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(54) Title: AUTOMATIC G-FACTOR CALIBRATION

(57) Abstract: The invention provides a method by which an instrument can automatically measure the absolute value of fluorescense polarization, FP, despite instrumental offsets, with no *a priori* knowledge what the actual FP value should be. The invention specifically provides a method for determining multiple G factors to accurately determine the FP values of each probe in samples containing two or more probes.

Automatic G-Factor Calibration

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention is directed toward biochemical assays, more particularly toward assays using fluorescence polarization detection.

2. Description of the Related Art

Fluorescence polarization (FP) assays are becoming popular, since they are homogeneous and relatively safe, with no radioactive material. A good discussion is provided in the recent review article by John Owicki entitled "Fluorescence Polarization and Anisotropy in High Throughput Screening: Perspectives and Primer", published in the Journal of Biomolecular Screening, Volume 5, No. 5, pp 297-306 (2000).

The technique has at its core the detection of relative intensity of fluorescence emission in two orthogonal states of polarization. Probe molecules are excited with linearly polarized light and, depending on the molecular rotation rate and the excitation lifetime, their fluorescence emission is preferentially polarized along the axis of the excitation beam to a greater or lesser extent. If the molecular rotation time is long compared with the excited-state lifetime, the polarization of the emission is more highly polarized; if the rotation time is short, the emission is more nearly random in polarization. Since chemical binding or other reactions alter the molecular rotation time, they alter the FP value and thus can be detected.

FP is defined by the equation

$$P \equiv [I_{parr} - I_{perp}] / [I_{parr} + I_{perp}] = [I_{parr}/I_{perp} - 1] / [I_{parr}/I_{perp} + 1] \quad [1]$$

There is a related concept termed fluorescence anisotropy (FA), which normalizes according to total fluorescence emission $I = I_{parr} + 2I_{perp}$ and is defined by the equation

$$r \equiv [I_{parr} - I_{perp}] / [I_{parr} + 2I_{perp}] = [I_{parr}/I - 1] / [I_{parr}/I + 2] \quad [2]$$

One can convert between P and r using the equations

$$P = 3r / (2 + r) \quad [3]$$

$$r = 2P / (3 - P) \quad [4]$$

and in general, instrumentation or assays that provide a measurement of P will provide a measurement of r as shown in equations [3] and [4]; and vice versa. Similarly, instrumentation that provides an improved ability to measure one, will also provide an improved ability to measure the other. For simplicity, this teaching refers to FP throughout but is equally applicable to FA.

Most fluorescence polarization (FP) measurement instruments, in particular those designed for use with microtitre plates (e.g., 96, 384 and 1536 well plates manufactured by Greiner, Nunc, Polyfiltronics, Corning), require the use of a "G-

factor" for accurate measurement of degree of polarization. The G-factor is a multiplicative correction made to measured polarization values that compensates for instrumental bias.

A G-factor correction is required because instruments typically do not read both polarization states with equal collection efficiency and/or response. This manifests itself as an FP offset. Typically, G is a multiplicative factor close to 1 applied to I_{perp}, before FP is calculated with the above equation:

$$FP = (I_{parr} - G \cdot I_{perp}) / (I_{parr} + G \cdot I_{perp}) \quad [5]$$

A typical example is with fluorescein, probably the most common fluorophore used in FP measurements. It is well known that fluorescein in an unbound state in solution will have an FP value of about 0.027, or 27 mP, at room temperature. A G-factor determination is carried out by measuring free fluorescein in solution and telling the instrument software that it should be reading 27 mP. The instrumental software then determines what G-factor correction is required to change the measured values to agree with the target value of 27 mP.

This is disadvantageous because it requires a priori knowledge of what the FP value should be, which may or may not be possible in the actual measurement conditions, due to interferences from other compounds in the well, and other factors.

SUMMARY OF THE INVENTION

The invention consists of a method by which an instrument can automatically measure the absolute value of FP despite instrumental offsets, with no *a priori* knowledge what the actual FP values should be. This is achieved through a measurement sequence requiring two measurements in addition to I_{parr} and I_{perp}.

Once this information has been obtained for a given probe and instrument, and the G-factor has been determined in this way, one can measure subsequent FP values accurately using only the conventional readings of I_{parr} and I_{perp}.

The invention provides a method for determining multiple G factors to accurately determine the FP values of each probe in multi-probe FP experiments, even if there is significant spectral cross-talk between the spectral bands used to detect the various probes. There is no need for a priori knowledge of the FP values for the probes involved. And, as in the single-probe case, determination of the G factors requires twice as many measurements as are normally taken, after which one can obtain accurately calibrated FP readings using only the normal set of measurements.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the apparent FP of fluoroscein as calculated using the prior art, and using symmetric calculations, for an instrument with unequal gain in the v and h detector channels; and unequal exposure for v and h excitation, as a function of the differential gain between the v and h detector channels.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

Throughout this discussion, the term probe and label are used interchangeably, to refer to a compound that fluoresces when exposed to excitation light. Band or spectral band are used to describe a range of wavelengths in the visible, ultraviolet, or infrared range.

In the description of the background art, I_{parr} was used to refer to the fluorescence intensity having the same state of polarization as the light used to excite the sample. In general, one may speak of the intensity of a given polarization component of the emission light, when excited using a specified excitation polarization, using the notation I_{xe}, where x refers to the excitation polarization state, and e refers to the emission polarization state. In this discussion, the subscripts h and v are used throughout. The h and v components need not literally be horizontal and vertical, but are the two principal components of polarization for the instrument.

Using this notation, I_{vh} indicates the intensity of horizontally polarized emission, when excited with vertically-polarized light. Conversely, I_{hv} indicates the intensity of vertically polarized emission when excited with horizontally-polarized light. Either would be referred to as I_{perp} in the prior art, such as in Equations [1] or [2]. Similarly, the intensity of h- or v-polarized emission when excited by light of the same polarization state, would be inidcated by I_{hh} and I_{vv}, respectively; and these would be referred to as I_{parr} in the prior art, such as in Equations [1] or [2].

In my co-pending patent application, Serial No. 09/395,661, entitled "Fluorescence Polarization Assay System and Method", dated November 23, 1998, a method and algorithm are presented that measures the absolute FP value, even if the instrument has polarization bias and would require G factors other than 1. Specifically, if one calculates the FP using the equation:

$$P = (I_{hh} - I_{hv} + I_{vv} - I_{vh}) / (I_{hh} + I_{hv} + I_{vv} + I_{vh})$$

[6]

one will obtain a measure of FP that is absolutely accurate despite systematic errors in the instrument. This result is due to the symmetric nature of the measurements, and a complete proof of this is provided in the patent application cited above. Throughout this discussion, such a method of measurement will be referred to as the symmety method, and a calculation of FP derived this way will be termed a symmetric calculation.

The symmetric calculation may include another term that accounts for differences in brightness between those measurements taken under conditions of h excitation, and those taken with v excitation exposure. These differences may arise from lamp drift, or sample aging, or other factors, that occur in the trim between the various measurements. Also, in many instruments that incorporate tilted dichroic elements there can be systematic changes in brightness associated with changing the excitation polarization state. The polarization-dependent behavior of tilted dielectric elements acts to shift the wavelength and/or intensity

of the excitation light that reaches the sample, altering the amount of emission flux that it produces.

One can obtain a perfect measurement of FP, despite such instrumental effects, by using the following equation:

$$P = [I_{hh} - I_{hv} + \gamma(I_{vv} - I_{vh})] / [I_{hh} + I_{hv} + \gamma(I_{vv} + I_{vh})] \quad [7]$$

where $\gamma = [(I_{hh} \cdot I_{hv}) / (I_{vv} \cdot I_{vh})]^{1/2}$. The result is that one can obtain a measurement of FP that is correct and absolutely calibrated, despite instrumental errors that would confound a measurement calculated using the prior-art method of Equation [1].

This is shown in Figure 1, which depicts various calculations of FP based on models using either the FP equation of the prior art, shown as 11; the symmetric equation, shown as 12; or the symmetric equation including the gamma correction factor, shown as 13. The calculations include 0.2% randomly distributed gaussian measurement noise in each measurement of intensity, and a systematic change of 10% brightness between h and v excitations. The curves 11, 12, and 13 show the calculated FP as a function of mismatch between the h- and v- channels that measure emission flux. The actual FP value for fluoroscein, the probe modeled here, is 0.027 at room temperature.

This figure shows that even if the detectors for the h- and v-polarization are very carefully matched to within a few percent, there are large errors when one uses the FP calculation of the prior art. Mismatches of

this magnitude are quite difficult to avoid. Yet typically one would like to know the actual FP to within a few mP, where one mP is 0.001. This is readily achieved by the symmetric equations, which essentially eliminate the effect of instrumental errors. For the highest accuracy, it is best to include the gamma correction factor. But while it is the optimum equation, comparable equations that merely approximate [6] and [7] can yield correction of instrumental errors that are significant, if imperfect; variant equations may therefore be used if the errors introduced are acceptable for the purpose at hand.

The present invention combines the following steps:

1) measurement of all four quantities $\{I_{vv}, I_{vh}, I_{hv}, \text{ and } I_{hh}\}$;

2) generation of an absolutely calibrated FP value despite the presence of various instrumental errors, using the symmetric calculation of equation [6], [7], or equivalents thereto;

3) derivation of a G factor from the experimental readings and the derived FP value;

4) use of the derived G factor to obtain accurate values of FP in subsequent experiments that utilize measurements of the set $\{I_{vv}, I_{vh}\}$ or the set $\{I_{hh}, I_{hv}\}$ but not both.

The calculation of FP using a G factor was described generally in equation [5], and may be written more explicitly as:

$$FP = (I_{hh} - G_h \cdot I_{hv}) / (I_{hh} + G_h \cdot I_{hv}) \quad [8a]$$

or

$$FP = (I_{vv} - G_v \cdot I_{vh}) / (I_{vv} + G_v \cdot I_{vh}) \quad [8b]$$

depending on whether the measurements are to be taken under h- or v-polarized excitation, respectively. Note that different G_x factors are required for the two cases. These may be calculated from the absolutely calibrated FP value using the equations:

$$G_h = [I_{hh} (1 - FP)] / [I_{hv} (1 + FP)] \quad [9a]$$

$$G_v = [I_{vv} (1 - FP)] / [I_{vh} (1 + FP)] \quad [9b]$$

A single symmetric measurement of FP provides all the values necessary to solve [9a] or [9b], from which one may calculate G_h and G_v. Then, depending on whether one wishes to take the subsequent measurements under conditions of v- or h-polarized excitation, one acquires intensity measurements $\{I_{vv}, I_{vh}\}$ or $\{I_{hh}, I_{hv}\}$, then uses Equation [8a] or [8b] to calculate FP.

It is equally possible to derive an equivalent set of factors, termed G' factors, which are used when a given observation set consists of $\{I_{hh}, I_{vh}\}$ or $\{I_{vv}, I_{hv}\}$, that is, when one observes the intensity of emission in a given polarization state, while varying the excitation state of polarization. The invention can be used here in analogous fashion to that just described, by first

performing a symmetric measurement of FP, then solving for G', and finally making subsequent measurements that utilize this factor in connection with $\{I_{hh}, I_{vh}\}$ or $\{I_{vv}, I_{hv}\}$ to calculate accurate values of FP.

It is possible to use this method with an instrument having any design whatever, so long as the instrument is capable of measuring the four intensity values referenced in the symmetric FP equation. Many commercially available instruments provide this capability, including the Analyst (LJL Biosystems, SunnyVale, CA).

Using the present invention, such an instrument could be automated to calculate the determination of G factors so that the user need not key in, or even know, the FP value of the probe being measured. A number of benefits accrue from this.

First, there is the convenience of not having to manually perform G-factor calibration. Second, there is improved data integrity since the prior-art method requires calibrating the instrument by use of a chemical standard. If this sample is not perfectly prepared, all subsequent measurements will be systematically shifted due to miscalibration. The present invention does not derive its calibration from the sample, and does not require that the sample exhibit any particular FP value in order that the method work accurately. Third, the invention is useful in cases where the target FP value is not known. This is especially valuable when developing new probes for which there is not a well-known FP value. Indeed, an instrument incorporating the present invention provides such a value. Fourth, because

the invention generates FP values that are absolutely calibrated, it provides a way to detect changes in sample chemistry that would go undetected using the G factor methods of the prior art. That is, changes that systematically change the chemical standard and the test samples in the same fashion would not be observed if one calibrated to the standard, as taught by the prior art. The present invention provides a way to detect such shifts, which usually indicate an unwanted change in the experimental conditions.

More generally, the invention provides a way to measure FP with the full accuracy and self-calibration of the symmetric method, without the speed penalty that would otherwise be incurred from measuring all four quantites $\{I_{vv}, I_{vh}, I_{hv}, \text{and } I_{hh}\}$. After the symmetric measurement is performed once and the appropriate G-factor is derived, the instrument can be used with that probe in a conventional manner requiring two readings per sample, instead of four. This doubles the plate reading speed for subsequent measurements, compared with a full symmetric approach.

The invention for automatic G-factor calculation can also be applied when measuring more than one probe per sample. The topic of multi-probe fluorescence polarization measurements is addressed in my co-pending application "Multi-label fluorescence polarization assay system and method", filed the same day as the present application, the contents of which are hereby included in full and made a part of this application.

In general, one needs a total of 4N pieces of data to make a symmetric calculation of FP for N probes. These data comprise the various combinations of excitation polarization state, emission polarization state, and spectral band. These measurements are the raw data from which one calculates the FP for each probe. However, if one were to take the values obtained at the spectral band corresponding principally to a given probe, and plug them into the FP equations of the prior art, one would not obtain the desired result, namely an accurate value of FP for that probe. Yet when one uses the symmetric calculation together with the methods taught in "Multi-label fluorescence polarization assay system and method", the multi-probe measurement is inherently self-calibrating, with no need for G factors or *a priori* knowledge about the FP properties of the probes being measured.

In practicing the present invention with multiple-probe samples, one takes a single full dataset comprising 4N pieces of data, from which an instrumental calibration is derived using the symmetric multi-label calculation; G factors are developed for each probe; subsequent readings are taken with a smaller set of 2N pieces of data and processed to yield accurately calibrated values of FP using the G factors for each probe. The process is entirely analogous to the method described above for single-label experiments.

This first requires one to determine the instrumental response matrix. This matrix, also called the cross-talk matrix, describes the degree to which a given probe is detected when the instrument is seeking to measure each of the different probes in turn.

Mathematically, one writes the instrument's response to probe k when set to read probe j as a_{jk}, and one can write the instrumental response function for all probes and instrumental settings as a matrix **A** populated with elements a_{jk}. In such a matrix, the diagonal members represent the response of the instrument to the target probe, while off-diagonal members represent cross-talk. For this reason, the **A** matrix is also called the cross-talk matrix for the probes and instrument involved. The degree of isolation between flux from different probes is never perfect due to instrumental limitations and the spectral response of the probes. In the usual case where probes have partially overlapping spectra there can be moderate to severe spectral cross-talk.

The matrix **A** is typically measured using control samples that contain pure samples, i.e. only a single probe each. One may use these to characterize a set of four matrices $\{\mathbf{A}_{hh}, \mathbf{A}_{hv}, \mathbf{A}_{vh}, \mathbf{A}_{vv}\}$ for all possible excitation and emission polarization states, for instruments whose response is known to vary as a function of polarization. This can be caused by factors such as polarization-dependent transmission in dichroic mirror elements, which shifts the spectral response and thus alters the contents of matrix **A**. The set of matrices may then be used to derive accurate values of the probe contributions, and thus of FP, despite polarization-dependent cross-talk in the overall assay.

The method for measuring A is now described in detail. If we denote a given measurement in terms of the excitation polarization, the emission polarization, and the spectral band selection as m_{xeb}, a symmetric dual-label measurement requires that one acquire the set

$\{m_{vv1}, m_{vh1}, m_{vv2}, m_{vh2}, m_{hv1}, m_{hh1}, m_{hv2}, m_{hh2}\}$. Note that the third subscript on m indicates the spectral band b, not the target probe p.

One can model the reading m_{xeb} as

$$m_{xeb} = F \cdot (s_{xe1} \cdot a_{xeb1} + s_{xe2} \cdot a_{xeb2}) \quad [10]$$

where:

a_{jkxe} is the instrumental responsivity in band j to flux from probe k for excitation polarization x and emission polarization e;

F is an exposure correction factor which reflects the relative amount of excitation flux for (and integration time, if the measurement is an integrating type), for that measurement compared to an nominal value;

s_{xep} indicate the flux produced under the nominal excitation flux, in excitation polarization state x into emission polarization state e, by sample probe p.

To determine the contents of **A**, one typically takes measurements of control samples which have only a single probe species. From measurements of the same sample in each of the spectral bands, one obtains a_{xebp} for all bands; i.e. one obtains a row in of **A** for that set of excitation and emission states. By repeating for samples that have single pure species of each probe, one obtains all rows in **A**. This normalizes the analysis so unit values of **S** correspond to the fluxes produced by the control samples.

The process is then repeated for all remaining combinations of excitation and emission polarization states, to obtain the full set $\{\mathbf{A}_{hh}, \mathbf{A}_{hv}, \mathbf{A}_{vh}, \mathbf{A}_{vv}\}$.

It is important that the **A** matrices be precisely determined. While individual measurements of any type contain noise, one can virtually eliminate the effect of exposure fluctuations, or other random sources of error, in estimating **A**, by repeating this measurement several times. For example, lamp flicker will introduce a random noise through the F factor. This can be eliminated by measurement averaging, which is not unwieldy since the determination of **A** is undertaken once for a given combination of probes on a given instrument.

Turning to the calculation of FP, one first rewrites the equivalent of [1] in terms of s_{xep} in order to accommodate the formalism of a multi-label experiment:

$$FP_p = (s_{hhp} - G_{hp}s_{hvp}) / (s_{hhp} + G_{hp}s_{hvp}) \quad [11a]$$

or equivalently

$$FP_p = (s_{vvp} - G_{vp}s_{vhp}) / (s_{vvp} + G_{vp}s_{vhp}) \quad [11b]$$

where $G_{hp}$and G_{vp}denote the G factors for probe p when measuring under conditions of h- and v-polarized light, respectively.

The symmetric equation of FP for a given probe is

$$FP = (s_{hhp} - s_{hvp}+ s_{vvp} - s_{vhp}) / (s_{hhp} - s_{hvp}+ s_{vvp} - s_{vhp}) \quad [12]$$

and the symmetric calculation may further incorporate a correction for changes in exposure or excitation flux between the h- and v-excited flux measurements, as

$$FP = [s_{hhp} - s_{hvp} + \gamma_p(s_{vvp} - s_{vhp})] / [s_{hhp} - s_{hvp} + \gamma_p(s_{vvp} - s_{vhp})] \quad [13]$$

where $\gamma_p \equiv [(s_{hhp} \cdot s_{hv}) / (s_{vvp} \cdot s_{vhp})]^{1/2}$. As these equations make clear, the contribution to intensity from each probe species s_{xep} is analogous to the intensity I_{xe} in a single-label experiment.

One needs to determine s_{xep} from the measurements m_{xeb} in order to derive either a symmetric FP or simple FP measure. As equation [10] shows, the reading m obtained in a given band is the sum of signals from the probe p primarily associated with band b, along with signals from the other probe(s), weighted according to coefficients a_{jkxe}. While this equation shows a two-label case, extension to more probes is straightforward.

To achieve this, one may write the measurements as a vector **M** with members m_b and the flux as vector **S** with members s_p. By inverting **A**, one can calculate the value of s_{xep} from the associated m_{xeb} as

$$\mathbf{S} = \mathbf{A}^{-1}\,\mathbf{M} \quad [14]$$

Fundamentally, equation [14] describes a process which converts data about intensity levels in various spectral bands, into data about various probes. This operation yields data for each probe that is independent of the presence of the other probes, within one's measurement error and errors in derivation of the

A matrices. This separates the coupled multi-probe measurements m_{xeb} into independent probe measurements s_{xep}. From that point forward, the s_{xep} values for each probe may be treated like the I_{xe} values obtained in a single-probe experiment.

Summarizing, the procedure for determining G factors in multi-probe experiments is the following:

a) obtain values for **A** for a given set of excitation and emission states using control samples of individual pure species as described above;

b) repeat step 1) for all combinations of excitation and emission states used, storing the various matrices as $\{\mathbf{A}_{xe}, \ldots\}$, and inverting to yield $\{\mathbf{A}^{-1}_{xe}, \ldots\}$

c) using data from the pure samples just obtained or from multi-label samples, obtain values for **M** for all combinations of probes, and excitation and emission states;

d) calculate **S** from equation [14] for each combination of excitation and emission states used;

e) calculate FP for each probe from the **S** values using a symmetric calculation such as equation [12], [13], or an equivalent;

f) determine the G_{xp}values for each probe, for a chosen excitation state x.

Once the G factors are determined, one may obtain subsequent FP as follows:

g) obtain values for **M** for all bands and excitation states of polarization, for a given emission state of polarization x;

h) calculate **S** from equation [14] using the appropriate $\mathbf{A}_{xe}$ matrix;

i) determine FP from equation [11a] or [11b], depending on the excitation state of polarization.

As in the single-probe case, it is possible to derive an analogous set of G' factors which apply when one measures the s_{xep} values at a given emission state of polarization, for both excitation states of polarization.

The techniques and methods herein described may be used separately, or in combination with one another and with techniques known in the prior art of instrument design and fluorescence polarization measurement.

Thus, while there have been shown and described and pointed out fundamental novel features of the invention as applied to preferred embodiments thereof, it will be understood that various omissions and substitutions and changes in the form and details of the devices illustrated, and in their operation, may be made by those skilled in the art without departing from the spirit of the invention. For example, it is expressly intended that all combinations of those elements and/or method steps which perform substantially

the same function in substantially the same way to achieve the same or substantially the same results are within the scope of the invention. Substitutions of elements from one described embodiment to another are also fully intended and contemplated. It is also to be understood that the drawings are not necessarily drawn to scale but that they are merely conceptual in nature. It is the intention, therefore, to be limited only as indicated by the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. A method of determining a G-factor correction to the measured fluorescence polarization of a first sample which contains at least a first probe comprising the following steps:

illuminating the sample to effect fluorescence emission from the first probe with a beam of excitation light that is linearly polarized along a first axis;

measuring the intensities of a first component of the fluorescence emission that is polarized along the first axis and a second component of the fluorescence emission that is polarized orthogonal to the first axis while the sample is illuminated with the beam of excitation light to obtain a measured fluorescence polarization of the first probe;

switching the state of polarization of the beam of excitation light to a polarization state wherein said beam is linearly polarized along a second axis substantially orthogonal to the first axis;

measuring the intensities of a third component of fluorescence emission that is polarized along the first axis and a fourth component that is polarized orthogonal to the first axis while the sample is

illuminated with the beam of excitation light that is linearly polarized along the second axis; and

calculating the absolute fluorescence polarization of the first probe based on the measurements of the intensities of the first, second, third and fourth components; and

calculating the G-factor correction for the first probe from the absolute fluorescence polarization and the measured fluorescence polarization.

2. The method of claim 1 wherein the step of calculating the absolute fluorescence polarization of the first probe includes a correction for changes in exposure flux between the measurements of the intensities of the first and second components.

3. The method of claim 1 wherein the step of calculating the absolute fluorescence polarization of the first probe includes a correction for changes in excitation flux between the measurements of the intensities of the first and second components.

4. The method of claim 1 further including the steps of obtaining the measured fluorescence polarization of the first probe for additional samples; and

calculating the absolute fluorescence polarizations of the first probe for the additional samples from the G-factor correction for the first probe and the measured fluorescence polarizations of the first probe for the additional samples.

5. The method of claim 4 wherein the step of calculating the absolute fluorescence polarization of the additional samples includes a correction for changes in exposure flux between the measurements of the intensities of the first and second components.

6. The method of claim 1 wherein the step of calculating the absolute fluorescence polarization of the additional samples includes a correction for changes in excitation flux between the measurements of the intensities of the first and second components.

7. The method of claim 1 wherein the sample contains two or more probes which exhibit fluorescence polarizations further comprising the steps of:

illuminating the sample to effect fluorescence emission from a second probe with a beam of excitation light that is linearly polarized along a third axis;

measuring the intensities of a first component of the fluorescence emission from the second probe that is polarized along the third axis and a second component

of the fluorescence emission that is polarized orthogonal to the third axis while the sample is illuminated with the beam of excitation light to obtain a measured fluorescence polarization of the second probe;

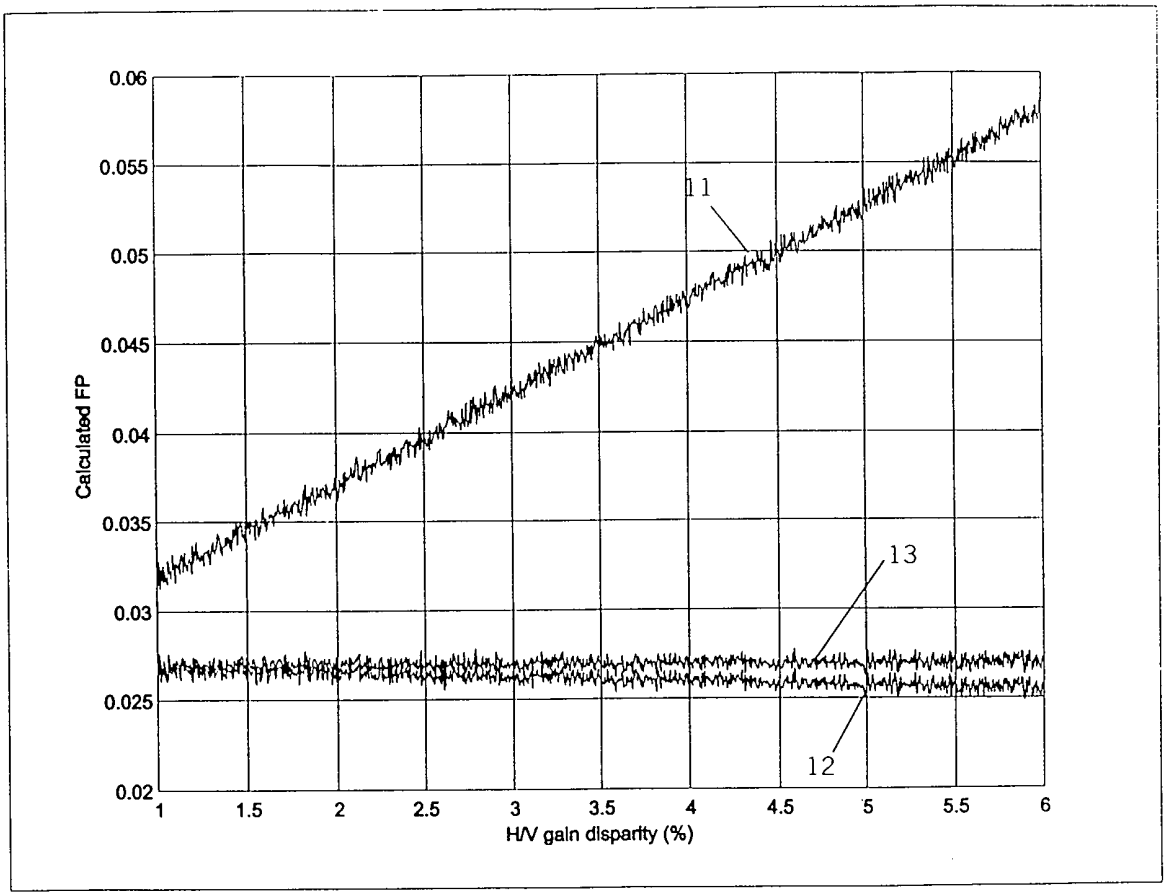
switching the state of polarization of the beam of excitation light to a polarization state wherein said excitation beam is linearly polarized along a fourth axis substantially orthogonal to the third axis;

measuring the intensities of a third component of fluorescence emission that is polarized along the third axis and a fourth component of fluorescence emission that is polarized orthogonal to the third axis while the sample is illuminated with the excitation beam linearly polarized along the fourth axis; and

calculating the absolute fluorescence polarization of the second probe based on the measurements of the intensities of the first, second, third and fourth components of fluorescence emission; and

calculating the G-factor correction for the second probe from the absolute fluorescence polarization and the measured fluorescence polarization of the second probe.

8. The method of claim 7 wherein the third axis is the same as the first axis, and the fourth axis is the same as the second axis.


0.06
0.055
0.05
0.045
0.04
0.035
0.03
0.025
0.02
Calculated FP
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
H/V gain disparity (%)
11
13
12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06227

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 21/64
US CL : 250/458.1

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. : 250/458.1, 459.1, 461.1, 461.2

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,025,917 A (TOYONAGA et al.) 15 February 2000 (15.02.00), column 2, lines 38-67.	1-8

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

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Constantine Hannaher Telephone No. (703) 308-4850

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Continuation of B. FIELDS SEARCHED Item 3:
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