Cell assay using a two-photon-excited europium chelate

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Abstract: We report application of two-photon excitation of europium chelates to immunolabeling of epidermal growth factor receptor (EGFR) cell surface proteins on A431 cancer cells. The europium chelates are excited with two photons of infrared light and emit in the visible. Europium chelates are conjugated to antibodies for EGFR. A431 (human epidermoid carcinoma) cells are labeled with this conjugate and imaged using a multiphoton microscope. To minimize signal loss due to the relatively long-lived Eu3+ emission, the multiphoton microscope is used with scanning laser two-photon excitation and non-scanning detection with a CCD. The chelate labels show very little photobleaching (less than 1% during continuous illumination in the microscope for 20 minutes) and low levels of autofluorescence (less than 1% of the signal from labeled cells). The detection limit of the europium label in the cell assay is better than 100 zeptomoles.

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Here we report the practical application of a two-photon-excited europium chelate reporter to a cell-based assay using a microscope combining scanned excitation and CCD detection. Photobleaching, autofluorescence, and detection limits are quantified. The assay targets EGFR phosphorylation against background light. The narrow emission bands of lanthanide ions present opportunities for multiplexing. Lanthanide chelates do not self-quench, allowing for many labels to be conjugated to a molecular probe. Recent work has examined methods to achieve upconverted emission from lanthanide chelates (near infrared excitation and visible emission) either through direct excitation of lanthanide ions such as trivalent erbium or thulium or by two-photon excitation of a sensitizing molecule followed by efficient energy transfer to a lanthanide ion such as europium [5–12]. Like organic dyes, the lanthanide chelates are small in size yet the lanthanide ions are immune to photobleaching like the much larger quantum dots [13,14] and upconverting phosphors [15,16]. Photobleaching of a sensitizing antenna is an issue discussed in section 3.4. Upconverted emission from lanthanide chelates tends to exhibit minimal autofluorescent background from the biological matrix. Photobleaching and autofluorescence are significant limitations for fluorescent reporters today. Other advantages that stem from near infrared excitation include deeper penetration of thick biological samples and potentially lower phototoxicity.

Here we report the practical application of a two-photon-excited europium chelate reporter to a cell-based assay using a microscope combining scanned excitation and CCD detection. Photobleaching, autofluorescence, and detection limits are quantified. The assay targets EGFR on A431 (human epidermoid carcinoma) cells. Elevated expression of EGFR is a tumor marker for squamous carcinomas.

2. Experiment

2.1. Excitation method

The excitation pathway for the two-photon-excited emission from our Eu chelate is shown in Fig. 1. Europium emission is produced through two-photon excitation of naphthoyltrifluoroacetone (NTA, the sensitizer) followed by energy transfer to the Eu

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levels. This energy transfer is generally believed to occur through the triplet state [17,18]. We use the strong emission on the hypersensitive $^5D_0 \rightarrow ^7F_2$ transition at ~615 nm for microscopic imaging.

![Energy Transfer Diagram](image)

Fig. 1. Schematic of energy transfer from sensitizer to Eu$^{3+}$.

Our sensitized Eu chelate is formed in situ by simply adding a solution of NTA to cells, which have a EuDOTA streptavidin conjugate on their surface. Because the DOTA is conjugated through one of its carboxylates, the DOTA chelate covers only 7 coordination sites on the Eu$^{3+}$ ion. This leaves 2 coordination sites open to be filled by solvent or in this case NTA.

Figure 2 shows spectra of the EuDOTA chelate before and after conjugation to streptavidin (SA) and with NTA added. These spectra were taken with a conventional fluorimeter (single photon excitation with Perkin Elmer 650-10S). The EuDOTA spectrum does not change qualitatively when it is conjugated to SA. The EuDOTA emission is excited at 395 nm, which corresponds to an f-f transition of Eu$^{3+}$. Consequently, the spectrum, which features a dominant peak at 590 nm, is relatively weak. When NTA is added and the excitation wavelength is changed to 370 nm, the emission becomes approximately 100 times stronger and the dominant peak shifts to 615 nm. Although the direct f-f excitation of the EuDOTA is somewhat weak, it is quite sufficient for titration of the EuDOTA streptavidin conjugate.

Our strategy of producing a sensitized Eu chelate in situ allows us to use a relatively inexpensive, commercially available bifunctional ligand for conjugation to the biomolecular probe and obviates any possible complications involving the sensitizing moiety during conjugation.

2.2. Multiphoton microscope

Figure 3 shows the experimental apparatus for multiphoton microscopy. The most significant aspect of this microscope is the use of scanned excitation and non-scanned detection using a CCD. Multiphoton and other nonlinear microscopies use a scanned laser beam for excitation since the optical response is nonlinear with the laser power density. Thus, much higher detection efficiency is possible by scanning a focused spot of high intensity rather than using illumination with a larger spot and lower intensity. In most cases, imaging is achieved using the same scanning mechanism and detection a photomultiplier, as is performed with confocal microscopy. When using fluorescent dyes for multiphoton microscopy, for example, the lifetime of the dye is quite short (in the nanosecond range). When using lanthanide emitters however, the lifetimes are typically in the range of hundreds of microseconds, which is long compared to a typical single pixel dwell time for a laser-scanning microscope. In principle,
one could slow the scan rate when using a lanthanide emitter. However maintaining a high laser intensity on one pixel for longer periods of time can lead to thermal damage of the sample. Furthermore, the image acquisition time in this case is limited by the emission rate of the lanthanide as opposed to adjusting the image acquisition time to achieve a desired signal-to-noise ratio. Our microscope uses scanned laser excitation and non-scanned detection with a CCD [19], a configuration usually used with multifocal multiphoton microscopy [20] to speed image acquisition. Here we use this configuration to avoid loss of light due to the limited dwell time on a given pixel in a confocal arrangement. Since each pixel of the CCD is continuously illuminated by the imaged lanthanides such loss of light is avoided.

Fig. 2. Spectra of Eu DOTA-NHS before conjugation (a) and after conjugation to streptavidin with and without NTA added (b). These spectra were taken in a conventional fluorimeter.

![Schematic of multiphoton microscope.](image)

The light source for the microscope was a Spectra Physics Tsunami Ti:sapphire laser tuned to 740 nm. The beam was passed through a telescope (not shown) to provide an appropriate beam size and convergence. A pair of mirrors controlled by galvanometers was used to provide the scanning. Two lenses in a 4-f configuration are used to image the plane of the galvanometer mirror pair onto the back aperture of the microscope objective. A dichroic mirror was used to reflect the infrared beam into the microscope while transmitting the visible light onto the CCD (Q Imaging Retiga EXi). The emission was filtered using a combination of a 600 nm long pass filter and a 620 nm short pass filter. An additional 640 nm short pass filter completely blocks the 740 nm laser radiation. The 615 nm Eu³⁺ emission is selectively captured in the 600-620 nm window created by the filter set.
2.3. Preparation of streptavidin conjugates

As a chelating ligand for the work, we use 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). Conjugates of EuDOTA to streptavidin (SA) were prepared by two different methods. Both protocols involved a bifunctional DOTA-NHS (N-hydroxysuccinamide) ligand obtained from Macrocyclics. The two protocols differed in whether the Eu\(^{3+}\) was inserted in the DOTA macrocycle before or after conjugation to the streptavidin.

In the first method, the Eu\(^{3+}\) was inserted into the bifunctional DOTA before conjugation to SA. We dissolved 22 mg of DOTA-NHS, provided as the PF\(_6\) salt, in 1 mL water and the pH adjusted to between 5 and 6 with NaOH. A molar equivalent of EuCl\(_3\) was added and allowed to react for 30 minutes. A 50-µL aliquot of the reaction mixture was then added to 200 µL of streptavidin (5 mg/mL) in carbonate coupling buffer (pH 9). The coupling reaction between the NHS and lysine residues on streptavidin was allowed to proceed for 2 hours. The unreacted EuDOTA-NHS was separated from the SA conjugate by size exclusion chromatography on a Sephadex column using a PBS (phosphate buffered saline) elution buffer (pH 7.2). Comparison of the EuDOTA SA conjugate emission spectrum with that of the initial EuDOTA-NHS solution (with known DOTA concentration) indicated that the conjugate contained 4 EuDOTA chelates per SA on average. The SA concentration was determined spectrophotometrically. We assume that the insertion of Eu\(^{3+}\) into the DOTA was 90% complete to obtain the number four chelates per SA. A potential problem with the first method is that the NHS may hydrolyze during insertion of Eu\(^{3+}\) prior to the coupling reaction. The half-life of NHS in water is 4 to 5 hours and hydrolysis occurs even faster in basic pH. Therefore the time for the Eu\(^{3+}\) insertion into DOTA must be strictly limited. Consequently, the insertion at pH 5-6 may have been incomplete in 30 minutes.

We have used a second method that avoids hydrolysis of the NHS during insertion of Eu\(^{3+}\) by inserting the Eu\(^{3+}\) after conjugation to SA. 20 mg of DOTA-NHS was dissolved in 0.5 mL of water and the solution pH adjusted to 5 with NaOH. A 100-µL aliquot of this solution was added to 200 µL of streptavidin (5 mg/mL) in coupling buffer and allowed to react for 2 hours. The unreacted DOTA-NHS was separated from the SA conjugate by size exclusion as before except that the elution was performed with TRIS buffer. Use of TRIS buffer rather than PBS was very important to the post-conjugation insertion of Eu\(^{3+}\) since EuPO\(_4\) is insoluble. Emission spectra indicated that the Eu\(^{3+}\) was successfully inserted into the DOTA conjugated to SA as evidenced by the EuDOTA emission spectrum. Comparison of the conjugate spectrum to that of a known concentration of EuDOTA-NHS showed that we were able to achieve a higher level of labeling (6-7 EuDOTA per SA) using the second conjugation method.

2.4. Cell assay protocol

A schematic diagram of the overall assay is shown in Fig. 4. The A431 cells fixed with methanol were incubated with a monoclonal anti-EGFR primary antibody obtained from Neomarkers. The cells were subsequently incubated with a biotinylated secondary antibody

![Fig. 4. Schematic representation of cell-based assay using europium chelates](#146881 - $15.00 USD)

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(anti-mouse). Antibody dilutions, incubation times, blocking and rinsing were carried out as recommended in the protocols provided by the antibody vendors. The final step was incubation with the EuDOTA streptavidin conjugates described above. Prior to microscopic imaging, the cells were rinsed with TRIS buffer and deionized water. The cells were then blanketed with a layer of NTA by evaporation of a 5 µL drop of 0.1 mM NTA ethanol on top of the cells. This produces a layer of sensitizing molecules that absorb two 740 nm photons and transfer energy to Eu$^{3+}$, which then emits radiation at 615 nm. We also performed the assay with a FITC-SA conjugate using a conventional fluorescence microscope for comparison with the Eu$^{3+}$ labels.

After taking the initial images, we optionally covered the cells with a drop of 0.1 M KHP (potassium hydrogen phthalate) solution. Only a small portion of the entire cell sample is subjected to KHP treatment. This allows for examination of other cells in the sample at a future time. We hypothesize that the low pH of the KHP solution releases Eu$^{3+}$ from the DOTA macrocycle by protonating its acetates and nitrogens, similar to the strategy of Hemmila et al. [1] except without the surfactant. The presence of excess NTA may assist in removal of the Eu$^{3+}$ from the DOTA. The Eu$^{3+}$ might then be directly complexed by NTA resulting in significantly better sensitization. Immediately upon addition of the KHP, the image becomes significantly brighter for a short time, but then the image intensity decreases. We ascribe the drop in intensity to release of the europium label from the cell surface as evidenced by the blurring of the contrast and a gradual drop in the signal level. The persistence of a lower signal level in a stable image after a few minutes may be due to a portion of the europium complex that remains adsorbed on the cell surface. The net result of the KHP treatment is not necessarily a brighter image but a more stable image as dry samples were not stable over long periods in the laser due to thermal effects. This optional step in the protocol provides evidence that the signal was indeed derived from the europium label without taking spectra and also allowed us to perform photostability measurements without complications due to thermal effects. Although we don’t have independent confirmation of the lanthanide species under each condition it is quite reasonable to expect that the lanthanide remains in the DOTA macrocycle when only NTA is applied since the Eu$^{3+}$ contrast is strongly localized, as is typical for antibody-based surface labeling. When KHP is added, the contrast becomes quite diffuse, indicating that the lanthanide is no longer localized by the antibody complex.

3. Results

Figure 5 shows images of A431 cells as (a) a bright field image, (b) a two-photon-excited luminescent image after application of the NTA layer, (c) an overlay of brightfield and two-photon-excited images, and (d) an image after KHP addition.

Figure 6 shows images of cells that were subjected to the same protocols as the cells in Fig. 4 except that the primary antibody was omitted. The image illustrates the specificity of the assay as well as the lack of autofluorescence. Figure 7 features bare cells examined in the multiphoton microscope that have been blanketed with NTA. This image further underscores the lack of autofluorescence for the two-photon-excited lanthanide assay.

3.1. Determination of detection limit

We have determined the detection limit for the europium label through signal-to-noise ratio measurements on a known quantity of the EuDOTA label with NTA. A solution of the europium label was prepared at 0.01 molar concentration. Serial dilution was used to prepare solutions at concentrations of $1 \times 10^{-4}$ and $1 \times 10^{-5}$ molar concentrations. Aliquots of these solutions (5 microliters) were applied to a microscope slide using a micropipette to cover an area of 1 cm$^2$. The diluted label concentrations and area information are used to derive a label concentration (5 \times 10^5 femtomoles/cm$^2$ for 100-fold dilution; this dilution is the basis for the remaining calculations). The width of an area viewed by a single pixel on the camera was determined through calibration using a resolution test target and found to be 0.118 microns, or
an area of $1.4 \times 10^{-10}$ cm$^2$. Thus there were 70 zeptomoles in the area viewed by a single CCD pixel. The fact that this size is below the diffraction limit is not important because we are determining signal levels per area and blurring does not change this number. CCD signals in counts per second were measured while the laser power was maintained at a constant level. This measurement yields a signal size of ~75 counts for the solution containing 70 zeptomoles in the area viewed by a single CCD pixel. Since a signal level of 75 counts is well above the read noise and dark current of the camera, we can detect below 100 zeptomoles of the europium chelate label.
It is not too surprising that detection is possible at the 100 zeptomole level. Because the experimental method does not descan the emitted Eu\textsuperscript{3+} luminescence, there is no loss of efficiency in light collection due to a pixel dwell time that is less than the luminescence lifetime. In addition, the lanthanide complex has two qualities that allow for a low detection limit. First, the absorption cross section is greatly enhanced by energy transfer from the NTA. Second, the quantum efficiency of Eu\textsuperscript{3+} can be relatively high when protected by ligands such as DOTA and NTA. Images acquired with and without the addition of KHP have similar brightness indicating that the exact ligand surrounding the Eu\textsuperscript{3+} is not critical. Although multiphoton excitation is less efficient than single photon excitation, single dye molecule detection has been demonstrated using two-photon excitation [21], which is roughly 5 orders of magnitude better than our detection limit. On comparing two-photon excitation of a dye molecule with the lanthanide chelate, the dye has a much shorter lifetime, and hence more detected photons per second, while the lanthanide chelate is quite insensitive to photobleaching and autofluorescence. The difference in lifetime and the energy transfer efficiency may be the dominant factors in the worse detection limit for the lanthanide chelate.

It is also of interest to know the quantity of label on the cells. Using the above calibration of counts per femtomole and cell areas known from the calibration for the imaged area per pixel, we find signal levels of 0.69 ± 0.36 femtomoles label per cell. There are roughly 6 to 7 chelate molecules per probe molecule. Thus the calibrated system method yields 0.11 ± 0.05 femtomoles of probe per cell.

For comparison, we can consider the total quantity of probe applied to the cells for imaging. The purified stock probe EuDOTA SA conjugate solution had a concentration of 0.5 mg/mL. This number was verified two ways: (1) through spectrophotometric measurement of the protein concentration at 280 nm, and (2) through comparison of the different fractions of the 1 mg/mL streptavidin sample after label conjugation and purification. This solution was diluted by a factor of 20 and a 0.3 mL aliquot was applied to the slide well of prepared cells. The number of cells per slide well was determined to be 5 × 10\textsuperscript{4} cells per well using a hemocytometer. Microscopic examination of the cell density supports this number. The molecular weight of the streptavidin is 53,000 (provided by the vendor). If all of the labeled probe bound to the cells, the maximum labeled probe on a cell was 2.8 femtomoles. This value is 2.7 femtomoles larger than the number determined from the system calibration method in the previous paragraph. This is not surprising because most of the conjugated probe typically washes off the cells after incubation.

Finally, we can compare the measured signal levels with the number of probe molecules per cell. The number of EGF receptors (EGFR) on A431 cells is about 2 × 10\textsuperscript{6} [22]. It is possible for more than one probe to bind to each receptor site. This amplification is due primarily to the multiple biotin molecules on the secondary antibody, which provide multiple binding sites for the streptavidin-label conjugates. In addition, some primary antibodies may have more than one secondary antibody bound to them. The extent of multiple binding of the
The streptavidin-label conjugate to the secondary antibody is influenced by the steric hindrance of the label. The chelating molecule for our label is quite small, with a molecular weight of 404 Daltons. The vendor for the secondary antibody (biotinylated donkey anti-mouse IgG from Jackson ImmunoResearch Laboratories, Inc.) has found that up to 10 conjugate molecules can bind to a single primary antibody receptor site for small conjugates, which is the case for us. Thus an upper bound for the number of probe molecules is $2 \times 10^7$, or 0.03 femtomoles/cell. This approximate calculation is 0.08 femtomoles smaller than 0.11 femtomoles/cell calculated from the calibrated system method.

3.2. Comparison with FITC labeling

We have compared our results with those obtained using fluorescein isothiocyanate (FITC), a widely used fluorescent reporter. The cell assays for FITC and the europium chelates were performed using the same concentrations and conditions for the application of the primary antibody, secondary antibody, and the streptavidin-label conjugate that we used for the europium chelate label. Images of the positive and negative controls for the FITC assay are shown in Fig. 8. The images for the FITC-labeled cells are quite similar to those obtained using the europium chelate label in Fig. 5, except that the europium chelate label has no discernable background in the negative control assay in Fig. 6. Fluorescein is one of the brightest optical labels known, and is often used for single molecule detection experiments (one molecule is $1.7 \times 10^{-3}$ zeptomoles). Because of photobleaching however, the imaging time for single molecules is limited.

3.3. Determination of autofluorescence

Figures 4 and 5 illustrate that autofluorescence is extremely low. We quantified the autofluorescence by taking an image of cells without the Eu\(^{3+}\) chelate reporter using a very long exposure time (1.2 minutes). Taking a ratio of the signal from labeled cells to the signal in the absence of reporter, we obtain a ratio of $7 \times 10^{-3}$, which is less than 1% of the signal from labeled cells.

3.4. Determination of photobleaching

Figure 9 demonstrates the photostability of the cell images. The average pixel intensity for a two-photon-excited luminescence image of cells treated with KHP is plotted as a function of time. The intensity remains stable over a twenty minute period with a standard deviation of 1%. While the europium cannot photobleach, the organic sensitizer should be susceptible to photobleaching. One possible rationale for the reduced photobleaching observed in Fig. 9 is related to the long-lived triplet state, which is generally believed to be the source of photobleaching. As shown in Fig. 1, energy transfer can occur rapidly between the triplet state and the nearby lanthanide ion that it sensitizes. Thus the coupling of the triplet state to the lanthanide ion can shorten the triplet state lifetime and reduce the amount of photobleaching. Another factor for these studies is that the excess NTA could compensate for photobleaching.
4. Conclusions

We have demonstrated imaging of EGFR receptors on A431 cells using an multiphoton microscope specially adapted to use of lanthanide chelates in which excitation is performed with a scanning laser beam and non-scanning detection is performed with a CCD camera. The assay provides a detection limit of approximately 100 zeptomoles of lanthanide chelate, photobleaching of ~1% over twenty minutes of illumination, and autofluorescent background of less than 1%.

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