Time-Resolved Luminescence Microscopy and Microarray Using Europium Chelate Labels

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The luminescent Eu$^{3+}$ complex, DTBTA-Eu$^{3+}$, is highly stable in terms of ligand-metal dissociation, and can be labelled to amino groups of proteins and other bio-molecules. Owing to the long lifetime of Eu$^{3+}$ luminescence, time-resolved measurement of DTBTA-Eu$^{3+}$ removes background fluorescence and enhances the sensitivity of the labelled materials. This property was applied to time-resolved luminescence microscopy and DNA microarray. In these systems the high sensitivity and reliable quantification of the image was proved. In time-resolved images, autofluorescence of tissues and cells was dramatically reduced. In addition, due to the high stability of the metal complex towards photo-bleaching and ozone oxidation, which had been serious problems in the conventional normal fluorescence microscopy and microarray using organic dye labels, deterioration of the images on time elapse turned out to be drastically reduced in the time-resolved imaging, compared to the normal fluorescence images.

Keywords europium luminescent complex; time-resolved luminescence microscopy; time-resolved microarray

1. Introduction

The luminescence of Eu$^{3+}$ complexes has several distinct features that fluorescence of conventional organic dyes do not have: (i) large Stokes shift (excitation in 300-350 nm and red emission at ca. 615 nm), (ii) Line-like sharp emission profile (half width ~10 nm), (iii) long lifetime (several hundreds $\mu$s – more than 1 ms). Owing to such a long lifetime, time-resolved measurement of the Eu$^{3+}$ label allows effective removal of background fluorescence due to cuvette or coexisting materials in the solution, and enhances the sensitivity [1-3]. In the author’s group, several Eu$^{3+}$ as well as Tb$^{3+}$ complexes were developed [4,5], which have a group for covalent binding as a luminescence label to amino groups of bio-molecules, and were applied to time-resolved luminescence measurement in immunoassay, DNA hybridization assay [6], signal amplified Invader-based immunoassay [7], and several other new techniques in SNPs (single nucleotide polymorphism) detection, chromatography, and electrophoresis [8]. In this research, time-resolved luminescence microscopic imaging using Eu$^{3+}$ luminescence was developed and applied to immunostaining of oxidative stress markers in tissues. The same principle was also applied to DNA microarray, and in both systems, the superior sensitivity, quality of the image, and quantitative reliability have been proved.

2. Time-resolved measurement of Eu$^{3+}$ luminescence

2.1 Luminescence properties of Eu$^{3+}$ complexes

Not all Eu$^{3+}$ complexes are luminescent, but Eu$^{3+}$ becomes strongly luminescent when suitable ligands are attached. In order for a complex to be luminescent, the ligand must have aromatic group(s) with suitable energy gaps for UV light absorption and energy levels must be suitable for efficient ligand-to-metal energy transfer. Numerous luminescent Eu$^{3+}$ complexes are reported, and the ligands range to a wide variety. To mention some of the typical ligands, $\beta$-diketonate, oligo-pyridyl and other nitrogen-containing aromatic rings, and EDTA (ethylenediamine tetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), TTHA (triethylenetetraminehexaacetic acid) and other analogous polyamino polyacids having aromatic ring(s) bound to one of the ethylene carbon atoms. Many other ligands are reported, however they extend to a too wide range to introduce in this limited article space. Only four Eu$^{3+}$ complexes and a Tb$^{3+}$ complexes are shown in Fig. 1, which were developed in the author’s group. All of the complexes except TP-crown have a binding group for amino groups of biomolecules, i.e., chlorosulphonyl, succinimidyl, and dichlorotriazyl groups. Different from the red emission (ca. 615 nm) of Eu$^{3+}$ complexes, Tb$^{3+}$ complexes emit green light at ca. 545 nm when irradiated with UV light. All the complexes in Fig. 1 are strongly luminescent, except TP-crown, which is highly stable in terms of the metal-ligand dissociation due to the diazacrown and terpyridyl chelate structures, however its luminescence is rather weak, and is not used as a luminescent label.

The physical properties of the luminescence of Eu$^{3+}$ complexes are specific to Eu$^{3+}$ ion, and the luminescence spectra are scarcely dependent on the ligand. The luminescence properties are totally different from those of the conventional organic fluorescent dyes such as fluoresceine (FITC), rhodamine (RBITC), and cyanine dyes (Cy 3 and Cy 5 in Fig. 2),

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and make lanthanide complexes, not only of Eu$^{3+}$ and Tb$^{3+}$ but of Sm$^{3+}$ and Dy$^{3+}$ as well, a distinct class of luminescence materials for highly sensitive time-resolved detection of the labelled target in bio-assay.

![Fig. 1](image1.png)

*Fig. 1* Several Eu$^{3+}$ and Tb$^{3+}$ luminescent complexes developed as labels for bio-analysis.

![Fig. 2](image2.png)

*Fig. 2* Conventional organic dyes used as fluorescent labels in bio-analysis and biotechnology.

In Table 1, the luminescence properties of [Eu($\beta$-NTA)$_3$] ($\beta$-NTA: naphthyltrifluoroacetylacetonate) and two typical organic dyes are summarized. The lifetimes of Eu$^{3+}$ complexes are generally long, being in the range of several hundreds $\mu$s to more than 1 ms. This should be compared to the lifetimes of fluorescent organic dyes, which are several to several tens ns as shown in Table 1. The Stokes shift that is the wavelength difference between the excitation and emission, is generally very large for Eu$^{3+}$ and other lanthanide complexes, in the range of more than 200 nm, which is contrasted to those of conventional organic dyes, in the range of only 20 to 50 nm.
Table 1. Luminescence properties of a Eu\(^{3+}\) complex and fluorescent organic dyes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lifetime (ns)</th>
<th>Stokes shift (nm)</th>
<th>(\lambda_{\text{ex,max}}) (nm)</th>
<th>(\lambda_{\text{em,max}}) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein (FITC)</td>
<td>4.5</td>
<td>28</td>
<td>492</td>
<td>520</td>
</tr>
<tr>
<td>Rhodamin B (RBITC)</td>
<td>2</td>
<td>35</td>
<td>550</td>
<td>585</td>
</tr>
<tr>
<td>[Eu((\beta)-NTA)](_3^+)</td>
<td>500 000</td>
<td>276</td>
<td>339</td>
<td>615</td>
</tr>
</tbody>
</table>

The distinct luminescence properties of Eu\(^{3+}\) complexes are typically observed in the excitation and emission spectra of BHHCT-Eu\(^{3+}\) in Fig. 3, and the comparison with those of FITC in Fig. 3 confirmed the obvious spectral distinction between these two classes of compounds.

In addition, the emission band width of lanthanide complexes is generally narrow (fwhm ~ 10 nm), which should be compared to the broad peak profiles of organic dyes (fwhm over 50 nm). This contrast is also observed in Fig. 3. The two figures in Fig. 3 show the typical spectral features generally observed for the two classes of compounds.

Such specific luminescence properties of Eu\(^{3+}\) complexes are due to the excitation and emission mechanism shown in Fig. 4.

The UV excitation light is absorbed by the ligand via ligand \(\pi - \pi^*\) transition of the aromatic ring(s). The absorbed energy is transferred from \(S_1\) of the ligand to the Eu\(^{3+}\) \(^5D_0\) level via the ligand triplet state \(T_1\), and the Eu\(^{3+}\) ion finally emits luminescence via the transition from \(^5D_0\) to one of the \(^7F\) manifolds. The most strong emission of Eu\(^{3+}\)-BHHCT in Fig. 3 observed at ca. 615 nm corresponds to the transition from \(^5D_0\) to \(^7F_2\) and the small peak at the foot is due to the transition from \(^5D_0\) to \(^7F_1\). In all of the luminescent Eu\(^{3+}\) complexes, the emission wavelengths of the Eu\(^{3+}\) peaks are not significantly different, being scarcely dependent on the ligand, since the emission is based basically on the transitions \(^5D_0\) - \(^7F_n\). The transition \(^5D_0\) - \(^7F_2\) is always the strongest emission among several Eu\(^{3+}\) peaks in 590 to 700 nm, but the
relative emission intensity ratios of the transitions from $^5D_0$ to several $^7F_n$ levels depend on the coordination symmetry around the metal ion and thus the ligand type. The emission peak profiles are always sharp as observed in Fig. 3, and this is explained by the fact that the emission transition is basically that of the metal ion. The long lifetime is caused mainly by the final f-f transition which is not easily disturbed by the environment, but also such a multiple-step energy transitions involving $T_1$ state contributes. Since the mechanism involves $T_1$ state, the luminescence is not fluorescence. It is phosphorescence, but is usually called luminescence. Although lifetimes are reported for a number of Eu$^{3+}$ and other Ln$^{3+}$ luminescent complexes, the lifetime of each step other than the final f-f transition is rarely reported. The final radiative f-f transition is by far dominantly long and constitutes most of the total lifetime.

2.2 Principle of time-resolved measurement of Eu$^{3+}$ luminescence

The principle of time-resolved measurement is shown in Fig. 5. By employing the initial delay time (200 $\mu$s in Fig. 5) after the pulse excitation, most of the background fluorescence due to co-existing materials having several ns lifetime decays, and after 200 $\mu$s only Eu$^{3+}$ luminescence remains. Therefore, the measurement is started after 200 $\mu$s and is continued for several hundreds $\mu$s. After having the recovering time, the pulse excitation and the time-resolved measurement is repeated and the signal intensities of the counting time are accumulated. By such a time-resolved measurement, most of the background signal is removed and a good S/N ratio is obtained. With such a measurement, very small signals that is usually buried in the background noise fluctuation and cannot be detected as a significant signal in normal fluorometry, can be detected and thus the sensitivity of the analysis is drastically improved.

![Measurement principle of time-resolved fluorometry](image)

**Measurement principle of time-resolved fluorometry**

Fig. 5 The principle of time-resolved luminescence measurement for Eu$^{3+}$ complexes.

This principle has been applied to many bio-assays including immunoassay and DNA hybridization assay using BHHCT-Eu$^{3+}$ [4], BPTA-Tb$^{3+}$ [5], and DTBTA-Eu$^{3+}$ [9] (Fig. 1) as the label. For all of the immunoassays of over 20 proteins and small chemicals in serum, the detection limits were improved one to two orders of magnitude, compared to those reported in the literatures, and the superiority of the time-resolved measurement of Eu$^{3+}$ and Tb$^{3+}$ complexes has been proved. In these applications, it was also proved that labeling of such heavy metal complexes to proteins does not cause any significant deteriorating effect to the biological interactions involved in the assay, such as antigen-antibody binding ligand-protein binding, or DNA hybridization.

3. Time-resolved DNA microarray using DTBTA-Eu$^{3+}$

Since time-resolved measurement of labelled Eu$^{3+}$ complexes effectively removes background fluorescence, time-resolved detection system for DNA microarray was developed. Such system was expected to give high sensitivity, and more reproducible and quantitative analysis was expected, since Eu$^{3+}$ complexes are more stable than organic dyes in terms of photo-bleaching and ozone-oxidation. Ozone-oxidation is not a serious problem in time-resolved
immunoassay using Eu\(^{3+}\) complexes, but in DNA microarray, where a very trace amount of DNA and the labelling reagent are used, photo-bleaching of the label is often serious in the presence of trace of ozone produced in other optical instruments and copying machines in the same room. Actually in our comparative experiment, luminescence of DTBTA-Eu\(^{3+}\) (Fig. 1) was stable and the intensity did not change significantly for more than 5 min and even after 5 min the intensity decrease was only a few % until 30 min, whereas the most common dye used in commercial DNA microarrays, Cy3 and Cy5 (Fig. 2), rapidly decreased and the fluorescence intensity decayed less than 50 % in the initial 1 min. These high stability to oxidation under photoradiation and also to photobleaching even in the absence of ozone are more or less common properties to lanthanide complexes in general, but DTBTA-Eu\(^{3+}\) has, in addition, a superior property that other Eu\(^{3+}\) complexes do not have, i.e., the chelate structure is remarkably stable compared to other Eu\(^{3+}\) chelate complexes, and the metal ion does not dissociate significantly from the ligand in most environment. This property is strongly required when Eu\(^{3+}\) chelate is used in phosphate buffer, in DNA solution, or in tissue staining. In the last case, relatively dense tissue materials sometimes cause dissociation of Eu\(^{3+}\) from the ligand and the luminescence is decreased. Phosphate ion has high affinity to lanthanide ions and often metal dissociation and phosphate-coordination occur in phosphate buffer or DNA solutions. This is often a serious problem in DNA assays, where a large amount of DNA phosphate causes decrease of the Eu\(^{3+}\) luminescence. Therefore, development of a Eu\(^{3+}\) complex that is endurable in DNA analysis had been the target of research. The high stability of DTBTA-Eu\(^{3+}\) was found to suppress the luminescence decrease to a minimum in DNA analysis.

We have developed the time-resolved microarray detector shown in Fig. 6 [10]. The detector is basically a microscope but with higher magnification to observe and quantify the luminescence of each spot with a diameter of ~600 \(\mu\)m.

![Fig. 6 The emission mechanism of Eu\(^{3+}\) complexes. BP: band-path filter.](image)

The UV light (337 nm) of the He-Cd laser was irradiated to the DNA spots on the quartz glass slide support via a UV optical fiber. The quartz material of the support is necessary in order to avoid the background fluorescence caused when usual glass was irradiated with UV light. The excitation light is chopped (1 ms window width, 50 Hz), and is synchronized to the image-intensifier attached CCD detector. The UV optical fiber is introduced to the DNA spots on the slide support with a specific angle, so that the light is reflected away on the support, and does not enter the object lens.

The principle of DNA microarray is schematically drawn in Fig.7. On the microarray, which is usually a glass slide, hundreds kinds of DNAs with known base sequences are chemically or physically attached as small spots (several to several hundreds \(\mu\)m in \(\phi\)). Therefore, each spot on the microarray has different single strand DNAs with known base sequences. The extracted cDNAs from cell A and cell B are labelled with fluorescent dyes with different colors so that the origin of the DNA can be recognized by the fluorescence color. The solutions of the labelled cDNAs are mixed and added to the whole areas of the spots on the microarray, and the hybridization (formation of the double helices) is allowed to proceed. If the cDNA solution contains a specific base sequence that is complementary to one of the DNAs on the microarray, hybridization takes place and fluorescence remains on the spot. Since the non-hybridized DNAs and excess DNAs are washed out after hybridization, existence or non-existence of known base sequences of DNA in the two cells is detected by measuring the fluorescence of each spot for the two colors. A non-fluorescent spot means that the base sequence complementary to the sequence on the spot does not exist in either cell. If only one color is detected on a spot, that means that only one of the cells corresponding to that color has the base sequence complementary to that of the spot. Usually, a comparative study of the two color intensities on a spot is used for diagnostic examination and other genetic researches.
The enlarged microarray images obtained on the time-resolved DNA detector using DTBTA-Eu$^{3+}$ label are shown in Fig. 8. The target DNA was $\lambda$-DNA, to which TFR DNA was used as the non-complementary DNA for comparison. The DNAs were spotted on a synthetic quartz support for time-resolved imaging, and on a usual glass for normal fluorescence microarray. The experiment was carried out with triplicate spots to each sample, and as expected, three almost equivalent spots were observed for $\lambda$-DNA, whereas no luminescence was observed for the spots where either TFR or no DNA was attached (right figures in Fig. 8). For comparison, the same DNAs were labelled with Cy5 and were spotted on a glass support, and the image was taken after hybridization (left figures in Fig. 8). In both systems, correct images were obtained, however, it is obvious that the contour of the time-resolved spot in Fig. 8 is by far clearer than that of the normal fluorescence spots even in the more enlarged picture in the upper right of Fig. 8. This is because in time-resolved image, the weak background caused by glass is removed.

With this time-resolved detector, the calibration curve is linear up to 6 orders of magnitude, which is contrasted to ca. 3 orders for commercial normal fluorometric microarray scanners using organic dye labels. Such wide dynamic range of time-resolved measurement is made possible by the large Stokes shift and the sharp emission peak profiles of Eu$^{3+}$ complexes as shown in Table 1. With the usual small Stokes shifts and the relatively broad emission peak profiles of organic dyes, part of the emitted light is reabsorbed by the same substance, causing concentration quenching, and thus the fluorescence intensity is levelled off at higher concentrations.

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**Fig. 7** Schematic drawing of a microarray and gene expression analysis of two cells.

**Fig. 8** Time-resolved images of DNA microarray spots. The target and the sample DNA are $\lambda$-DNA, to which TRF DNA is used as non-complementary DNA. The spot diameter is ca. 600 $\mu$m.
3. Time-resolved microscopy for tissue imaging using DTBTA-Eu$^{3+}$

Immunohistochemical staining and time-resolved imaging of rat intestine tissues were conducted on the lab-made time-resolved microscope, which has a Xe pulse lamp as the excitation source, several filters for selection of the excitation and emission wavelengths, and an electronically triggered shutter in front of the CCD camera for time-resolved measurement [11,12]. The method was applied to the study of oxidative stress in rats after LPS injection. LPS is a polysaccharide known to cause inflammation and oxidative stress to various organs. As the imaging targets to observe the proceeding of the oxidative stress in rat intestine after LPS injection, the oxidative-stress marker HNE (4-hydroxynonenal) and IgA were stained with the respective antibodies. The anti-HNE antibody was labeled with HRP and was observed with the conventional HRP substrate, 3,3'-diaminobenzidine (DAB, brown precipitate) in Fig. 9, whereas anti-IgA antibody was labelled with DTBTA-Eu$^{3+}$ (red luminescence at 615 nm). In order to grasp the relative geometrical distribution of the dyes in the tissue structures, nucleus was stained with DAPI (blue fluorescence at 460 nm), and the images of the DTBTA-Eu$^{3+}$ and DAPI were obtained with 335-380 nm excitation. In the time-resolved image of the labelled Eu$^{3+}$ complex in Fig. 9 (the lowest four images), the background weak fluorescence due to the tissue materials is effectively removed, as compared to the anti-HNE and anti-IgA + DAPI images in the upper and middle rows, and only the red spots of DTBTA-Eu$^{3+}$ are observed. This image is in a sense less informative, compared to the normal fluorescence images, since only the red spots are observed and their location within the tissue structure is not known. However, by comparison with the normal fluorescence images of DAB of the same tissue specimen, the localization of the red spots is possible as shown in Fig. 9. The time-resolved mode gives clearer images for very weak spots that with conventional imaging cannot be detected. For instance, the IgA image in the lowest row of 7 h after LPS injection in Fig. 9 shows that blurred weak red color gradually spreads over the entire tissue after 7 h. Such weak signal increase is difficult to observe in normal imaging. In this way, the use of both time-resolved and non-time-resolve modes for several multi-color imaging gives clearer and more informative images than conventional fluorescence imaging. The time-resolved spots are observed even for a very low-concentration component which cannot be observed significantly with normal fluorescence imaging.

The medical significance of the increase and decrease of HNE and IgA along the time elapse after LPS injection is detailed in the literature [11] and is not detailed here. However, the powerful capability of the time-resolved imaging can be understood in these figures. In addition, it should be emphasized here that the low-level HNE concentration in the rat serum was determined for the first time with time-resolved immunoassay using the Eu$^{3+}$ label. Such determination had not been possible with commercial ELISA kit for HNE assay.

Fig. 9 Immunohistochemical detection of IgA and HNE in the rat intestine after LPS injection.

The evaluation of the quantitative analysis of time-resolved images was carried out as compared to the normal images on the histochemical imaging of another oxidative stress marker, nitrotyrosine, in rat intestine. Higher signal to noise ratio was proved quantitatively for the time-resolved imaging [12].
References


