Title: BACKGROUND SUPPRESSION IN FLUOROMETRY

Abstract: In one aspect, the present invention relates to a direct method for real-time suppression of autofluorescence in time-domain or frequency-domain fluorometry. The method uses a gated detector and the sample is excited by a pulsed train. The detector is gated on following each excitation pulse after a suitable time delay for decay of the prompt autofluorescence.
TITLE OF THE INVENTION

BACKGROUND SUPPRESSION IN FLUOROMETRY

CROSS-REFERENCE TO RELATED APPLICATIONS

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STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND OF THE INVENTION

1. Field of the Invention

In one aspect, the present invention relates to a direct method for real-time suppression of autofluorescence in time-domain or frequency-domain fluorometry. The method uses a gated detector and the sample is excited by a pulsed train. The detector is gated on following each excitation pulse after a suitable time delay for decay of the prompt autofluorescence.

2. Description of the Related Art

A bibliography follows at the end of the Detailed Description of the Invention. The listed references are all incorporated herein by reference.

Fluorescent detection is used throughout the biosciences for numerous applications including clinical chemistry, DNA sequencing, FISH, flow cytometry, high throughput screening and cellular imaging [1-8]. In many instances the sensitivity is limited by interfering autofluorescence from the sample rather than detectability of the emission. This interference can be suppressed with gated detection [9-10] in which the detector is gated off during an excitation pulse, and gated on following a
suitable time delay which allows the scattered light and short-lived autofluorescence to decay. This method is frequently used with the long lifetime lanthanides in the so-called "time-resolved immunoassays" [11].

At present, methods to directly suppress or off-gate the short-lived interference are not available when using the frequency-domain method. In frequency-domain fluorometry, the sample is typically excited with sine wave modulated light and the detector is continuously on, so there is no useful temporal separation of the short and long lifetime emissions. Lack of a procedure for background suppression is disadvantageous because frequency-domain measurements are of current interest for developing simple and robust instruments for on-site measurements of a variety of analytes, blood chemistry and immunoassays. The need for gated detection in frequency-domain fluorometry has been increased by the introduction of long lifetime metal-ligand complexes (MLCs) as luminescence probes. These probes display lifetimes ranging from 20 ns to 10 μs [12-15], which is much longer than the typically ns decay times for autofluorescence. Because of their long lifetimes, these MLC probes can provide high sensitivity detection when used with gated detection.

The present invention provides a method for frequency-domain measurement of the time-resolved intensity decays of long-lived luminophores with real-time suppression of the short-lived interfering autofluorescence. The present method allows recovery of the lifetimes and amplitudes, and is not just a measurement of the integrated intensity of the long lifetime emission which is the basis of the so-called "time-resolved immunoassays" [11]. The intensity decay parameters are of interest because of the possibility of chemical sensing based on the decay times [16-18].

There have been two previous reports of correcting for background fluorescence in frequency-domain fluorometry [19-20]. These methods require a separate measurement of a blank sample displaying background, as well as a measurement of the sample
which also displays the background signal. The data from the sample are then corrected for the background using procedures appropriate for the frequency-domain data [19-20]. This approach allows correction for background signal even when its decay times are comparable to those displayed by the desired sample components. This is a more stringent requirement than is needed for suppression of prompt autofluorescence, and the method is more complex than necessary for suppression of short-lived components.

The present invention provides a frequency-domain method which can be used to directly measure the long-lived decay times with simultaneous suppression of the short decay time components. The method depends on the use of a train of excitation pulses, rather than a sine wave modulated light source. It is known that a pulse train can be used for excitation in frequency-domain fluorometry based on the harmonic content of the pulses [21-24]. As an example, a 1 MHz pulse train with a pulse width near 0.3 ns has useful harmonic content at each integer multiple of 1 MHz up to about 1 GHz [22].

The use of pulse train excitation provides the opportunity to turn off the detector during and immediately after the excitation pulse, at which time the emission contains the short-lived autofluorescence. This opportunity does not seem to be available with sine wave excitation. Methods to suppress single decay time components have been described [25-27], but these methods cannot be used to suppress autofluorescence and still recover the time-resolved parameters.

In frequency-domain fluorometry one compares the phase shift and relative modulation of a sample and reference. The reference is typically a slightly turbid solution which scatters light and provides a measure of the phase and modulation of the incident light. The use of a scattering reference poses a dilemma because gating of the detector will prevent detection of the scattered light, precluding comparison of the sample and reference. If one uses gating on the sample, but not on the reference, then the sample and reference emission are no longer
directly comparable. This comparison is fundamental to the measurements and used in all modern frequency-domain instruments. In the present invention, those difficulties are avoided by the use of a long lived luminophore as the reference. The use of short lifetime reference fluorophores was described previously as a method to correct for the wavelength-dependent time response of photomultiplier tubes (PMTs) [28-29]. If the decay time of the reference is known, the measured phase and modulation of the sample can be corrected to that which would have been observed with scattered light or with a zero decay time reference. However, such ns lifetime standards can not be used with the present background suppression method because their emission will not be detectable at longer times. The present invention utilizes a reference with an adequately long lifetime so that its emission is observable until the PMT is gated on. The sample and reference are observed with the same gated PMT, with the same gating time profile. The PMT is gated on after each excitation pulse, following a delay time suitable for decay of the autofluorescence. We show by simulations that the phase and modulation data can be used directly to recover the intensity decay parameters of the long lived luminescence.

SUMMARY OF THE INVENTION

The present invention relates to a method for determining the presence or concentration of an analyte, comprising the steps of:

a) providing a fluorescent reference molecule and a fluorescent sensing molecule;

b) exposing said sensing molecule to an analyte to form a mixture, wherein said analyte is capable of changing an aspect of the fluorescence emitted by the sensing molecule in a concentration-dependent manner;

c) exposing said reference molecule and said mixture to a pulsed radiation source which causes said reference and sensing molecules to emit fluorescence;

d) measuring an aspect of the fluorescence emitted by said
reference and sensing molecules following a delay time long enough to allow the decay of any autofluorescence from said mixture; and

e) correlating the step (d) measurement with the presence or concentration of said analyte in said mixture.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a simulated time-dependent intensity decay according to eq. 1, $\tau_s = 10$ ns, $\tau_g = 1000$ ns, $\alpha_s^0 = 1000$ and $\alpha_g^0 = 10$. The dashed line shows the gating function, with an on-time of $t_{on} = 100$ ns.

Figure 2 depicts the fractional intensity of the background (B) and sample (S) for various on-gating times ($t_{on}$). The intensity decay law is given by Equation 1.

Figures 3(A)-3(D) show the distortion of the frequency response by increased background. For these simulations, $\tau_B = 10$ ns and $\tau_s = 1000$ ns and $\alpha_s^0$ as shown in each panel. The goal of gated detection is to recover the background-free intensity decay (dashed line).

Figures 4(A)-4(C) show the effect of gated detection on the emission resulting from pulse train excitation. Top, no gated detection; middle, the gating function; bottom, signal seen by the detection with gating. For these simulations the various parameters were as follows: pulse repetition rate 0.07 MHz, $\tau_B = 10$ ns, $\alpha_B^0 = 100$, $\tau_s = 2000$ ns, $\alpha_s^0 = 1$, $\tau_R = 1000$ ns, $\alpha_R^0 = 1$, $t_{on} = 100$ ns, $\Delta t = 100$ ns, $t_{off} = 10,000$ ns, on-off ratio $7 \times 10^4$. In the top and bottom panel the solid lines represent the sample signal and the dashed lines the reference signal.

Figures 5(A)-5(D) show the simulated frequency-domain phase and modulation data with gated detection. The parameter values are the same as on Figure 3(D) except that $\tau_s$ was varied from 500 to 5000 ns. From top to bottom the lifetimes recovered from the least-squares analysis were 492, 996, 1996 and 5022 ns.

Figures 6(A)-6(C) show simulated frequency-domain phase and modulation data. The parameter values are the same as on Figure
3 except for $\tau_s = 5000$ ns and a pulse repetition rate of 0.01 MHz.

Figures 7(A)-7(C) show the effect of incomplete suppression of the background signal due to a large amplitude of $\alpha_b$. The assumed on-off ratio was $7 \times 10^4$.

Figures 8(A)-8(D) show the effect of incomplete background suppression because of an early gating-on time. For these simulations $\alpha_b^0 = 1000$, $\tau_b = 10$ ns, $\alpha_s^0 = 1$, $\tau_s = 1000$ ns, and the gating ratio was $7 \times 10^4$.

Figure 9 shows the dependence of observed fluorescence intensity on the on-gating time $t_{on}$. B refers to a background fluorescence, S to sample, and T to total (B+S) fluorescence. The insert shows the dependence of the S/B ratio on $t_{on}$. For $t_{on} = 180$ ns the signal intensity is $10^6$-fold higher than background.

Figures 10(A)-10(B) show the measurements of multi-exponential intensity decay with gated detection. The intensity decay was assumed to be a double-exponential with parameters $\tau_{s1} = 500$ ns, $\tau_{s2} = 3,000$ ns, $\alpha_{s1} = 0.9$ and $\alpha_{s2} = 0.1$. The background lifetime was $\tau_b = 10$ ns and amplitude $\alpha_b = 100$. The gating parameters were: $t_{on} = 100$ ns, $\Delta t = 10$ ns, $t_{off} = 9,000$ ns, On-Off ratio $7 \times 10^4$ and pulse repetition 0.1 MHz. In the top and bottom panel the solid lines are the sample signal and the dashed lines the reference signals.

Figure 11A shows simulated data for a double exponential decay with (---) and without background (---). Figure 11B shows the analysis of simulated data for a double-exponential decay with background and with gated detection. The decay, background and gating parameters are the same as on Figure 10.

Figure 12 shows the dependence of short component amplitude $\alpha_1$ on gating start time $t_{on}$. The decay, background and gating parameters are the same as on Figures 10 and 11.

Figures 13(A)-13(D) show time-domain anisotropy data. The top panel is the pulses and the second panel is the gating function. The third panel shows the data with insufficient gating when the on time is at 20 ns. The lowest panel shows
that the correct anisotropy decay for the long-lived sample is
obtained with a gating time of 40 ns, which is adequate to
eliminate the background fluorescence. The parameters were:
pulse repetition rate 0.07 MHz; \( \tau_s = 1000 \text{ ns} \); \( \alpha_s = 10 \); \( \tau_b = 10 \text{ ns} \);
\( \alpha_b = 1000 \); \( \theta_s = 1000 \text{ ns} \); \( \theta_b = 10 \text{ ns} \); \( r_b(s) = 0.2 \); \( r_b(B) = 0.2 \); \( \Delta t = 10 \text{ ns} \);
\( t_{off} = 10,000 \text{ ns} \); on-off ratio \( 10^3 \).

Figures 14(A)-14(F) show frequency-domain anisotropy data
for the sample with various gating times. In each case the
solid line shows the values expected for the sample without
background fluorescence. The top panels (A and B) show that the
data are seriously distorted by the presence of a background
component with a 10 ns correlation time. The data are shifted
towards the correct values for the 20 ns on-time. With the 40
ns on-time the data overlapped precisely with the expected
values. These data show that off-gating of the interfering
background allows measurement of the anisotropy decay of the
long lived sample.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention provides a way to
decrease the background or autofluorescence given off by a
sample in the course of assaying for the presence or
concentration of an analyte of interest. The present invention
may be used to detect any analyte for which a suitable
fluorescent sensing probe is available, i.e., a sensing molecule
whose fluorescent emission changes upon exposure to the analyte
in a concentration-dependent manner. Many such molecules are
well-known, and may be used to measure, for example, pH;
saccharides such as glucose, fructose and other cis-diols;
oxygen; blood gases; various electrolytes such as zinc,
potassium, carbonate, sodium, magnesium, etc.; proteins; nucleic
acids; tissue fluorescence, etc.

The fluorescent reference molecule used as a lifetime
reference in the present invention is chosen to have a
sufficiently long fluorescent lifetime so as to allow the decay
of any autofluorescence from the mixture of the sample and the
sensing molecule before the fluorescence of the reference molecule is analyzed. In other words, the lifetime of the reference molecule should generally be longer than the lifetime of the sample autofluorescence. Preferably, the lifetime of the reference molecule is at least about 20 ns, more preferably on the order of about 1 µs to about 1 ms. There are a wide range of suitable reference molecules [12-20], for example those sold by Fluka and other companies. These include transition metal-ligand complexes and lanthanide metal-ligand complexes.

However, the present invention contemplates the use of shorter lifetime reference molecules, on the order of about 2-10 ns, under certain circumstances. For example, such short lifetime reference molecules would be useful when the present invention is used to analyze tissue, as there would be a great deal of scattering off of the tissue. Hence, the emission from even a nanosecond lifetime reference molecule could be observable at the longer times used herein.

The reference molecule may be a distinct entity but having the same structure as the sensing molecule, provided that the reference molecule is not exposed to the analyte, e.g., is isolated in a separate compartment, is embedded in a matrix, or the like.

In practice, the sensing molecule is exposed to a sample which may contain an analyte of interest. The reference and sensing molecules are then exposed to a pulsed radiation source which causes the molecules to emit fluorescence. The choice of the radiation source will depend on a number of factors, such as the fluorescent characteristics of the reference and sensing molecules, the specific application, etc. Preferred sources include lasers, laser diodes, light emitting diodes, arc lamps, flash lamps, electroluminescent devices, sunlight and other light sources.

After a time delay long enough to allow most or all of the background fluorescent emission to decay, the fluorescent emission from the reference and sensing molecules is then analyzed. The time delay may be accomplished simply by
conventional gated detection.

The sensing molecule of the present invention will generally have a detectable quality that changes in a concentration-dependent manner when the macromolecule is exposed to the analyte to be measured. Many such qualities are known and may be used in the present invention. For example, the sensing molecule may include a luminescent (fluorescent or phosphorescent) label, an absorbance based label, etc. The sensing molecule may comprise an energy donor moiety and an energy acceptor moiety, each spaced such that there is a detectable change when the sensing molecule is exposed to the analyte.

Preferably, the detectable quality is a detectable spectral change. Such includes changes in fluorescent decay time (determined by time domain or frequency domain measurement), fluorescent intensity, fluorescent anisotropy or polarization; a spectral shift of the emission spectrum; a change in time-resolved anisotropy decay (determined by time domain or frequency domain measurement), etc.

When the reference and sensing molecules incorporate fluorescent indicator substituents, various detection techniques also are known in the art that can be used. For example, the present invention is applicable to fluorescence lifetime imaging microscopy. In that case, an imaging detector such as an image intensifier and a CCD camera would be gated in a way analogous to a photomultiplier tube.

Gated detection and the use of a reference molecule as described herein huse in time-domain fluorescence. For example, in some cases the scattering or autofluorescence from the sample will be substantial and will dominate the detected signal. This would cause increased data acquisition times with photon counting because most of the observed photons would be from the interfering signal. If the detector were gated on after the interfering signal decayed, then only the useful signal from the sensing molecule would be detected. However, the time-dependent decay would then be observed using a detector or photomultiplier
tube whose signal is increased as the voltage on the detector is
increased. In such an instance, accurate interpretation of the
data would require knowledge of the gating-on function of the
detector. That could be measured using a reference molecule
whose lifetime is long enough to yield useful signals during and
after the gating-on time.

The present invention may be utilized in any environment in
which analytes may be measured. In particular, the present
methods may utilize, e.g., nucleic acid arrays, multi-well
plates, etc.

Prior to describing the theory for frequency-domain
measurements with background suppression it is informative to
have a intuitive understanding of the method. Assume the
intensity decay of the sample following δ-function excitation is
given by a sum of exponential components

\[ I(t) = \sum_i \alpha_i^0 \exp(-t/\tau_i) \]  

(1)

In this expression \( \alpha_i^0 \) are the amplitudes at \( t = 0 \) associated
with each decay component. The superscript 0 is used to stress
the fact that the \( \alpha_i^0 \) value refers to \( t = 0 \). As will be shown
below, gated detection can alter the apparent values of \( \alpha_i \),
particularly when the sample (S) decay is a multi-exponential.

To illustrate the usefulness of background suppression we
assume the intensity decay of the sample displays two decay
times (\( \tau_B \) and \( \tau_S \)) associated with the emission from the
background (B) and from the sample (S). The intensity decay is
thus given by

\[ I(t) = \alpha_B^0 \exp(-t/\tau_B) + \alpha_S^0 \exp(-t/\tau_S) \]  

(2)

The goal of gated detection to measure the sample decay time \( \tau_S \)
without interference from the background component.

The usefulness of gated detection can be seen by
examination of a simulated intensity decay (Figure 1). In this
simulation we assumed that the sample displays two decay times
of $\tau_b = 10$ ns and $\tau_s = 1$ $\mu$s. Following $\delta$-function excitation
the observed intensity decay is given by

$$I_{obs}(t) = 1000 \exp(-t/10 \text{ ns}) + 10 \exp(-t/1000 \text{ ns})$$  \hspace{1cm} (3)

In this expression $\alpha_B^0 = 1000$ is the relative amplitude of the
short lifetime background (B) component with $\tau_B = 10$ ns, and $\alpha_S^0 = 10$
the relative amplitude of the long lifetime sample(s)
component with $\tau_S = 1000$ ns. The experimental goal is to measure
the longer decay time without interference from the short-lived
autofluorescence.

It is important to recall the meaning of the $\alpha_i$ values,
which are amplitudes in the intensity decay. When measuring a
steady state intensity one usually wants to know the fractional
contribution ($f_i$) of each component to the measured intensity.
If the sample is excited continuously and the detector is always
on, then the fractional intensities of the short-lived
background ($f_B$) and long-lived sample ($f_S$) decay components are
given by

$$f_B^0 = \frac{\alpha_B^0 \tau_B}{\alpha_B^0 \tau_B + \alpha_S^0 \tau_S} = 0.5$$  \hspace{1cm} (4)

$$f_S^0 = \frac{\alpha_S^0 \tau_S}{\alpha_B^0 \tau_B + \alpha_S^0 \tau_S} = 0.5$$  \hspace{1cm} (5)

The simulated intensity decay shows that a component of
interest, with a total intensity of 50% of the measure signal,
will have a minor amplitude in the time dependent decay (Figure
1). Stated conversely, the time-zero amplitude of the
background signal can be many-fold greater than the amplitude of
the component of interest.

Now consider that this time-dependent decay is observed
with a gated detector which has an on-off ratio of $10^5$. Such
ratios can be achieved and ratios as large as $7 \times 10^5$ have been
reported [10]. With a standard squirrel-cage PMT the rise time
of the gating on signal is typically 1-5 ns, which we will assume to be instantaneous compared to the longer components in the decay. Suppose the gate is turned on at a delay time $t_{on} = 50$ ns, and that the gating rise time is instantaneous. The fractional steady state intensities of each component are given by the integral by eq. 1 from $t_{on} = 50$ to infinity. Integrating each decay component separately and normalizing by the sum reveals that the fractional amplitudes are now $f_8 = 0.007$ and $f_9 = 0.993$ (Figure 2). The superscript zero has been dropped to reflect the use of gated detection and a change in the apparent $\alpha_i$ and $f_i$ values. Still greater suppression of the background occurs with $t_{on} = 100$ ns, which is the gating function shown as the dashed line in Figure 1. In this case $f_8 = 5 \times 10^{-5}$ and $f_9 = 0.99995$ (Table I). It is clear from these simulations that the amplitude ($\alpha_8$) and fractional intensity ($f_8$) of the background is progressively decreased as the delay time $t_{on}$ is increased. Hence, readily achievable PMT gating results in essentially complete elimination of the scattered light and/or autofluorescence from the sample. If measured in the time-domain the data obtained for times greater than 100 ns can now be analyzed by the usual method of non-linear least squares as applied to time-domain data [30]. The use of gated detection would eliminate the signal due to the short-lived autofluorescence, and result in faster data acquisition of the signal of interest. Hence gated detection can also be valuable for time-domain measurements by removal of the background signal rather than measurement and analysis of data which are corrupted by the background.

It is informative to examine the effects of short-lived autofluorescence on the frequency-domain data measured without gated detection. Figure 3 shows the frequency response expected for a single exponential decay of the sample ($t_s = 1000$ ns) with increasing amplitudes ($\alpha_8^0$) of the 10 ns background signal when measured without gated detection. As the amplitude of the background increases the frequency responses (––) become distorted from the response expected from the sample itself (—–)
In principle one could analyze the frequency-domain data in terms of two decay times, and thereby recover $\tau_s$ as one of the components from the multi-exponential analysis. In practice, the amplitude of the background can exceed that of the sample, making the signal of interest a minor component in the measured signal. Also, the fraction of the signal due to the component of interest contains more Poisson noise due to the higher intensity signal. For these reasons, it is preferable to eliminate the background signal prior to detection.

The gated concept can be applied to frequency-domain fluorometry. Consider a frequency-domain experiment in which the light source is a continuous pulse train (Figure 4). A pulse train is known to be useful for frequency-domain measurements when using the harmonic content method [21-24]. When using such a light source the frequency-domain data can be measured at every integer multiple of the pulse repetition frequency up to a frequency near $(t_p)^{-1}$, where $t_p$ is the pulse width of the incident light. The top panel of Figure 4 shows the signal expected without gated detection. The vertical dotted lines (· · ·) show the signal observed from the usual dilute scattering reference which displays a zero lifetime. The solid lines (---) show the intensity decay of the sample. The sample intensity decay is assumed to display a short-lived signal due to background, as well as a long decay time due to the sample. For these simulations we assumed the sample lifetime of interest was $\tau_s = 2000$ ns. The short-lived component is the vertical region of the decay, and the decay of the sample of interest is the angled region of this line. The dashed line shows the decay of the reference fluorophore with $\tau_r = 1000$ ns. For these simulations we assumed the reference did not display a short lived component, but this assumption is not necessary because gated detection is performed on both the sample and the reference.

When measuring the phase and modulation the sample (---) and reference (-- --) signals are alternatively observed using the same detector. The phase and modulation are typically
measured using cross correlation electronics at the desired
measurement frequency (ω in radians/sec) and one obtains the
same phase and modulation of the emission as if the excitation
source was modulated as a pure sine wave at frequency ω [21-24].
Because the detector is continuously on, one observes the total
emission from the short and long lifetime components, and the
observed phase (ϕ_{obs}) and modulation (m_{obs}) are distorted by the
presence of the short-lived background.

Now assume the detector is gated off during the excitation
pulse, and gated on after the autofluorescence has decayed, at
about 100 ns after the excitation pulse. The gating function
would be a sequence of rectangular gating pulses (Figure 4,
middle panel). In this case the short-lived components do not
contribute to the signal seen by the PMT. However, the gating
function will completely suppress the signal from the scattering
reference, so one cannot measure the phase difference and
modulation of the sample as compared to the scattering
reference. While in principle one could determine in the
arrival time of the pulses by other means, much of the precision
and freedom from artifacts in frequency-domain measurements
originates by comparing the scattering reference and the sample
with the same detector under the same experimental conditions.

The difficulty caused by suppression of the reference
signal by the gating function can be overcome using long
lifetime reference luminophores. Suppose the scattering
solution is replaced by a reference which displays a known
single exponential lifetime (τ_R). The phase and modulation of
the reference, relative to a scattering reference, is given by

$$ \phi_R = \tan^{-1} (\omega \tau_R) $$

$$ m_R = (1 + \omega^2 \tau_R^2)^{-1/2} $$

Suppose the sample is measured relative to this reference rather
than to the scattering solution. Then the observed phase angle
\( \phi_{\text{obs}} \) for the sample is shorter than the true value by \( \phi_R \) [28-29]. The actual phase angle (\( \phi \)) of the sample is given by

\[
\phi = \phi_{\text{obs}} + \phi_R
\]  

(8)

Similarly, the observed modulation of the sample (\( m_{\text{obs}} \)) is larger than the true value by a factor (\( m_R^{-1} \)). The actual modulation (\( m \)) is given by

\[
m = m_{\text{obs}} (1 + \omega^2 \tau_R^2)^{-1/2} = m_{\text{obs}} m_R
\]  

(9)

Correction of the measured phase and modulation values for a reference lifetime is a standard part of most frequency-domain data analysis programs. References with known lifetimes are used to correct for the color-dependent time response of PMTs.

Most fluorophores used as lifetime references have decay times of 1 to 10 ns [28-29]. Hence, except as noted above, these standards cannot be used with this gating method because their emission will have decayed prior to on-gating of the detector. This difficulty can be solved by using longer decay time references luminophores. In particular, the transition metal-ligand complexes display usefully long decay times near 1 \( \mu \)s, and frequently display single exponential decays in fluid solvents. Because of the long decay time the signal will persist after the detector is gated on at \( t = t_{\text{on}} \).

Examination of the bottom panel of Figure 4 reveals a useful result. The detector does not see the arrival time of the light pulse, but only the rise of the photocurrent at \( t = t_{\text{on}} \). Hence, comparing the reference and sample, with the same detector and gating function, yields data comparable to that observed with a reference fluorophore without gating.

Frequency-domain measurements can thus be performed with suppression of autofluorescence by using off-gating at times near the excitation pulse. For a single exponential decay the long lifetime can be obtained using eqs. 8 and 9.

Alternatively, the resulting data can be directly used in currently available frequency-domain software to recover the
multiple decay times without any modification. Minor changes in
the software are needed to recover the true time-zero
amplitudes.

5

THEORY

A detailed description of frequency-domain background
suppression requires expressions which describe the time-
dependent signals. Following δ-function excitation the observed
(obs) time-dependent decay is given by

\[ I_{\text{obs}}(t) = I_B(t) + I_S(t) \]  \hspace{1cm} (10)

where \( I_B(t) \) describes the intensity decay of the background
autofluorescence and \( I_S(t) \) the decay law of the sample in the
absence of autofluorescence. For simplicity with no loss of
generality we assume the autofluorescence decays with a single
decay time \( \tau_B \), and the sample itself displays a multi-exponential
decay. Then

\[ I_{\text{obs}}(t) = \alpha_B \exp(-t/\tau_B) + \sum_i \alpha_i \exp(-t/\tau_i) \]  \hspace{1cm} (11)

were \( \tau_i \) are the background-free decay times and \( \sum \alpha_i = 1.0 \). We
choose to normalize the \( \sum \alpha_i \) to 1.0 because the magnitude of the
short lived component is then easily seen as the excess
amplitude of \( I_{\text{obs}}(t) \) over 1.0.

Analysis of the frequency-domain data is accomplished by
comparing the measured phase (\( \phi_\omega \)) and modulation (\( m_\omega \)) at a given
frequency with those calculated (\( \phi_\text{calc} \) and \( m_\text{calc} \)) for an assumed decay
law \([31-32]\). The calculated values are found from the sine and
cosine transforms of the intensity decay. For on-gating at \( t = t_{\text{ON}} \) and off-gating at \( t = t_{\text{OFF}} \) these transforms are

\[ N_\omega = \frac{1}{2} \int_{t_{\text{ON}}}^{t_{\text{OFF}}} I_{\text{obs}}(t) \sin \omega t \, dt \]  \hspace{1cm} (12)
\[ D_\omega = \frac{1}{J} \int_{t_{ON}}^{t_{OFF}} I_{obs}(t) \cos \omega t \, dt \]  \hfill (13)

\[ J = \int_{t_{ON}}^{t_{OFF}} I_{obs}(t) \, dt \]  \hfill (14)

The calculated phase \( (\phi_{cw}) \) and modulation \( (m_{cw}) \) are given by

\[ \phi_{cw} = N_\omega / D_\omega \]  \hfill (15)

\[ m_{cw} = (N_\omega^2 + D_\omega^2)^{1/2} \]  \hfill (16)

where \( N_\omega, D_\omega \) and \( J \) are evaluated with assumed parameters values in eq. 11. Eqs. 12-14 are comparable to the standard expressions, except for integration from \( t_{ON} \) to \( t_{OFF} \) rather than \( t = 0 \) to infinity as is done without gated detection.

For simulation purposes we calculated the phase and modulation according to eqs. 12-16, for assumed values of \( t_{ON} \) and the parameters in eq. 11 \( (\alpha_b, \tau_b, \alpha_i \text{ and } \tau_i) \). These values were compared with the values expected without gated detection, with no background \( (\alpha_b = 0) \) calculated using eqs. 10-14 with \( t_{ON} = 0 \).

Simulations were performed to determine whether analysis of the data expected with background suppression could be used to recover the expected values of \( \alpha_i \) and \( \tau_i \) from the background-free decay.

The simulations were performed with different assumed decay laws \( (I(t)) \), time delays \( (t_b) \) and shapes of the on-gating function. After a suitable on-time the gate is turned off at \( t = t_{OFF} \). We assumed the shape of the on-gate was given by the error function complement shape, which is an integrated Gaussian. In this case the gating function is given by

\[ g(t) = \frac{1}{G} \left( 1 - \frac{1}{G} \left[ 1 - \frac{1}{2} \text{erfc} \left( \frac{t - t_{ON}}{\Delta t} \right) \right] \left[ \frac{1}{2} \text{erfc} \left( \frac{t - t_{OFF}}{\Delta t} \right) \right] \right) \]  \hfill (17)
where $G$ is the on-off gain ratio. This equation which is inserted into eqs. 12-14 for the integration. The value of $\Delta t$ was varied to simulate on-gating with various rise times. For the long assumed sample decay times the values of $\Delta t$ near 10 to 100 ns gave essentially a rectangular gating function where the signal is only detected between $t_{on}$ or $t_{off}$.

**Multi-Exponential Intensity Decays**

It is straightforward to recover a single decay time from the sample when using gated detection. In this case the recovered decay time is the long decay time of the sample, and the amplitude assumed equal to 1.0. The situation is slightly more complex for a multi-exponential decay. The amplitudes ($\alpha_i$) in a time-dependent decay (eq. 11) represent the amplitudes at $t = 0$. Since the detector is off at $t = 0$, the recovered time-zero amplitudes reflect the values at $t = t_{on}$. Fortunately, it is straightforward to calculate the $\alpha_i$ values at $t = 0$ ($\alpha_i^0$) using the recovered lifetimes and amplitudes. When using gated detection the observed amplitude ($\alpha_i^{obs}$) will be given by the integrated intensity of this component between $t = t_{on}$ and $t = t_{off}$. For a double exponential decay these amplitudes are proportional to

$$\alpha_i^{obs} = k \alpha_i^0 \left[ \exp(-t_{ON}/\tau_i) - \exp(-t_{OFF}/\tau_i) \right]$$  \hspace{1cm} (18)

$$\alpha_2^{obs} = k \alpha_2^0 \left[ \exp(-t_{ON}/\tau_2) - \exp(-t_{OFF}/\tau_2) \right]$$ \hspace{1cm} (19)

where $k$ is the proportionally constant. Hence the ratio of $\alpha_i^0$ to $\alpha_2^0$ can be calculated using

$$\frac{\alpha_i^0}{\alpha_2^0} = \frac{\alpha_i^{obs}}{\alpha_2^{obs}} \left[ \frac{\exp(-t_{ON}/\tau_2) - \exp(-t_{OFF}/\tau_2)}{\exp(-t_{ON}/\tau_1) - \exp(-t_{OFF}/\tau_1)} \right]$$  \hspace{1cm} (20)
normalized values of $\alpha_1^0$ and $\alpha_2^0$ are calculated by recalling $\alpha_1^0 + \alpha_2^0 = 1.0$. It is necessary to develop other expressions for non-exponential decays.

RESULTS

We simulated the frequency-domain data expected with gated detection (Figure 5). For these simulations we chose a pulse repetition rate of 0.07 MHz and the decay law shown in eq. 3, except the long sample decay time ($\tau_s$) was varied from 500 to 5000 ns. The gating on and off times were 100 and 10,000 ns, respectively, and the reference lifetime was $\tau_R = 1000$ ns. The solid lines shown in Figure 5 are the least fits to a single exponential decay. Except for $\tau_s = 5000$ ns (lowest panel), the data are well matched to the single exponential model, and the recovered lifetimes agree with the values assumed for the simulations. These results demonstrate that frequency-domain measurements can be performed with background suppression and that the data are essentially identical to those found in the absence of auto-fluorescence.

Somewhat surprising results were found for $\tau_s = 5000$ ns (Figure 5, bottom panel). The phase angles appear to be “noisy.” By further simulations we found that for decay times very different from the reference lifetime, and comparable to the spacing between the pulses, there were oscillations in the phase and modulation values. This is more clearly seen in Figure 6, which shows the result with $\tau_s = 5000$ ns for a more appropriate range of frequencies and a larger number of data points. The phase and modulation values oscillate around the values expected for a single exponential decay with $\tau_s = 5000$ ns. By variation of the assumed parameters we found that the amplitude and frequency of the oscillation depended on the $\tau_R$, $\tau_s$, $t_{on}$, and $t_{off}$. In practice, this does not seem to be a serious problem. The origin seems to mostly be truncation of the decay at $t = t_{off}$, but the value of $t_{on}$ also has an effect. This effect can be minimized by selecting a pulse repetition rate and values of $t_{on}$ and $t_{off}$ which allow observation of most of the intensity.
decay of the reference and sample. In this case the oscillations are minimal, as seen in the upper three panels of Figure 5.

5 Effect of Incomplete Suppression

In highly scattering samples it is possible that some of the autofluorescence will be observed even with gated detection. Hence we simulated the results expected for incomplete background suppression. If the background amplitude is too large to be suppressed then the frequency-domain data will be distorted (Figure 7). The distortion is typical of the presence of a short-lived component, which is a decreasing phase angle at high frequencies [19]. For these simulations we assumed the on/off gain ratio was $7 \times 10^4$. This value is 10-fold smaller than is readily achievable with gated PMTs [9], so that even high intensity autofluorescence can be suppressed.

The autofluorescence can also distort the frequency-domain data if the gate-on time is too short compared to the decay time of the background (Figure 8). Suppose the autofluorescence decay time is 10 ns. When the on-time is delayed to 150 ns the background signal is not seen in the frequency response. As the on-time is shortened to 100 or 90 ns, the presence of a short lived component is visually evident. In practice the on-time can be adjusted to longer times. It is straightforward to calculate the effect of the gating-on time on an assumed intensity decay. These calculations (Figure 9) show that, for the assumed parameter values, the amplitude of a background with $\tau_b = 10$ ns is not significant for on-times larger than about 70 ns, even for a high intensity background.

30 Multi-Exponential Sample Decays

The situation is somewhat more complex if the sample displays more than a single decay time. In this case the decay times will be accurately recovered, but the time-zero amplitudes $(\alpha_0)$ will be distorted. More specifically, the amplitude of the shorter sample decay time to be attenuated by gating, relative
to the attenuation of the longer decay time components. Following the usual normalization of the amplitudes, the apparent $\alpha_i$ and $f_i$ values of the shorter decay components will be lower than the true time-zero value.

We simulated this effect of gating when measuring a double exponential sample decays with decay times of $\tau_1$ and $\tau_2$. An intuitive presentation is shown in Figure 10. In the absence of gating (top) the sample shows a sharp spike due to the short lifetime autofluorescence. Following this spike the log intensity plot is curved showing the presence of multiple decay times. The lower panel (Figure 10) shows the effect of gating. The spike is removed from the sample decay. Importantly, the log intensity plot is still curved, showing that the data still contains information on the multiple decay times. We further showed that analysis of the simulated phase and modulation data with gating yields the expected decay times and amplitudes (Figure 11).

We questioned how the apparent amplitudes of the multi-exponential sample decay would be affected by gating. Hence we performed simulations with a constant value of $\tau_1 = 1000$ ns. The value of $\tau_2$ was varied from 500 ns to 50 ns (Table II). The detector was gated on at $t_{on} = 100$ ns. The simulated data were then analyzed as usual by non-linear least squares. As the shorter decay time was decreased the recovered amplitude ($\alpha_2$) also decreased below the simulated value of 0.5. For $\tau_2 = 500$ ns the amplitudes are distorted by about 1%. However, for $\tau_2 = 100$ ns the amplitude $\alpha_2$ is decreased to 0.20 (Table II). Hence, correction procedures are needed if the measured decay times are comparable to the gate-on time. In practice, autofluorescence decays in about 5 ns, so gate-on times as long as 100 ns will rarely be needed.

Fortunately, it is relatively easy to correct the distorted amplitudes. The basic idea is to use the recovered decay times and amplitudes to extrapolate back to $t = 0$. This extrapolation is contained in eq. 20. The corrected values $\alpha_2^0$ are listed in Table III. The calculated values of $\alpha_2^0$ are all accurately
recovered except for $\tau_2$ values shorter than the gate-on time (70 and 50 ns in Table II). In these cases the contribution of the short component to the data is not adequate to allow reliable recovery of $\alpha_2^0$.

We also considered a double exponential sample decay with $\tau_1 = 500$ ns and $\tau_2 = 3000$ ns. In this case we varied the gate-on time from 100 to 2000 ns. As the gate-on time become longer the amplitudes of $\alpha_1$ decreased (Figure 11). With gate-on times as long as 2000 ns we reliably recovered the amplitudes of the two decay times (Table III). This suggests the procedure is somewhat robust if at least one of the decay times is longer than the gating time. Alternatively one can measure the intensity decays for several gate-on times, and graphically extrapolate the $\alpha_i$ values to $t = 0$ (Figure 12).

The present invention also involves the suppression of autofluorescence in anisotropy measurements with gated detection. Figure 13 shows time-domain anisotropy data. The top panel is the pulses and the second panel shows the gating function. The third panel shows the data with insufficient gating when the on time is at 20 ns. That is somewhat too short for the assumed background of the interfering signal which had a 10 ns lifetime. The fourth panel shows that the correct anisotropy decay for the long-lived sample is obtained with a gating time of 40 ns, which is adequate to eliminate the background fluorescence. The parameters were as described above.

In Figure 14, frequency-domain anisotropy data for the sample with various gating times are shown. In each panel the solid line shows the values expected for the sample without background fluorescence. The top panels show that the data are seriously distorted by the presence of a background component with a 10 ns correlation time. The data are shifted towards the correct values for the 20 ns on-time. With the 40 ns on-time the data overlapped precisely with the expected values. These data show that off-gating of the interfering background allows measurement of the anisotropy decay of the long lived sample.

-22-
Such an assay would include measurement of the anisotropy decay time, correlation time, differential phase angle, modulation ratio or modulated anisotropy of the fluorescence emitted by the sensing molecule.

DISCUSSION

The data presented herein establish that the present invention provides a viable way to suppress frequency-domain autofluorescence. Construction of the electronics for frequency-domain background suppression as described herein is not difficult. Pulsed laser sources are now routinely used for frequency-domain measurements [22-24], particularly since the recent interest in multi-photon excitation [33-34]. In addition, it is now known that light emitting diodes (LEDs) can be modulated at frequencies in excess of 100 MHz [35-36]. Also, it is known that laser diodes can give pulse widths of 50 ps or less, and LEDs can provide pulse widths less than 2 ns [37]. Hence, frequency-domain background suppression as described herein can be accomplished with simple light sources and electronics.

Frequency-domain background suppression is needed in a variety of analytical and clinical applications of time-resolved fluorescence. For instance, phase-modulation measurements with long lived metal-ligand complexes are being developed for use in resonance energy transfer immunoassays, measurements of blood electrolytes and gases, bioprocess monitoring and in high throughput screening for drug discovery. In all these applications it would be valuable to make use of the high sensitivity of gated detection with the robustness of phase-modulation fluorometry. The present invention is useful in these important applications.
Table I. Effect of Off-Gating on the Fractional Intensities of the Background (B) and Sample (S)*

<table>
<thead>
<tr>
<th>Gating time (ns)</th>
<th>$f_B$</th>
<th>$f_S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>10 ns</td>
<td>0.271</td>
<td>0.729</td>
</tr>
<tr>
<td>25 ns</td>
<td>0.078</td>
<td>0.922</td>
</tr>
<tr>
<td>50 ns</td>
<td>0.007</td>
<td>0.993</td>
</tr>
<tr>
<td>75 ns</td>
<td>0.0006</td>
<td>0.9994</td>
</tr>
<tr>
<td>100 ns</td>
<td>0.00005</td>
<td>0.99995</td>
</tr>
<tr>
<td>200 ns</td>
<td>$2.5 \times 10^{-9}$</td>
<td>-</td>
</tr>
</tbody>
</table>

*The intensity decay parameters are given in eq. 1.
<table>
<thead>
<tr>
<th>Simulated</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \tau_1 ) (ns)</td>
<td>( \alpha_1 )</td>
</tr>
</tbody>
</table>
| 1000 | 0.5 | 500 | 0.5 | 994.7 | 0.509 | 522.4 | 0.491 | 0.487 | 1.2 (10.8)
| 1000 | 0.5 | 300 | 0.5 | 1004.6 | 0.533 | 323.8 | 0.467 | 0.506 | 1.0 (79.6)
| 1000 | 0.5 | 200 | 0.5 | 1002.1 | 0.592 | 220.1 | 0.408 | 0.513 | 1.3 (161.0)
| 1000 | 0.5 | 150 | 0.5 | 1006.6 | 0.632 | 175.1 | 0.368 | 0.529 | 1.5 (183.6)
| 1000 | 0.5 | 100 | 0.5 | 1002.0 | 0.715 | 117.7 | 0.285 | 0.561 | 1.2 (129.1)
| 1000 | 0.5 | 70 | 0.5 | 982.2 | 0.807 | 70.3 | 0.193 | 0.560 | 1.0 (40.0)
| 1000 | 0.5 | 50 | 0.5 | 996.7 | 0.910 | 90.8 | 0.090 | 0.804 | 0.9 (10.2)

\[ a \] For simulations the various parameters were as follows: pulse repetition rate 0.07 MHz, \( \tau_b = 10 \) ns, \( \alpha_0^b = 100 \), \( \tau_a = 1000 \) ns, \( \tau_{on} = 100 \) ns, \( \tau_{off} = 10,000 \) ns. \( \Delta t = 10 \) ns, on-off Ratio \( 7 \times 10^4 \). Background was presented only in the sample. The Gaussian noise in the simulations was \( \delta \phi = 0.3^\circ \) and \( \delta m = 0.007 \).

\[ b \] The values in < > brackets were held fixed at the indicated values during least squares analysis.

\[ c \] The value of amplitude at \( t = 0 \), calculated from eq. 20.

\[ d \] Values for one-exponential fit.
Table III. Recovered decay parameters of a double two-exponential intensity decay with various values of the on-gating time.\(^a\)

<table>
<thead>
<tr>
<th>(t_{\text{on}} ) (ns)</th>
<th>( \tau_1 ) (ns)</th>
<th>( \tau_2 ) (ns)</th>
<th>( \alpha_1 )</th>
<th>( \alpha_1^0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,000</td>
<td>441.1</td>
<td>2994.8</td>
<td>0.234(^b)</td>
<td>0.929(^c)</td>
</tr>
<tr>
<td></td>
<td>&lt;500&gt;</td>
<td>&lt;3000&gt;</td>
<td>0.248</td>
<td>0.893</td>
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<td>1,500</td>
<td>459.2</td>
<td>2943.6</td>
<td>0.390</td>
<td>0.903</td>
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<tr>
<td></td>
<td>&lt;500&gt;</td>
<td>&lt;3000&gt;</td>
<td>0.410</td>
<td>0.886</td>
</tr>
<tr>
<td>1,000</td>
<td>466.8</td>
<td>2770.2</td>
<td>0.585</td>
<td>0.888</td>
</tr>
<tr>
<td></td>
<td>&lt;500&gt;</td>
<td>&lt;3000&gt;</td>
<td>0.619</td>
<td>0.889</td>
</tr>
<tr>
<td>700</td>
<td>465.3</td>
<td>2613.7</td>
<td>0.696</td>
<td>0.883</td>
</tr>
<tr>
<td></td>
<td>&lt;500&gt;</td>
<td>&lt;3000&gt;</td>
<td>0.741</td>
<td>0.896</td>
</tr>
<tr>
<td>500</td>
<td>482.2</td>
<td>2640.8</td>
<td>0.755</td>
<td>0.873</td>
</tr>
<tr>
<td></td>
<td>&lt;500&gt;</td>
<td>&lt;3000&gt;</td>
<td>0.787</td>
<td>0.889</td>
</tr>
<tr>
<td>300</td>
<td>494.9</td>
<td>2724.7</td>
<td>0.833</td>
<td>0.887</td>
</tr>
<tr>
<td></td>
<td>&lt;500&gt;</td>
<td>&lt;3000&gt;</td>
<td>0.849</td>
<td>0.898</td>
</tr>
<tr>
<td>100</td>
<td>501.1</td>
<td>2929.5</td>
<td>0.873</td>
<td>0.885</td>
</tr>
<tr>
<td></td>
<td>&lt;500&gt;</td>
<td>&lt;3000&gt;</td>
<td>0.876</td>
<td>0.888</td>
</tr>
</tbody>
</table>

\(^a\)For these simulations the various parameters were as follows:
pulse repetition rate 0.01 MHz, \(\tau_b = 10\) ns, \(\alpha_b = 100\), \(\tau_R = 1000\) ns, \(\alpha_R = 1\), \(\tau_1 = 500\) ns, \(\alpha_1^0 = 0.9\), \(\tau_2 = 0.1\), \(t_{\text{off}} = 9.000\), \(\Delta t = 10\) ns, On-Off ratio = 7 x 10^4.

\(^b\) \(\alpha_2 = 1-\alpha_1\)

\(^c\) The value of amplitude at \(t = 0\), calculated from eq. 20.
References Cited


Cytometry **11**:126-131.


19. Lakowicz, J. R., Jayaweera, R., Joshi, N., and Gryczynski,


25. Lakowicz, J. R., and Cherek, H., 1981, Phase-sensitive fluorescence spectroscopy. A new mixture to resolve fluorescence lifetimes or emission spectra of components in


37. IBH, Inc., Glasgow, United Kingdom, Nano LED pulsed light source, product literature, 1999.
What is claimed is:

1. A method for determining the presence or concentration of an analyte, comprising the steps of:
   a) providing a fluorescent reference molecule and a fluorescent sensing molecule;
   b) exposing said sensing molecule to an analyte to form a mixture, wherein said analyte is capable of changing an aspect of the fluorescence emitted by the sensing molecule in a concentration-dependent manner;
   c) exposing said reference molecule and said mixture to a pulsed radiation source which causes said reference and sensing molecules to emit fluorescence;
   d) measuring an aspect of the fluorescence emitted by said reference and sensing molecules following a delay time long enough to allow the decay of any autofluorescence from said mixture; and
   e) correlating the step (d) measurement with the presence or concentration of said analyte in said mixture.

2. The method of claim 1, wherein the intensity decay of the fluorescence emitted by the sensing molecule is measured in step (d).

3. The method of claim 1, wherein the decay time of the fluorescence emitted by the sensing molecule is measured in step (d).
4. The method of claim 1, wherein the phase and modulation at a given frequency of the fluorescence emitted by the sensing molecule is measured in step (d).

5. The method of claim 1, wherein the anisotropy decay time of the fluorescence emitted by the sensing molecule is measured in step (d).

6. The method of claim 1, wherein the anisotropy correlation time of the fluorescence emitted by the sensing molecule is measured in step (d).

7. The method of claim 1, wherein the anisotropy differential phase angle of the fluorescence emitted by the sensing molecule is measured in step (d).

8. The method of claim 1, wherein the anisotropy modulation ratio of the fluorescence emitted by the sensing molecule is measured in step (d).

9. The method of claim 1, wherein the modulated anisotropy of the fluorescence emitted by the sensing molecule is measured in step (d).

10. The method of claim 1, wherein the reference molecule has a fluorescent lifetime of at least about 2 ns.
11. The method of claim 10, wherein the reference molecule has a fluorescent lifetime of at least about 20 ns.

12. The method of claim 10, wherein the reference molecule has a fluorescent lifetime of about 1 μs.

13. The method of claim 10, wherein the reference molecule has a fluorescent lifetime of about 1 ms.

14. The method of claim 10, wherein the reference molecule is a metal-ligand complex.

15. The method of claim 14, wherein the reference molecule is a lanthanide metal-ligand complex.

16. The method of claim 1, wherein the analyte is selected from the group consisting of an electrolyte, a saccharide, oxygen, a blood gas, a protein, a nucleic acid, and tissue fluorescence.

17. The method of claim 1, wherein the radiation source is selected from the group consisting of a laser, a laser diode, a light emitting diode, a flash lamp or an electroluminescent device.

18. The method of claim 1, wherein the reference molecule and the sensing molecule are distinct entities having the same
structure, and the reference molecule is isolated from the analyte.

19. The method of claim 1, wherein step (d) utilizes fluorescence lifetime imaging microscopy.

20. The method of claim 1, wherein nucleic acid arrays are utilized.

21. The method of claim 1, wherein multi-well plates are utilized.
FIGURE 3
FIGURE 6

\[ \tau = 4376 \text{ ns} \]
\[ \chi^2_R = 272.3 \]
FIGURE 8
FIGURE 9
FIGURE 11
Simulated Data:

- $\tau_1 = 500\text{ns}, \alpha_1 = 0.9$
- $\tau_2 = 3000\text{ns}, \alpha_2 = 0.1$

FIGURE 12
FIGURE 13
FIGURE 14
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
   IPC(7) : Please See Extra Sheet.
   US CL : Please See Extra Sheet.
   According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
   Minimum documentation searched (classification system followed by classification symbols)
   U.S. : 436/501, 544, 546, 63, 68, 81, 82, 164, 172, 800, 809; 435/7.1, 288.3, 288.4, 288.7, 968
   Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
   Documentation from foreign patent offices:

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>A</td>
<td>US 5,212,099 A (MARCUS) 18 May 1993, abstract.</td>
<td>1-21</td>
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<td>A</td>
<td>US 5,863,401 A (CHEN) 26 January 1999, abstract.</td>
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<tr>
<td>A</td>
<td>US 5,624,847 A (LAKOWICZ et al) 29 April 1997, abstract.</td>
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<tr>
<td>A</td>
<td>US 5,618,732 A (PEASE et al) 8 April 1997, abstract.</td>
<td>1-21</td>
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</table>

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
   "A" document defining the general state of the art which is not considered to be of particular relevance
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   "O" document referring to an oral disclosure, use, exhibition or other means
   "P" document published prior to the international filing date but later than the priority date claimed
   "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
   "X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
   "Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
   "&" document member of the same patent family

Date of the actual completion of the international search: 16 SEPTEMBER 2000
Date of mailing of the international search report: 16 NOV 2000

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   Washington, D.C. 20231
   Facsimile No. (703) 305-3230

Authorized officer
   MONIQUE T. COLE

Telephone No. (703) 306-0661

Form PCT/ISA/210 (second sheet) (July 1998)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):
G01N 33/48, 33/50, 33/20, 21/64, 21/75, 21/76, 33/566, 33/533, 33/53

A. CLASSIFICATION OF SUBJECT MATTER:

US CL.:
436/501, 544, 546, 63, 68, 81, 82, 164, 172, 800, 809; 435/7.1, 288.3, 288.4, 288.7, 968