , and  $^4\text{G}_{5/2} \rightarrow ^6\text{H}_{9/2}$  transitions of Sm(III); the constant-intensity emission at 614 nm arises from the  $^5\text{D}_0 \rightarrow ^7\text{F}_2$  transition of the Eu(III) contaminant.

It is well known that the emission intensity of the luminescent lanthanide(III) ions is especially strong in ethanol solutions containing certain  $\beta$ -diketones<sup>24</sup>. It was therefore of interest to compare the emission intensities of equal concentrations of the SmMac complex in the gadolinium-containing aqueous cofluorescence solution and in ethanol solutions containing luminescence-inducing  $\beta$ -diketones, such as HTTFA and HTFNA. The following solutions were prepared: (a) a Gd-containing cofluorescence solution of SmMac ( $1.0 \times 10^{-4} \text{ M}$ ), described in Section 2.4; (b) a solution of SmMac ( $1.0 \times 10^{-4} \text{ M}$ ) in anhydrous ethanol, containing 1,1,1-trifluoro-4(2-thienyl)-2,4-butanedione (HTTFA,  $4.0 \times 10^{-4} \text{ M}$ ), and (c) a solution of SmMac ( $1.0 \times 10^{-4} \text{ M}$ ) in anhydrous ethanol, containing 1,1,1-trifluoro-4(2-napthyl)-2,4-butanedione (HTFNA,  $4.0 \times 10^{-4} \text{ M}$ ). The luminescence spectra of the solutions were obtained with a SPEX 1692T spectrofluorometer and the results are illustrated in Figure 3 and Figure 4.

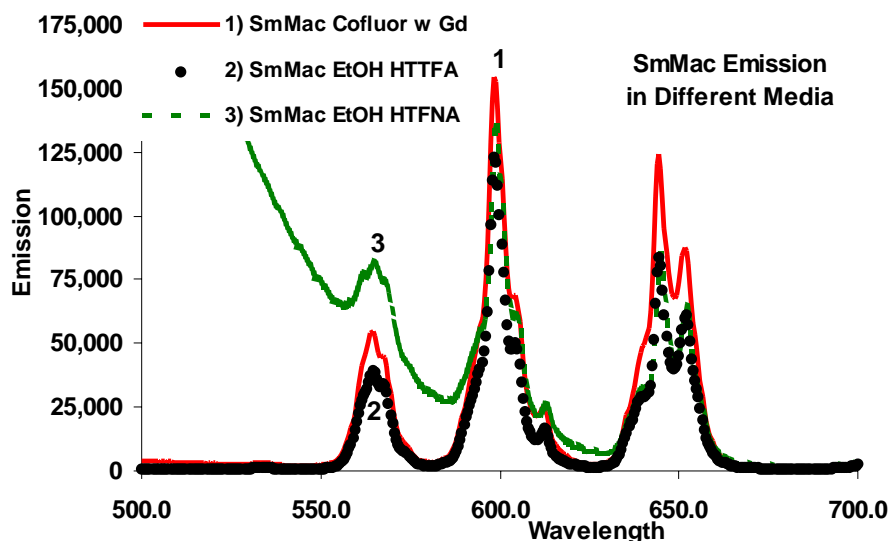


Figure 3. Emission spectra (excitation, 367 nm) of  $[\text{Sm-macrocycle}(\text{acetate})_2](\text{acetate})$  ( $1.0 \times 10^{-4} \text{ M}$ ) in: 1) a Gd-containing optimized cofluorescence solution, 2) an ethanol solution containing HTTFA ( $4.0 \times 10^{-4} \text{ M}$ ), and 3) an ethanol solution containing HTFNA ( $4.0 \times 10^{-4} \text{ M}$ ).

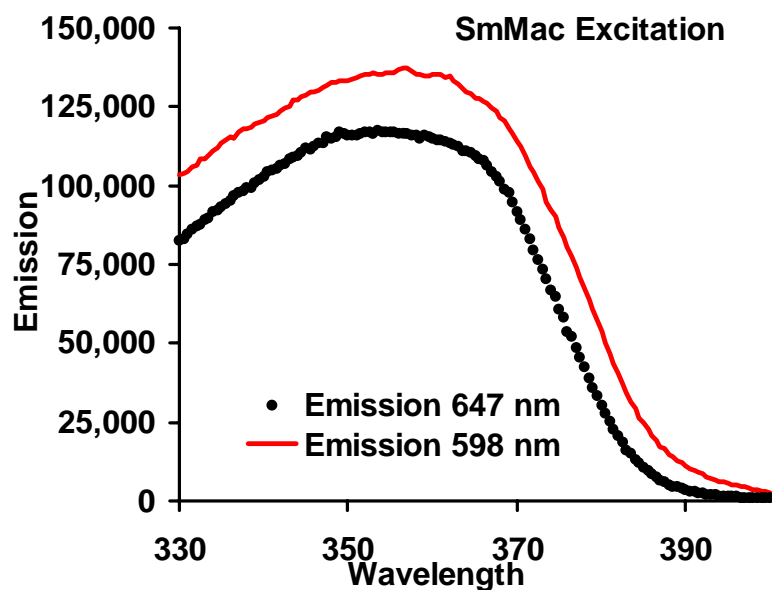


Figure 4. Excitation spectra of [Sm-macrocycle(acetate)<sub>2</sub>](acetate) ( $1.0 \times 10^{-4}$  M) in an ethanol solution containing HTTFA ( $4.0 \times 10^{-4}$  M), for emission of 598.5 and 647.0 nm, respectively.

### 3.3. Simultaneous Enhancement of the Luminescence of the SmMac and EuMac Complexes by Gadolinium(III) in Aqueous Micellar Solutions.

A series of experiments was performed to determine conditions leading to the simultaneous luminescence enhancement of the SmMac and EuMac complexes by gadolinium(III) in aqueous micellar solutions. The following Gd-containing cofluorescence solution were prepared: (a) SmMac ( $5.0 \times 10^{-6}$  M), (b) EuMac ( $5.7 \times 10^{-8}$  M), (c) SmMac ( $5.0 \times 10^{-6}$  M) and EuMac ( $1.4 \times 10^{-7}$  M), (d) SmMac ( $5.0 \times 10^{-6}$  M) and EuMac ( $5.7 \times 10^{-8}$  M). The protocol for the preparation of these solution was the same as that described in Section 2.4. The emission and excitation spectra were obtained with the SLM-8000 instrument as described above in Section 2.2 except the slits (both excitation and emission) were set as described below.

The emission and excitation spectra of a Gd-containing cofluorescence solution prepared from a mixture of the EuMac ( $1.4 \times 10^{-7}$  M) and SmMac ( $5.0 \times 10^{-6}$  M) are shown respectively in Figure 5 and Figure 6. At this concentration ratio, the emissions of the EuMac and SmMac in the combined solution have very similar intensities. For comparison, the superimposed emission spectra of two Gd-containing cofluorescence solutions, one with EuMac alone ( $1.4 \times 10^{-7}$  M) and the other with SmMac alone ( $5.0 \times 10^{-6}$  M), are shown in Figure 7 and the corresponding excitation spectra are shown in Figure 8. At this concentration ratio, the EuMac alone and the SmMac alone have very similar emission intensities. The major peaks of Figure 5 are also found in Figure 7. Clearly the EuMac peak at 620 nm has very little overlap with the 599 nm peak of the SmMac and negligible overlap with the combined 645 and 652 nm emissions of the SmMac. The peak at 614 nm is due to europium ion contamination in the gadolinium chloride, as previously mentioned in Section 3.1. These long wavelength emissions of the SmMac and EuMac are clearly separable from those of conventional ultraviolet-excited DNA dyes including DAPI.

Thus the use of two functionalized lanthanide macrocycles together, or in conjunction with other fluorophores, increases the number of measurable analytes and in the case of ultraviolet-excited dyes simplifies the instrumentation required for excitation of the luminescence and/or fluorescence.

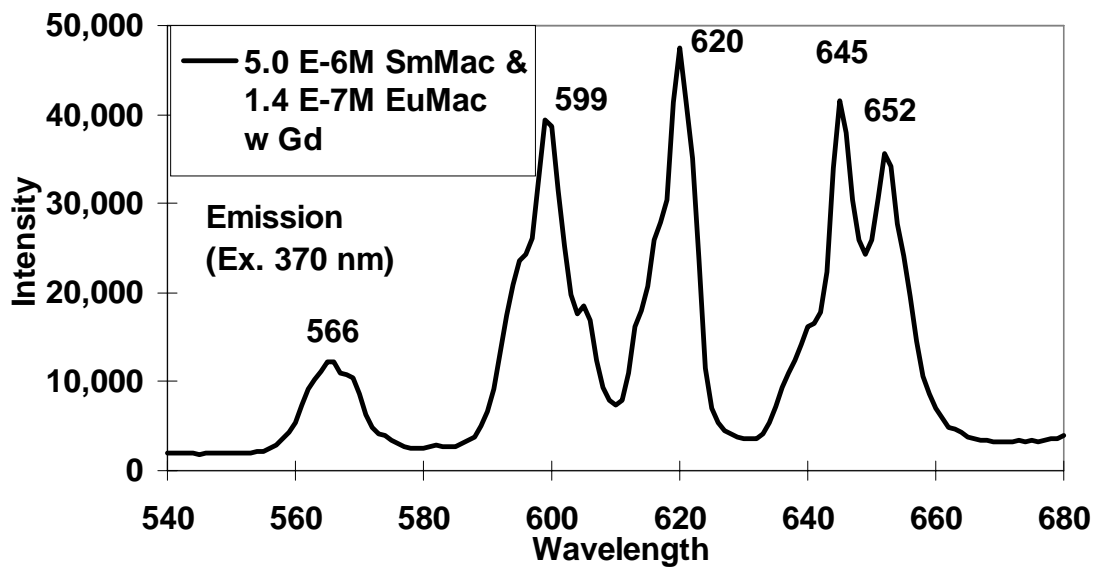


Figure 5. Emission spectrum (excitation, 370 nm) of a gadolinium-induced cofluorescence solution containing  $5.0 \times 10^{-6}$  M [Sm-macrocycle(acetate)<sub>2</sub>](acetate) and  $1.4 \times 10^{-7}$  M [Eu-macrocycle(acetate)<sub>2</sub>](acetate); all other components as in Table 2. The SmMac and EuMac complexes were combined prior to micelle formation and their concentrations were chosen to provide approximately equal emission intensities in the mixture. The  $^5D_0 \rightarrow ^7F_2$  (619 nm) emission of the EuMac species is well separated from the neighboring  $^4G_{5/2} \rightarrow ^6H_{7/2}$  (599 nm), and  $^4G_{5/2} \rightarrow ^6H_{9/2}$  (644 and 652 nm) emissions of the SmMac, so that the intensities of each emission can be measured independently. The excitation and emission slits of the SLM 8000 spectrofluorometer were respectively 16 and 2 nm.

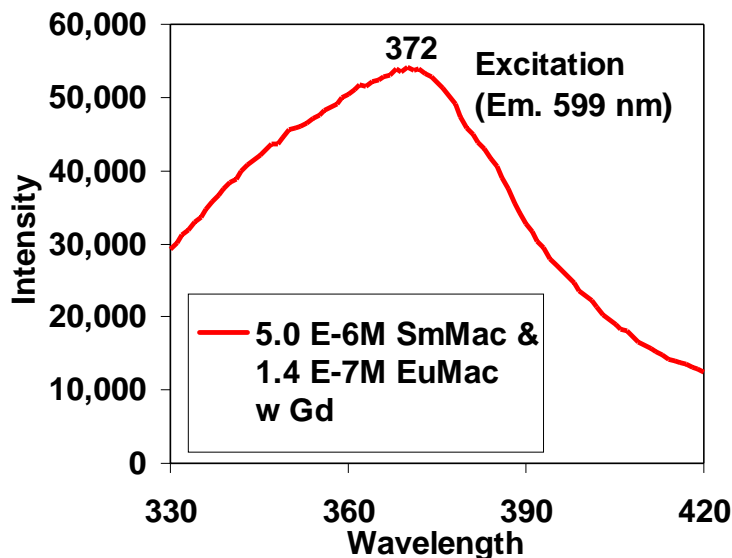


Figure 6. Excitation spectrum of the SmMac complex (emission, 599 nm) in a gadolinium-induced cofluorescence solution containing  $5.0 \times 10^{-6}$  M [Sm-macrocycle(acetate)<sub>2</sub>](acetate) and  $1.4 \times 10^{-7}$  M [Eu-macrocycle(acetate)<sub>2</sub>](acetate). All other components had the concentrations given in Table 2 and the SmMac and EuMac were combined prior to micelle formation. The excitation and emission slits of the SLM 8000 spectrofluorometer were 8 and 4 nm, respectively. The excitation spectrum of the EuMac complex is nearly identical.

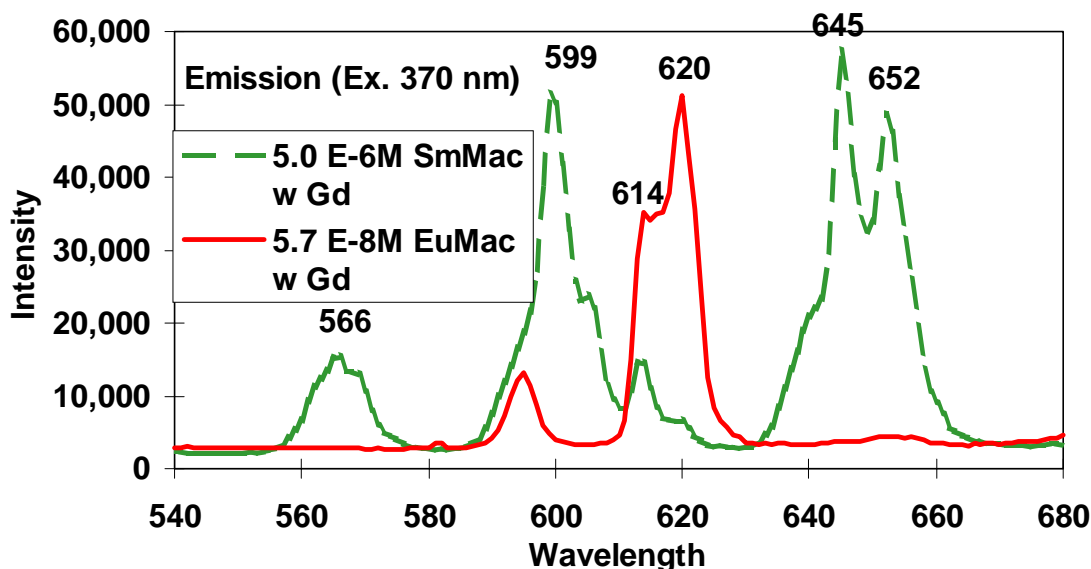


Figure 7. Composite of the emission spectra (excitation, 370 nm) of two gadolinium-induced cofluorescence solutions, one containing SmMac ( $5.0 \times 10^{-6}$  M) alone and the other containing EuMac ( $5.7 \times 10^{-8}$  M) alone. The concentrations of the macrocyclic complexes were chosen to provide approximately equal emission intensities. The excitation and emission slits of the SLM 8000 spectrofluorometer were respectively 16 and 2 nm.

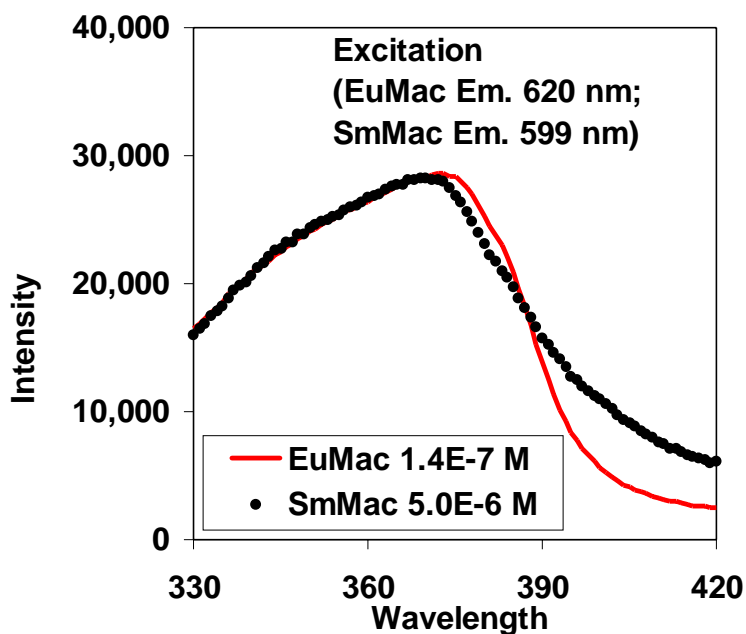


Figure 8. Excitation spectra of [Eu-macrocycle(acetate)<sub>2</sub>](acetate) alone ( $1.4 \times 10^{-7}$  M) and [Sm-macrocycle(acetate)<sub>2</sub>](acetate) alone ( $5.0 \times 10^{-6}$  M) in the gadolinium-containing aqueous cofluorescence solutions. The excitation and emission slit widths of the SLM 8000 spectrofluorometer were respectively 8 and 4 nanometers. The SmMac spectrum was normalized to have the same intensity as the EuMac spectrum at 366 nm--the wavelength that can be used to simultaneously excite both lanthanide macrocycles.

#### 3.4. Enhancement of the Luminescence of the TbMac Complex by Gadolinium(III) in Aqueous Micellar Solutions.

The effect of Gd(III) as energy transfer donor on the luminescence intensity of the TbMac triacetate complex in aqueous micellar solutions was investigated in a series of experiments that utilized the materials listed in Section 2.1 and followed the protocol described in Section 2.4, with the substitution of TbMac for SmMac and of 1,1,1-trimethyl-5,5,5-trifluoro-2,4-pen-



tanedione (pivaloyltrifluoroacetone, HPTFA) for HTTFA. The optimum luminescence intensity was observed when the solution contained HPTFA ( $8 \times 10^{-4}$  M), in conjunction with the other components listed in Table 2, each at the concentration shown in that Table.

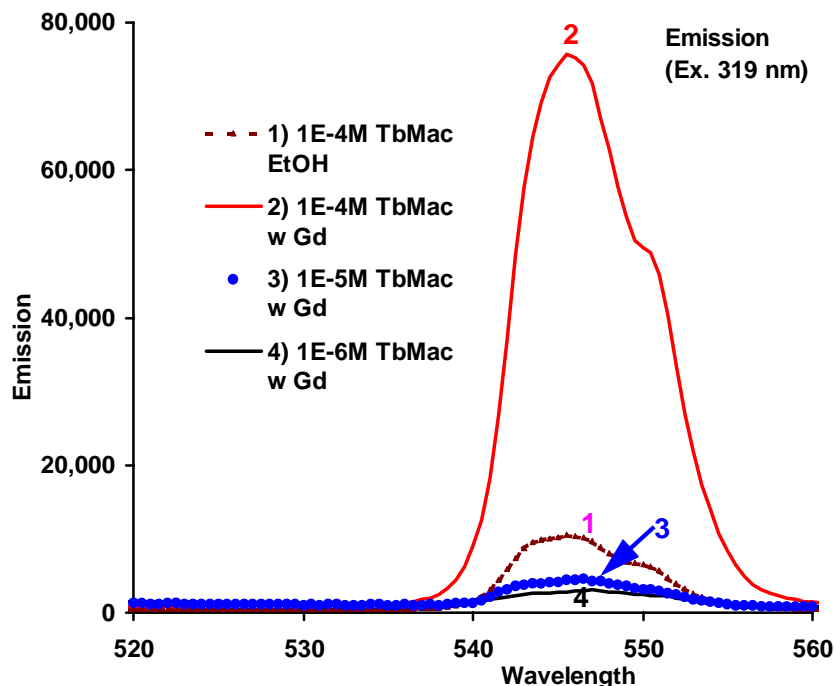


Figure 9. Emission spectra (excitation, 319 nm) of: (1) an ethanol solution of TbMac,  $1.0 \times 10^{-4}$  M with only HPTFA ( $8 \times 10^{-4}$  M), (2) (3) and (4) Gd-containing cofluorescence-optimized solutions of TbMac,  $1.0 \times 10^{-4}$  M,  $1.0 \times 10^{-5}$  M,  $1.0 \times 10^{-6}$  M, respectively.

simultaneously excited and share both the excitation filter and the dichroic mirror, the only necessary difference being the emission filter. Thus, precise measurements of the EuMac-to-SmMac ratio, and also of the ratio of the individual macrocycles to DAPI should be possible. These ratios, which are independent of both object (chromosome) size and of reasonable fluctuations in excitation intensity should serve as excellent descriptors for nucleic acid measurements including: chromosome painting, comparative genomic hybridization, and FISH. The EuMac and SmMac can also be used as tags for proteins.

The cofluorescence-enhanced luminescence of the terbium macrocycle, achieved under different conditions, adds to the existing palette of markers a useful new narrow-band, long-lifetime species emitting in the green. The TbMac cofluorescence effects opens the possibility that other functionalized lanthanide macrocycle tags may be developed in the future. The fluorophore additions reported here will permit a significant increase in the number of species that can be observed and quantitated by luminescence without the need for time-gated or other sophisticated instrumentation.

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A set of optimized cofluorescence solutions containing different concentrations of the TbMac complex ( $1.0 \times 10^{-4}$  M,  $1.0 \times 10^{-5}$  M,  $1.0 \times 10^{-6}$  M) were prepared, and their emission spectra were obtained with a SPEX 1692T spectrofluorometer. For comparison, a solution of TbMac ( $1.0 \times 10^{-4}$  M) in ethanol containing only HPTFA ( $4.0 \times 10^{-4}$  M) was also prepared and examined under the same conditions. Figure 9 shows the emission spectra of TbMac in the ethanol/HPTFA solution and in the optimized cofluorescence solutions containing Gd(III), illustrating that the luminescence of TbMac is greatly enhanced by the presence of Gd(III) in an aqueous micellar system.

This comparatively long wave excitation of a Tb(III) complex at 319 nm should permit the use of some commercial fluorescence objectives rather than requiring very expensive quartz objectives.

#### 4. CONCLUSIONS

A new combination of three fluorescent tags has been described. The DNA-specific stain DAPI can be combined with two narrow emission functionalized lanthanide macrocycles. All three fluorophores can be

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