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(54) **DEVICE AND METHOD FOR DETERMINING THE CONCENTRATION OF A SUBSTANCE**

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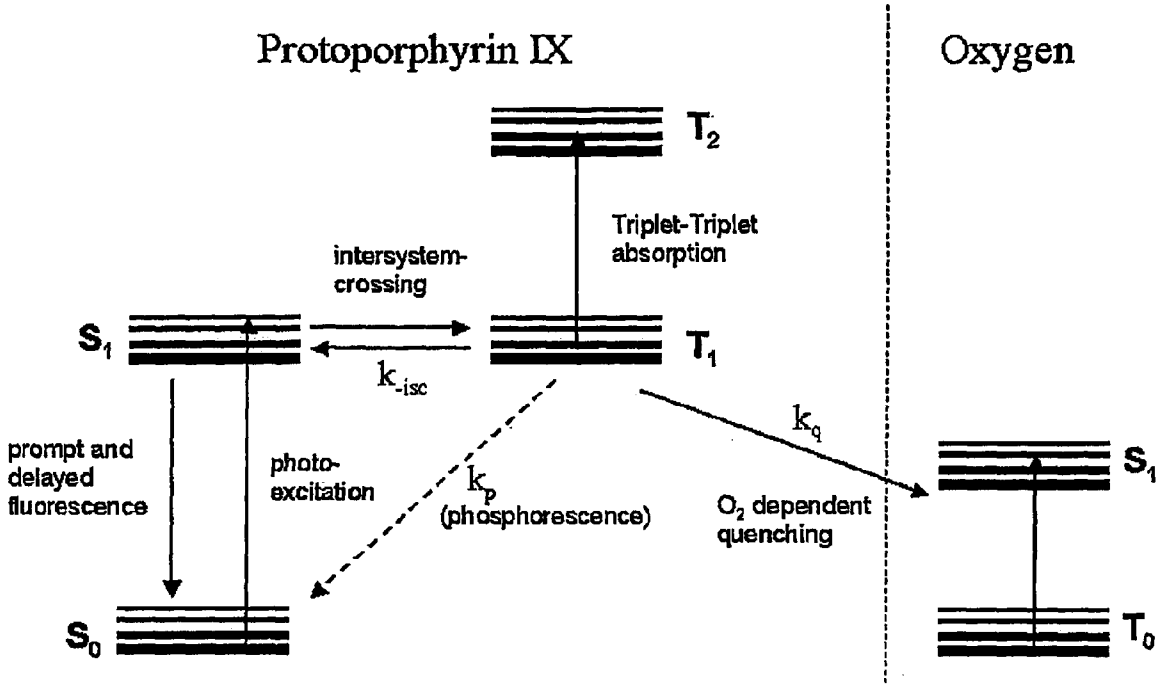
(57) **ABSTRACT**

The invention provides a method for determining a concentration of a substance in a compartment comprising exciting an endogenous compound, or a functional part, derivative, analogue or precursor thereof, measuring the lifetime of the luminescence and/or transient absorption exhibited by said compound, functional part, derivative, analogue and/or precursor, and correlating said lifetime with the concentration of said substance.



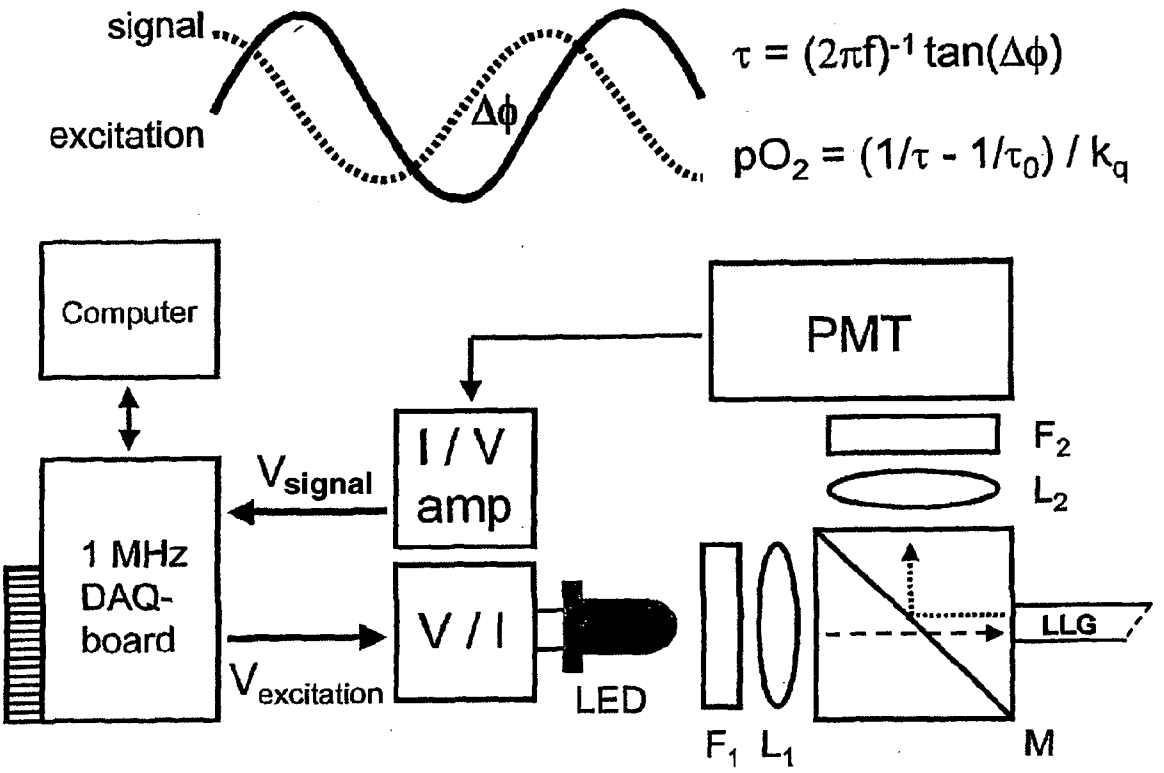
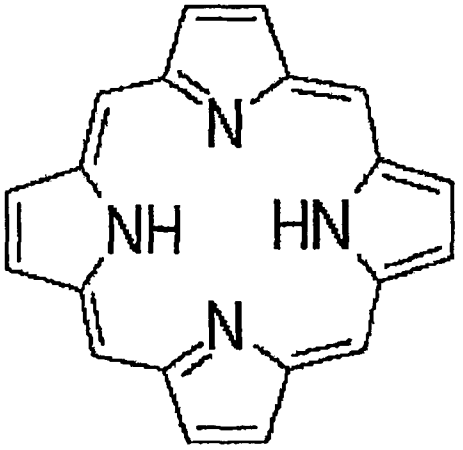
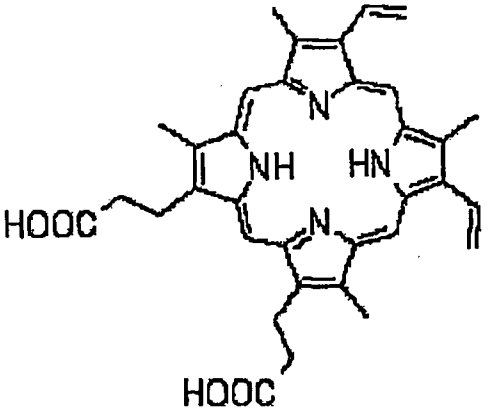

signal
excitation
Δϕ
τ = (2πf)-1 tan(Δϕ)
pO2 = (1/τ - 1/τ0) / kq
Computer
PMT
I / V amp
F2
L2
Vsignal
1 MHz DAQ-board
V / I
LLG
Vexcitation
LED
F1 L1
M

FIG. 1




porphine




protoporphyrin IX

FIG. 2

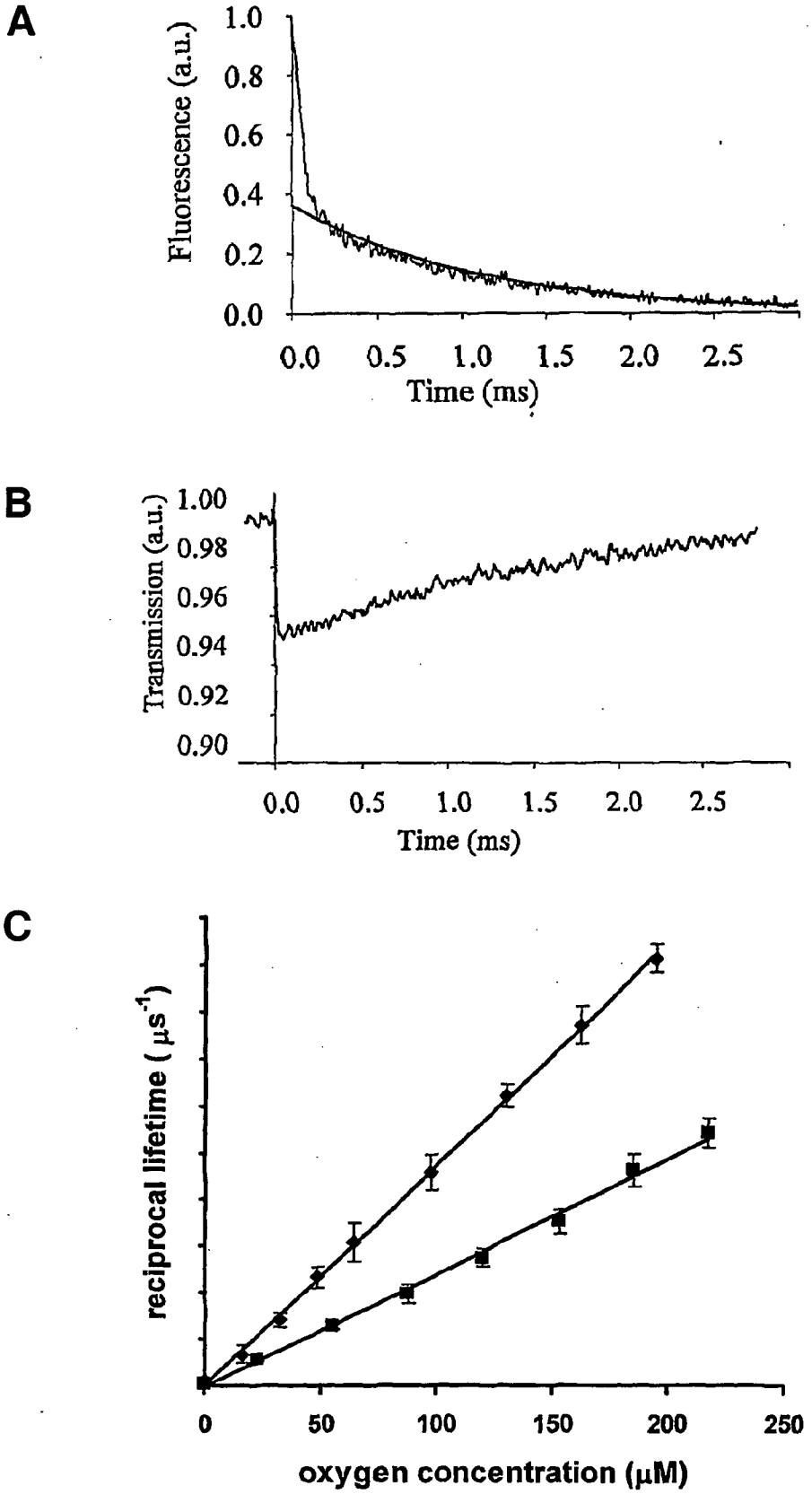

A
Fluorescence (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
0.0 0.5 1.0 1.5 2.0 2.5
Time (ms)
B
Transmission (a.u.)
1.00
0.98
0.96
0.94
0.92
0.90
0.0 0.5 1.0 1.5 2.0 2.5
Time (ms)
C
reciprocal lifetime (μs-1)
0 50 100 150 200 250
oxygen concentration (μM)

FIG. 3

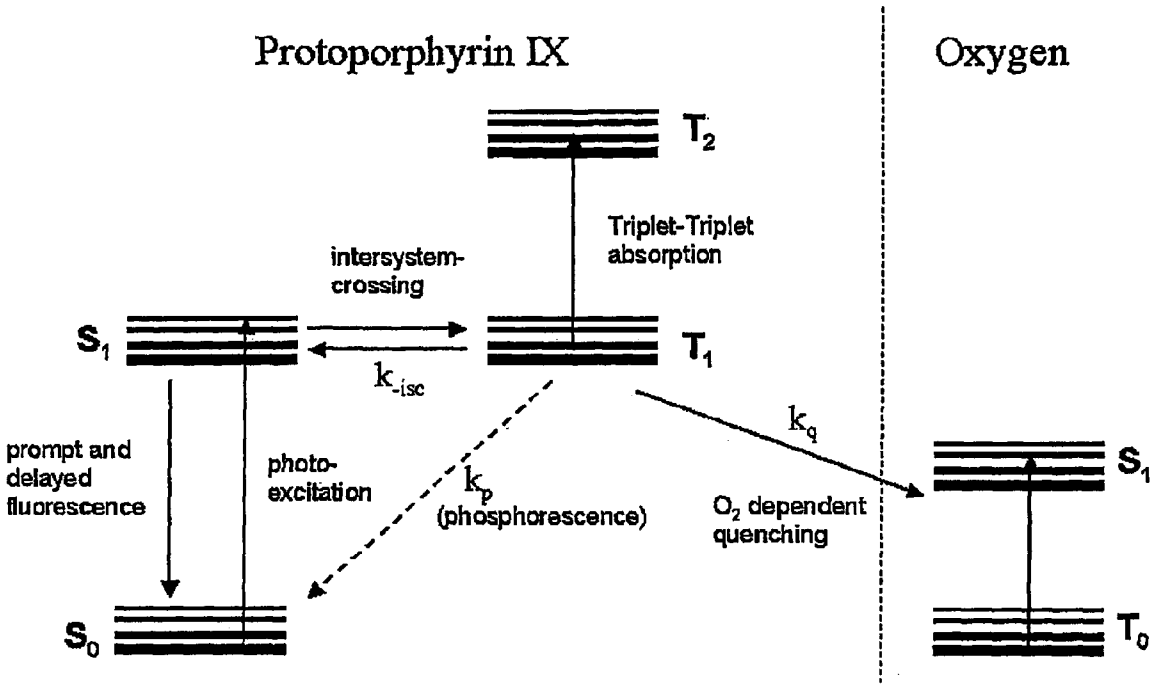

Protoporphyrin IX
Oxygen
T2
Triplet-Triplet absorption
intersystem-crossing
S1
T1
k-isc
kq
prompt and delayed fluorescence
photo-excitation
kp
(phosphorescence)
O2 dependent quenching
S1
S0
T0

FIG. 4

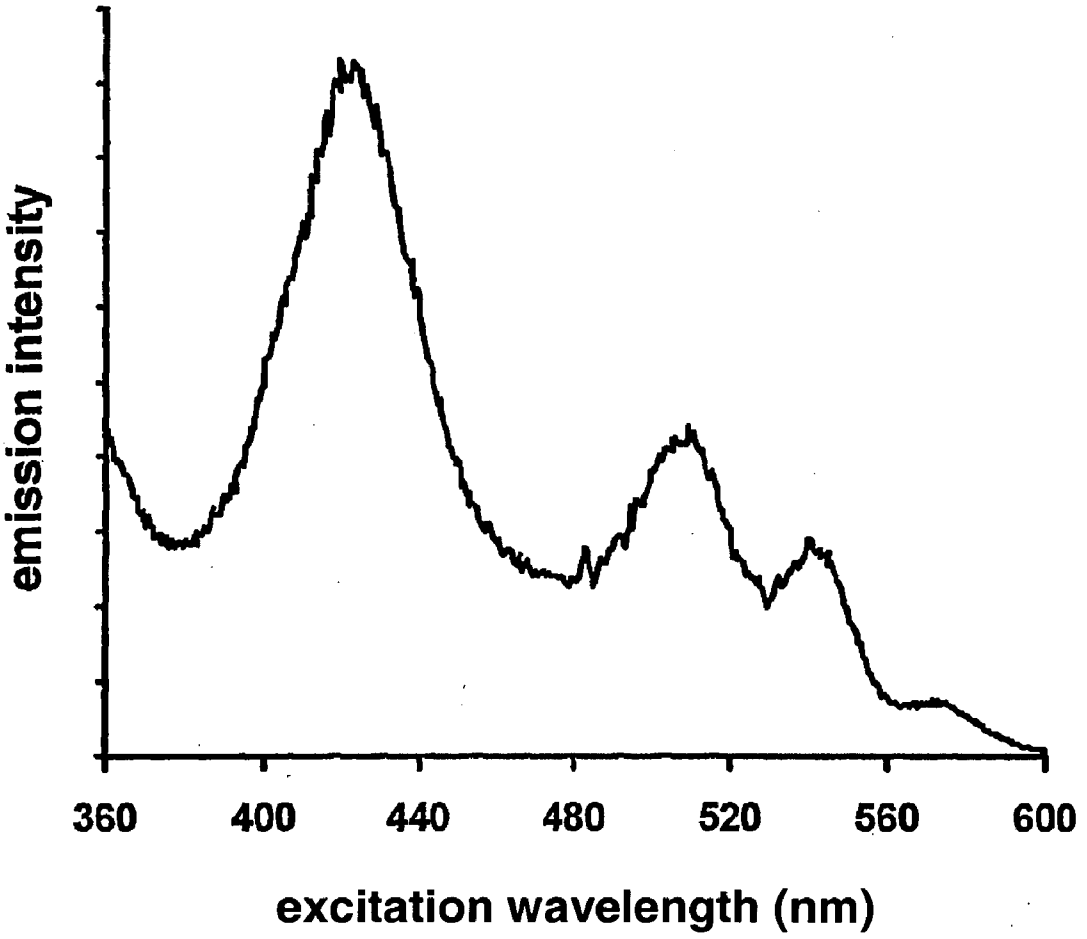

emission intensity
360
400
440
480
520
560
600
excitation wavelength (nm)

FIG. 5

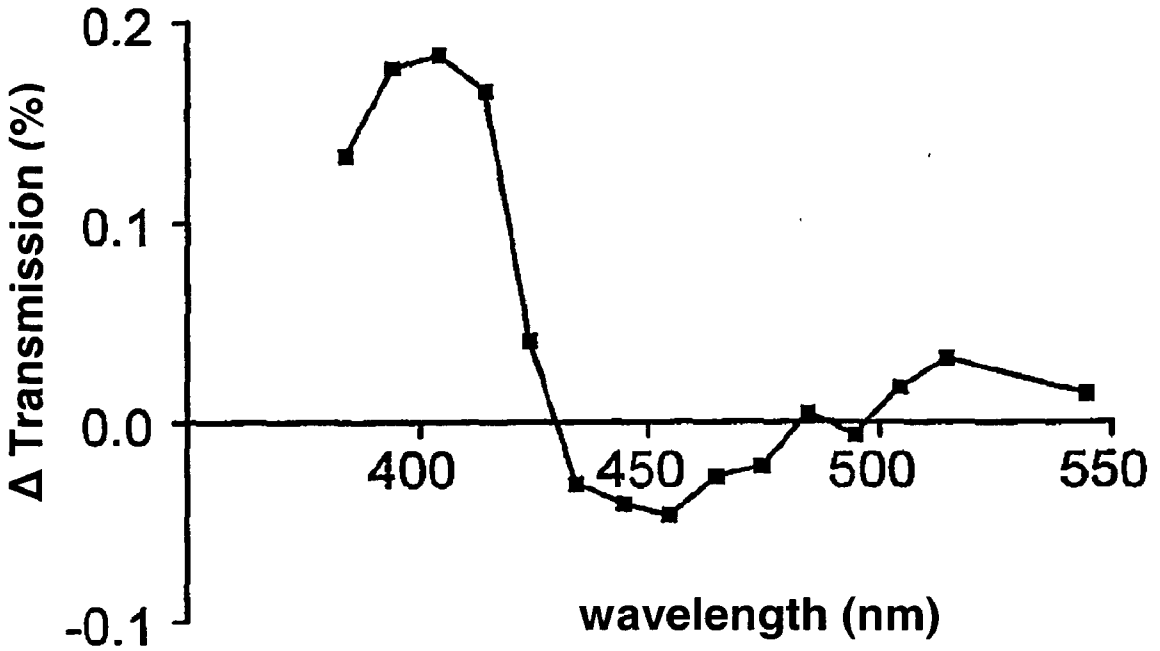



FIG. 6

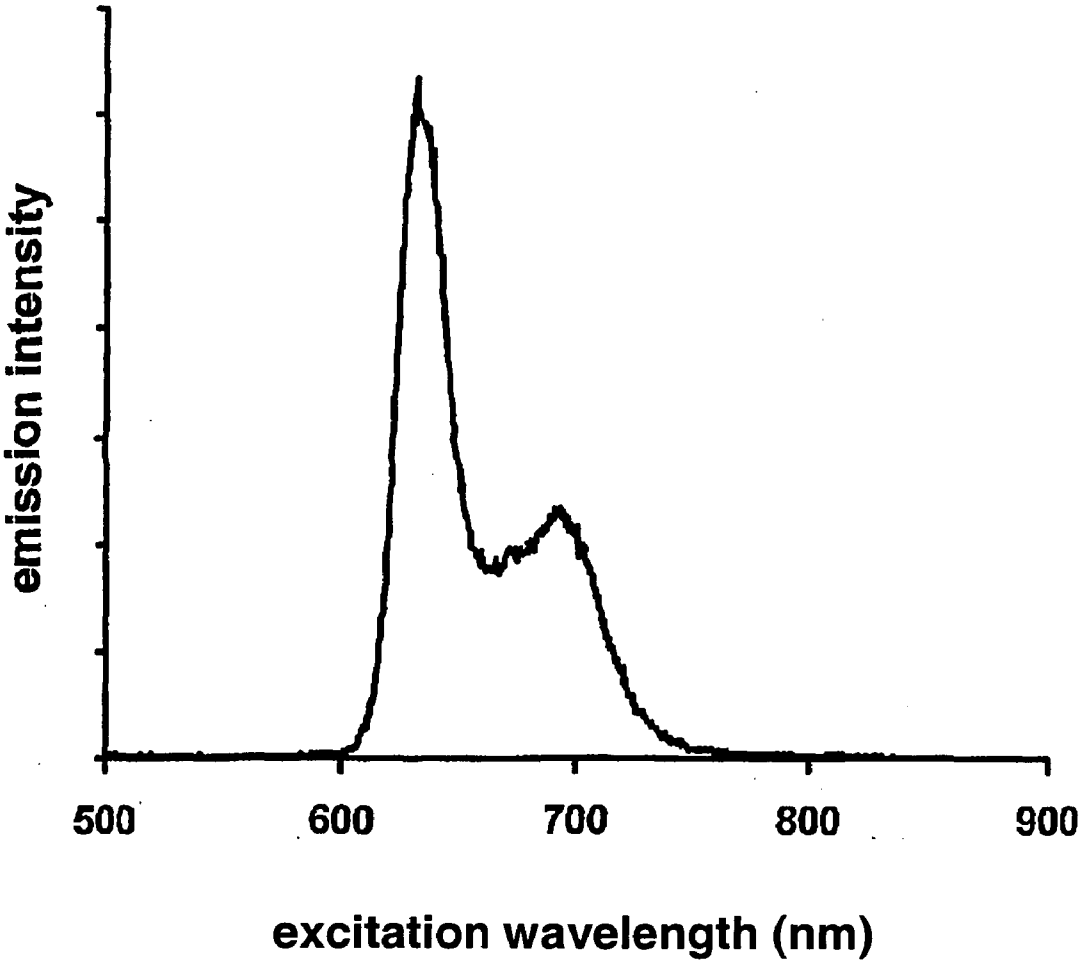

emission intensity
500
600
700
800
900
excitation wavelength (nm)

FIG. 7

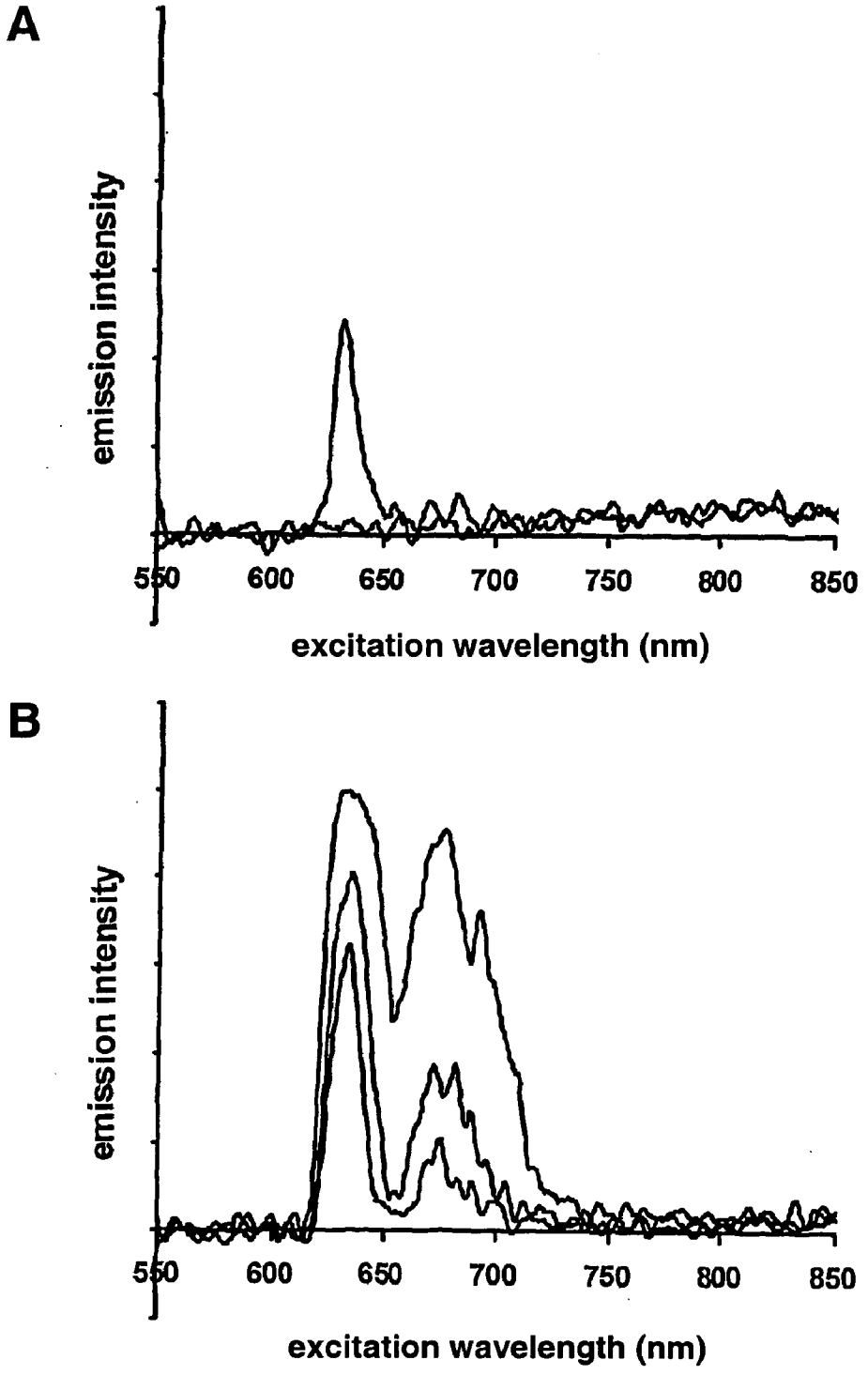
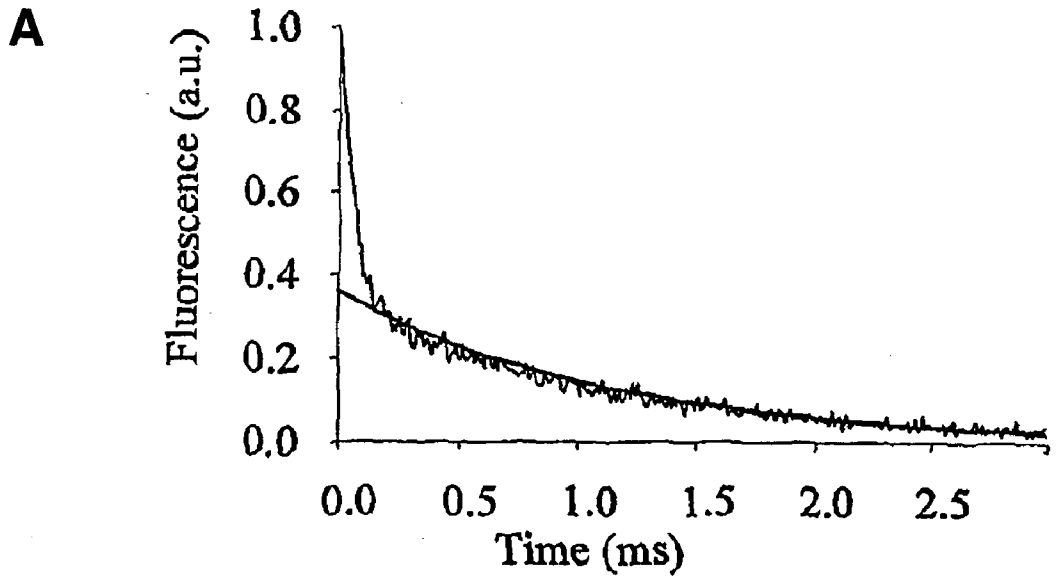

A
emission intensity
550
600
650
700
750
800
850
excitation wavelength (nm)
B
emission intensity
550
600
650
700
750
800
850
excitation wavelength (nm)

FIG. 8


A
Fluorescence (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.5
1.0
1.5
2.0
2.5
Time (ms)

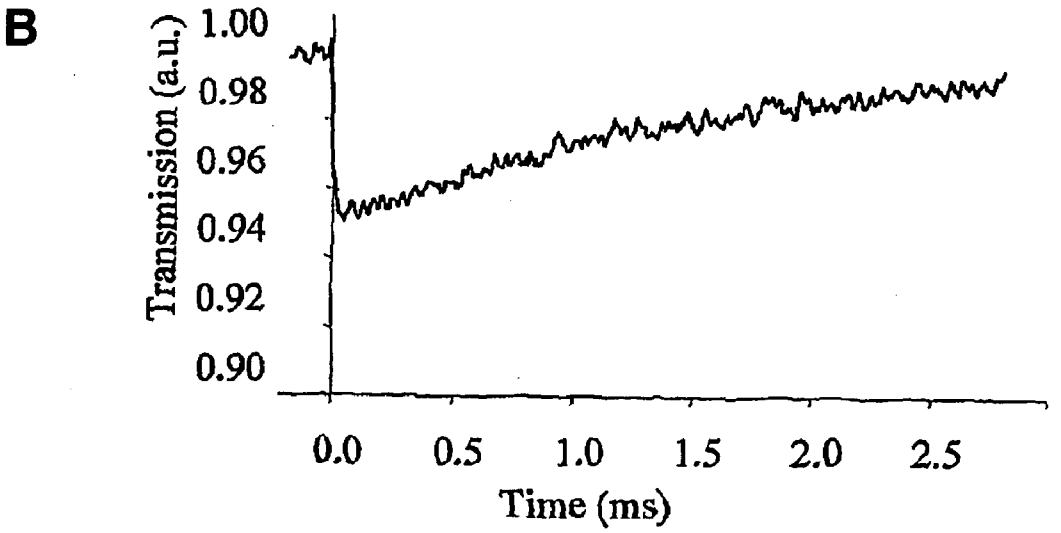

B
Transmission (a.u.)
1.00
0.98
0.96
0.94
0.92
0.90
0.0
0.5
1.0
1.5
2.0
2.5
Time (ms)

FIG. 9

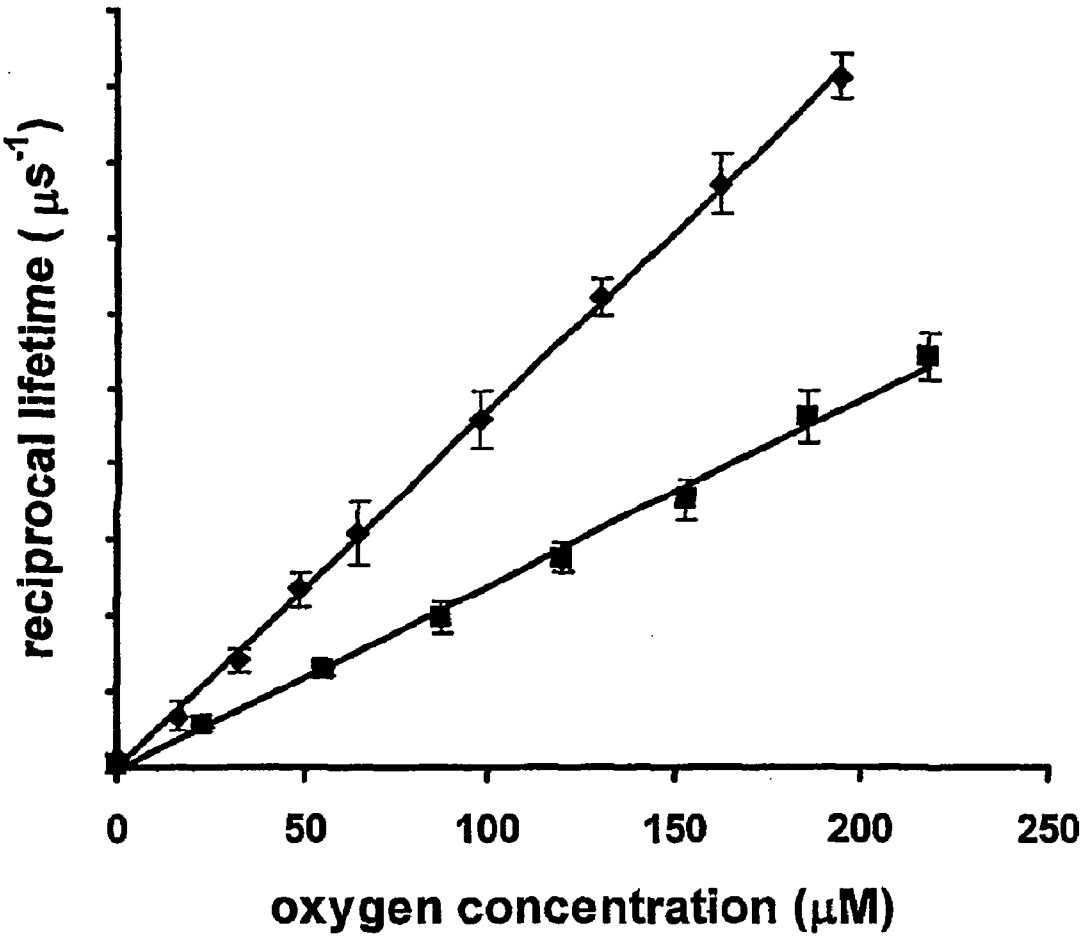

reciprocal lifetime ( μs-1 )
0
50
100
150
200
250
oxygen concentration (μM)

FIG. 10

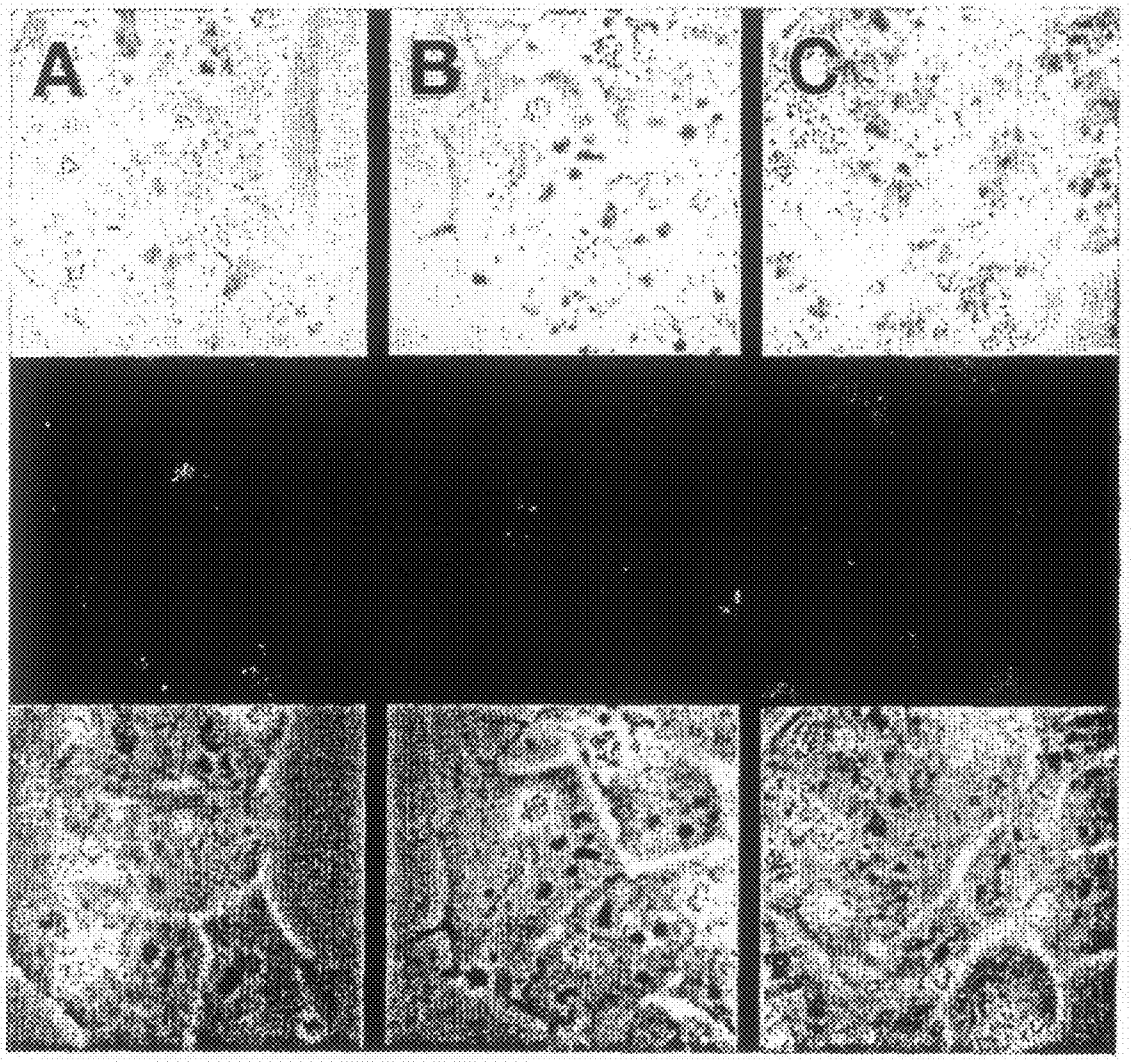



FIG. 11

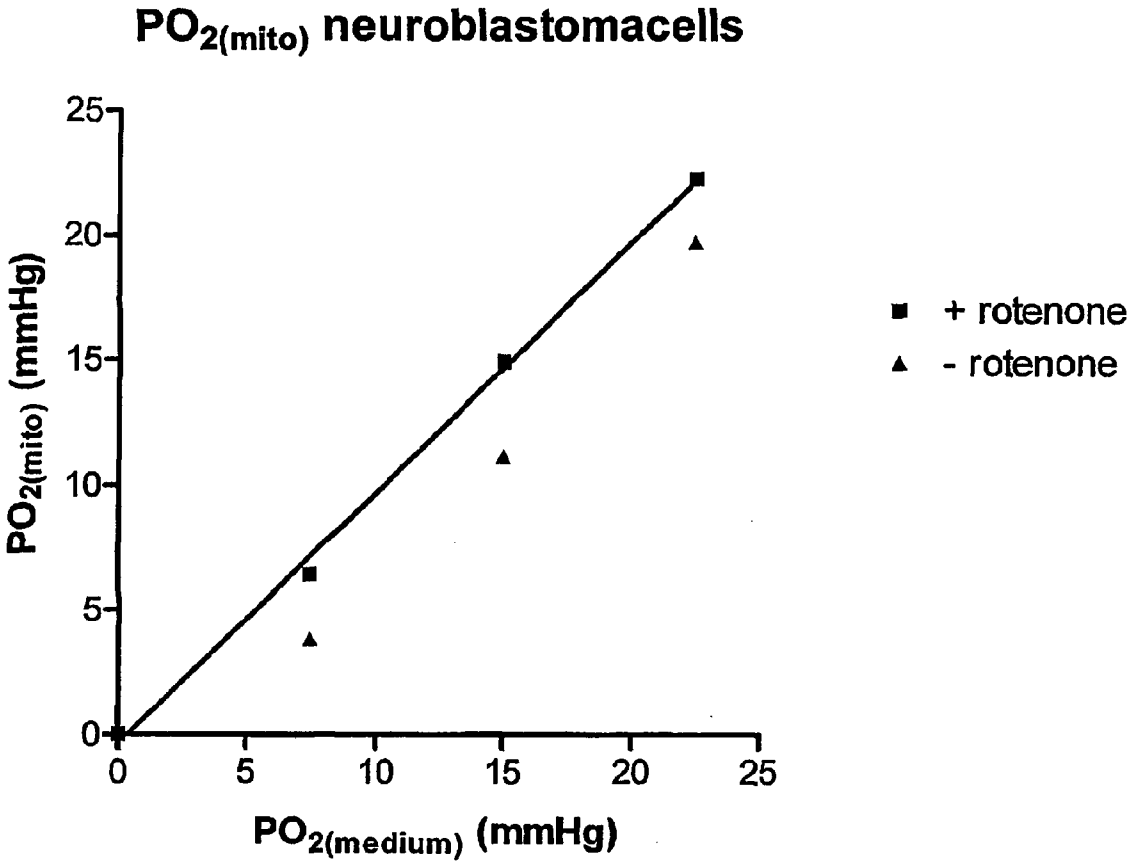

PO2(mito) neuroblastomacells
PO2(mito) (mmHg)
PO2(medium) (mmHg)
0
5
10
15
20
25
+ rotenone
- rotenone

FIG. 12

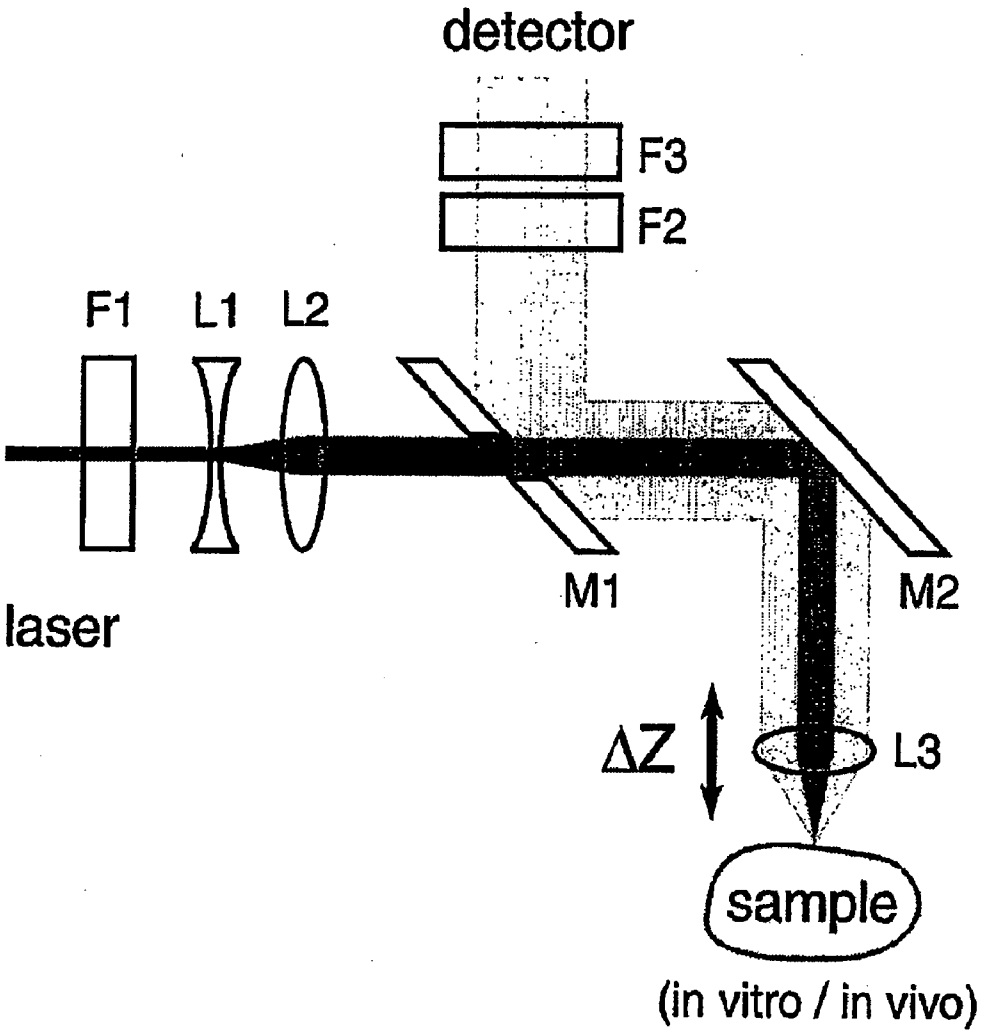

detector
F3
F2
F1
L1
L2
M1
M2
laser
ΔZ
L3
sample
(in vitro / in vivo)

FIG. 13

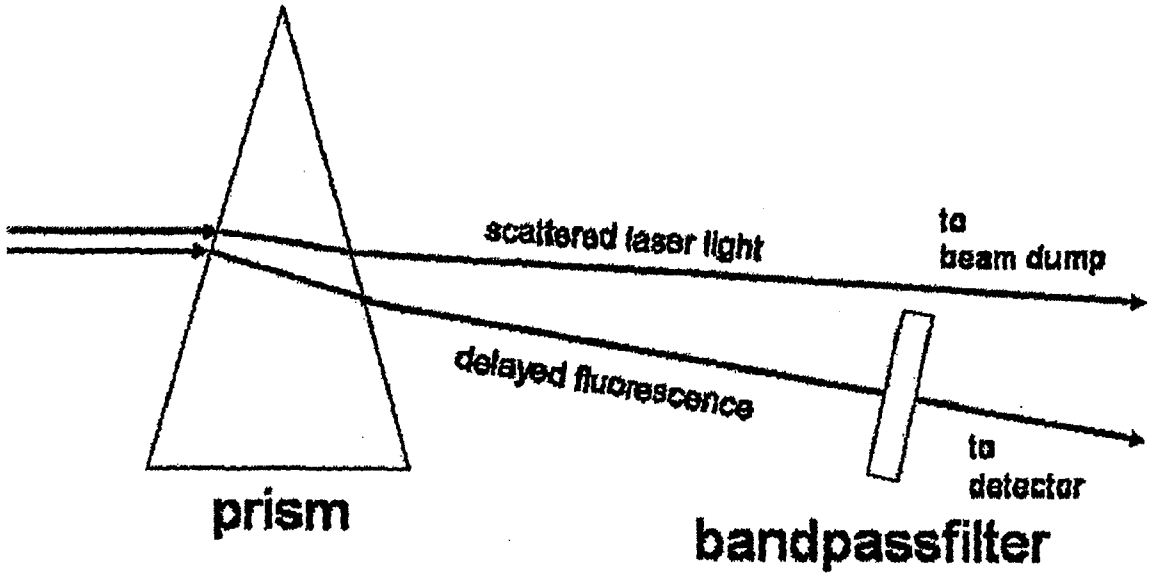

scattered laser light
to
beam dump
delayed fluorescence
to
detector
prism
bandpassfilter

FIG. 14

# DEVICE AND METHOD FOR DETERMINING THE CONCENTRATION OF A SUBSTANCE

**[0001]** The invention relates to the field of medicine. More specifically, the invention relates to monitoring the concentration of a substance.

**[0002]** Health control, diagnosis of disease and/or monitoring of treatment of disease often involves measurement of various parameters. One parameter is the concentration of a certain substance, such as oxygen, within at least part of an organism. Local tissue oxygenation is an important parameter in the diagnosis and treatment of a wide range of diseases. Measurements of the amount of oxygen present in a specific part of a subject are for instance carried out during perioperative monitoring in the operating room and intensive care and for diagnosis of a wide range of clinical disorders in which tissue oxygenation lies central to the development and cure of disease. Examples include diagnosis of cardiovascular disease, monitoring healing of decubitus and diabetic wounds, monitoring hyperbaric correction of radiation wounds and assessment of success of bypass surgery. Monitoring of tissue oxygen pressure ($pO_2$) during critical illness is considered a major need in the adequate treatment of intensive care patients (Siegemund et al., 1999). Assessment of tumor oxygenation is an example wherein measuring of local tissue oxygenation is helpful for the choice of treatment, as oxygen is an important determinant for success of radiotherapy. Hence, the concentration of oxygen in a tumor is preferably determined in order to determine whether radiotherapy is recommended. Local oxygen measurements are also applicable for the assessment of organ viability for transplantation.

**[0003]** Dioxygen is a molecule of utmost biological importance because of its role as the primary biological oxidant. Therefore, oxygen plays a key role in the oxidation/reduction reactions that are coupled to cellular respiration and energy supply. Adequate measurement of oxygen concentrations in biological samples like cells, tissues and whole organs is important to gain insight in the determinants of oxygen supply and utilisation under normal and pathological conditions. It is interesting to note that the clinical interest in methods providing information about blood-flow and oxygen delivery at the (sub-)organ level (e.g. microcirculatory) is growing. This is amongst other things because of increasing insight into the role of the microcirculation in pathogenesis, and the importance of adequate tissue perfusion as end-point of treatment (Siegemund et al., 1999).

**[0004]** Various techniques have been developed for direct or indirect oxygen measurements in tissue, each having its specific advantages and disadvantages (for a review on this subject see J. M. Vanderkooi et al., 1991). Conventionally, measurements of tissue oxygenation have been made by use of oxygen electrodes and spectrophotometry of the hemoglobin or myoglobin molecule. Reflection spectrophotometry records the difference in absorption and scattering between a standard reference sample and a tissue sample. The method is based on the illumination of a tissue sample by light with a known spectral content and detection of the diffusely reflected light from the tissue at several different wavelengths. The spectral difference between illumination light and detected light contains information about the wavelength-dependent absorption and scattering within the tissue. The reference sample, used for correction of non-ideal apparatus behavior, can be anything with well-known absorption properties, but a white sample (no absorption) is mostly used. The relative tissue absorbency [$E_{r(tissue)}$] can be described by the following equation:

$$[E_{r(tissue)}]=\log(I_{r(standard)}/I_{r(tissue)}) \quad (1)$$

where $I_{r(standard)}$ and $I_{r(tissue)}$ are the intensity of the diffusely reflected light from the white standard and the tissue, respectively. Since the absorption spectra of oxygenated and deoxygenated hemoglobin show marked differences that can easily be detected by RS, this technique is widely used for measurement of hemoglobin saturation in tissue. In order to derive more or less quantitative data with RS, it is necessary to take into account the influence of tissue optical parameters other than the hemoglobin related ones. Different approaches for developing an appropriate analysis algorithm are possible. One described approach is based on the use of isobestic points (the intersection points of the curves of oxygenated and deoxygenated hemoglobin) as reference points within the calculation (Sato, 1979). Dummler used a somewhat different approach for his derivation of an algorithm (Dummler, 1988) based on the two-flux theory of Kubelka and Munk (Kubelka, 1931, Kessler, 1992). The EMPHO, the Erlangen Microlightguide spectrophotometer (Frank, 1989) (EMPHO II, Bodenseewerk Gerätetechnik, Überlingen, Germany) and the O2C (Lea Medizin Technik, Giesen, Germany) are spectrophotometers using improved Dummler algorithms for hemoglobin saturation measurements.

**[0005]** Drawback of these conventional techniques is that they are either mechanically disruptive (insertion of oxygen electrodes) or qualitative (spectrophotometry). These constrains have led to the development of alternative methods. One of the most promising techniques in this respect has been the use of oxygen dependent quenching of phosphorescent dyes for measurements in the microcirculation (Vanderkooi et al., 1987; Sinaasappel & Ince, 1996; Sinaasappel et. al., 1999).

**[0006]** Wilson and Vanderkooi (Vanderkooi, 1987) introduced the oxygen-dependent quenching of phosphorescence of metallo-porphyrin compounds for biological oxygen concentration measurements. The technique is based upon the principle that a metallo-porphyrin molecule that has been excited by light can either release this absorbed energy as light (phosphorescence) or transfer the absorbed energy to oxygen (without light emission). This results in an oxygen dependent phosphorescence intensity and lifetime. The relationship between the lifetime and the oxygen concentration is given by the Stern-Volmer relationship (Vanderkooi, 1989). Calibration constants associated with the Stern-Volmer relationship allow oxygen concentrations to be calculated from the measured lifetimes. The measurement of lifetimes allows quantitative measurements without the influence of tissue optical properties.

**[0007]** For in vivo measurements, Pd-porphyrin is bound to albumin to form a large molecular complex that after injection into the circulation remains confined, at least for a certain time, inside the blood vessels. This allows microvascular $pO_2$ measurements to be made using a phosphorimeter. A phosphorimeter is a device that measures the phosphorescence decay after a pulse of light (time-domain device) or determines the phase-shift between a modulated excitation source and the emitted phosphorescence (frequency-domain device). Several of these systems have been described in literature (Mik, 2002; Coremans, 1993; Sinaasappel, 1996

and Vinogradov, 2002). Attached to a microscope phosphorescence lifetime measurements allow the measurement of $pO_2$ in single blood vessels in the microcirculation. Use of fiber phosphorimeters allows measurement of microvascular $pO_2$ ($\mu pO_2$) without having to resort to microscope techniques. A fiber phosphorimeter has been developed for measurement of μpO2 in large animal models of shock and sepsis (Sinaasappel, 1999; Van Iterson, 1998), as well as in mice (Van Bommel, 1998) and the analysis of the decay kinetics has been improved to provide more reliable calculation of $pO_2$ values from the decay kinetics (Mik, 2002). A multichannel implementation of this phosphorimeter allows simultaneous detection of $\mu pO_2$ at different sites and different organs. In general, the use of multi-fiber technology is, besides imaging, a way to detect special information in optical spectroscopy. FIG. **1** shows schematically an example of a frequency-domain phosphorimeter of which the light source is a very cost-effective light emitting diode (LED).

**[0008]** An advantage of lifetime measurements is the independence of the concentration of the chromophore, making quantitative measurements possible in vivo, where the precise concentration of said chromophore cannot be predicted. An important drawback of this technique is however that it relies on injection of palladium-porphyrin into the circulation, making this technique unsuitable for clinical settings because of long-term toxicity. The use is limited to pre-clinical applications. Moreover, this technique is only suitable for measuring oxygen levels in the microcirculation. Since the molecules are large and cell-impermeable, this technique cannot be applied for intracellular oxygen measurements without disrupting the intracellular compartment by micro-injection (Hogan, 1999).

**[0009]** A kind of semi-quantitative oxygen measurement using non-specific protein phosphorescence has been used for oxygen measurements in mitochondrial suspensions. This was based on oxygen-dependent quenching of the phosphorescence of the amino acid tryptophan (Vanderkooi et al., 1990). Unfortunately, this phosphorescence cannot be used for quantitative oxygen measurements because of the complex decay kinetics arising from the different tryptophan containing proteins (Vanderkooi et al., 1987b). The use of tryptophan phosphorescence for in vivo applications is furthermore limited because of the excitation in the UV region (283 nm), resulting in extremely shallow penetration depths in tissue, besides the well-known photo-toxicity of this high energetic light.

**[0010]** Although both oxygen-dependent quenching of phosphorescence and hemoglobin saturation measurements give information about the microvascular oxygenation status, they do not provide a direct measurement of the adequacy of tissue oxygenation. The latter is highly dependent on factors like tissue oxygen consumption and diffusion distances within the tissue. Additional measurements of e.g. oxygen extraction and $CO_2$ production are therefore often required.

**[0011]** More direct spectroscopic determinations of tissue oxygenation are also possible. One of the oldest, and most widely used, is NADH-fluorimetry. The measurement of tissue bioenergetics is commonly used for measurement of the adequacy of tissue oxygenation. Oxidative phosphorylation occurring in the mitochondria of cells is the main site for the production of ATP. In the final step of the electron transport chain, reduced pyridine nucleotides (NADH) is oxidized to $NAD^+$ and $H_2O$, utilizing molecular oxygen. In contrast to $NAD^+$, NADH emits blue fluorescence (around 450 nm) when illuminated with ultraviolet light (around 360 nm). This allows spectroscopic determination of relative tissue NADH levels. The fluorescence intensity of NADH is therefore an optical indicator of cellular metabolism.

**[0012]** Measurement of the fluorescence intensity of endogenous mitochondrial NADH in situ can thus be used as a direct measure of tissue bioenergetics. Since for the conversion of mitochondrial NADH to $NAD^+$ the availability of molecular oxygen is mandatory, lack of oxygen results in accumulation of NADH and subsequent increase in fluorescence intensity. The fluorescence intensity is for instance imaged using sensitive photographic or video techniques and can be used to study the regional heterogeneity of tissue dysoxia on organ surfaces in vitro and in vivo. Unwanted influence of the absorbance of hemoglobin can be corrected by use of a two-wavelength method (Coremans, 1997).

**[0013]** However, even with proper calibration, exact quantification of the NADH levels remains impossible (Masters, 1993). One of the reasons is the contribution of cytosolic NADH and NADPH to the total fluorescence signal.

**[0014]** Hence, although oxygen is one of the most important biological molecules, concentration measurements in vivo remain cumbersome. The same kinds of problems arise when the concentration of another substance is measured.

**[0015]** It is an object of the present invention to provide an alternative method for determining a concentration of a substance. Preferably a method is provided wherein at least one of the above mentioned disadvantages is overcome.

**[0016]** The invention provides a method for determining a concentration of a substance in a compartment comprising:

**[0017]** exciting an endogenous compound of said compartment, or a functional part, derivative, analogue and/or precursor of said compound, wherein said compound, functional part, derivative, analogue and/or precursor, if excited, exhibits a luminescence and/or transient absorption, the lifetime of which is dependent on said substance,

**[0018]** measuring the lifetime of luminescence and/or transient absorption exhibited by said compound, functional part, derivative, analogue and/or precursor, and

**[0019]** correlating said luminescence lifetime with said concentration of said substance.

**[0020]** According to the present invention an endogenous compound of an organism, or a precursor, functional part, derivative and/or analogue thereof, is suitable for concentration measurements of a given substance, since it is possible to excite an endogenous compound or a precursor, functional part, derivative and/or analogue thereof in order to exhibit a luminescence and/or a transient absorption, the lifetime of which is dependent on the concentration of said substance. Hence, the lifetime of said luminescence and/or transient absorption is correlated to the concentration of said substance. An endogenous compound is defined as a compound which is naturally present in said compartment, without artificial interference by man, or which is essentially the same kind of compound as a compound which is naturally present in said compartment. Preferably said compound is identical to a compound which is naturally present in said compartment. In one embodiment said endogenous compound comprises an administered compound which is essentially the same kind of compound as a compound which is naturally present in said compartment. In another embodiment said compound is present as a result of a conversion of a precursor into at least one compound which is naturally present in said compart-

ment, or which is essentially the same kind of compound as a compound which is naturally present in said compartment. Hence, in one embodiment an endogenous compound is derived from a precursor.

**[0021]** It is of course possible to provide a compartment with a compound which is the same kind of compound as an endogenous compound. This is for instance done to increase the concentration of said endogenous compound. Hence, a method of the invention is not limited to exciting compounds which are already naturally present in a compartment. Exciting an administered compound which is essentially the same kind of compound as an endogenous compound, or which is a functional part, derivative and/or analogue of an endogenous compound, is also within the scope of the present invention. Hence, one embodiment of the invention comprises exciting an endogenous compound, or a functional part, derivative and/or analogue of an endogenous compound, which has been administered to a compartment. Additionally, or alternatively, a method of the invention comprises exciting an endogenous compound which is already naturally present within said compartment. Yet another embodiment of the invention comprises administering a precursor of an endogenous compound, which is capable of being converted into at least one endogenous compound, and exciting a compound derived from said precursor. In one embodiment said precursor is excited.

**[0022]** A functional part of a compound is defined as a part which has the same kind of properties in kind, not necessarily in amount. Preferably said functional part exhibits a luminescence and/or transient absorption property which is the same—in kind, not necessarily in amount—as said compound. Most preferably said functional part comprises the same delayed fluorescence and/or triplet-triplet absorption properties as said compound in kind, not necessarily in amount. A functional derivative of a compound is defined as a compound which has been altered such that the luminescence and/or transient absorption properties of said compound are essentially the same in kind, not necessarily in amount. A derivative can be provided in many ways, for instance by addition, deletion and/or substitution of at least one atom or group, by an esterification, et cetera.

**[0023]** A person skilled in the art is well able to generate analogous compounds. An analogue has essentially the same luminescence and/or transient absorption properties of said compound in kind, not necessarily in amount.

**[0024]** As used herein, the phrase "endogenous compound" also encompasses a functional part, derivative and/or analogue of an endogenous compound.

**[0025]** A compartment is defined as an area with properties that make it distinguishable from other areas. Said compartment for instance comprises an organism as a whole, or a part of an organism such as for instance an organ, a tissue, a cell, an organelle, a tumor and/or the microcirculation of an organism, or a part of said organ, tissue, cell, organelle, tumor and/or microcirculation. In a preferred embodiment said compartment comprises a mitochondrion. In one embodiment said compartment comprises a part of an organ, tissue or cell. With a method of the present invention it is possible to measure a concentration of a substance in several parts of an organ, tissue, or cell, such that concentration of a substance at several sites is determined. In one preferred embodiment a concentration gradient is determined.

**[0026]** In yet another embodiment said compartment comprises an in vitro compartment, such as for instance a culture medium, a cell suspension, a bioreactor or a tissue or organ cultured in vitro. In one embodiment said compartment comprises an enclosed area, such as an organism, cell, organelle (preferably a mitochondrion) or bioreactor. In an alternative embodiment said compartment is not enclosed. Examples of such compartments are parts of a tissue, organ and/or tumor. Although no exact borders of such compartment are present, usually tissue present within 20 cm, preferably within 15 cm of a given site of interest is considered. In one embodiment said compartment comprises a tumor, because information about the concentration of a substance such as oxygen in a tumor is desired in order to determine whether a certain treatment such as irradiation and/or photodynamic therapy is suitable. In one preferred embodiment a concentration gradient through at least part of an organ, wound and/or tumor is determined.

**[0027]** With a method of the invention it is possible to measure the concentration of any substance capable of influencing a luminescence lifetime and/or transient absorption lifetime of an endogenous compound, or a functional part, derivative, analogue and/or precursor thereof, that has been excited. In a preferred embodiment said substance comprises oxygen. The invention is further exemplified by the preferred embodiments relating to determination of oxygen concentration. It is to be understood however that a method of the invention is also applicable to determining a concentration of another substance capable of influencing a luminescence lifetime of an excited endogenous compound.

**[0028]** In order to determine oxygen concentration within an organism, phosphorescent dyes such as metallo-porphyrins are currently often injected into the circulation. However, as already mentioned, such methods have the disadvantage of long-term toxicity. With a method of the invention, wherein an endogenous compound and/or a precursor thereof is used, this problem is circumvented.

**[0029]** Preferably, a method of the invention is provided wherein said endogenous compound comprises a compound capable of being excited to a triplet state since molecular oxygen is a molecule of which the ground state is a triplet state. Oxygen is therefore capable of quenching an excited triplet state. Hence, a compound capable of being excited to a triplet state is particularly suitable for determining an oxygen concentration with a method of the present invention. As used herein, quenching an excited triplet state means causing relaxation of an excited triplet state to occur at a rate that is higher than the rate of spontaneous relaxation. Spontaneous relaxation means relaxation without the presence of a substance capable of accelerating relaxation. For instance, in the presence of oxygen the lifetime of an excited triplet state is shortened as compared to the lifetime of an excited triplet state in the absence of oxygen.

**[0030]** Luminescence for instance comprises phosphorescence and/or fluorescence. Fluorescence and phosphorescence lifetime measurements are based on the fact that after pulsed excitation the emitted signal does not vanish instantaneously, but decays with a certain lifetime. Energy transfer between the excited molecules and quencher molecules in its environment causes shortening of the luminescence lifetime. Preferably said luminescence comprises delayed fluorescence. Delayed fluorescence is a phenomenon which occurs in the case of a bi-directional intersystem-crossing. For instance, repopulation of a S1 state from a T1 state results in delayed fluorescence. Delayed fluorescence presents itself as another component of fluorescence besides prompt fluores-

cence, having a decay time equal to the lifetime of a triplet state if the time needed for intersystem-crossing is much shorter than the lifetime of the T1 state. Compared to prompt fluorescence, delayed fluorescence is measured much longer after a molecule has been photo-excited, thus avoiding interference of the emitted light pulse and the measured fluorescence.

**[0031]** Transient absorption is defined as a temporary absorption change after photoexcitation. Such temporary absorption change is measured using any method known in the art. In one preferred embodiment said transient absorption comprises triplet-triplet absorption. A preferred method of the invention therefore comprises measuring a triplet-triplet absorption. This is for instance performed with a MicroScan. Triplet-triplet absorption from the first excited Triplet state (T1) to the second excited triplet state (T2) is a process that can only occur after previous population of the first excited Triplet state and during the existence of this T1 state. If for example the T1 to T2 transition occurs with the absorption of light of a certain wavelength λ, than a transient absorption of light of wavelength λ is observed after photo excitation of the compound. This transient absorption has a lifetime equal to the T1 lifetime and is therefore also a means to measure the T1 lifetime. Triplet-triplet absorption measurements require a second light source (with another wavelength as the main excitation source).

**[0032]** In one aspect of the invention said endogenous compound comprises a porphyrin. A porphyrin chelated to an iron atom constitutes the haem molecule. Haem is one of the central molecules involved in oxygen transport (haemoglobin and myoglobin) and oxygen utilisation (cytochromes in the mitochondrial respiratory chain). Porphyrins are derivatives of porphine. Porphine possesses a ringsystem (FIG. **2**) with four pyrolrings and is a chemically very stable molecule that can be found as "chemical fossil" in oil. Porphine and its derivatives are of biological importance because of their central role in most vital processes were oxygen turnover takes place. For example in plants derivatives of porphine are key substances in the photosynthesis process. This is the process were oxygen is produced out of carbon dioxide and light. In mammals on the contrary, porphine derivatives like heme and cytochrome C play central roles in oxygen transport and oxygen consumption.

**[0033]** Preferably, said endogenous compound comprises a protoporphyrin. An even more preferred embodiment provides a method of the invention wherein said compound comprises protoporphyrin IX or a functional part, derivative and/or analogue thereof. Protoporphyrin IX (PpIX) is the final precursor in the synthesis of haem and present in many cells and tissues. Protoporphyrin IX (PpIX, structure formula in FIG. **2**) is synthesized inside the mitochondria were it becomes heme after inclusion of an iron atom by the enzyme ferrochelatase. Since the ferrochelatase activity is rather slow (speed limiting step), adding the precursor 5-aminolevulinic acid (ALA) results in a temporary rise in intramitochondrial PpIX levels. Hence, if desired, the level of PpIX in a compartment such as for instance a cell and/or tissue is easily enhanced by administration of 5-aminolevulinic acid (ALA), a precursor of the haem biosynthetic pathway. Additionally, or alternatively, the level of PpIX in a compartment is enhanced by administration of PpIX. A study of Chantrell et al. reports that PpIX dimethyl ester does not show measurable phosphorescence in the visible range (Chantrell et al., 1977). Therefore, this molecule was not expected to be useful for monitoring a concentration of a substance like oxygen. However, according to the present invention, protoporphyrin IX emits delayed fluorescence after excitation. Protoporphyrin IX possesses an excited triplet state that is quenched by a substance like for instance oxygen, making its lifetime dependent on said substance. After excitation of PpIX, delayed fluorescence is observed. Moreover, triplet-triplet absorption is measurable. A use of a porphyrin or a functional part, derivative and/or analogue thereof for determining a concentration of a substance in a compartment is therefore also herewith provided. Said porphyrin preferably comprises protoporphyrin IX. In one preferred embodiment said porphyrin comprises a clinically used photodynamic agent, preferably (but not limited to) photofrin, which is currently used for photodynamic therapy against, amongst other things, tumor cells. This provides the advantage that oxygen concentration measurements is possible during therapy with a method of the invention using the therapeutic agent itself.

**[0034]** Without being bound to theory, a working model for state transitions, quenching and measurement modes for PpIX is shown in the Jablonski diagram in FIG. **4**. Most often the population of the triplet state is achieved through excitation of the molecule from the ground state $S_0$ into an excited singlet state ($S_1$ or higher), followed by intersystem crossing from $S_1$ to $T_1$. Because the spontaneous $T_1 \rightarrow S_0$ transition is spin-disallowed, the rate of occurrence is much less than the spin-allowed $S_1 \rightarrow S_0$ transition. This results in relatively long triplet state lifetimes in the order of μs to ms. Molecular oxygen, a molecule of which the ground state is a triplet state, is a quencher of an excited triplet state. If a molecule while it is in the $T_1$ state collides with an oxygen molecule, the oxygen absorbs the energy from the excited molecule. This event results in a relaxation of the excited molecules at a rate higher than the rate of spontaneous relaxation. At sufficiently low concentrations of excited molecules, the relationship between the $T_1$ lifetime and the oxygen concentration is given by the Stern-Volmer relationship:

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_q[O_2] \qquad (2)$$

where τ is the $T_1$ lifetime, $\tau_0$ is the $T_1$ lifetime in the absence of oxygen and $k_q$ is the rate constant of quenching by oxygen. Quantitative oxygen concentration measurements are possible by means of $T_1$-lifetime measurements.

**[0035]** In general, $T_1$-lifetimes are determined in several ways. In FIG. **4** three different modes of $T_1$-lifetime measurements are shown: phosphorescence, triplet-triplet absorption and delayed fluorescence. In the case of exogenous phosphorescent dyes, phosphorescence lifetimes are measured by measuring the decay of the emitted light after pulsed excitation. However, PpIX does not show measurable phosphorescence (Chantrell et al., 1977). Triplet-triplet absorption relies on the measurement of the transient increase in absorption after photo excitation and population of the triplet state. Triplet-triplet absorption is also suitable for measuring a triplet lifetime of PpIX. One embodiment therefore provides a method of the invention wherein measuring said transient absorption lifetime comprises measuring triplet-triplet absorption.

**[0036]** In view of the fact that PpIX does not show measurable phosphorescence, PpIX was not considered in the art to be suitable for monitoring a concentration of a substance like

for instance oxygen. However, according to the present invention, PpIX is nevertheless suitable since it shows delayed fluorescence and triplet-triplet absorption with an oxygen dependent lifetime. In contrast to phosphorescence, delayed fluorescence is not red-shifted compared to the prompt fluorescence. Delayed fluorescence of PpIX has not been described in the art.

**[0037]** In vitro studies by the present inventors have shown that PpIX shows a type of delayed fluorescence with a decay time comparable to the decay of the $T_1$ state. The decay of the $T_1$ state was determined by measurement of the light transmission through the sample, the transmission being the reverse of the Triplet-Triplet absorption (FIGS. **3**A and **3**B). The sample consisted of a solution of PpIX bound to albumin. Moreover, experiments showed that the decay time of the delayed fluorescence is dependent on the oxygen concentration (FIG. **3**C). FIG. **4** shows a working model for state transitions, quenching and measurement modes.

**[0038]** PpIX is the final precursor in the synthesis of haem used for haemoglobin, myoglobin and cytochromes, all key substances in the transport and/or utilization of oxygen. This makes the use of PpIX as oxygen sensor even more attractive because it provides a unique method for measurement of an oxygen concentration at the place where the availability of oxygen is the most important (i.e. intracellular and inside the mitochondria). Moreover, delayed fluorescence measurements are easier to implement in vivo and in clinical use than absorption measurements. With a method of the invention it has become possible to measure the amount of a substance like oxygen directly in a cell and/or organelle, without the need of addition of exogenous, toxic compounds to an organism and without the need to indirectly deduce said concentration from for instance the concentration of said substance in the bloodstream or in an intercellular environment.

**[0039]** In one aspect a method of the invention is provided wherein an endogenous compound or a functional part, derivative, analogue and/or precursor thereof is photo-excited. This is a usual way of exciting a compound and a lot of equipment for photo-exciting is available in the art. An example of a photo-exciting device is described in Shonat et al, 1997, incorporated herein by reference. However, in other embodiments an endogenous compound or a functional part, derivative, analogue and/or precursor thereof is excited by other means, like for instance electromagnetic radiation.

**[0040]** Since protoporphyrin IX is naturally present within cells, it has become possible to determine a concentration of a substance, such as for instance oxygen, within a cell. A method of the invention is therefore provided wherein said compartment comprises a cell. In one embodiment said compartment comprises an organelle. Even more preferably said compartment comprises a mitochondrion, since protoporphyrin IX is naturally present in mitochondria. Hence, a method of the invention is particularly suitable for determining oxygen concentration in mitochondria. This is a preferred application of the invention since the availability of oxygen in mitochondria is a measure of tissue bioenergetics. Since mitochondria normally consume oxygen, a low concentration of oxygen within mitochondria is indicative for tissue bioenergetics. Tissue bioenergetics is therefore preferably assessed by determining a mitochondrial oxygen concentration with a method of the present invention. One preferred embodiment of the present invention involves determining mitochondrial oxygen concentration after a period of tissue dysoxia in order to determine whether tissue cells are still viable or whether these cells are prone to apoptosis. If a mitochondrial oxygen concentration appears to be low, it indicates that bioenergetics still take place and that cells are still viable. If however mitochondrial oxygen concentrations appear to be high, bioenergetics hardly—if at all—take place indicating that cells are prone to apoptosis. Preferably, 5-aminolevulinic acid is administered to cells, resulting in accumulation of protoporphyrin IX inside the mitochondria. According to this embodiment, luminescence and/or transient absorption lifetime of said accumulated PpIX is measured in order to determine mitochondrial oxygen concentration. Afterwards, a more diffuse fluorescence and/or transient absorption is observed in the cytosol and oxygen concentration throughout the cell is preferably measured. In one preferred embodiment oxygen concentration in mitochondria of a cell is determined within four hours, more preferably within two hours, even more preferably within one hour after administration of 5-aminolevulinic acid to said cell, because during this period PpIX primarily accumulates inside mitochondria. In one embodiment oxygen concentration is determined in other parts of said cell after four hours.

**[0041]** A method of the invention is suitable for determining the concentration of a substance such as oxygen in a tissue or organ, or in a certain part of a tissue or organ. Important applications are for instance measurements of oxygen concentration in the heart, the brain and/or the retina of the eye, preferably during surgery. Oxygen concentration in the brain and/or heart is for instance measured in order to determine whether a stroke and/or myocardial infarction has occurred. In one embodiment oxygen concentrations in several different parts of a certain tissue or organ are determined in order to obtain an overall impression, and/or to measure a $pO_2$ gradient. A method of the invention is therefore provided wherein said compartment comprises at least part of a tissue.

**[0042]** Another application of a method of the invention is determination of oxygen concentration at a tumor site. In this embodiment oxygen concentration within a tumor is determined. Information about oxygen concentration at a tumor site is for instance required for determining whether a certain kind of treatment, such as irradiation and/or photodynamic therapy, is suitable. For instance, little oxygen is present at a solid tumor site. Irradiation is therefore not likely to be effective at such site. Therefore, once it is determined with a method of the invention that an individual is suffering from a solid tumor with little oxygen, irradiation therapy is preferably not applied. Instead, alternative treatment is preferred. Hence, therapy is adapted to information about oxygen concentration, which information is obtained by a method of the invention. In a further embodiment, oxygen concentration at a tumor site and/or around a tumor site is monitored with a method of the invention in order to monitor progress of disease and/or therapy.

**[0043]** In one embodiment a concentration of a substance such as for instance oxygen at a location of interest is measured by providing an organism with an endogenous compound, and/or with a precursor thereof, which is coupled to a moiety capable of specifically binding said location of interest. Said moiety for instance comprises an antibody or a functional part, derivative and/or analogue thereof. For instance, if the oxygen concentration in a tumor is to be measured, an endogenous compound or a precursor thereof is preferably coupled to an antibody capable of specifically binding a tumor-specific antigen. A tumor-specific antigen is an antigen that is present on a tumor cell while it is less

(preferably not) present on normal cells. Said endogenous compound or precursor coupled to a tumor-specific antibody will accumulate in and/or around said tumor. This results in an increased concentration of said endogenous compound and/or precursor in and/or around said tumor, facilitating oxygen concentration measurement in and/or around said tumor. Likewise, in other embodiments the concentration of a substance is specifically measured at any location of interest, using an endogenous compound and/or precursor thereof that is coupled to a moiety capable of specifically binding said location of interest.

**[0044]** In yet another aspect an endogenous compound such as a porphyrin, preferably protoporphyrin IX, is administered to the circulation of an individual. Alternatively, or additionally, a precursor is administered which is converted in vivo into at least one metabolite that is essentially the same kind as—preferably identical to—an endogenous compound and which, if excited, exhibits a luminescence and/or transient absorption of which the life time is dependent on the concentration of a given substance. In one embodiment 5-aminolevulinic acid is administered, which is metabolized into protoporphyrin IX in vivo. When an endogenous compound or a precursor thereof is administered to the circulation of an individual, said molecule is in one embodiment bound, for instance to albumin, to form a large molecular complex that remains confined, at least for a certain time, inside the circulation. Said administered compound which is essentially the same kind as an endogenous compound, and/or whose metabolite is essentially the same kind as an endogenous compound, is not or to a lesser extent toxic as compared to exogenous compounds such as for instance palladium-porphyrin. Administration of said compound is therefore not, or to a lesser extent, involved with (harmful) side reactions. In one embodiment a method of the invention is therefore provided wherein said compartment comprises the (micro)circulation.

**[0045]** Another application of a method of the invention is the use of an endogenous compound or a functional part, derivative, analogue and/or precursor thereof, for determining the concentration of a substance in a culture medium. In one embodiment a certain kind of tissue, cell and/or organism is cultured in a culture medium. In order to determine the concentration of a substance within said tissue, cell and/or organism, an endogenous compound of said tissue, cell and/or organism or a functional part, derivative, analogue and/or precursor thereof is excited. Subsequently, the lifetime of luminescence and/or transient absorption is measured. In one embodiment said culture medium comprises a cell suspension.

**[0046]** It is possible to administer to said culture medium a suitable compound which, when excited, displays a luminescence and/or transient absorption, the lifetime of which is dependent on the concentration of a certain substance, or a compound which is converted in vivo into at least one metabolite that is essentially the same kind as—preferably identical to—an endogenous compound. Said administered compound for instance comprises a compound which is essentially the same kind as—preferably identical to—an endogenous compound. However, said compound need not be naturally present in said cultured tissue, cell and/or organism. In one embodiment a porphyrin, preferably protoporphyrin IX, or a precursor thereof such as for instance 5-aminolevulinic acid is administered to a culture medium, such as a bioreactor, in order to monitor oxygen concentration with a method of the invention, comprising exciting said porphyrin and measuring the lifetime of delayed fluorescence. Preferably, said oxygen concentration is measured at several time points, such that the availability of oxygen is monitored over time.

**[0047]** In a preferred embodiment the lifetime of luminescence and/or transient absorption is compared with a reference. A reference curve (also called a calibration curve) is for instance generated, from which kq and $\tau_0$ are derived. Once kq and $\tau_0$ are determined, a luminescence lifetime is correlated with the concentration of a substance, preferably by the Stern-Volmer relationship. Additionally, or alternatively, a reference curve is preferably generated in order to correlate the lifetime of transient absorption to the concentration of a given substance. In one embodiment, a compartment is successively provided with various concentrations of a substance in order to generate a reference curve. Additionally, or alternatively, several similar compartments are provided with various concentrations of a substance. According to this embodiment, luminescence and/or transient absorption lifetime is determined at various concentrations of said substance. Many alternative methods of generating a reference curve are known in the art, which are suitable for a method of the present invention.

**[0048]** In order to generate a reference curve, a luminescence and/or transient absorption lifetime is preferably determined at least two concentrations of said substance. Preferably however, luminescence and/or transient absorption lifetime is determined at least three concentrations of said substance, more preferably at least four concentrations of said substance. The more luminescence and/or transient absorption lifetime vs substance concentration values are measured, the more accurate a reference curve will be. A reference curve is for instance generated by plotting luminescence lifetime and/or transient absorption lifetime versus concentration of a substance. Of course, said reference curve need not to be physically plotted. It is for instance also possible to store measured reference values, for instance in a (computer) database. A formula representing a reference curve is for instance calculated. In one embodiment a measured luminescence lifetime and/or transient absorption lifetime is entered into said database, after which an algorithm calculates and discloses the correlated substance concentration.

**[0049]** Preferably, a calibration curve is generated using the same kind of compartment(s) as the compartment(s) wherein the concentration of at least one substance is to be measured. Moreover, said reference curve is preferably generated using the same kind of substance(s) as the substance(s) whose concentration(s) is/are to be measured. Once a calibration curve is generated, it is preferably used to correlate a measured luminescence and/or transient absorption lifetime with a concentration of a substance. In one embodiment, a calibration curve is generated before the concentration of a substance in a compartment is determined. However, once a calibration curve has been generated, it is not necessary to generate another calibration curve each time before a concentration of a substance is determined. For instance, once kq and $\tau_0$ have been determined it is preferably repeatedly used for correlating said lifetime to the concentration of a certain substance.

**[0050]** In one aspect of the invention a luminescence lifetime is measured in the time-domain, meaning that said lifetime is measured after a pulse of light. In another aspect said lifetime is measured in the frequency-domain, meaning that continuous excitation takes place. The phase-shift between a

modulated excitation source and the emitted luminescence is measured. For instance, phosphorescence and/or delayed fluorescence is capable of being measured in the frequency domain. Measurement of said lifetime in the frequency domain is usually cheaper. On the other hand, measurement of said lifetime in the time domain is possible with a higher intensity of light.

**[0051]** A method of the invention is suitable for being performed with single-photon excitation. However, a preferred embodiment provides a method of the invention wherein multi-photon excitation is applied, such as for instance two-photon, three-photon or four-photon excitation. Multi-photon excitation involves excitation with multiple photons instead of one. The multiple photons for instance have one half of the energy of a single photon (in case of two-photon excitation). The multiple photons have one third of the energy of a single photon (in case of three-photon excitation), or one fourth of the energy of a single photon (in case of four-photon excitation), and so on. Multi-photon excitation is preferred because it allows for deeper tissue penetration and a more precise and confined selection of an excitation volume as compared to single-photon excitation, due to the non-linear multi-photon effect. Hence, with multi-photon excitation inner parts of a compartment, such as for instance inner parts of a tissue or organ, are more easily examined. Multi-photon excitation facilitates determination of a concentration gradient, for instance from an outer surface of a tissue until an inner part of such tissue or vice versa. Moreover, since multi-photon excitation allows for a more precise and confined selection of an excitation volume, damage to surrounding tissue is more easily avoided.

**[0052]** In one preferred embodiment a method of the invention is used for an "optical biopsy". This means that a certain part of interest, such as a small part of a certain tissue, is investigated but not excised. A characteristic such as for instance an oxygen concentration of said part of interest is determined using a method of the invention specifically directed to said part of interest, while said part of interest remains at its original site. For instance, at least part of a tissue of an organism is investigated while said part remains in said organism. This is preferably performed using multi-photon excitation because multi-photon excitation allows for a precise selection of an excitation volume.

**[0053]** In a preferred embodiment, two-photon excitation is applied. The principles and advantages of two-photon excitation are outlined in (Mik, 2004), which is incorporated herein by reference. In contrast to single-photon excitation, two-photon excitation is a non-linear optical process in which a compound is excited by two photons instead of a single photon with a double energy (or half the wavelength). By considering the excitation as the rate-limiting step in a chemical reaction consisting of a single-step termolecular process involving one molecule and two photons, one derives the rate of production of excited-state molecules, $R_{TPE}$:

$$R_{TPE} = \frac{\delta}{2}\frac{I}{A}CP^2 \quad (3)$$

**[0054]** where δ is the two-photon cross-section, l the path-length, A the cross-sectional area of the beam (multiplying l by A defines the interaction volume), C the molar concentration of the excitable compounds and P the power of the excitation beam. In phosphorescence measurements, the intensity of the signal is proportional to $R_{TPE}$, therefore equation (3) can be rewritten in terms of signal intensity versus excitation power:

$$I_0 \propto CP^2 \quad (4)$$

**[0055]** where $I_0$ is the measured phosphorescence intensity at time zero, i.e. directly after the excitation pulse. In equation 4, constants influencing the absolute value of $I_0$, like the molecular constants, excitation geometry and detection efficiency are omitted. These constants are intensity independent so that the proportionality sign describes the relation between $I_0$ and $P^2$. The non-linear behavior of TPE provides a means of selective excitation within a 3-dimensional space, and the quadratic dependence of emission intensity versus excitation power is regarded as proof of the two-photon nature of the studied phenomena.

**[0056]** The invention furthermore provides a device for determining a concentration of a substance in a compartment comprising:

**[0057]** means for exciting an endogenous compound or a functional part, derivative, analogue and/or precursor thereof, wherein said compound, part, derivative, analogue and/or precursor, if excited, exhibits a luminescence and/or transient absorption of which the lifetime is dependent on said substance, and

**[0058]** means for measuring said lifetime.

**[0059]** Preferably, equipment for optical spectroscopy comprises an illumination light source, an optical system (for instance comprising filters, mirrors and lenses) and a detection unit. The detector for instance comprises a sensitive CCD camera, photomultiplier tube and/or spectrophotometer. Several descriptions of optical systems are described in the literature (Carlsen et al, 2002; Baxter et al, 1997; Green et al, **1988**). An example of a frequency domain phosphorescence lifetime measurement device is described in Shonat et al, 1997, incorporated herein by reference. Non-limiting examples of a device of the invention are outlined in the Examples. In a preferred embodiment a combination of a prism and a bandpassfilter is used, at least partly preventing a high amount of excitation light to reach the filters in order to avoid possible disturbance of a delayed fluorescence signal as a result of fluorescence and/or phosphorescence of the filters themselves. In a further preferred embodiment a device of the invention comprises a fast shutter in front of a PMT, preferably a pockel cell, in order to prevent distortion of the first 20 to 30 μs of a signal which would otherwise occur if a PMT is gated by switching the voltages of the second and third dynodes during the laser pulse. Alternatively a semi-conductor device, preferably an avalanche-photodiode is used, which is cheaper.

**[0060]** In one preferred embodiment a device according to the invention comprising an imaging device capable of oxygen mapping, preferably a CCD camera and/or a diode array, is used in order to allow imaging of a specific location.

**[0061]** Reference measurements are preferably performed for quantitative measurements, in order to take account of possible influences of tissue optical properties on the signal.

**[0062]** The invention is further explained in the following examples. The examples do not limit the scope of the invention; they merely serve to exemplify the invention.

## EXAMPLES

### Example 1

**[0063]** The spectra of prompt and delayed luminescence were recorded using a LS50B luminescence spectrometer

(Perkin-Elmer, Wellesley, Mass., USA). Prompt fluorescence was measured using the fluorescence mode with excitation source correction. Delayed luminescence was recorded in the phosphorescence mode, using varying delay times with respect to the excitation flash and a gate width of 100 μs. The measurements were made at room temperature. Excitation and emission wavelengths and slit widths will be specified in the results section. Spectra were recorded with either air-saturated samples or samples containing zero oxygen. Adding a sufficient amount of ascorbic acid (20 μl of 200 mM solution) to the already ascorbate oxidase containing samples (1 unit ascorbate oxidase per ml, 3 ml total sample volume) created the zero-oxygen conditions. This method of reducing oxygen levels is explained in more detail below. The amounts of ascorbate oxidase and ascorbic acid used did not interfere with the readings of the spectra.

**[0064]** The experiments concerning comparison of triplet-triplet absorption kinetics with delayed fluorescence lifetimes, and the measurement of transient absorption spectra, were performed using a LFDL-3/Remote flash lamp pumped dye laser (Candela Laser Corporation, Wayland, Mass.). This system provided pulses with a duration of approximately 1 μs at 505 nm at a repetition frequency of 10 Hz. The output of the laser was directly focussed on the sample, consisting of a quartz cuvette containing the PpIX solution. The used detector was a R928 (HAMAMATSU, Hamamatsu City, Japan) photomultiplier tube (PMT) with a C1392-09 (HAMAMATSU, Hamamatsy City, Japan) gated socket. The detector was coupled to a monochromator (Oriel 77320) in order to select the emission wavelength of interest. The output of the PMT was fed into an oscilloscope (Tektronix **2440**, TEKTRONIX INC., Beaverton Oreg., USA) and transferred to a computer by the serial bus. The wavelength-dependent transient absorption was measured using a white light source and scanning of the monochromator. These experiments were carried out at room temperature (20° C.).

**[0065]** Calibration experiments with varying oxygen concentrations were performed with a different set-up. A XeCl excimer laser (Lambda Physik LPX 110i, Göttingen, Germany), operated at 10 Hz and producing 50 mJ pulses was used to pump a dye laser (Lambda Physik, LPD 3002) operating at 405 nm. The output of the dye laser was focussed on a quartz optical fiber with a core of 0.6 mm (Ensign Bickford Optics, Avon, Conn.) using a 3 cm F/1.2 quartz lens. The fiber was coupled to the reaction vessel (described below) used for the calibration experiments. The detector was the same R928 PMT with C1392-09 socked, switched off during 5 μs gate width. The detector was coupled to the reaction vessel by a VIS-type liquid light guide with a 5 mm optical core (Oriel, Stratford, USA). Instead of the monochromator three 630 nm long pass glass filters were used for filtering of the emission light. The laser pulse was fired 1 μs after off gating of the PMT, the repetition rate was 10 Hz. Per measurement 64 traces were averaged on a digital oscilloscope (Tektronix TDS-350, Tektronix Inc., Beaverton Oreg., USA). Data were transferred to a computer by serial bus and lifetime analysis was performed using LabView 5.1 graphical programming software (National Instruments, Austin, Tex., USA). Mono-exponential fitting was performed using a Marquard-Levenberg non-linear fit.

**[0066]** To perform delayed fluorescence lifetime measurements at varying oxygen concentrations the oxygen concentration in the PpIX solution was varied using the ascorbate oxidase/ascorbic acid enzymatic reaction. Calibration experiments, needing precisely controlled oxygen concentrations, were performed using a specially made reaction vessel. The vessel had to be airtight, allow continuous mixing of the PpIX solution, temperature control, continuous temperature monitoring and physical access to the content. The latter was necessary to allow injection of aliquots of ascorbic acid solution but should not go at the expense of an interfering oxygen back-diffusion into the sample. It consisted of two glass parts, a bottom part and a top part. Both parts were interconnected by screw lock. An airtight connection was assured by a teflon ring surrounding the connection site. The bottom of the reaction vessel was flat, to allow continuous stirring of the content by a magnetic stirrer. The top part contained three capillary entries: one allowing insertion of a small thermocouple, one for the insertion of the light guide from the excitation source and the latter for injection of ascorbic acid. The capillaries had a length of 2 cm and a lumen of 1 mm diameter. The diffusion barrier was large enough to prevent measurable oxygen back diffusion within an hour, an adequate time span for calibration experiments. This was checked by oxygen dependent quenching of phosphorescence of Pd-meso-tetra (4)-carboxyphenyl porphine starting at varying oxygen concentrations below 40 μM. The reaction vessel was mounted in a temperature-controlled water jacked on top of a magnetic stirring device. The total content of the reaction vessel, after insertion of the magnetic stirrer, was 30.7 ml. Injection of 10 μl of a 200 mM solution of ascorbic acid resulted therefore in 32.5 μM oxygen steps ($PO_2$ steps of approximately 20 mmHg). Prior to the experiments the reaction vessel was filled with pre-heated, room-air equilibrated PpIX solution. Special care was taken to remove all air bubbles from the solution. Calibration experiments were performed at 22° C. and 37° C.

**[0067]** Chemicals

**[0068]** Pd-meso-tetra(4)-carboxyphenyl porphine was purchased from Porphyrin Products (Porphyrin Products Inc., Logan, Utah, USA). Protoporphyrin IX disodium salt (PpIX) was purchased from Sigma (Sigma Chemical CO., St. Louis, Mo., USA). Two regimens of creating PpIX solutions were used. In the first regimen, 8 mg/ml PpIX was dissolved in distilled water brought at a pH of 8.0 by titration with 1M TRIS base. From this solution 0.5 ml was added to 50 ml of a human albumin solution (40 gr/l) in phosphate buffered saline (PBS). This mixture was brought to a pH of 7.4 by titration with HCl. The PpIX is dissolved in an albumin solution to obtain a complex, mimicking the environmental circumstances in cells and tissue (Takemura et al., 1991). The experiments concerning triplet-triplet absorption were performed with PpIX solution prepared following this protocol. Since dissolving PpIX according to the protocol above takes rather long (PpIX is usually not completely dissolved after several hours), during the course of the study we looked for a more efficient way of preparing the PpIX solutions. In the second regimen, 4.0 gram of bovine serum albumin (BSA, Sigma Chemical CO. St. Louis Mo. USA) was dissolved in 200 ml PBS. To increase the buffer capacity, needed to prevent pH changes when adding aliquots of ascorbic acid to the solution, 800 mg HEPES was added. PpIX was dissolved in methanol (6.07 mg PpIX in 10 ml methanol) and 2 ml of this PpIX solution was immediately added to the albumin solution, resulting in a final concentration of approximately 10 μM PpIX. PpIX solutions according to the second regimen were used for the recording of the shown spectra and calibration experiments, unless stated otherwise.

**[0069]** Results

**[0070]** Metallo-porphyrins used for oxygen concentration measurements in vivo can usually be effectively excited at several different wavelengths. For example Pd-porphyrin, the most widely used phosphorescent dye for in vivo measurements, can be effectively excited around 400 nm (the Soret maximum) and 530 nm (the Q-band). Generally, light with a longer wavelength penetrates deeper into tissue, the reason why usually excitation at 530 nm is favoured for in vivo measurements, although the excitation efficiency at 400 nm is much higher. FIG. **5** shows the fluorescence emission versus the excitation wavelength of PpIX bound to albumin. Two peak emissions, one around 400 nm and one around 510 nm are prominently present. The excitation wavelengths of the used lasers are indicated in the figure for convenience. As will become apparent, both wavelengths are effective for delayed fluorescence measurements.

**[0071]** In order to locate an appropriate wavelength for triplet-triplet absorption measurements, the transient transmission spectrum was recorded. FIG. **6** shows the transient transmission spectrum of a 20 μM PpIX solution as function of the transmission wavelength. The maximum at 400 nm is caused by depletion of the ground state by the laser pulse, the minimum at 450 nm is due to population of the $T_1$ level and absorption to the $T_2$ level. These results are in good agreement with previous studies (Chantrell et al., 1977; Bonnett et al., 1983; Sinclair et al., 1980).

**[0072]** To identify the type of delayed luminescence that was observed after pulsed excitation of PpIX solutions, prompt and delayed luminescence spectra were recorded. FIG. **7** shows the prompt fluorescence spectrum, with its characteristic peak at 636 nm. Delayed luminescence spectra, recorded using varying delays after the excitation flash, are shown in FIG. **8**. FIG. **8**A shows the delayed luminescence in an air-saturated sample. Delayed luminescence is hardly detectable 30 μs after the excitation and is totally vanished after a delay of 100 μs. In contrast, FIG. **8**B shows that under zero oxygen conditions delayed luminescence can be detected even after a 1 ms delay. From FIG. **8**B it is also evident that the spectrum of the delayed luminescence is qualitatively the same as the prompt fluorescence spectrum shown in FIG. **7**. Especially the red shift, characteristic for phosphorescence, is absent. We therefore identify the delayed luminescence as delayed fluorescence.

**[0073]** To be useful for quantitative oxygen measurements, the delayed fluorescence lifetime should be an appropriate representative of the $T_1$ lifetime. To test this, the delayed fluorescence lifetime was compared to the lifetime of transient Triplet-Triplet absorption in a deoxygenated sample. FIG. **9** shows the decay of the triplet state measured with both delayed fluorescence and Triplet-Triplet absorption. Panel A displays the decay curve measured by delayed fluorescence at 636 nm after pulsed excitation at 505 nm. The fast decaying first part of the curve is an artefact introduced by the excitation source. Panel B contains the corresponding decay trace as measured by Triplet-Triplet absorption at 470 nm.

**[0074]** From FIG. **8** it is already noticeable that the lifetimes of the delayed fluorescence are highly dependable upon the oxygen concentration in the solution. A quantitative relationship between the lifetime and the oxygen concentration is mandatory if delayed fluorescence lifetimes are to be used for oxygen concentration measurements. To test the applicability of the Stern-Volmer relationship we measured delayed fluorescence lifetimes at varying oxygen concentrations. These experiments were performed using the described reaction vessel. Starting at a high oxygen concentration (the sample was equilibrated with room air) the oxygen concentration was lowered in steps of 32.5 μM as described in the Materials and Methods section. The Stern-Volmer relationship predicts a linear relationship between the reciprocal lifetime ($1/\tau$) and the oxygen concentration. FIG. **10** shows the measured values of the reciprocal lifetime versus the oxygen concentration at 22 and 37° C. By performing a linear fit procedure on these data, the quenching constant $k_q$ was determined. The best-fit results are also shown in FIG. **10**. At 22° C. $k_q$ was found to be $243\pm5\ M^{-1}\mu s^{-1}$ and at 37° C. this value was $471\pm7\ M^{-1}\mu s^{-1}$. Measured values for the decay time at zero oxygen conditions, $\tau_0$, were $1.4\pm0.1$ ms and $1.0\pm0.1$ ms for 22° C. and 37° C. respectively. From FIG. **10** it is clear that a good linearity between the reciprocal values of the lifetimes and the oxygen concentration exists, as is confirmed by correlation coefficients of 0.9882 and 0.9924 for 22° C. and 37° C. respectively. Moreover, no significant departure from linearity could be detected by a Runs test, providing p-values of 0.07 and 0.79 for 22° C. and 37° C. respectively. These results show that, at least over the tested oxygen concentration range, the Stern-Volmer is accurate in quantifying the relationship between the delayed fluorescence lifetimes and oxygen concentrations. As a check, a calibration experiment with a PpIX solution prepared according to the first regimen was run. The result was comparable to the calibrations performed with solutions according to the second regimen (data not shown), indicating that the reported phenomena are independent of the followed preparation procedure.

**[0075]** Discussion

**[0076]** The main findings of this study can be summarized as follows: 1) PpIX shows delayed luminescence besides the already known prompt fluorescence. 2) The emission spectrum of the delayed luminescence overlaps the spectrum of the prompt fluorescence and a red shift is absent, therefore the delayed luminescence is classified as delayed fluorescence. 3) The lifetime of this delayed fluorescence is a representative of the lifetime of the first Triplet state. 4) Oxygen is a known quencher of the Triplet state of PpIX and this study shows that the delayed fluorescence lifetime is also oxygen dependent. 5) Moreover, it is shown that the Stern-Volmer relationship describes quantitatively the dependence of the delayed fluorescence lifetime on the oxygen concentration. These findings show that oxygen-dependent quenching of delayed fluorescence provides an exciting new method to measure oxygen concentrations, since it allows non-invasive tissue- and intracellular oxygen concentration measurements by an endogenous compound such as for instance a porphyrin.

Example 2

**[0077]** In this example we demonstrate the feasibility of the proposed method for measuring intramitochondrial oxygen levels in living cells.

Equipment

**[0078]** In this Example a method of the invention is for instance performed in the time-domain using pulsed excitation from an experimental high power tuneable laser. The laser of this Example consists of a doubled flash-lamp pumped Nd-YAG laser pumping an optical parametric oscillator (OPO). This results in a tuneable laser providing 10 mJ pulses of 6 ns duration. The laser is coupled to a quart cuvette

containing the studied samples using a glass fiber. Perpendicular to the laser beam is a detector consisting of coupling lens, monochromator and photomultiplier tube (PMT). The photomultiplier (Hamamatsu R928) is working in photon-counting mode and is gated during laser excitation by reversing the polarities of the second and third dynode. The current from the PMT is voltage converted using a fast-switching integrator (integration time 3.5 μs and reset time 0.5 μs). The voltage is digitised at a sample rate of 250 kHz using a data-acquisition board in a PC. The signal of 64 pulses is averaged before applying a mono-exponential fit procedure to the measured decay curves. The lifetime typically varies from 20 ms at high oxygen levels to 700 ms at zero-oxygen conditions.

Results

**[0079]** First the intracellular distribution of protoporphyrin IX as a function of time after the administration of 5-aminolevulinic acid (ALA) was investigated. Therefore neuroblastomacells were incubated with ALA during varying periods of time. Cells were observed using a Leica fluorescence microscope with appropriate filterset. FIG. **11** shows the distribution of the PpIX fluorescence at three different time points (2, 4 and 8 hours for panel A, B and C respectively). At least until four hours, the PpIX fluorescence shows a spotty appearance corresponding to a mitochondrial pattern. At 8 hours a more diffuse fluorescence is observed located in the cytosol.

**[0080]** To demonstrate the ability to measure intramitochondrial oxygen levels, calibration experiments were performed in suspensions of neuroblastoma cells ($4*10^6$ cells/ml) after 4 hours incubation with ALA. Extracellular oxygen levels were controlled using a rotational cell oxygenator and gas flow controllers. Intramitochondrial oxygen measurements were performed before and after administration of rotenone. Rotenone is a blocker of complex 1 of the mitochondrial respiratory chain and therefore inhibits mitochondrial oxygen consumption. If the measurement is indeed mitochondrial of nature, adding rotenone will cause a decrease of intracellular oxygen gradients until ultimately the intramitochondrial oxygen level is the same as the extracellular oxygen level. For the measurement this implies that adding rotenone will cause an increase in the measured intramitochondrial oxygen concentration. FIG. **12** shows the results of such a measurement. It is clear that adding rotenone causes the predicted effect, thus the PpIX signal is mitochondrial of nature. Moreover, the signal can be calibrated, making quantitative measurements possible.

**[0081]** From this example it is concluded that after administration of ALA a time window exists in which PpIX accumulates inside the mitochondria. Moreover, it is concluded that quantitative intramitochondrial oxygen measurements are possible in living cells.

Example 3

**[0082]** An example of an experimental two-photon set-up is given in FIG. **13**. In this example, excitation is achieved using a Q-switched laser operating at 1064 nm (Laser 1-2-3, Schartz Electro-Optics Inc., Orlando, Fla., USA). The laser provides pulses of approximately 10 ns duration and an energy ranging from 10 mJ per pulse for in vitro experiments to 100 mJ per pulse in in vivo experiments. The bundle diameter of the laser beam is slightly expanded to a final diameter of 5 mm by a beam expander, before being directed to the focusing lens by an optical mirror with an enhanced silver reflection surface (Opto Sigma, Santa Anna, Calif., USA). The focusing lens is a single plan-convex lens with a focal length of 2.0 cm. Based on Gaussian beam optics, the bundle diameter of 5 mm combined with a lens with a focal length of 2.0 cm results in a focal spot size of 8 μm and a focus length of 94 μm (in air). Assuming a refractive index in tissue of 1.4, the measurement volume is approximately a cylinder with diameter of 10 μm and a length of 130 μm. The focusing lens is connected to a micrometer-screw for manual adjustment of the focal plane, thereby allowing longitudinal measurements to be made. For in vivo application, the reading of the micrometer screw is multiplied by the refractive index of tissue, assumed to be 1.4. Emission light is collected by the same lens and directed towards the photo detector by two mirrors. Selection of the phosphorescence light is achieved by two 700±20 nm band pass filters (Oriel, Stratford, Conn., USA), positioned in series before the cathode of the photomultiplier tube (PMT, type R928, Hamamatsu, Hamamatsu City, Japan). The output of the PMT is voltage-converted by a current-to-voltage converter with subsequent wide-band amplifier (30 MHz) and fed into a digital oscilloscope (Tektronix **2440**, Tektronix Inc., Beaverton, Oreg., USA). To increase signal-to-noise ratio, luminescent traces are averaged on the oscilloscope. For instance, an average of 32 traces is used. The resulting averaged traces are transferred to a computer by serial bus for data-collection and analysis using software, for instance written in LabView (National Instruments, Austin, Tex., USA).

Example 4

**[0083]** Luminescence lifetimes can be measured both in the time-domain as well as in the frequency-domain. In the time-domain the real decay curve is measured after photo excitation with a short pulse of light. In the frequency-domain the (continuous) excitation light is modulated with a known frequency and the lifetime can be determined from the phase-shift between excitation and emission light. Both methods have their specific advantages and disadvantages:

| Time-domain | Frequency-domain |
|---|---|
| Pros: | Pros: |
| No disturbance by prompt fluorescence | Lock-in amplification (high S/N-ratio) |
| No influence on oxygen tension | Relatively cheap |
| Cons: | Cons: |
| Background light needs to be taken care of | Possible disturbance by prompt fluorescence |
| Expensive | Oxygen consumption |

**[0084]** Technical improvements in the time domain are described below.

Optics:

**[0085]** A monochromator is preferably used instead of filters in order to avoid possible disturbance of the delayed fluorescence signal as a result of fluorescence and/or phosphorescence of the filters themselves. Unfortunately monochromators have low transmission efficiency and a gain in performance is achieved by using a different optical system. A cost-effective solution is a use of band-pass filters com-

bined with an optical system that at least partly prevents a high amount of excitation light to reach the filters. An example of this embodiment is shown in FIG. **14**.

Detectors:

**[0086]** Considering the low signal levels PMT's are a good choice. Due to the high energetic laser pulse and the resulting high amount of prompt fluorescence the detector and electronics are preferably protected against damage. In one embodiment gating of the PMT is performed by switching the voltages of the second and third dynodes during the laser pulse. This causes distortion of the first 20 to 30 μs of the signal, diminishing adequate measurement of short lifetimes. A dedicated microchannelplate PMT is therefore a preferred option. An alternative is using a fast shutter in front of a standard PMT, e.g. a pockel cell. An even cheaper alternative is the use of semi-conductor devices like avalanche-photodiodes.

## LEGENDS OF THE FIGURES

**[0087]** FIG. **1**: Schematic example of frequency-domain phosphorimeter. A sinusoidal voltage ($V_{excitation}$) with a frequency of 2000 Hz is generated by a data acquisition board (PCI-MIO-16E1, National Instruments). The light output of the green LED is modulated by $V_{excitation}$ through a voltage-to-current converter. The excitation light is filtered by a 530 broadband bandpass filter (F**1**) and focussed into a liquid light guide (LLG, Oriel) by a lens (L**1**). The emission light returning from the sample is directed to the detector by a dichroic mirror (M). L**2** is a coupling lens and F**2** is a 700 nm bandpass filter. The detector is a red-sensitive photomultiplier tube (PMT, Hamamatsu R928). The current from the PMT passes a current-to-voltage converter and is amplified to generate a signal ($V_{signal}$) that can be sampled by the DAQ-board. The phase-shift between $V_{excitation}$ and $V_{signal}$ is determined by software, for instance written in LabView (such as version 5.1, National Instruments). The phosphorescence lifetime ($\tau$) is calculated from $\Delta\Phi$, allowing the calculation of the oxygen tension ($pO_2$) by the Stern-Volmer relationship, with $\tau_0$ the lifetime under zero-oxygen conditions and $k_q$ the quenching constant.

**[0088]** FIG. **2**: Structure formulas of porphine and protoporphyrin IX.

**[0089]** FIG. **3**: Panel A: The delayed fluorescence at zero oxygen conditions measured in a solution of PpIX bound to albumin. Panel B: The Triplet-Triplet absorption at 470 nm also at zero oxygen, same sample as A. Panel C: Reciprocal lifetime of delayed fluorescence as a function of oxygen concentration at two different temperatures.

**[0090]** FIG. **4**: Jablonski diagram showing, schematically, the energy states and state-transitions of PpIX and its interaction with dioxygen. $S_0$-$S_2$ denote singlet states. $T_0$-$T_2$ denote triplet states. $K_q$ and $k_p$ are the rate-constants of the occurrence of quenching and phosphorescence respectively. In the diagram, parentheses and the broken arrow depict the absence of detectable phosphorescence. $K_{isc}$ is the rate constant of the $T_1 \rightarrow S_1$ intersystem crossing.

**[0091]** FIG. **5**: Emission intensity of PpIX fluorescence as a function of the excitation wavelength.

**[0092]** FIG. **6**: The transient transmission of 20 μM PpIX in 4% albumin as function of the transmission wavelength after pulsed excitation at 505 nm. The maximum at 400 nm is caused by depletion of the ground state by the laser pulse, the minimum at 450 nm is due to population of the $T_1$ level and absorption to the $T_2$ level.

**[0093]** FIG. **7**: Prompt fluorescence emission spectrum of PpIX bound to albumin. Excitation wavelength was 405±2.5 nm. The emission was detected with a 4 nm slit width of the monochromator.

**[0094]** FIG. **8**: Delayed luminescence spectra of PpIX bound to albumin. The emission spectra recorded at varying delays after the excitation flashes are shown. The used gate-width was 100 μs. All spectra are the result of summation of 10 consecutive runs. The spectra shown in **8**A were recorded in an air-equilibrated sample. The spectra in **8**B show the increase in delayed luminescence after deoxygenation of the sample (see text for details).

**[0095]** FIG. **9**: Panel A: The delayed fluorescence at zero oxygen conditions measured in a solution of PpIX bound to albumin. Panel B: The Triplet-Triplet absorption at 470 nm also at zero oxygen, same sample as A.

**[0096]** FIG. **10**: Reciprocal lifetime of delayed fluorescence as a function of oxygen concentration at two different temperatures.

**[0097]** FIG. **11**: Microscopy in ALA incubated neuroblastoma cells. From up to down the image shows phase-contrast wide field, PpIX fluorescence and a combination image. Panel A: 2 hours ALA incubation. Panel B: 4 hours ALA incubation. Panel C: 8 hours ALA incubation.

**[0098]** FIG. **12**: Intramitochondrial oxygen measurement in a cell suspension of neuroblastoma cells. Rotenone is a blocker of the mitochondrial oxygen consumption. After administration of rotenone, the intramitochondrial $PO_2$ is assumed to be the same as the extracellular $PO_2$.

**[0099]** FIG. **13**: Schematic diagram of the experimental set-up. The laser provided pulses of 10 ns with a wavelength of 1064 nm at a repetition rate of 10 Hz. F**1** is a 1064 nm laser line bandpass filter. L**1** and L**2** form a beam-expander resulting in a beam width of approximately 5 mm. Mirror M**1** is a standard optical mirror with a central bore-hole for passing of the laser-beam. Mirror M**2** has an enhanced silver surface. L**3** is a lens with a focal length of 2 cm. This distance of this lens to the sample can be varied in the z-plane (AZ) for adjustment of the measurement depth. Filters F**2** and F**3** are 700 nm bandpass filters. The detector is a red-sensitive photomultiplier tube, the output is fed into a digital oscilloscope.

**[0100]** FIG. **14**: A combination of a prism and a bandpass filter is a cost-effective alternative to a monochromator and provides even better transmission efficiency.

## REFERENCES

**[0101]** Baxter W T, Davidenko J M, Loew L M, Wuskell J P, and Jalife J. Technical features of a CCD video camera system to record cardiac fluorescence data. *Ann Biomed Eng* 25: 713-725, 1997.

**[0102]** Bonnett, R., C. Lambert, E. J. Land, P. A. Scourides, R. S. Sinclair and T. G. Truscott. The triplet and radical species of haematoporphyrin and some of its derivatives. *Photochem Photobiol* 38: 1-8, 1983.

**[0103]** Carlsen H, Moskaug J O, Fromm S H, and Blomhoff R. In vivo imaging of NF-κB activity. *J Immunol* 168: 1441-1446, 2002.

**[0104]** Chantrell, S. J., C. A. Meauliffe, R. W. Munn, A. C. Pratt and E. J. Land. 1977. Excited states of protoporphyrin IX dimethyl ester: reaction of the triplet with carotenoids. *J.C.S. Faraday* 173: 858-864.

**[0105]** Coremans JMCC, Ince C, and Bruining H A. NADH fluorimetry and diffuse reflectance spectroscopy on rat heart. In: *Medical Optical Tomography: Functional Imaging and Monitoring*. Edited by Müller et al. Washington, SPIE—The International Society for Optical Engineering, p. 589-617, 1993.

**[0106]** Coremans J M, Ince C, Bruining H A, and Puppels G J. (Semi-)quantitative analysis of reduced nicotinamide adenine dinucleotide fluorescence images of blood-perfused rat heart. *Biophys J* 72: 1849-1860, 1997.

**[0107]** Dümmler W. Bestimmung von Hämoglobin-Oxygenierung und relativer Hämoglobin-Konzentration in biologische systemen durch auswertung von remissionspektren met hilfe der Kubelka-Munk-theorie. Friedrich-Alexander-Universität, Erlangen-Nürnberg, 1988.

**[0108]** Frank K H, Kessler M, Appelbaum K, and Dummler W. The Erlangen micro-lightguide spectrophotometer EMPHO 1. *Phys Med Biol* 34: 1883-1900, 1989.

**[0109]** Green T J, Wilson D F, Vanderkooi J M, and DeFeo S P. Phosphorimeters for analysis of decay profiles and real time monitoring of exponential decay and oxygen concentrations. *Anal Biochem* 174: 73-79, 1998.

**[0110]** Hogan M C. (1999). Phosphorescence quenching method for measurement of intracellular $pO_2$ in isolated skeletal muscle fibers. *J Appl Physiol* 86(2): 720-724.

**[0111]** Kessler M, Frank K H, Höper J, Tauschek D, and Zündorf J. Reflection Spectrometry. In: *Oxygen Transport to Tissue XIV*. Edited by Erdmann W. and Bruley D. F. New York, Plenum Press, p. 203-212, 1992.

**[0112]** Kubelka P, and Munk F. Ein beitrag zur optik der farbanstriche. *Z. Technische Physik* 11a: 76-77, 1931.

**[0113]** Masters B R. Functional imaging of cells and tissues: NADP(H) and flavoprotein redox imaging. In: *Medical Optical Tomography: Functional Imaging and Monitoring*. Edited by Müller et al. Washington, SPIE—The International Society for Optical Engineering, p. 555-575, 1993.

**[0114]** Mik E G, Donkersloot C, Raat N J H, and Ince C. Excitation pulse deconvolution in luminescence lifetime analysis for oxygen measurements in vivo. *Photochem Photobiol* 76: 12-21, 2002.

**[0115]** Mik E G, Van Leeuwen T G, Raat N J, and Ince, C. Quantitative determination of localized tissue oxygen concentration in vivo by two-photon excitation phosphorescence lifetime measurements. *J Appl Physiol* 97:1962-1969, 2004.

**[0116]** Sato N, Takenobu K, Motoaki S, Kawano S, Abe H, and Hagihara B. Measurement of hemoperfusion and oxygen sufficiency in gastric mucosa in vivo. *Gastroeneterology* 76: 814-819, 1979.

**[0117]** Shonat R D, Wachman E S, Niu W, Korestsky A P, Farkas D L. Near-simultaneous hemoglobin saturation and oxygen tension maps in mouse brain using an AOTF microscope. *Biophys J* 73: 1223-1231, 1997.

**[0118]** Sinclair, R. S., D. Tait and T. G. Truscott. 1980. Triplet states of protoporphyrin IX and protoporphytin IX dimethyl esther. *J.C.S. Faraday I* 76: 417-425.

**[0119]** Siegemund M, van Bommel J, Ince C. (1999). Assessment of regional tissue oxygenation. *Intens Care Med* 25: 1044-1060.

**[0120]** Sinaasappel M, and Ince C. Calibration of Pd-porphyrin phosphorescence for oxygen concentration measurements in vivo. *J Appl Physiol* 81: 2297-2303, 1996.

**[0121]** Sinaasappel M, van Iterson M, and Ince C. Microvascular oxygen pressure in the pig intestine during hemorrhagic shock and resuscitation. *J Physiol* 514: 245-253, 1999.

**[0122]** Sinclair, R. S., D. Tait and T. G. Truscott. 1980. Triplet states of protoporphyrin IX and protoporphytin IX dimethyl esther. *J.C.S. Faraday I* 76: 417-425.

**[0123]** Sterenborg, H. J. C. M., M. E. Janson and M. J. C. van Gemert. A novel frequency domain fluorescence technique for determination of triplet decay times. *Phys Med Biol* 44: 1419-1426, 1999.

**[0124]** Takemura T, Ohta N, Nakajima S, Sakata I. The mechanism of photosensitization in photodynamic therapy: phosphorescence behavior of porphyrin derivatives in saline solution containing human serum albumin. *Photochem Photobiol* 54: 683-688, 1991.

**[0125]** Van Bommel J, Maas M A W, Sinaasappel M, and Ince C. Intestinal microvascular PO2 measurement with Pd-porphyrin phosphorescence in the mechanically ventilated mouse. *Ad. Exp Med Biol* 454: 189-193, 1998.

**[0126]** Vanderkooi J M, Maniara G, Green T J, and Wilson D F. An optical method for measurement of dioxygen concentration based upon quenching of phosphorescence. *J Biol Chem* 262: 5476-5482, 1987a.

**[0127]** Vanderkooi J M, Calhoun D B, Englander S W. On the prevalence or room-temperature protein phosphorescence. *Science* 236: 568-569, 1987b.

**[0128]** Vanderkooi J M, and Berger J W. Excited triplet states used to study biological macromolecules at room temperature. *Biochim Biophys Acta* 976: 1-27, 1989.

**[0129]** Vanderkooi J M, Erecinska M, Silver I A. Oxygen in mammalian tissue: methods of measurement and affinities of various reactions. *Am J Physiol* 260: C1131-50, 1991.

**[0130]** Vinogradov S A, Fernandez-Seara M A, Dupan B W, and Wilson D F. A method for measuring oxygen distributions in tissue using frequency domain phosphorometry. *Comp Biochem Physiol A Mol Integr Physiol* 132: 147-152, 2002.

**[0131]** Van Iterson M, Sinaasappel M, Burhop K, Trouwborst A, and Ince C. Low-volume resuscitation with a hemoglobin-based oxygen carrier after hemorrhage improves gut microvascular oxygenation in swine. *J Lab Clin Med* 132: 421-431, 1998.

**1**. A method for determining a concentration of a substance in a compartment, the method comprising:

exciting an endogenous compound or a functional part, derivative, analogue and/or precursor of said compound, wherein said endogenous compound or functional part, derivative, analogue and/or precursor, if excited, exhibits a luminescence and/or transient absorption of which the lifetime is dependent on said substance,

measuring the lifetime of the luminescence and/or transient absorption exhibited by said compound, functional part, derivative, analogue and/or precursor, and

correlating said lifetime with said concentration.

**2**. The method according to claim **1**, wherein said substance comprises oxygen.

**3**. The method according to claim **1**, wherein said luminescence and/or transient absorption comprises delayed fluorescence and/or triplet-triplet absorption.

**4**. The method according to claim **1**, wherein said compound comprises a compound capable of being excited to a triplet state.

**5**. The method according to claim **1**, wherein said compound comprises a porphyrin.

**6**. The method according to claim **1**, wherein said compound comprises a protoporphyrin.

**7**. The method according to claim **5**, wherein said compound comprises protoporphyrin IX.

**8**. The method according to claim **1**, wherein said compound is photo-excited.

**9**. The method according to claim **1**, wherein said compartment comprises a cell.

**10**. The method according to claim **1**, wherein said compartment comprises an organelle.

**11**. The method according to claim **10**, wherein said organelle comprises a mitochondrion.

**12**. The method according to claim **1**, wherein said compartment comprises at least part of a tissue.

**13**. The method according to claim **1**, wherein said compartment comprises an organ.

**14**. The method according to claim **1**, wherein said compartment comprises a tumor.

**15**. The method according to claim **1**, wherein said compartment comprises the microcirculation.

**16**. The method according to claim **12**, wherein said tissue is present in a culture medium.

**17**. The method according to claim **1**, wherein said compartment comprises a cell suspension.

**18**. The method according to claim **1**, wherein said lifetime is compared with a reference.

**19**. The method according to claim **1**, wherein said lifetime is measured within four hours.

**20**. The method according to claim **1**, wherein said lifetime is measured in the time-domain.

**21**. The method according to claim **1**, wherein said lifetime is measured in the frequency-domain.

**22**. The method according to claim **1**, wherein multi-photon excitation is applied.

**23**. The method according to claim **1**, wherein two-photon excitation is applied.

**24**. A device for determining a concentration of a substance in a compartment, the device comprising:

means for exciting an endogenous compound or a functional part, derivative, analogue and/or precursor thereof, wherein said endogenous compound, part, derivative, analogue and/or precursor, if excited, exhibits a luminescence and/or transient absorption of which the lifetime is dependent on said substance, and

means for measuring said lifetime.

**25**. The device according to claim **24**, comprising a combination of a prism and a bandpass filter.

**26**. The device according to claim **24**, comprising a fast shutter in front of a PMT.

**27**. The device of claim **24**, comprising a semi-conductor device.

**28**. The device of claim **24**, comprising an imaging device capable of oxygen mapping.

**29**.-**31**. (canceled)

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