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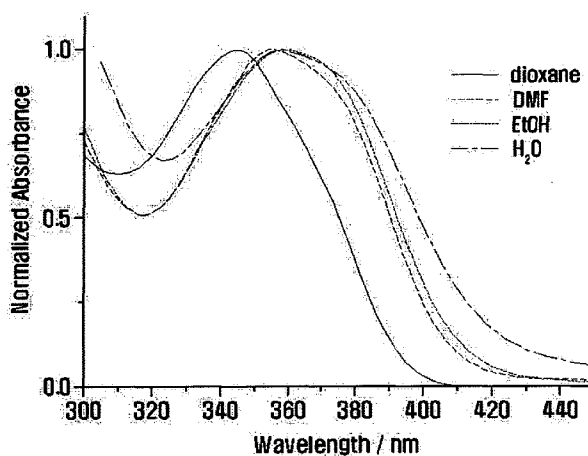
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(54) Title: TWO-PHOTON PROBE FOR REAL-TIME MONITORING OF INTRACELLULAR CALCIUM IONS, METHOD
FOR PREPARING THE PROBE AND METHOD FOR REAL-TIME MONITORING OF INTRACELLULAR CALCIUM IONS
USING THE PROBE

【Figure 1】



(57) Abstract: A two-photon probe for real-time monitoring of intracellular calcium ions is provided. The two-photon probe is very suitable for real-time imaging of intracellular calcium ions, shows 20~50-fold TPEF enhancement in response to Ca²⁺, has a dissociation constant (K_{dTP}) of 0.14 ± 0.02 to 0.25 ± 0.03 μ M, and emits 5-fold stronger TPEF than currently available one-photon fluorescent Ca²⁺ probes. Unlike the previously available probes, the two-photon probe can selectively detect dynamic levels of intracellular free Ca²⁺ in live cells and living tissues without interference from other metal ions and from the membrane-bound probes. Moreover, the two-photon probe is capable of monitoring the calcium waves at a depth of 100-300 μ m in live tissues for 1,100-4,000 s using two-photon microscopy (TPM) with no artifacts of photo-bleaching. Further provided are a method for preparing the two-photon probe and a method for real-time monitoring of intracellular calcium ions using the two-photon probe.

【Invention Title】

TWO-PHOTON PROBE FOR REAL-TIME MONITORING OF INTRACELLULAR CALCIUM IONS, METHOD FOR PREPARING THE PROBE AND METHOD FOR REAL-TIME MONITORING OF INTRACELLULAR CALCIUM IONS USING THE PROBE

【Technical Field】

The present invention relates to a two-photon probe for real-time monitoring of intracellular calcium ions, a method for preparing the two-photon probe, and a method for real-time monitoring of intracellular calcium ions using the two-photon probe. More particularly, the present invention relates to a two-photon probe suitable for real-time imaging of intracellular calcium ions due to its high two-photon absorption efficiency, ability to selectively recognize the membrane and calcium ions and high photostability, a method for preparing the two-photon probe, and a method for real-time monitoring of intracellular calcium ions using the two-photon probe.

【Background Art】

Calcium is a versatile intracellular signal messenger controlling numerous cellular functions. The Ca^{2+} -signalling system operates in many different ways to regulate various cellular processes that function over a wide dynamic range. Calcium triggers exocytosis within microseconds and drives the gene transcription and proliferation in minutes to hours.

To understand these functions, fluorescence imaging with fluorescent probes such as Oregon Green 488 BAPTA-1 (OG1) and fura-2 have most often been used. However, use of these probes with one-photon microscopy requires excitation with short wavelength light (~350-500 nm) that limits their application in tissue imaging

owing to shallow penetration depth ($< 100 \mu\text{m}$), photobleaching, photodamage, and cellular auto fluorescence.

Two-photon microscopy (TPM) overcomes these shortcomings. One-photon microscopy (OPM) employs one high-energy photon for excitation, whereas TPM employs two lower energy, near-infrared photons to produce an excited fluorescent substance. TPM has the advantages of localized excitation, increased penetration depth ($> 500 \mu\text{m}$), lower cellular autofluorescence and self-absorption, as well as reduced photodamage and photobleaching, when compared to OPM. Thus, TPM allows imaging deep inside tissues for a long period of time without interference from artifacts of surface preparation that can extend $> 70 \mu\text{m}$ into the tissue slice interior.

However, most of the fluorescent probes presently used for TPM have small TP action cross sections ($\Phi\delta$), demanding impractically high concentrations of probe and/or laser power. Furthermore, the fluorescence signals from membrane-bound probes can cause significant errors such as mistargeting because the fluorescence quantum yield is higher in the membrane than in the cytosol.

To the best of our knowledge, no study on two-photon dyes capable of selectively imaging calcium ions in real time has been reported. Therefore, there is a need to develop an efficient TP probe that can visualize the calcium waves deep inside the live tissue without photobleaching or mistargeting problems.

【Disclosure】

【Technical Problem】

Therefore, it is a first object of the present invention to provide a two-photon (TP) probe that has the advantages of significant TP cross section for bright TPM image at low probe concentration, high selectivity for Ca^{2+} ions, possible discrimination between the cytosolic and membrane-bound probes due to different emission spectra arising from the polarity of environments, and high photostability,

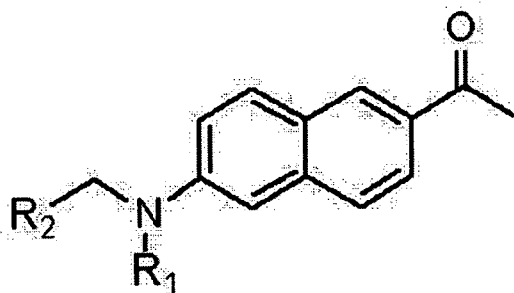
thus being suitable for real-time imaging of intracellular calcium ions.

It is a second object of the present invention to provide a method for preparing the two-photon probe.

It is a third object of the present invention to provide a method for real-time monitoring of intracellular calcium ions using the two-photon probe.

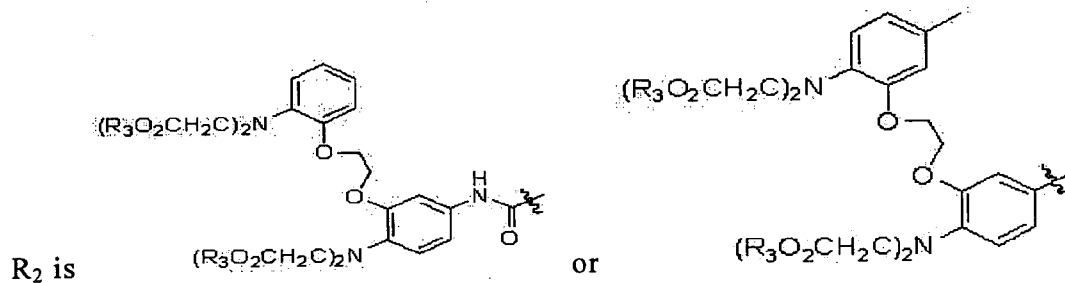
【Technical Solution】

In accordance with one aspect of the present invention, the first object can be accomplished by the provision of a two-photon probe for real-time monitoring of intracellular calcium ions, represented by Formula 1:



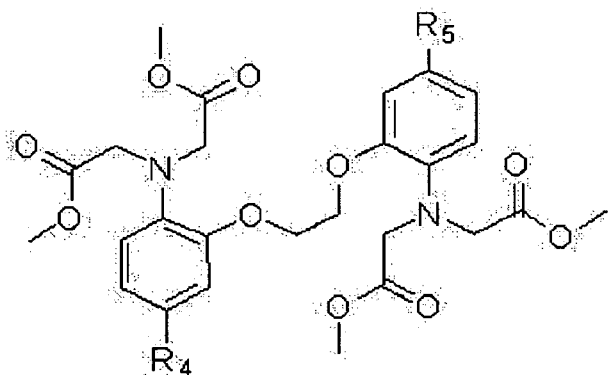
(1)

wherein R_1 is CH_3 or H , and



$(R_3 = H \text{ or } CH_2OCOCH_3)$.

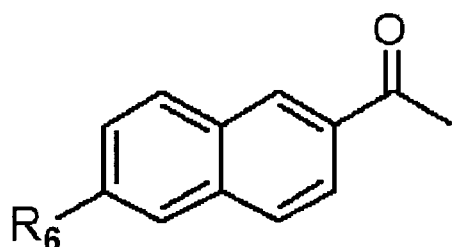
In accordance with another aspect of the present invention, the second object can be accomplished by the provision of a method for preparing the two-photon probe for real-time monitoring of intracellular calcium ions, the method comprising reacting a compound of Formula 2:



(2)

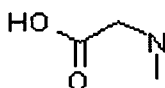
wherein R_4 is NH_2 or CHO and R_5 is H or CH_3 , with a compound of Formula

3:



(3)

wherein R_6 is NH_2 or



In accordance with yet another aspect of the present invention, the third object can be accomplished by the provision of a method for real-time monitoring of intracellular calcium ions, the method comprising the steps introducing the two-photon probe into cells of interest and imaging two-photon excited fluorescence emitted from the two-photon probe.

In an embodiment, the intracellular calcium ion concentration may be quantitatively determined by Equation 1:

$$[\text{Ca}^{2+}] = K_d[(F - F_{\min})/(F_{\max} - F)] \quad (1)$$

where K_d is the dissociation constant of the two-photon probe for Ca^{2+} , F is the observed two-photon fluorescence intensity, $F_{\min}$ is the minimum fluorescence intensity, and $F_{\max}$ is the maximum fluorescence intensity.

In another embodiment, the two-photon excited fluorescence images may be collected at wavelengths ranging from 500 to 620 nm.

The two-photon probe of the present invention is suitable for real-time imaging of intracellular calcium ions because it has the advantages of significant TP cross section for bright TPM image at low probe concentration, high selectivity for Ca^{2+} ions, possible discrimination between the cytosolic and membrane-bound probes due to different emission spectra with the polarity of environments (*e.g.*, hydrophilic and hydrophobic environments), and high photostability.

The two-photon probe of the present invention uses 2-acetyl-6-(dimethylamino)naphthalene as the TP chromophore and O,O'-bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (BAPTA) as the Ca^{2+} ion chelator.

The two-photon probe of the present invention shows significant TP cross section and large spectral shifts with the solvent polarity, allowing the detection of the two-photon excited fluorescence (TPEF) of the probe- Ca^{2+} complex separately from that of membrane-bound probes. In addition, the two-photon probe of the present invention is capable of imaging the calcium waves in live cells and living tissue at $> 100 \mu\text{m}$ depth for a long period of time without mistargeting and photobleaching problems.

Considering the cell permeability of the two-photon probe according to the present invention, R_3 in Formula 1 is preferably $\text{CH}_2\text{OCOCH}_3$. The replacement of the hydrogen atoms with $\text{CH}_2\text{OCOCH}_3$ in R_3 is performed by reacting bromomethyl acetate and triethylamine with the compound of Formula 1 ($\text{R}_3 = \text{H}$).

In the real-time monitoring method of the present invention, the two-photon excited fluorescence images can be collected using wavelengths between 500 nm and 620 nm and intracellular free Ca^{2+} only can be selectively detected with minimum contribution from the membrane-bound two-photon probes, as will be described below.

Unlike prior art methods, the intracellular calcium ions can be quantitatively

detected as well as qualitatively analyzed by the real-time monitoring method of the present invention.

【Advantageous Effects】

The two-photon probe of the present invention is very suitable for real-time imaging of intracellular calcium ions, shows 44-fold TPEF enhancement in response to Ca^{2+} , has a dissociation constant (K_d^{TP}) of $0.25 \pm 0.03 \mu\text{M}$, and emits 5-fold stronger TPEF than Oregon Green 488 BAPTA-1 (OG1) upon complexation with Ca^{2+} . Unlike the previously available probes, the two-photon probe of the present invention can selectively detect dynamic levels of intracellular free Ca^{2+} in live cells and living tissues without interference from other metal ions and from the membrane-bound probes. Moreover, the two-photon probe of the present invention is capable of monitoring the calcium waves at a depth of 100-300 μm in live tissues for longer than 1,100 s using TPM with no artifacts of photo-bleaching.

【Description of Drawings】

FIG. 1 shows absorption spectra of the compound of Formula 7 in 1,4-dioxane, DMF, ethanol and H_2O ;

FIG. 2 shows emission spectra of the compound of Formula 7 in 1,4-dioxane, DMF, ethanol and H_2O ;

FIG. 3 shows one-photon emission spectra of the compound of Formula 4 in the presence of free Ca^{2+} at various concentrations;

FIG. 4 shows one-photon absorption spectra of the compound of Formula 4 in the presence of free Ca^{2+} at various concentrations;

FIG. 5 shows two-photon action spectra of the compound (●) of Formula 4 and OG1 (○) in the presence of free $39 \mu\text{M}$ Ca^{2+} ;

FIG. 6 shows two-photon emission spectra of the compound of Formula 4 in

the presence of free Ca^{2+} at various concentrations;

FIG. 7 shows linear Hill plots for the complexation of the compound of Formula 4 with Ca^{2+} ;

FIG. 8 shows titration curves of the compound of Formula 4, which were fitted to Equation 5;

FIG. 9 shows one-photon emission spectra of the compound of Formula 4 in the presence of free Mg^{2+} at various concentrations;

FIG. 10 is a linear Hill plot for the complexation of the compound of Formula 4 with free Mg^{2+} at various concentrations;

FIG. 11 is a one-photon fluorescence titration curve for the complexation of the compound of Formula 4 with free Mg^{2+} at various concentrations;

FIG. 12 is a graph showing the reactivity of the compound of Formula 4 for various metal ions;

FIG. 13 is a graph showing the effect of pH on the reactivity of the compound of Formula 4;

FIG. 14 shows a pseudo colored TPM image of cultured astrocytes labeled with the compound of Formula 7;

FIG. 15 shows two-photon excited fluorescence spectra from the hydrophobic and hydrophilic domains of the astrocytes;

FIG. 16 shows a one-photon fluorescence intensity image labeled with the compound of Formula 7;

FIG. 17 shows a pseudocolored lifetime image of astrocytes labeled with the compound of Formula 7;

FIG. 18 shows the lifetime distribution of the astrocytes labeled with the compound of Formula 7;

FIG. 19 shows the results of single point analysis for the region indicated by the white arrow in FIG. 16;

FIG. 20 shows pseudo colored TPM images of astrocytes labeled with the compound of Formula 7, which were collected at 360-460 nm;

FIG. 21 shows pseudo colored TPM images of astrocytes labeled with the compound of Formula 7, which were collected at 500-620 nm;

FIG. 22 shows pseudo colored TPM Images of astrocytes labeled with the compound of Formula 7, which were taken after 110 s;

FIG. 23 shows pseudo colored TPM Images of astrocytes labeled with the compound of Formula 7, which were taken after 220 s;

FIG. 24 shows the time courses of the calcium waves in different locations of the astrocytes labeled with the compound of Formula 7;

FIG. 25 shows the time courses of the calcium waves in different locations of the astrocytes labeled with the compound of Formula 7;

FIG. 26 shows pseudo colored TPM images of an acute rat hypothalamic slice stained with the compound of Formula 7, which were taken after 195 s;

FIG. 27 shows pseudo colored TPM images of an acute rat hypothalamic slice stained with the compound of Formula 7, which were taken after 214 s; and

FIG. 28 shows spontaneous Ca^{2+} transients recorded in soma (1), astrocyte process (2), and neighboring cell (3).

【Mode for Invention】

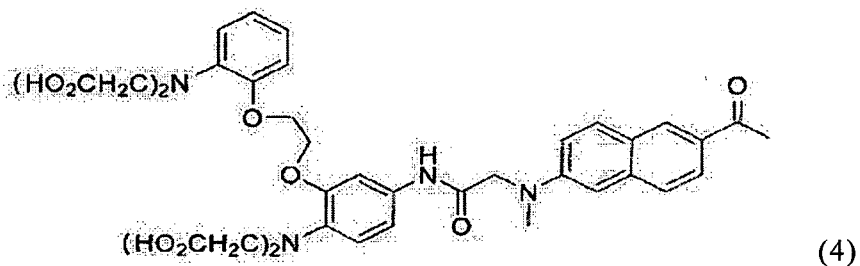
Hereinafter, the present invention will be explained in more detail with reference to the following examples. However, these examples serve to provide further appreciation of the invention but are not meant in any way to restrict the scope of the invention.

EXAMPLES

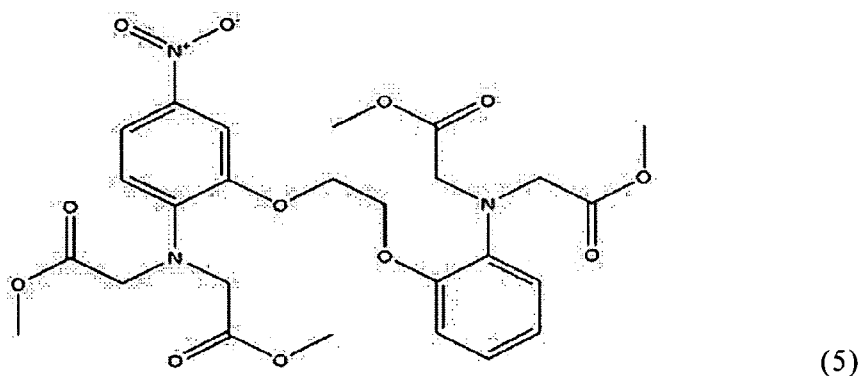
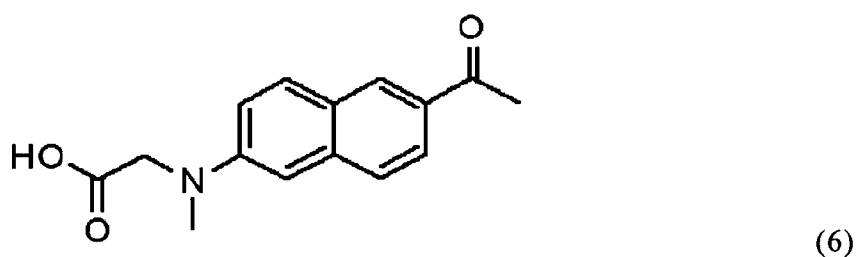
Preparative Example 1: Synthesis of the two-photon probe of the present

invention

In this example, the compound of Formula 4 was synthesized by the following procedure.

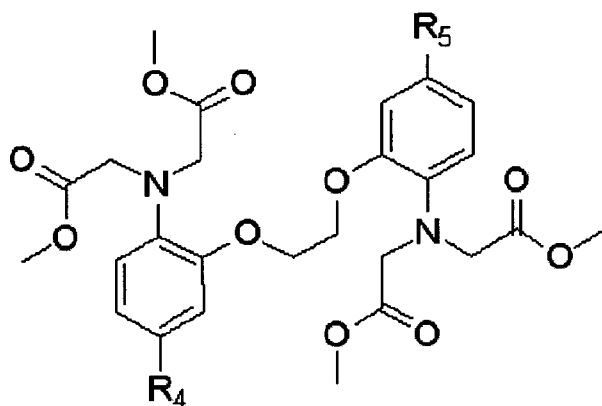


Preparative Example 1.1: Preparation of 5-nitro-BAPTA-tetramethyl ester (Formula 5) and 6-acetyl-2-[N-methyl-N-(carboxymethyl)amino]naphthalene (Formula 6)



The compounds were prepared by the literature methods (R. Pethig, M. Kuhn, R. Payne, E. Adler, T.-H. Chen, L. F. Jaffe, *Cell Calcium* **1989**, 10, 491-498 and H. M. Kim, C. Jung, B. R. Kim, S.-Y. Jung, J. H. Hong, Y.-G. Ko, K. J. Lee, B. R. Cho, *Angew. Chem. Int. Ed.* **2007**, 46, 3460-3463).

Preparative Example 1.2: Preparation of 5-amino-BAPTA-tetramethyl ester

(Formula 2)

(2)

(R₄ = NH₂, R₅ = H).

A mixture of the compound (2.2g, 3.8 mmol) of Formula 5 and 5% Pd on carbon (90 mg) in ethanol was shaken under hydrogen for 5 h. The reaction mixture was filtered and washed with hot ethanol, and the solvent was removed in vacuo. The product was purified by column chromatography using ethyl acetate/hexane (2:1) as the eluent.

Yield: 1.1g (53%); mp 121 °C; IR (KBr): 3438, 3346, 1753 cm⁻¹; ¹H NMR (300 MHz, CDCl₃): δ 6.87 (m, 4H), 6.77 (d, 1H, *J* = 9 Hz), 6.28 (d, 1H, *J* = 3.0 Hz), 6.22 (dd, 1H, *J* = 9, *J* = 3 Hz), 4.26 (m, 4H), 4.15 (s, 4H), 4.06 (s, 4H), 3.59 (s, 6H), 3.56 (s, 6H), 3.51 (br s, 2H); ¹³C NMR (100 MHz, CDCl₃): δ = 172.4, 172.3, 152.2, 150.6, 142.7, 139.4, 131.5, 122.5, 121.6, 119.2, 113.3, 113.2, 107.8, 101.8, 67.3, 67.2, 53.9, 53.5, 51.9, 51.7 ppm; Anal. Calcd for C₂₆H₃₃N₃O₁₀: C, 57.03; H, 6.07; N, 7.67. Found: C, 57.11; H, 6.05; N, 7.60.

Preparative Example 1.3: Preparation of compound of Formula 4

A mixture of the compound (0.38g, 1.48 mmol) of Formula 6 and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (0.34g, 1.80 mmol) in DMF (5 mL) was stirred for 20 min. To this mixture, the compound (0.90g, 1.64 mmol) of Formula 2 and 4-dimethylaminopyridine (26 mg, 0.22 mmol) were added and stirred for 12 h

under N₂. The product was extracted with chloroform, dried over MgSO₄, and the solvent was removed in vacuo. The product was purified by column chromatography using hexane/ethyl acetate (2:3) as the eluent. It was further purified by recrystallization from EtOH.

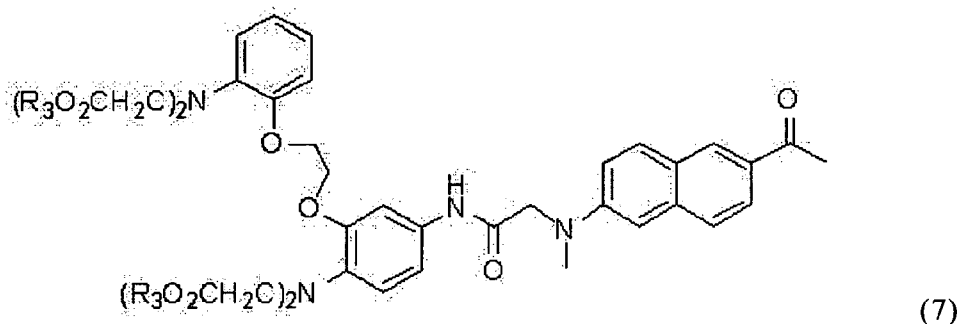
Yield: 0.62g (53%); mp 148 °C; IR (KBr): 3260, 1755, 1662 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 8.35 (d, 1H, *J* = 2 Hz), 8.18 (s, 1H), 7.98 (dd, 1H, *J* = 9, *J* = 2 Hz), 7.87 (d, 1H, *J* = 9 Hz), 7.70 (d, 1H, *J* = 9 Hz), 7.27 (d, 1H, *J* = 2 Hz), 7.16 (dd, 1H, *J* = 9, *J* = 2 Hz), 7.06 (d, 1H, *J* = 2.0 Hz), 6.88 (m, 6H), 4.25 (s, 4H), 4.12 (s, 4H), 4.10 (s, 6H), 3.56 (s, 6H), 3.53 (s, 6H), 3.24 (s, 3H), 2.68 (s, 3H); ¹³C NMR (100 MHz, CDCl₃): δ = 198.0, 172.2, 172.0, 168.0, 150.8, 150.5, 149.3, 139.4, 137.3, 136.4, 132.3, 132.2, 131.6, 130.4, 127.0, 126.6, 125.2, 122.5, 121.7, 119.4, 119.2, 116.7, 113.3, 112.9, 107.7, 106.1, 77.4, 67.5, 67.1, 59.5, 53.6, 51.9, 51.8, 40.4, 26.8 ppm; Anal. Calcd for C₄₁H₄₆N₄O₁₂: C, 62.59; H, 5.89; N, 7.12. Found: C, 62.52; H, 5.93; N, 7.07.

This ester (0.50g, 0.64 mmol) was hydrolyzed by the method as described above. The resulting precipitate was collected, washed with distilled water, and purified by crystallization from MeOHCHCl₃-petroleum ether.

Yield: 0.27g (58%); mp 145 °C; IR (KBr): 3250, 2910, 1745, 1660 cm⁻¹; ¹H NMR (400 MHz, CD₃OD): δ 8.39 (d, 1H, *J* = 2 Hz), 7.86 (d, 1H, *J* = 9 Hz), 7.85 (dd, 1H, *J* = 9, *J* = 2 Hz), 7.65 (d, 1H, *J* = 9 Hz), 7.46 (d, 1H, *J* = 2 Hz), 7.24 (dd, 1H, *J* = 9, *J* = 2 Hz), 6.96 (m, 7H), 4.31 (s, 4H), 4.30 (s, 2H), 3.91 (s, 8H), 3.24 (s, 3H), 2.63 (s, 3H); ¹³C NMR (100 MHz, CD₃OD): δ = 199.2, 173.9, 173.8, 170.0, 150.6, 150.5, 149.9, 149.8, 138.7, 138.0, 137.9, 135.3, 134.2, 130.8, 130.7, 126.3, 126.2, 125.8, 124.1, 123.6, 121.3, 118.7, 118.4, 116.2, 113.0, 112.5, 105.8, 66.8, 66.5, 56.3, 54.5, 39.2, 25.3 ppm. Anal. Calcd for C₃₇H₃₈N₄O₁₂: C, 60.82; H, 5.24; N, 7.67. Found: C, 60.72; H, 5.34; N, 7.59

Preparative Example 2: Synthesis of the two-photon probe of the present invention

In this example, the compound of Formula 7 was synthesized by the following procedure.



(R₃ = CH₂OCOCH₃).

A mixture of the compound (0.11g, 0.15 mmol) of Formula 4, bromomethyl acetate (0.24g, 1.55 mmol), and Et₃N (0.14g, 1.05 mmol) in CHCl₃ (5 mL) was stirred under N₂ for 24 h. The solution was removed in vacuo and the crude product was purified by column chromatography using ethyl acetate/hexane (3:1) as the eluent. It was further purified by recrystallization from MeOH to obtain a pale yellow solid.

Yield: 85 mg (56%); mp 137 °C; IR (KBr): 1759, 1710, 1665 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 8.36 (d, 1H, *J* = 2 Hz), 8.22 (s, 1H), 7.98 (dd, 1H, *J* = 9, *J* = 2 Hz), 7.88 (d, 1H, *J* = 9 Hz), 7.71 (d, 1H, *J* = 9 Hz), 7.31 (d, 1H, *J* = 2 Hz), 7.17 (dd, 1H, *J* = 9, *J* = 2 Hz), 7.07 (d, 1H, *J* = 2 Hz), 6.88 (m, 6H), 5.62 (s, 4H), 5.60 (s, 4H), 4.29 (s, 4H), 4.18 (s, 4H), 4.15 (s, 4H), 4.12 (s, 2H), 3.25 (s, 3H), 2.69 (s, 3H), 2.05 (s, 6H), 2.04 (s, 6H); ¹³C NMR (100 MHz, CDCl₃): δ = 198.0, 170.4, 170.2, 169.7, 168.1, 151.0, 150.6, 149.3, 138.8, 137.3, 135.7, 132.9, 132.3, 131.5, 130.3, 127.0, 126.6, 125.2, 123.2, 121.9, 120.4, 120.0, 116.7, 113.8, 113.1, 107.7, 106.5, 79.5, 79.4, 77.5, 77.4, 77.1, 67.5, 67.2, 59.5, 53.5, 40.4, 26.7, 20.9 ppm; Anal. Calcd for C₄₉H₅₄N₄O₂₀: C, 57.76; H, 5.34; N, 5.50. Found: C, 57.70; H, 5.31; N, 5.52.

Example 1: Measurements of absorption and emission spectra

The absorption spectra of the compound of Formula 7 were recorded on a Hewlett-Packard 8453 diode array spectrophotometer, and the fluorescence spectra of the compound were obtained with Amico-Bowman series 2 luminescence spectrometer with a 1 cm standard quartz cell. The fluorescence quantum yield of the compound was determined by using Coumarin 307 as the reference by the literature method (J. N. Demas, G. A. Crosby, *J. Phys. Chem.* **1971**, 75, 991-1024.). FIGs. 1 and 2 show the absorption (FIG. 1) and emission (FIG. 2) spectra of the compound in 1,4-dioxane, DMF, ethanol and H₂O. The absorption and emission maxima of the compound in various solvents are shown in Table 1.

【Table 1】

Compound	Solvent (E_T^N)*	$\lambda_{\max}^{(l)}$ (nm)	$\lambda_{\max}^{fl}$ (nm)
Formula 7	1,4-dioxane (0.164)	345	413
	DMF (0.386)	355	440
	Ethanol (0.654)	359	473
	H ₂ O (1.000)	362	495

*: The numbers in the parenthesis are normalized empirical parameter of solvent polarity (C. Reichardt, *Chem Rev.* **1994**, 94, 2319-2358).

The absorption and emission spectra of the compound of Formula 7 showed gradual red shifts with the solvent polarity in the order 1,4-dioxane < DMF < ethanol < H₂O. The effect was greater for the emission (82 nm) than for the absorption spectrum (17 nm), thus indicating the utility of the compound of the Formula 7 as a polarity probe. In addition, $\lambda_{\max}^{fl}$ of the compound of Formula 7 in DMF was similar to that of the membrane-bound probes, suggesting that it can be used as the model for membrane-bound probe.

FIGs. 3 and 4 show one-photon emission (FIG. 3) and absorption (FIG. 4)

spectra of the compound (1 μ M, 30 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) of Formula 4 in the presence of free Ca^{2+} at various concentrations (0-39 μ M); FIG. 5 shows two-photon action spectra of the compound (●) of Formula 4 and OG1 (○) in the presence of free 39 μ M Ca^{2+} ; and FIG. 6 shows two-photon emission spectra of the compound (1 μ M, 30 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) of Formula 4 in the presence of free Ca^{2+} at various concentrations.

When Ca^{2+} was added to the compound of Formula 4 in MOPS buffer solution (30 mM, pH 7.2), the fluorescence intensity increased dramatically as a function of metal ion concentration without affecting the absorption spectra (FIGs. 3 and 4), probably due to the blocking of the photo-induced electron transfer (PET) process by the complexation of metal ion.

A nearly identical result was observed in the two-photon process (FIGs. 5 and 6). The fluorescence enhancement factor $[(F - F_{\min})/F_{\min}]$ of the compound of Formula 4 was 40 in the presence of 39 μ M Ca^{2+} , nearly 3-fold larger than the value of 14 previously reported for OG1 (R. Rudolf, M. Mongillo, R. Rizzuto, T. Pozzan, *Nat. Rev. Mol. Cell Biol.* **2003**, 4, 579-586). Moreover, linear Hill plots determined for Ca^{2+} binding with slopes of 1.0 indicated 1:1 complexation between the probe and the cations (FIG. 7) (K. A. Connors, *Binding Constants* John Wiley & Sons, Inc.: New York, 1987).

Example 2: Determination of Dissociation Constants (K_d)

A series of calibration solutions containing various $[\text{Ca}^{2+}]$ was prepared by mixing two solutions (solution A containing 10 mM K_2EGTA and solution B containing 10 mM CaEGTA) in various ratios. Both solutions contained the compound (1 μ M) of Formula 4, 100 mM KCl, 30 mM MOPS, and they were adjusted to pH 7.2.

To determine the K_d for Ca^{2+} -the compound of Formula 4, the fluorescence spectrum was recorded with 2.0 mL of solution A (0 μ M free Ca^{2+}) at 20 °C. Then, 203

$\mu\ell$ of this solution was discarded and replaced by 203 $\mu\ell$ of solution B (39 μM free Ca^{2+}), and the spectrum was recorded. This brings the CaEGTA concentration to 1.00 mM and the $[\text{Ca}^{2+}]_{\text{free}}$ to about 0.017 μM with no change in the concentration of the probe or of the total EGTA. The $[\text{Ca}^{2+}]_{\text{free}}$ is calculated from the K_d of EGTA for Ca^{2+} (150.5 nM) using Equation 2:

$$[\text{Ca}^{2+}]_{\text{free}} = K_d^{\text{EGTA}} \times \frac{[\text{CaEGTA}]}{[\text{K}_2\text{EGTA}]} \quad (2)$$

Further, iterations attained 0.038, 0.065, 0.101, 0.150, 0.230, 0.350, 0.601, 0.800, 1.00, 1.30, 2.50, 5.30, 10.0, and 20.0 μM free Ca^{2+} by successively discarding 223, 251, 285, 327, 421, 479, 667, 420, 350, 412, 905, 1028, 926, and 992 $\mu\ell$ of solution A and replacing each with an equal volume of solution B.

When a 1:1 metal-ligand complex is formed between the compound of Formula 4 and Ca^{2+} , the equilibrium can be described by Equation 3:

$$[\text{LM}]^2 - ([\text{L}]_0 + [\text{M}]_0 + K_d)[\text{LM}] + [\text{L}]_0[\text{M}]_0 = 0 \quad (3)$$

where L and M represent the compound of Formula 4 and Ca^{2+} , respectively.

The total probe and metal ion concentration are defined as $[\text{L}]_0 = [\text{L}] + [\text{LM}]$ and $[\text{M}]_0 = [\text{M}] + [\text{LM}]$, respectively. With $[\text{L}]_0$ and $[\text{M}]_0$, the value of K_d is given by:

$$[\text{LM}] = \frac{([\text{L}]_0 + [\text{M}]_0 + K_d) - \sqrt{([\text{L}]_0 + [\text{M}]_0 + K_d)^2 - 4[\text{L}]_0[\text{M}]_0}}{2}$$

(4);

$$(F - F_{\min}) = \left(\frac{([\text{L}]_0 + [\text{M}]_0 + K_d) - \sqrt{([\text{L}]_0 + [\text{M}]_0 + K_d)^2 - 4[\text{L}]_0[\text{M}]_0}}{2[\text{L}]_0} \right) (F_{\max} - F_{\min})$$

(5)

where F is the observed fluorescence intensity, $F_{\min}$ is the minimum fluorescence intensity, and $F_{\max}$ is the maximum fluorescence intensity. The K_d value that best fits the titration curve (FIG. 8) with Equation 5 was calculated by using the Excel program as reported (J. R. Long, R. S. Drago, *J. Chem. Ed.* **1982**, 59, 1037; K. Hirose, *J. Incl. Phenom. Macrocycl. Chem.* **2001**, 39, 193).

In order to determine the K_d^{TP} for the two-photon process, the TPEF spectra were obtained with a DM IRE2 Microscope (Leica) excited by a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 780 nm and output power 1230 mW, which corresponded to approximately 10 mW average power in the focal plane. The TPEF titration curves (FIG. 8) were obtained and fitted to Equation 3.

The dissociation constants (K_d^{OP}) were calculated from the fluorescence titration curves (FIG. 8), as reported (J. R. Long, R. S. Drago, *J. Chem. Educ.* **1982**, 59, 1037-1039).

One-photon emission spectra of the compound of Formula 4 (30 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) in the presence of free Mg^{2+} (0-30 mM), a Hill plot for the complexation of the compound of Formula 4 with free Mg^{2+} (0-30 mM), and a one-photon fluorescence titration curve for the complexation of the compound of Formula 4 with free Mg^{2+} (0-30 mM) were obtained and are shown in FIGs. 9, 10 and 11, respectively.

The K_d^{OP} values of the compound of Formula 4 for Ca^{2+} and Mg^{2+} were $0.27 \pm 0.01 \mu\text{M}$ and $6.8 \pm 0.7 \text{ mM}$, respectively. A similar value was determined in the two-photon process [$K_d^{\text{TP}} (\text{Ca}^{2+}) = 0.25 \pm 0.03 \mu\text{M}$]. The compound of Formula 4 showed modest response toward Zn^{2+} and Mn^{2+} , much weaker response toward Mg^{2+} , Fe^{2+} and CO^{2+} , and no response toward Cu^{2+} (FIG. 12).

Because the intracellular concentrations of free Mn^{2+} is negligible and chelatable Zn^{2+} is essentially nonexistent except in specialized areas such as the

hippocampal CA3 region, the probe of the present invention can selectively detect the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) without interference from other metal ions. Furthermore, the compound of Formula 4 is pH-insensitive in the biologically relevant pH range (FIG. 13).

Example 3: Observations of two-photon action spectra

The TP action spectra of the Ca^{2+} complexes with the compound of Formula 4 and OG1 in buffer solutions indicated a value of 110 GM at 780 nm for $\Phi\delta$ for the compound of Formula 4- Ca^{2+} , 5-fold larger than that of OG1- Ca^{2+} . Thus, TPM images for samples stained with the compound of Formula 4 would be much brighter than those stained with commercial probe. In addition, the two-photon fluorescence enhancement factor (TFEF) estimated from the two-photon titration curve was 44 (Table 2), a value that allowed detection of Ca^{2+} by TPM.

【Table 2】

compd ^[a]	$\lambda_{\text{max}}^{(1)}$ [b]	$\lambda_{\text{max}}^{(2)}$ [b]	Φ [c]	$K_d^{\text{OP}}/K_d^{\text{TP}}$ [d]	$\text{TFEF}^{\text{OP}}/\text{TFEF}^{\text{TP}}$ [e]	$\lambda_{\text{max}}^{(2)}$ [f]	δ [g]	$\Phi\delta$ [h]
ACal-AM	362	495	0.060 ^[i]			nd ^[j]	nd ^[j]	nd ^[j]
ACal	365	498	0.012			nd ^[j]	nd ^[j]	nd ^[j]
ACal+ Ca^{2+}	365	498	0.49	0.27 ^[k] /0.25	40/44	780	230	110
OG1+ Ca^{2+}	494	523	0.66	0.17 ^[l] /nd	14 ^[l] /nd	800	37	24

[a] All data were measured in 30 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2 in the absence and presence (39 μM) of free Ca^{2+} .

[b] λ_{max} of the one-photon absorption and emission spectra in nm.

[c] Fluorescence quantum yield, $\pm 10\%$.

[d] Dissociation constants for Ca^{2+} in μM measured by one- (K_d^{OP}) and two-photon (K_d^{TP}) processes, $\pm 12\%$.

[e] Fluorescence enhancement factor, $(F - F_{\text{min}})/F_{\text{min}}$, measured by one-

(FEFOP) and two-photon (FEFTP) processes.

[f] $\lambda_{\max}$ of the two-photon excitation spectra in nm.

[g] The peak two-photon cross section in $10^{-50} \text{ cm}^4\text{s/photon (GM)}$, $\pm 15\%$.

[h] Two-photon action cross section.

[i] $\Phi = 0.27 \pm 0.02$ (compound of Formula 4) and 0.22 ± 0.02 (compound of Formula 7) in DMF.

[j] The two-photon excited fluorescence intensity was too weak to measure the cross section accurately.

[k] K_d^{OP} value of the compound of Formula 4 for Mg^{2+} was $6.8 \pm 0.7 \text{ mM}$.

[l] *A Guide to Fluorescent Probes and Labeling Technologies*, 10th ed., (Ed.: R. P. Haugland), Molecular Probes, Eugene, OR, **2005**.

The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique (S. K. Lee, W. J. Yang, J. J. Choi, C. H. Kim, S.-J. Jeon, B. R. Cho, *Org. Lett.* **2005**, 7, 323-326).

Example 4: Observation of cells using the two-photon probe of the present invention

Astrocytes were taken from cerebral cortices of 1-day-old rats (Sprague-Dawley; SD). Cerebral cortices were dissociated in Hank's balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD, USA) containing 3 U/ml papain (Worthington Biochemical Corporation, NJ, USA) and plated in 75 mm flasks. To prepare purified astrocytes culture, flasks were shaken for 6 h on a shaker at 37°C , and the floating cells that were displaced into the media were removed. Astrocytes were passaged with 5 min exposure to 0.25% trypsin and replated onto poly-D-lysine-coated glass coverslips at $50\sim 100 \text{ cells/mm}^2$ and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with penicillin/streptomycin and 10% fetal

bovine serum (FBS; Gibco) in a CO₂ incubator at 37°C. After 7-15 days in vitro, astrocytes were washed three times with serum-free media, and then incubated with 2 μM of the compound of Formula 7 in serum-free media for 20 min at 37°C. The cells were washed three times with phosphate buffered saline (PBS; Gibco) and then imaged after further incubation in colorless serum-free media for 15 min.

The pseudo colored TPM images of cultured astrocytes labeled with the compound (2 μM) of Formula 7 showed intense spots and homogeneous domains (FIG. 14). It has been estimated that the image was attributed to the TPEF emitted from the intracellular Ca²⁺ complex with the compound of Formula 4 and membrane-bound probes because the fluorescence quantum yields of the compound of Formula 4-Ca²⁺ in MOPS buffer (0.49) and the compound of Formula 7 in DMF (0.27) are much higher than those of the compound of Formula 4 (0.012) and the compound of Formula 7 (0.060) in MOPS buffer (Table 2), and the compound of Formula 7 in DMF has been assumed to be a good model for the membrane-bound probes due to the similarity in $\lambda_{\text{max}}^{\text{fl}}$.

The TPEF spectra of the intense spots and homogeneous domains showed emission maxima at 445 (FIG. 15, dotted line) and 494 nm (FIG. 15, solid line), respectively. Moreover, the emission band represented with dotted line was asymmetrical and could be fitted to two Gaussian functions with emission maxima at 445 and 488 nm, respectively, whereas the emission band represented with solid line could be fitted to a single Gaussian function with maximum at 500 nm. Compared with the emission spectra recorded in MOPS buffer (FIG. 3), the shorter wavelength band of the dissected spectrum was significantly blue shifted while the longer wavelength band remained similar. The spectral shift suggests that the probes may be located in two regions of differing polarity.

To assess the polarity of environments, lifetime images of astrocytes labeled

with the compound of Formula 7 were obtained.

Astrocytes were grown on coverslip in 10% FBS containing DMEM. Cells were washed briefly in PBS and incubated with 2 μ M of the compound of Formula 7 for 20 min at 37 °C. Cells were washed with PBS three times, fixed with formaldehyde (3.7% in PBS) for 10 min, washed with PBS three times and then mounted with mounting solution. The fluorescence decays were resolved by time-correlated single-photon counting (TCSPC) using an SPC830 acquisition board (Becker & Hickl, Berlin) synchronized with a Leica TSC-SP2-AOBS confocal microscope. The results are shown in FIGs. 16 to 19.

Referring to these figures, the intense spot exhibited an excited state lifetime of 1.8 ns, more than 2-fold longer than the upper extreme of the lifetime distribution curve centered at ~0.8 ns. This result may reflect two distinct environments populated by the probe of the present invention, a hydrophilic one that is likely to be cytosolic, which emits red light with a shorter lifetime, and a hydrophobic one, likely to be membrane-associated, with longer-lived blue emission.

The intracellular Ca^{2+} could be detected with minimum errors due to the membrane-bound probes. The spectrum of the shorter wavelength band in the dissected Gaussian function (FIG. 15, dotted line) decreased to baseline at ~500 nm, indicating that the TPEF emitted from the membrane-bound probes contributes negligible interference at $\lambda > 500$ nm. Similarly, if it is considered that the compound of Formula 7 in DMF is as a model for membrane-bound probe, the fluorescence at $\lambda > 500$ nm accounts for only 5% of the total emission band (FIG. 2). Consistently, the TPEF image collected at 500-620 nm is homogeneous without the intense spots (FIG. 21), whereas that collected at 360-460 nm clearly shows them (FIG. 20). Therefore, the intracellular Ca^{2+} can be selectively detected by using the detection window at 500-620 nm.

To demonstrate the utility of this probe, $[\text{Ca}^{2+}]_i$ waves in live cells and tissue

were monitored. The TPM images of cultured astrocytes labeled with the compound (2 μM) of Formula 7 revealed the spontaneous Ca^{2+} signal propagation from the astrocytic process (1) to soma (2) to terminal (3) with a speed of $7.5 \pm 2.2 \mu\text{m/s}$ ($n = 5$ astrocytes) (FIGs. 22 and 24). The spontaneous increase in $[\text{Ca}^{2+}]_i$ also propagated between astrocytes, as indicated by the delayed activity in the neighboring astrocyte (FIGs. 23 and 25).

The speed of propagation of spontaneously occurring waves was $1.8 \pm 1.1 \mu\text{m/s}$ ($n = 7$ astrocytes), a result consistent with reported data (H. R. Parri, T. M. Gould, V. Crunelli, *Nat. Neurosci.* **2001**, *4*, 803-812). Thus, the compound of Formula 4 is clearly capable of visualizing the intra- and intercellular calcium waves in cultured astrocytes using TPM.

Example 5: Observations of acute rat hypothalamic slices

Slices were prepared from the hippocampi and the hypothalmi of 2-day-old rat (SD). Coronal slices were cut into 400 μm thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 138.6 mM NaCl, 3.5 mM KCl, 21 mM NaHCO_3 , 0.6 mM NaH_2PO_4 , 9.9 mM D-glucose, 1 mM CaCl_2 , and 3 mM MgCl_2). Slices were incubated with the compound (10 μM) of Formula 7 in ACSF bubbled with 95% O_2 and 5% CO_2 for 30 min at 37°C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope.

To obtain the TPM images of the CA1 region in the presence of 20 μM N,N,N',N'-terakis(2-pyridyl)ethylenediamine (TPEN), a membrane permeable Zn^{2+} chelator that can effectively remove Zn^{2+} by chelation without causing toxic effect (C. M. Matias, N. C. Matos, M. Arif, J. C. Dionisio, M. E. Quinta-Ferreira, *Biol. Res.* **2006**, *39*, 521-530), a 20 mM stock solution of TPEN was prepared by dissolving 8.5 mg of TPEN in 1.0 mL of ethanol (M. E. Quinta-Ferreira, C. M. Matias, *Brain Res.*

2004, 1004, 52-60). A 1.0 μl of this solution was added to 1.0 mL of ACSF to prepare 20 μM TPEN in ACSF. After taking the TPM image of the hippocampal slice labeled with the compound of Formula 7, the ACSF solution in the glass-bottomed dish was removed with a micropipette, 1 mL of 20 μM TPEN in ACSF was added, and then TPM image was obtained.

FIGs. 26 and 27 shows pseudo colored TPM images of an acute rat hypothalamic slice stained with the compound (10 μM) of Formula 7 taken after 195 s (FIG. 26) and 214 s (FIG. 27), and FIG. 28 shows spontaneous Ca^{2+} transients recorded in soma (1), astrocyte process (2), and neighboring cell (3) in FIGs. 26 and 27.

Referring to FIG. 28, the spontaneous Ca^{2+} waves in the soma could be clearly visualized with a frequency of about 16 mHz ($n = 4$ slices) for more than 1100 s without appreciable decay. Furthermore, the spikes at the astrocyte process appeared slightly before those in the soma, confirming the previous finding that the signals propagate progressively from the process to the soma (J. Y. Koh, S. W. Suh, B. J. Gwag, Y. Y. He, C. Y. Hsu, D. W. Choi, *Science* **1996**, 272, 1013-1016).

Similar results were reported for TTX treated thalamus slices stained with fura-2 (H. R. Parri, T. M. Gould, V. Crunelli, *Nat. Neurosci.* **2001**, 4, 803-812), except that the image revealed damaged cells on the tissue surface and was not as clear as the TPM image presented in the present invention. Also, the TTX fluorescence intensity decayed appreciably after 500 s.

The improved TPM image of tissue labeled with the compound of Formula 4 obtained at $\sim 170 \mu\text{m}$ depth for a prolonged observation time underlines the high photo-stability and low photo-toxicity of the probe according to the present invention in addition to the capability of deep tissue imaging.

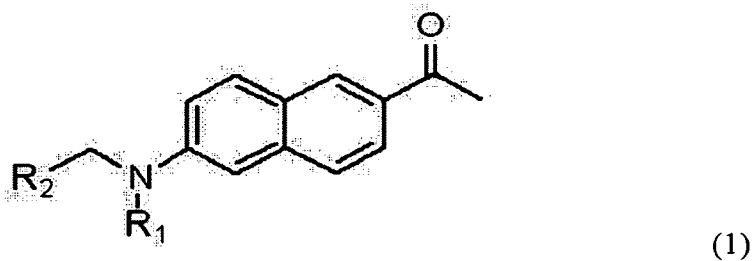
Finally, the spikes at the process became very weak after 700 s, probably because it has moved away from the focal point under the microscope. As can be seen

from the data in FIG. 28, a similar calcium wave was also observed in a different cell.

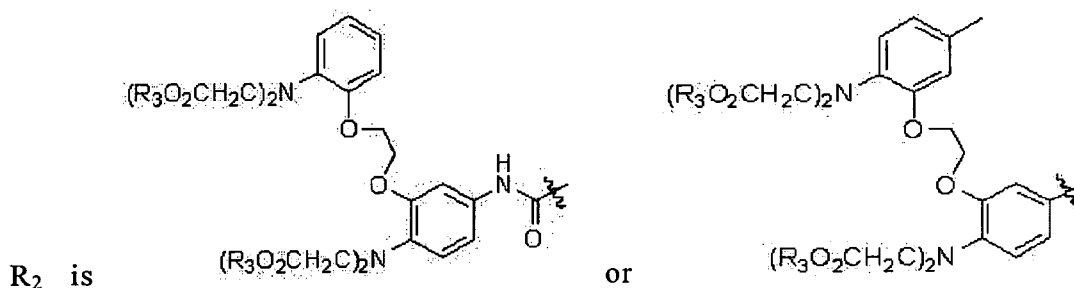
【CLAIMS】

【Claim 1】

A two-photon probe for real-time monitoring of intracellular calcium ions, represented by Formula 1:



wherein R₁ is CH₃ or H, and



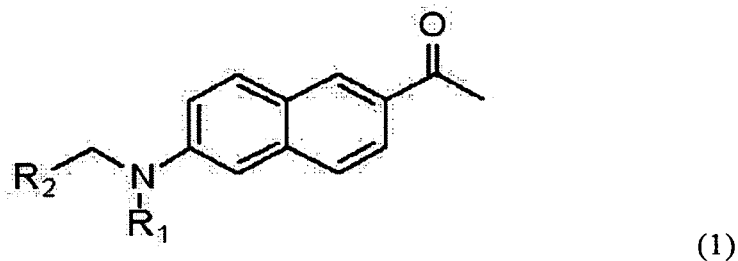
(R₃ = H or CH₂OCOCH₃).

【Claim 2】

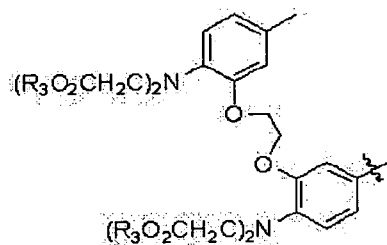
The two-photon probe according to claim 1, wherein R₃ is CH₂OCOCH₃.

【Claim 3】

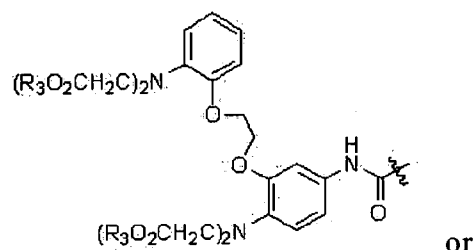
A method for preparing a two-photon probe for real-time monitoring of intracellular calcium ions, represented by Formula 1:



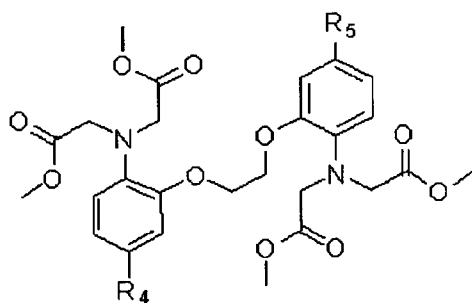
wherein R_1 is CH_3 or H , and R_2 is



($R_3 = \text{H}$ or $\text{CH}_2\text{OCOCH}_3$),



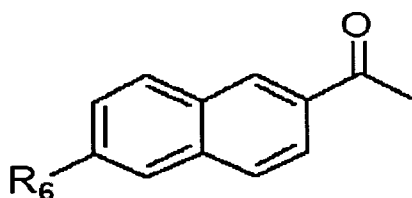
the method comprising reacting a compound of Formula 2:



(2)

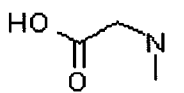
wherein R_4 is NH_2 or CHO and R_5 is H or CH_3 , with a compound of Formula

3:



(3)

wherein R_6 is NH_2 or



【Claim 4】

The method according to claim 3, wherein the hydrogen atoms in R_3 are replaced with $\text{CH}_2\text{OCOCH}_3$ by reacting bromomethyl acetate and triethylamine with the compound of Formula 1 ($R_3 = \text{H}$).

【Claim 5】

A method for real-time monitoring of intracellular calcium ions, the method

comprising the steps introducing the two-photon probe according to claim 1 or 2 into cells of interest and imaging two-photon excited fluorescence emitted from the two-photon probe.

【Claim 6】

The method according to claim 5, wherein the intracellular calcium ion concentration is quantitatively determined by Equation 1:

$$[\text{Ca}^{2+}] = K_d[(F - F_{\min})/(F_{\max} - F)] \quad (1)$$

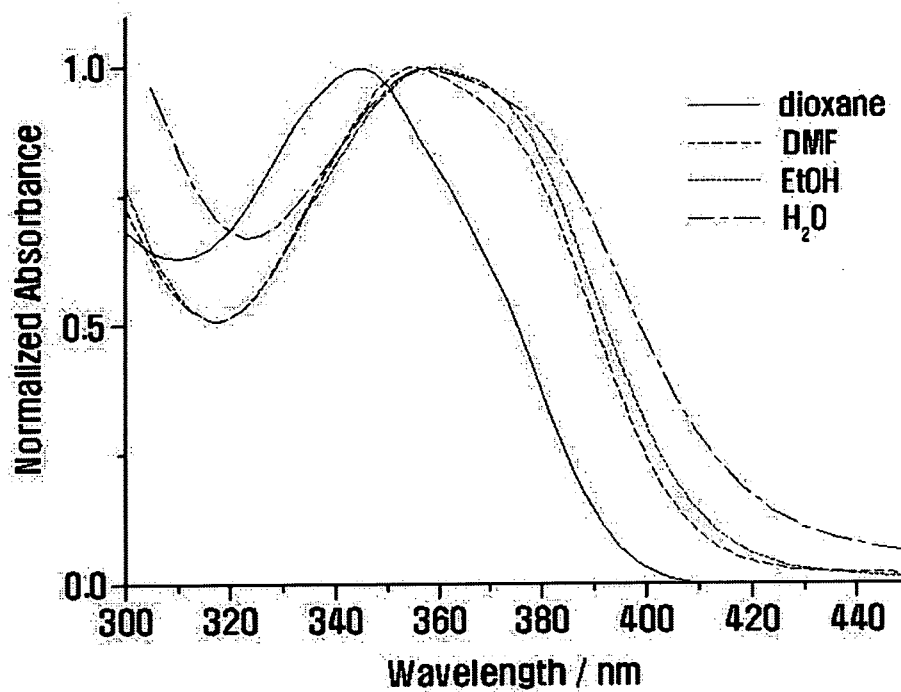
where K_d is the dissociation constant of the two-photon probe for Ca^{2+} , F is the observed two-photon fluorescence intensity, $F_{\min}$ is the minimum fluorescence intensity, and $F_{\max}$ is the maximum fluorescence intensity.

【Claim 7】

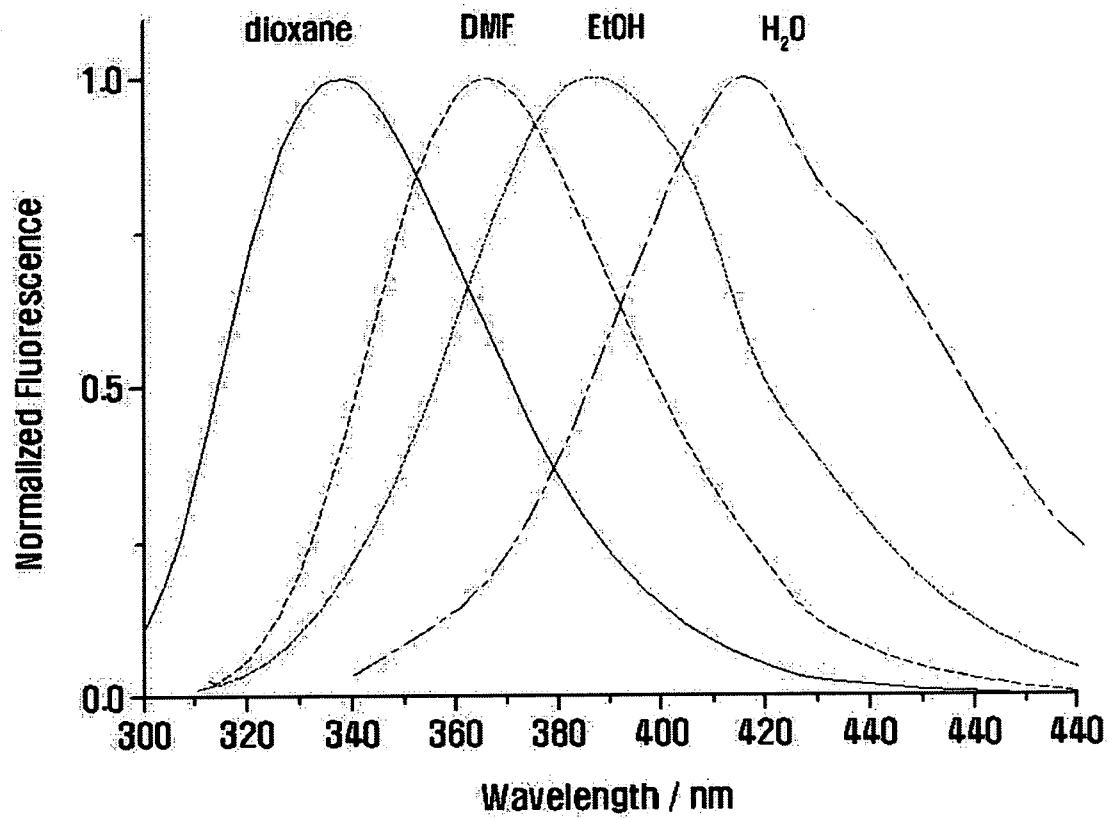
The method according to claim 5, wherein the two-photon excited fluorescence images are collected at wavelengths ranging from 500 to 620 nm.

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【Figure 1】

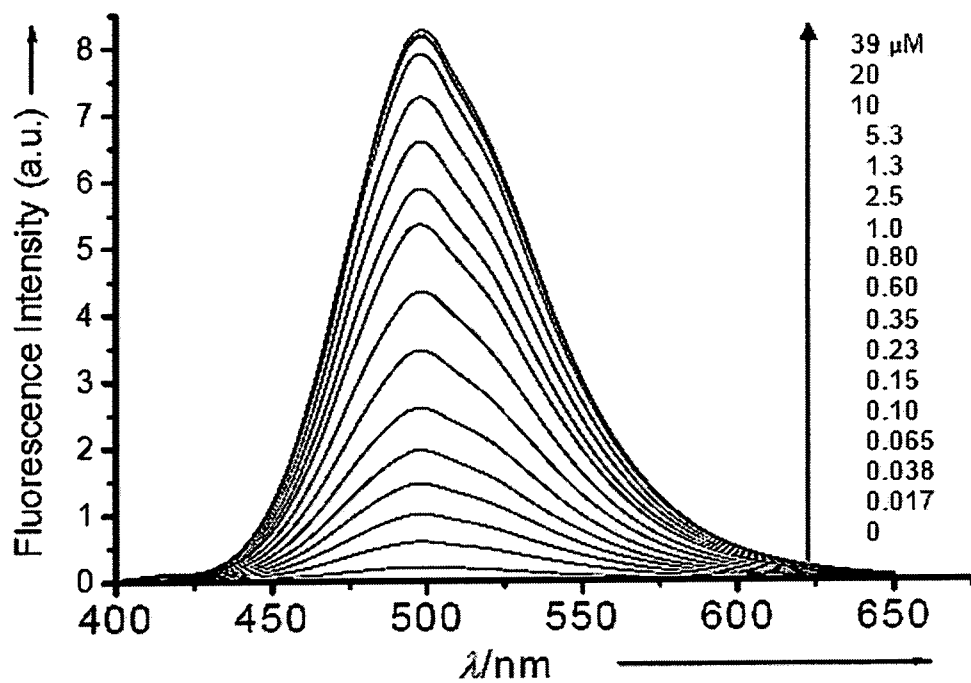


【Figure 2】

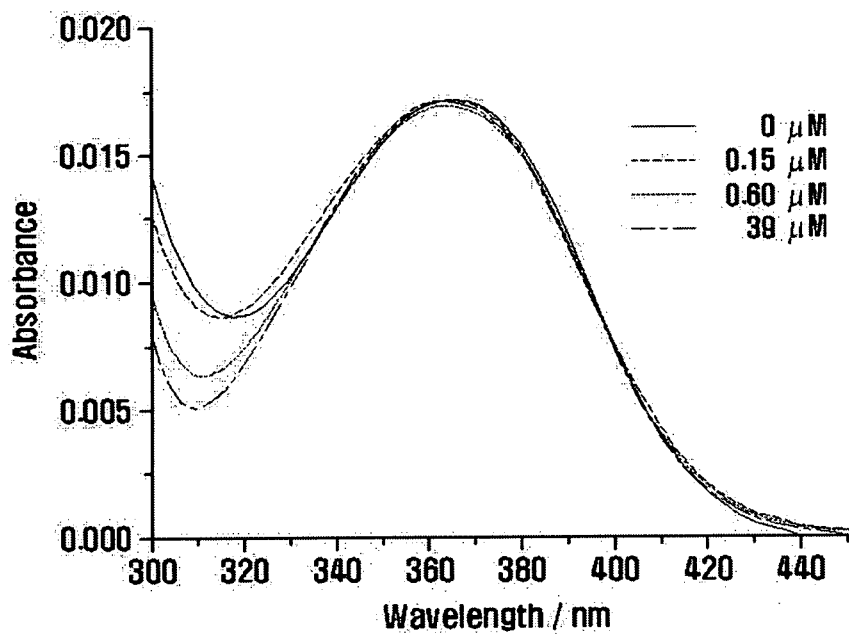


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【Figure 3】

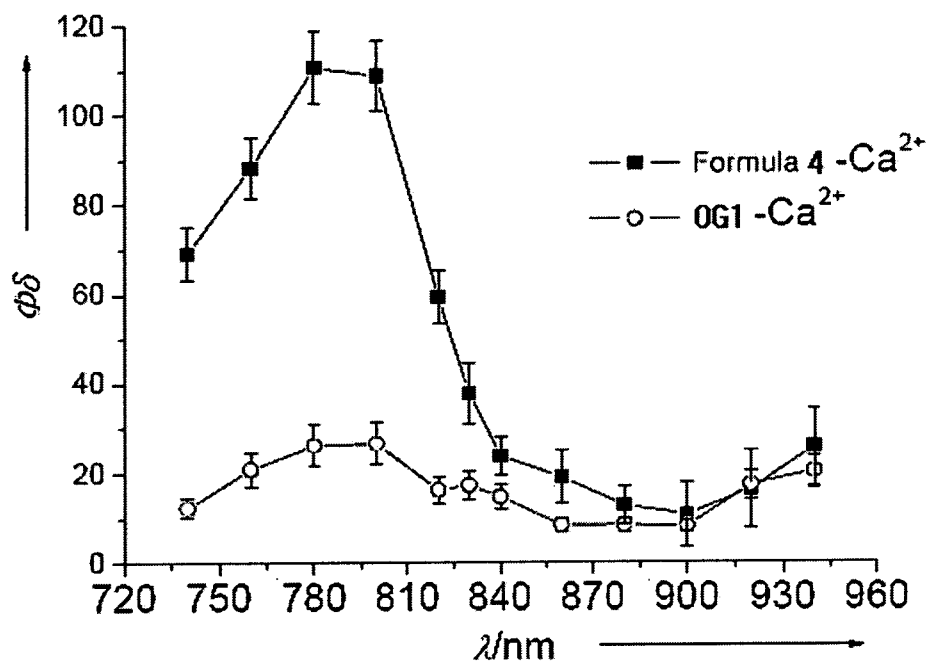


【Figure 4】

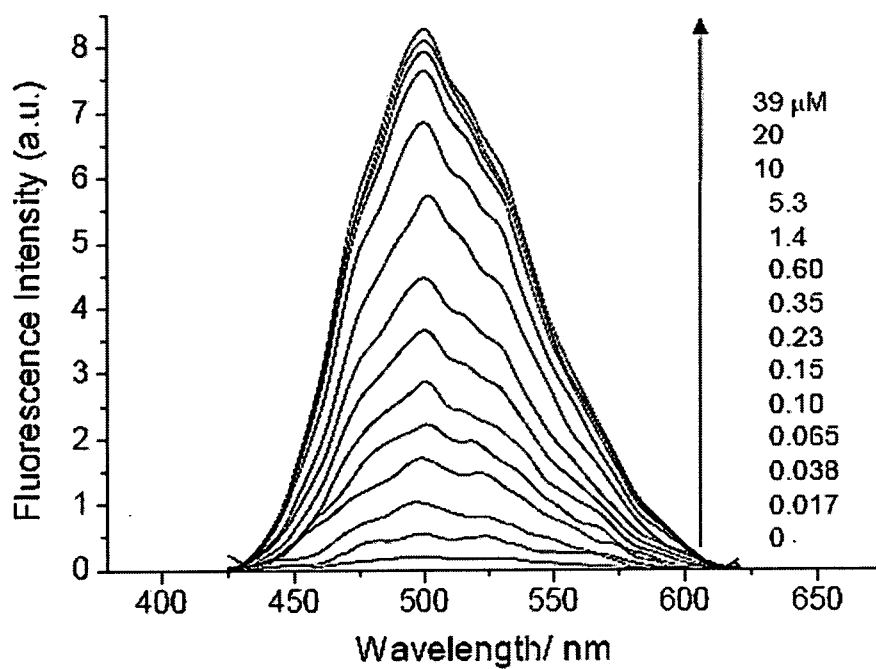


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【Figure 5】

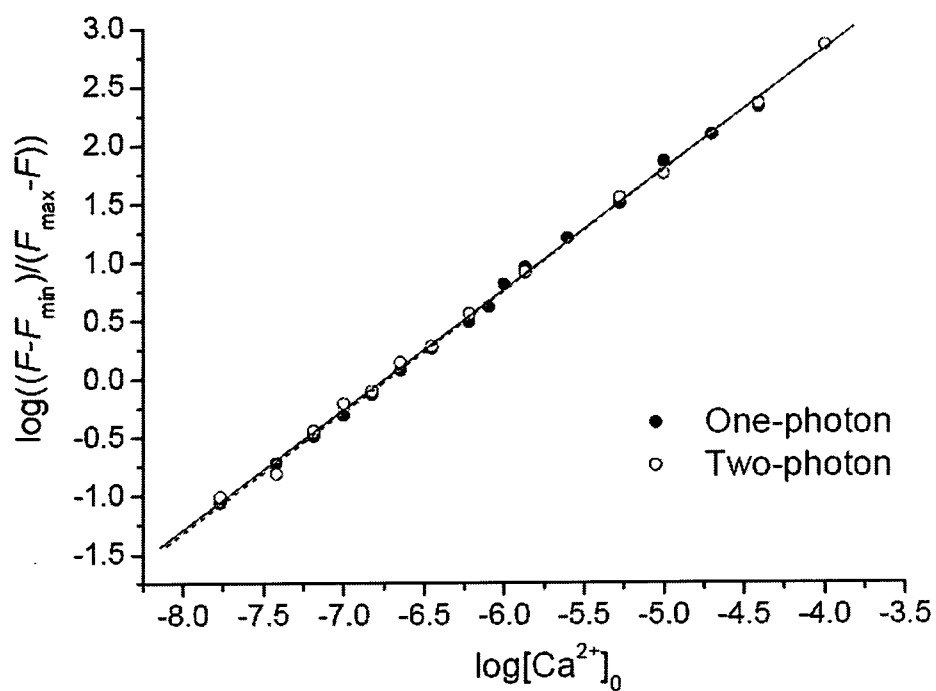


【Figure 6】

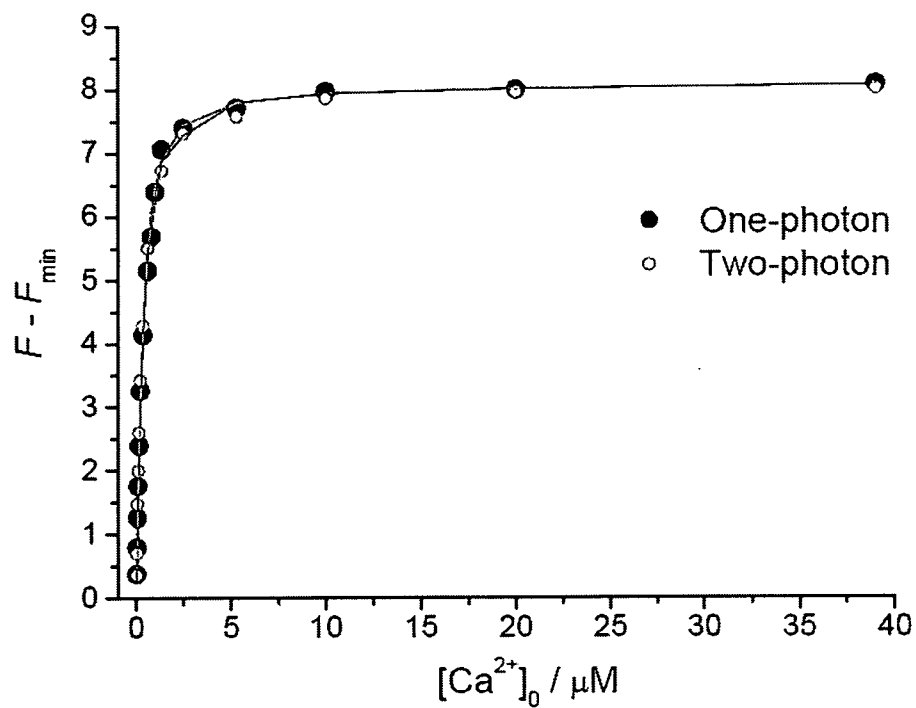


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【Figure 7】

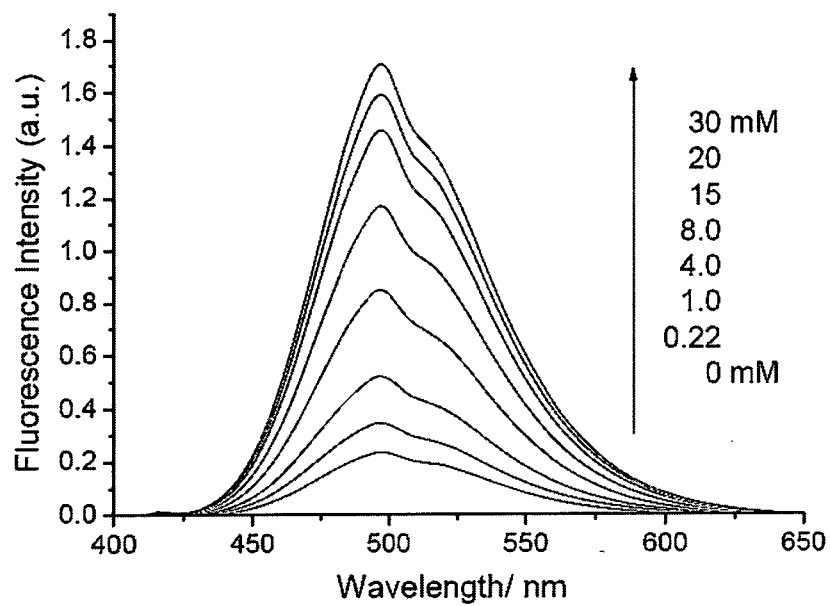


【Figure 8】

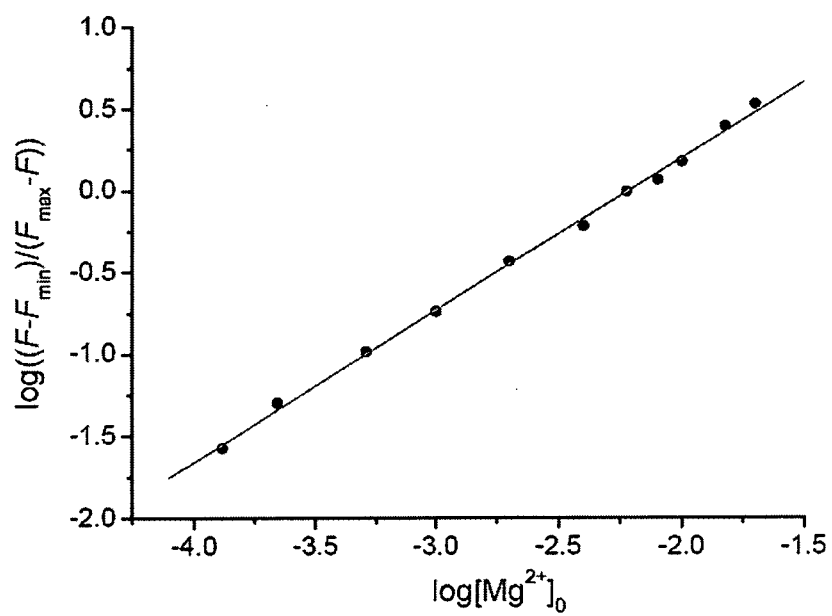


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【Figure 9】

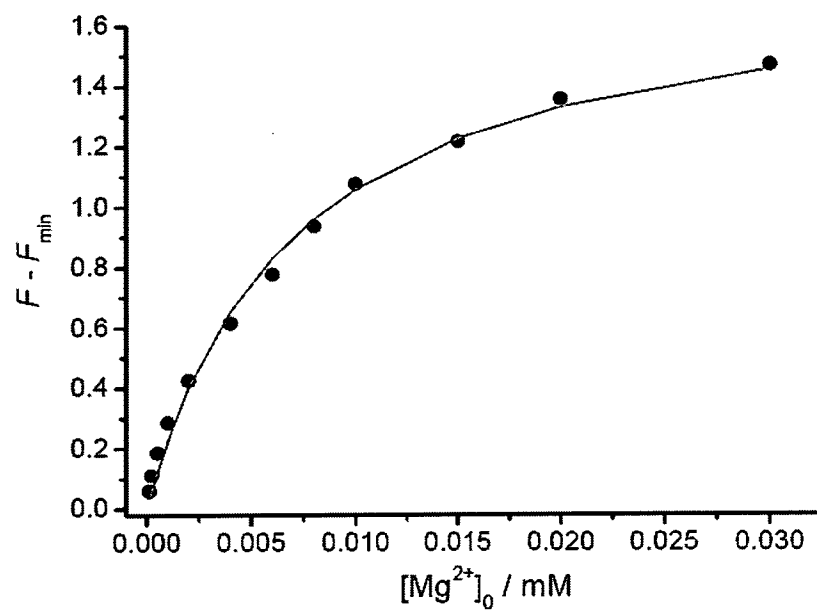


【Figure 10】

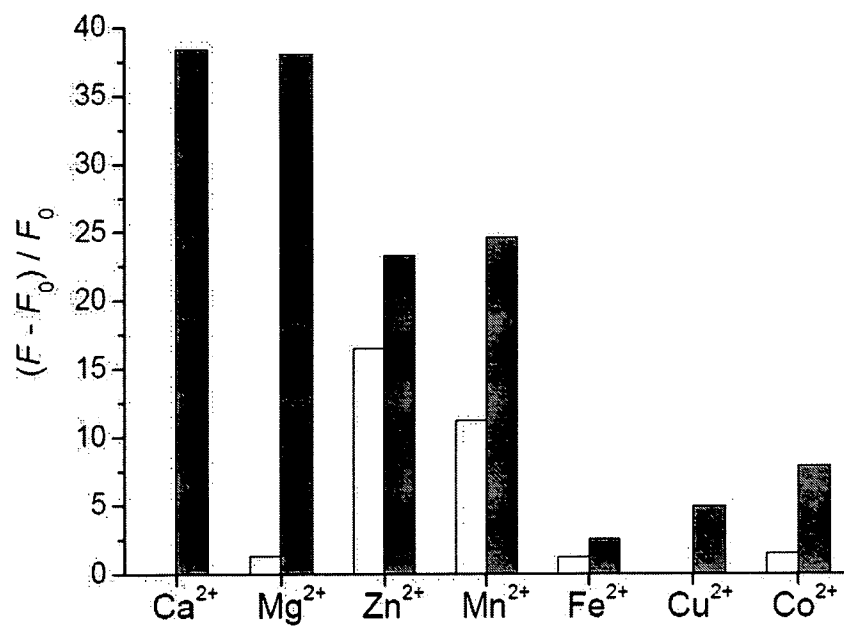


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【Figure 11】

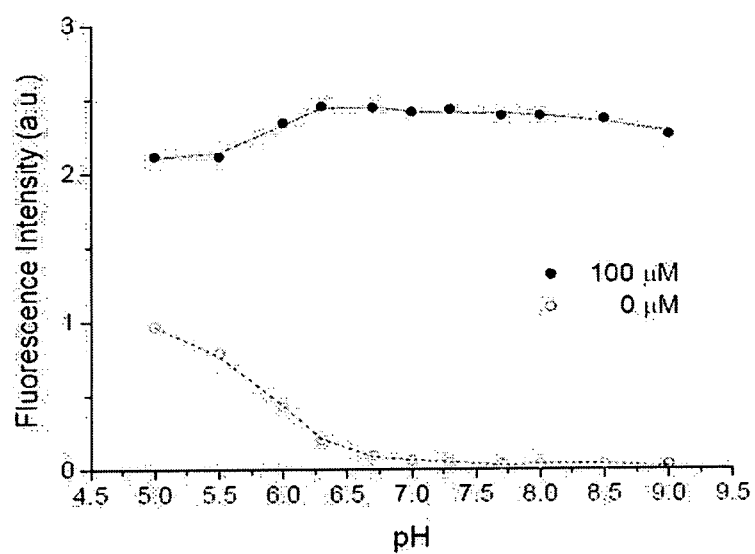


【Figure 12】



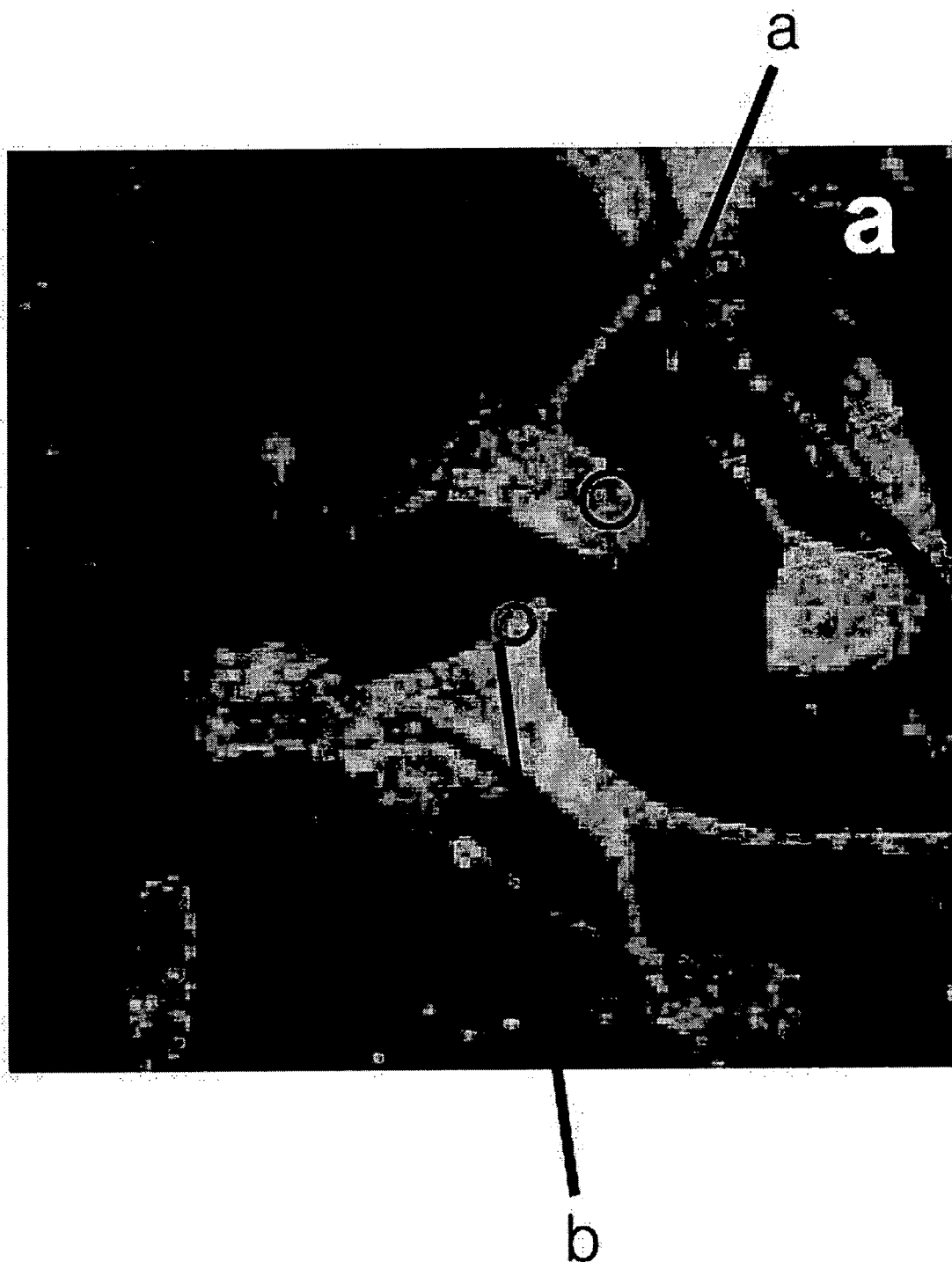
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【Figure 13】



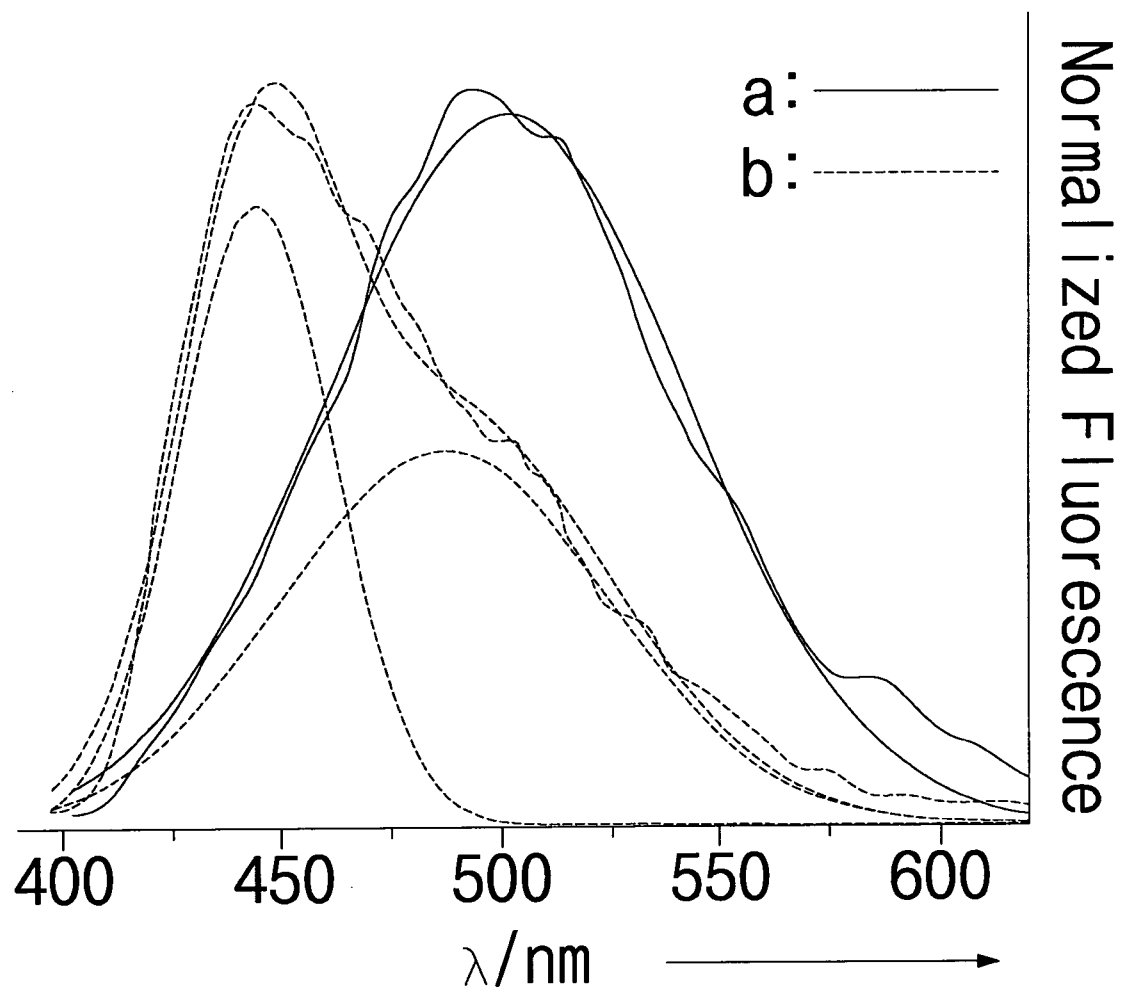
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【Figure 14】



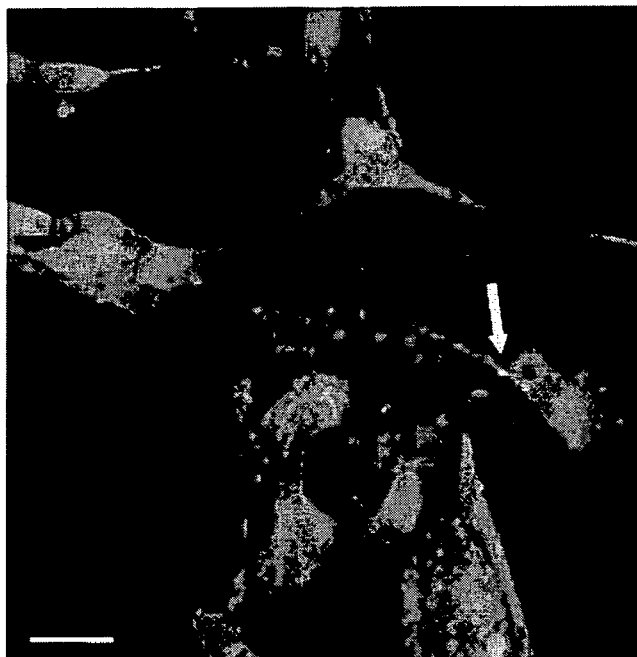
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【Figure 15】

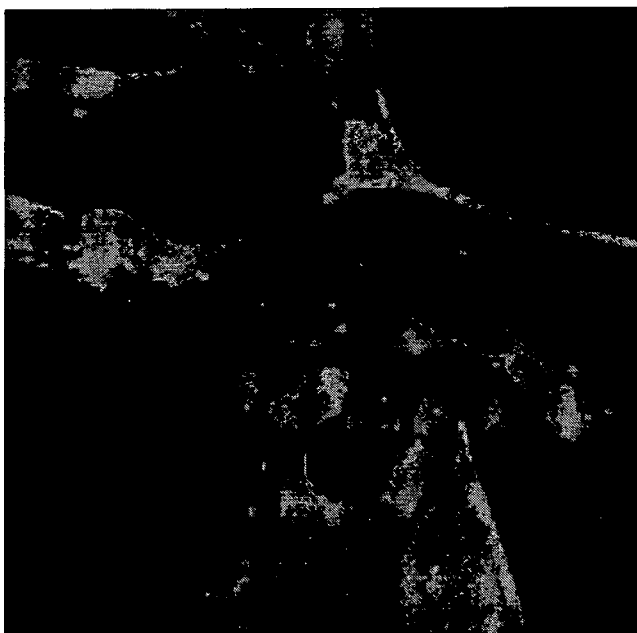


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【Figure 16】

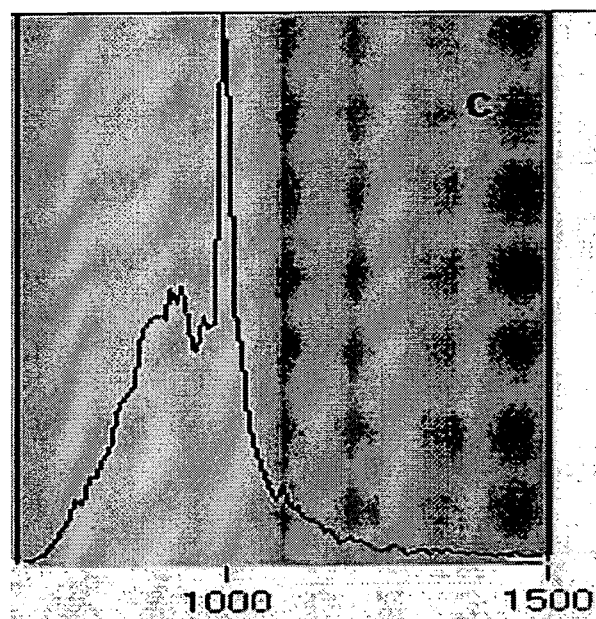


【Figure 17】

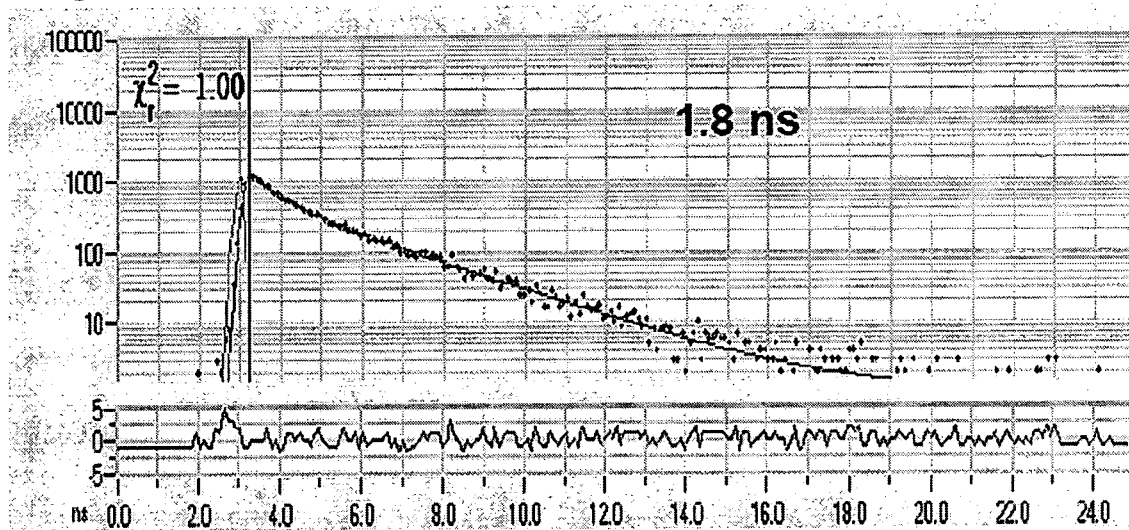


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【Figure 18】

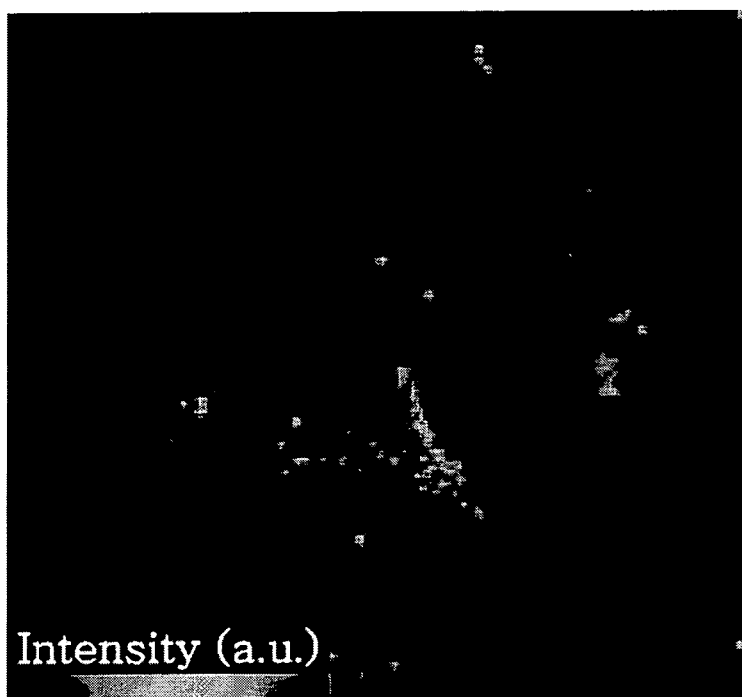


【Figure 19】

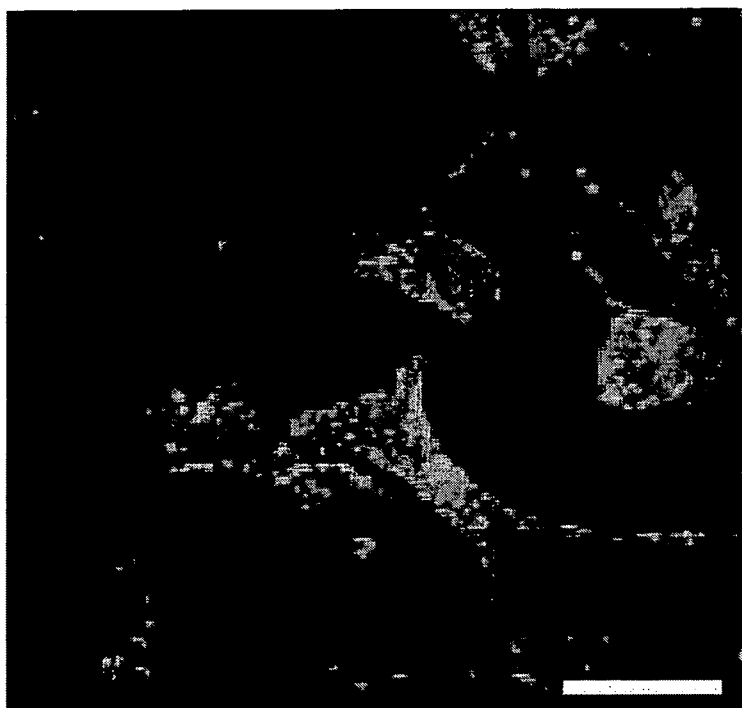


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【Figure 20】

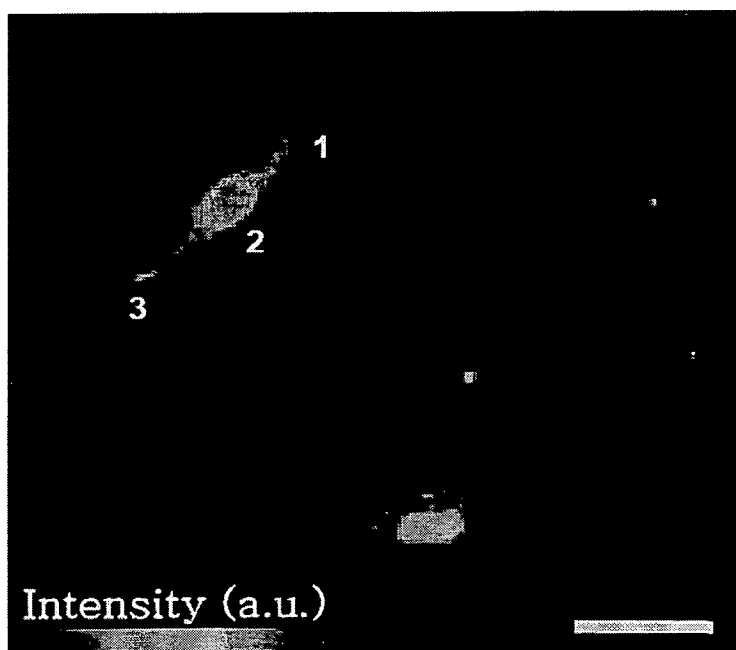


【Figure 21】

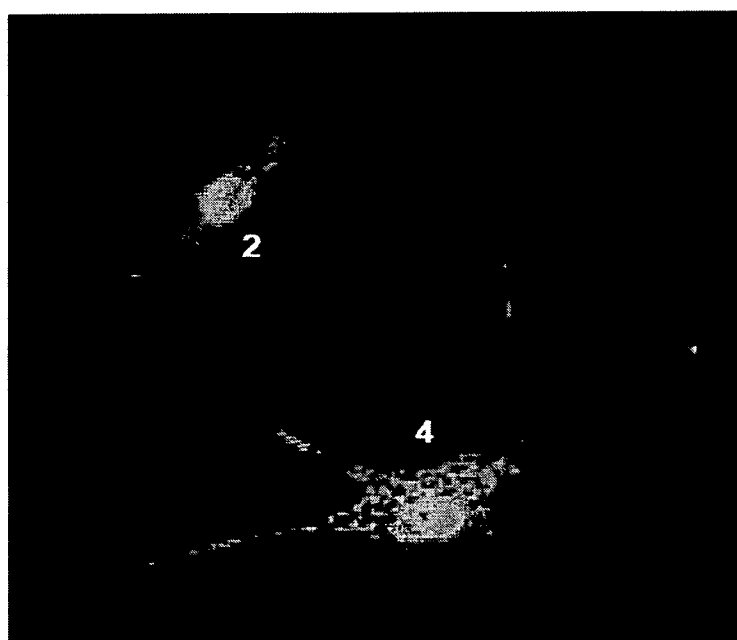


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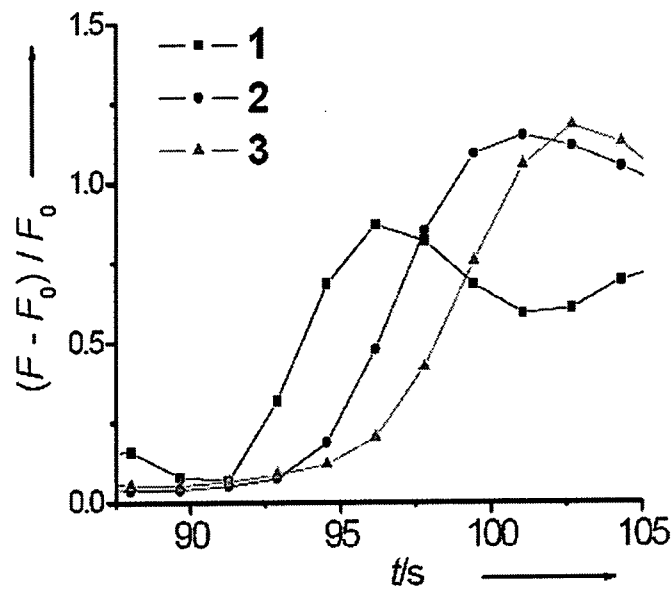
【Figure 22】



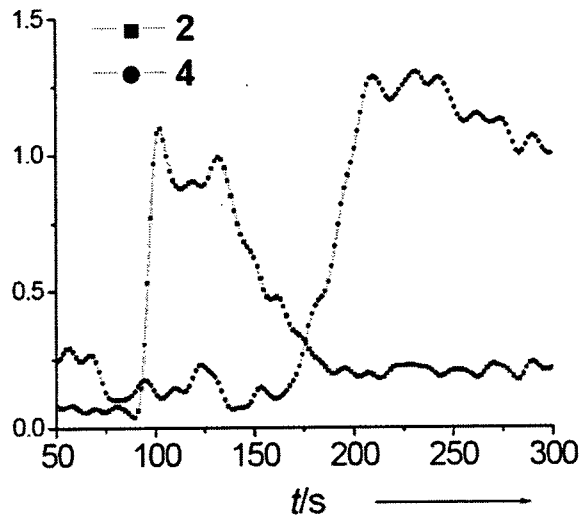
【Figure 23】



【Figure 24】

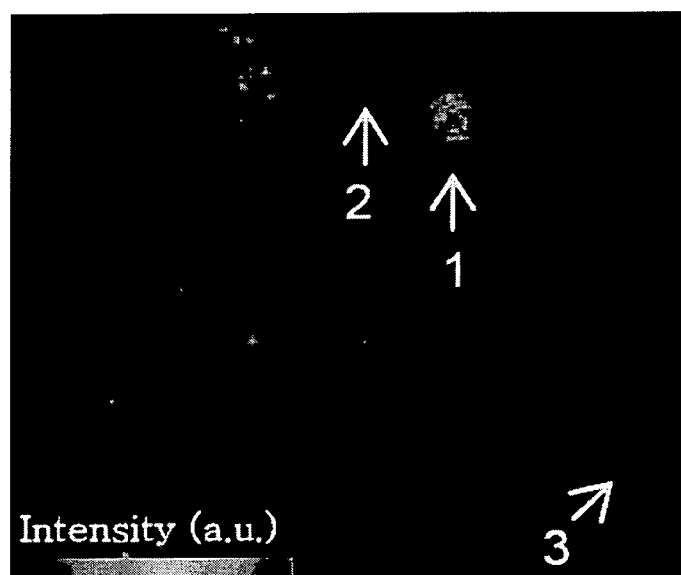


【Figure 25】

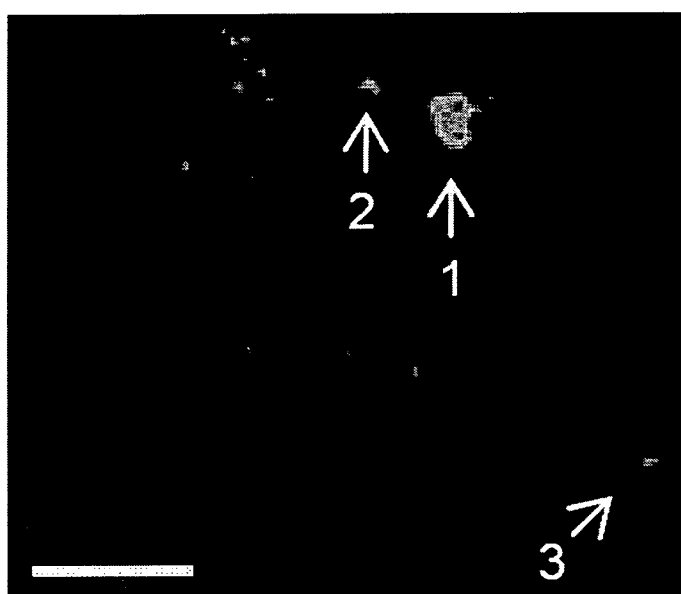


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【Figure 26】

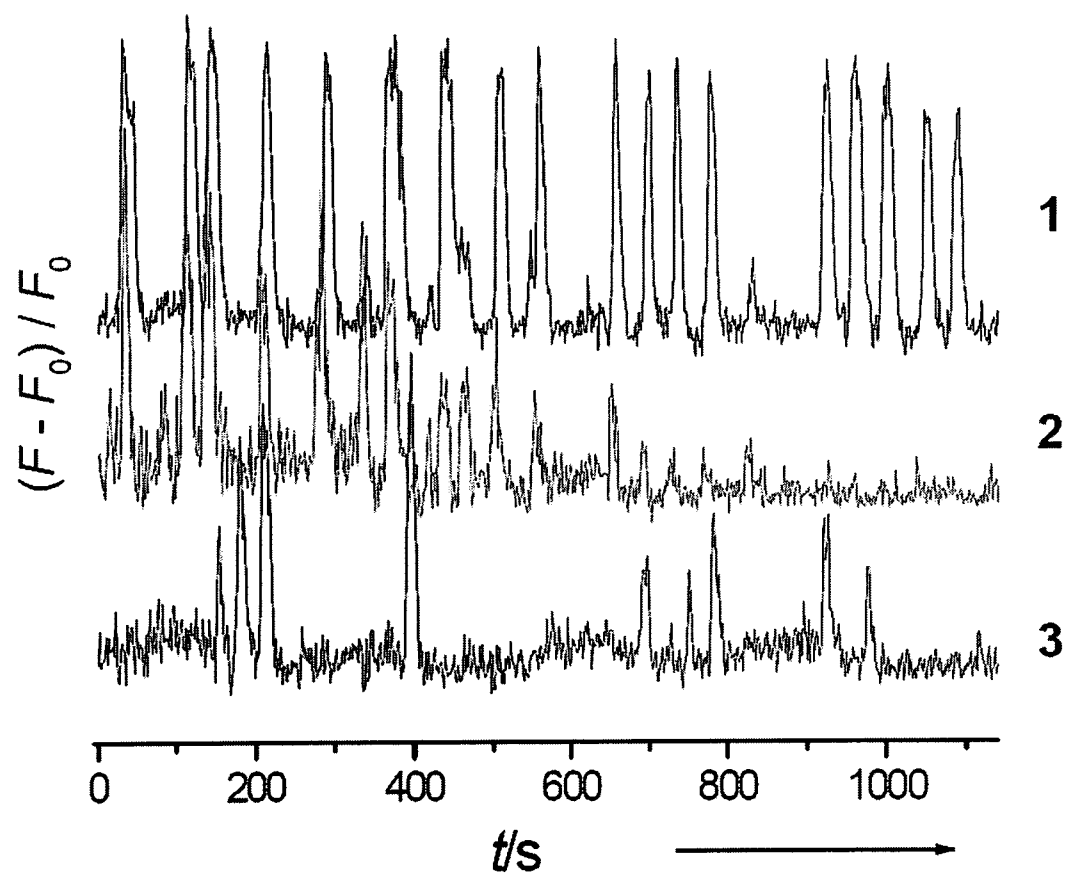


【Figure 27】



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【Figure 28】



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/000352**A. CLASSIFICATION OF SUBJECT MATTER*****C09B 29/095(2006.01)i***

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 :C09B 29/095

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility Models and Applications for Utility Models since 1975

Japanese Utility Models and Applications for Utility Models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,603,209 A (ROGER Y. TSIEN et al.) 29 July 1986 See abstract and claims 1-30	1-7
A	US 5,049,673 A (ROGER Y. TSIEN et al.) 17 September 1991 See abstract and claims 1-11	1-7
A	US 5,516,911 A (ROBERT E. LONDON et al.) 14 May 1996 See abstract and claims 1-5	1-7
A	EP 342891 A1 (SMITHKLINE BECHMAN CORPORATION) 23 November 1989 See abstract and claims 1-6	1-7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

28 MAY 2008 (28.05.2008)

Date of mailing of the international search report

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CHUNG, DOO HAN

Telephone No. 82-42-481-8400



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

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