Compensation method for quantitative observation of multicolor fluorescence with nonlinear mapping

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ABSTRACT

In the field of biology, compensation of multiple fluorescence is necessary for quantitative measurement of samples. Conventional methods depend on a linear combination model irrespective of the model's adequacy. Therefore, proper compensation has not been performed in some situations. To overcome this problem, we propose a method for performing nonlinear mapping with emphasis on the distribution of samples on a plane of which the base vectors are references of fluorescence. This paper describes an experiment with measurement of multiple fluorescence and compensation using a conventional method and our proposed method. Results show that multiple fluorescence is not always able to assume a linear combination model. Moreover, we confirmed that the presented method is independent of linearity of multiple fluorescence.

Keywords: fluorescent dyes, emission spectrum, multicolor fluorescence compensation, nonlinear transformation

1. INTRODUCTION

In the field of biology, fluorophores are widely used for protein labeling. A fluorophore yields fluorescence with longer wavelength than the exciting light. Therefore, it is easy to observe only the labeled proteins using optical filters. Moreover, to observe two or more targets simultaneously for purposes such as knowing the mutual interaction, multiple fluorescent staining can be used. Using fluorophores that yield different fluorescence enables an observer to view and distinguish multiple targets simultaneously.

The problem of crosstalk is not avoided with optical filters because fluorescence is broadening widely for the frequency domain. In general, to solve this problem, it is compensated with a method based on a linear combination model of fluorescence spectra. However, correct compensation is not likely to be done using the conventional method, which assumes linearity, because multiple fluorescence has negligible nonlinearity.

We describe an example with an actual measurement value. We prepared FITC and Alexa-488 as commonly used fluorochromes for this measurement. We made a water solution of these fluorochromes and measured their fluorescence spectra using a fluorescence spectrophotometer. The light wavelength used for excitement was 488 [nm]. Figure 1 presents the results. The spectra of FITC and Alexa-488 are drawn respectively as dotted lines and dashed lines. The ideal curve as the summation of two spectra is drawn as a chain line; the spectrum of the actually mixed sample is drawn as a solid line. The observed spectrum is expected to fit the ideal curve if the spectra of multiple fluorescence are based on a linear combination model. Nevertheless, fluorescence with stronger intensity than the ideal curve was observed at each wavelength. In such cases, it is not compensated correctly using the conventional method.

Although there is such a problem, conventional compensation methods, which are based on a linear combination model, have been used for a long time[1-7] . Even if an increase or decrease is caused by nonlinearity of fluorescence, it is available for qualitative observation such as measurement of marker existence.

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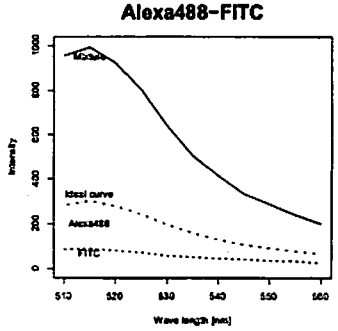

Figure 1. Discrimination ratio for the ratio of approach to destination

However, by virtue of studies of genome analysis and live cell imaging, the information that can be obtained from biological samples has increased. Quantitative measurement of multiple fluorescence has been necessary to process the information using computers. Nevertheless, not only data analysis, simulating the behavior of biological systems on computers using an appropriate model can be considered if quantitative measurements are used.

Quantitative measurement is necessary for setting model parameters. For the reason given above, we intend to construct a method that can carry out quantitative measurement of stained samples with multiple fluorochromes. First, we produced a water solution of FITC, Alexa-488, Alexa-532 and their mixtures, and measured their fluorescence spectra. Then we confirmed linearity of multiple fluorescence related to the concentration of each fluorochrome. Next, we propose a method to estimate the concentration of each reagent using nonlinear mapping. Finally, to verify the availability of our method, we compare the results to those obtained using the conventional method.

2. CONVENTIONAL METHOD

2.1 Linear Unmixing

Linear unmixing is a method used to estimate the contribution of each component dye from spectra. The scanning wavelength is the illumination wavelength or the observation wavelength; the other is fixed. Let λ be the scanning wavelength, L be the number of these values, and $\boldsymbol{x}$ be the L-dimension column vector of observations at each wavelength. Let $\boldsymbol{s}_i$ $(i = 1, \cdots, N)$ be the i-th dye's fluorescence spectra as L-dimension column vectors. As the reference spectra, these values are determined by fluorescence of singly stained samples. The reference matrix is represented as the following.

$$S = (\boldsymbol{s}_1, \cdots, \boldsymbol{s}_n).$$

In the framework of the linear unmixing, $\boldsymbol{x}$ is assumed that it is linear combination of S and $\boldsymbol{y} = (y_1, \cdots, y_n)^{\mathrm{T}}$ as follows.

$$\boldsymbol{x} = S\boldsymbol{y}.,$$

In that equation, y_i is the relative intensity of the i-th dye to the reference spectrum $\boldsymbol{s}_i$ and $^{\mathrm{T}}$ denotes the transpose of a vector or a matrix.

In addition, $\boldsymbol{y}$ is calculated as

$$\boldsymbol{y} = S^{-1}\boldsymbol{x}, \tag{1}$$

where S^{-1} is the inverse matrix or the general inverse of S.

When L is equal to N, S is a square matrix; it is easy to solve the inverse matrix. Even when L is not equal to N, S^{-1} is solved as the general inverse with a singular value decomposition (SVD).[8]

For compensation with a linear unmixing, $\boldsymbol{s}_i$ need not be mutually orthogonal, as long as the variables are linearly independent. This method is based on a linear combination model of spectra. In other words, it is assumed that fluorescence spectra are mutually independent.

2.2 Adequacy of a linear combination model

According to the model described above, sample points should distribute on a plane of which the base vectors are s_i. Figure 2 shows an example of the distribution of sample points on such a plane. The horizontal axis and the vertical axis respectively denote elements of the base vector s_1 and s_2; each point represents a sample point. The points are distributed like a rectangle because the elements of s_1 and s_2 are independent of each other. In such a situation, each element exactly represents the intensity of each dye's fluorescence. For that reason, compensation based on a linear combination model is successful.

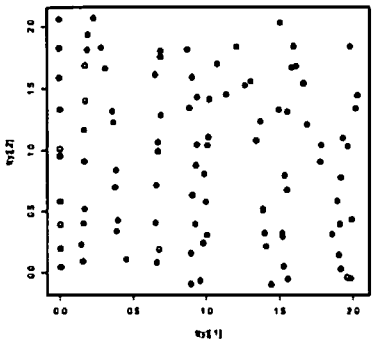

Figure 2. Example of distribution of sample points when the model based on a linear combination model is valid

On the other hand, Figure 3 shows an example of the distribution of sample points when the model based on a linear combination model is not valid. A strong correlation exists between elements of s_1 and s_2; each element does not show a correct intensity of each dye's fluorescence. Linear unmixing is not available in such a situation. In the next section, we propose a method to avoid this problem.

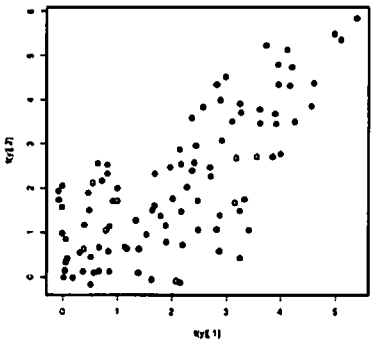

Figure 3. An example of distribution of sample points when the model based on a linear combination model is not valid

3. PROPOSED METHOD

3.1 Overview

It can be considered that the distribution of sample points like that shown in Fig. 3 is a stretched one resembling that shown in Fig. 2. Then, to transform it to a rectangle form, we compensate this turn of the distribution of sample points.

For purposes of illustration, we assume that the number of used dyes N is 2. The sample points are expected to be distributed on the edge of rectangle including the original point when the concentration of a dye or another one is 0. Then, s_1 and s_2 respectively correspond to these edges. Moreover, the rectangle is stretched for the opposing corner of the original point; the sample points for which both dyes' fluorescence are strong and distributed near the corner. We let $m = (m_1, m_2)^{\mathrm{T}}$ be the vector indicating such a point. This is defined similarly to the definition of s_1 and s_2, for example, a preliminary measurement and so on.

The transformation is not linear. Therefore, the transformation must be considered for each dye. Let $p = (p_1, p_2)^{\mathrm{T}}$ be a sample point of a rectangular distribution. We consider a transformation for the first dye as

$$f_1(p) = -p_2\{p_1(m - s_2) + s_2 - p_1 s_1\} + p_1 s_1. \tag{2}$$

Then, let $f_1^{-1}(p')$ the inverse function of $f_1(p)$, the compensated elements of s_1 for a sample point x are achieved to calculate $f_1^{-1}(x)$. For the second dye, we can achieve a transformation to interchange the suffix of formula (??).

3.2 Calculation of prediction error

To validate the model adequacy, we calculate the prediction error with leave-one-out cross validation.[9] Leave-one-out cross validation is one method for estimating generalization error based on "resampling" Cross validation is useful when training data are few.

Let n be the number of samples. In leave-one-out cross validation, $n - 1$ samples are used for regression analysis, and the remaining sample is used to estimate the generalization error. The Prediction Error Sum of Squares (PRESS) is calculated as

$$\mathrm{PRESS} = \sum_{k=1}^{n} (\hat{d}_{ik} - \delta_{ik}), \tag{3}$$

where $\hat{d}_{ik}$ is the predicted δ_{ik} with the data except the k-th value. Let Q^2 be the index of predictive accuracy, calculated as

$$Q^2 = 1 - \frac{\mathrm{PRESS}}{\sum_{k=1}^{n} (\delta_{ik} - \bar{\delta}_i)}, \tag{4}$$

where $\bar{\delta}_i$ is mean of δ_{ik}.

If the estimated values match the actual measurements completely, PRESS is 0, and Q^2 will be 1. Therefore, to the degree that parameters are better estimated, they will be closer to 1 Q^2.

4. EXPERIMENTAL RESULTS

To verify the usability of the proposed method, we performed some experiments applying the method with compensation of spectra observed from a water solution of fluorescent dyes.

4.1 Experimental Setup

4.1.1 Equipment

We constructed a fully automatic measurement system using a fluorescence spectrophotometer (F-4500; Hitachi Ltd.), a customized auto sampler (AS-3000; Hitachi Ltd.), and a custom-built computer for controlling the equipment and for data acquisition. To prevent bleaching of dyes, the storage of the auto sampler is light-shielded.

The system structure is depicted in Fig. 4.

Samples are prepared automatically using the auto sampler. Each sample's spectral fluorescence is measured using the following procedure.

1. A sample is run into a flow cell in the fluorescence spectrophotometer.
2. The auto-sampler transmits a trigger signal to the fluorescence spectrophotometer.

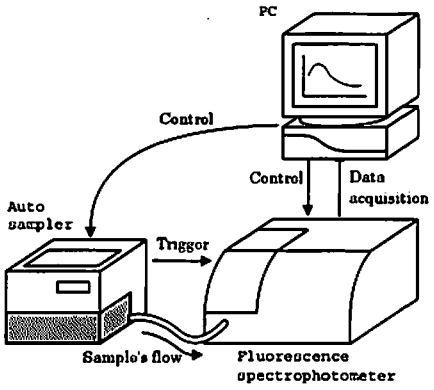

Figure 4. System structure

3. The fluorescence spectrophotometer receives the signal, and then starts measurement.
4. The system flow path of is washed with 99.5% ethanol and rinsed with pure water after the measurement.

This series of procedures is repeated for each prepared sample.

4.1.2 Reagent Solution

We used Alexa 488, Alexa 532, and Fluorescein (FITC). As solvent, we used PBS buffer for the Alexa series, and DMSO for FITC. These solutions were diluted with pure water to the specific density described in Table 1. We used two combinations of set with these three dyes, and measured 100 samples, which are combinations of 10 levels of concentration for each dye. Changing the volume ratio of the solutions, we achieved different partial concentration of samples. The specific volume ratio is also presented in Table 1.

Table 1. Each dye's concentration

Reagent	Concentration	Volume ratio
Alexa-488	20μg/ml	0/20, 1/20, 2/20, 3/20,
Alexa-532	20μg/ml	4/20, 5/20, 6/20, 7/20,
FITC	1μg/ml	8/20, 9/20

4.1.3 Measuring condition

The measuring conditions of the fluorescence spectrophotometer are presented in Table 2.

We interpolated the observed data using spline interpolation. Then we trimmed them to the range presented in the Table 2. We consider the trimmed data as the spectra observed with a single-wavelength light source.

4.2 Verifying linearity

In this section, we verify the spectrum linearity observed from single samples and mixture samples.

4.2.1 Single samples

Figure 5 shows the observed spectrum of each dye at every level of concentration. Particularly, the scale of the ordinate in the graph of Alexa532 is larger than other ones. Because 488 nm of the light source wavelength is not appropriate for Alexa532, its fluorescence is weak. When a single dye is used, fluorescence is linearly related to the dye concentration, but the influence of noise affects the results. Especially, for Alexa532, the noise is comparatively strong because its fluorescence is weak.

Figure 6 shows result of linear regression.

Table 2. Measuring conditions

Parameter	Value
Light source	150 W xenon lamp
Bandpass	Ex: 5.0 nm, Em: 5.0 nm
Measured wavelength range	Ex: 300 to 600 nm, Em: 400 to 700 nm
Trimmed range for analysis	Ex: 488 nm, Em: 510 to 560 nm
Wavelength step size	Ex: 5.0 nm, Em: 5.0 nm
Wavelength scan speed	30,000 nm/min
The voltage of the photomultiplier tube	700 V
Response	0.01 s

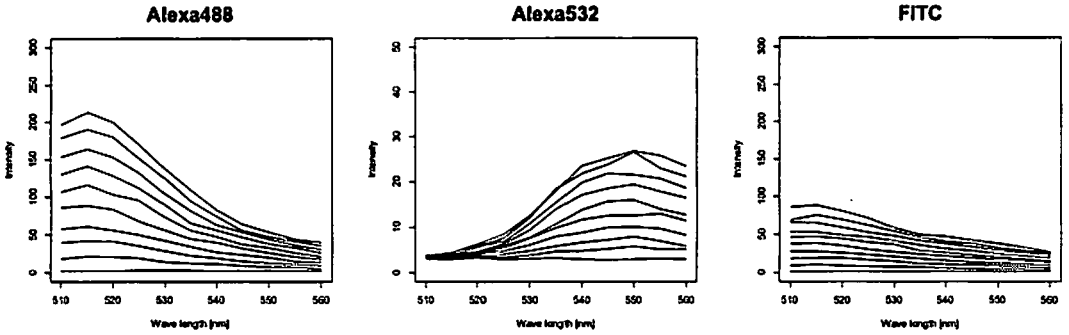

Figure 5. Spectral intensity of each level of concentration

4.2.2 Mixed samples

Figure 7 shows the observed spectrum of mixture samples and its ideal curve.

The ideal curve as the summation of two spectra is drawn as a chain line. The observed spectrum is drawn as a solid line. For purposes of reference, each single dye's spectral fluorescence is drawn as a dotted line or dashed line. The observed spectrum must fit the ideal curve if the mixture of fluorescence is based on a linear combination model.

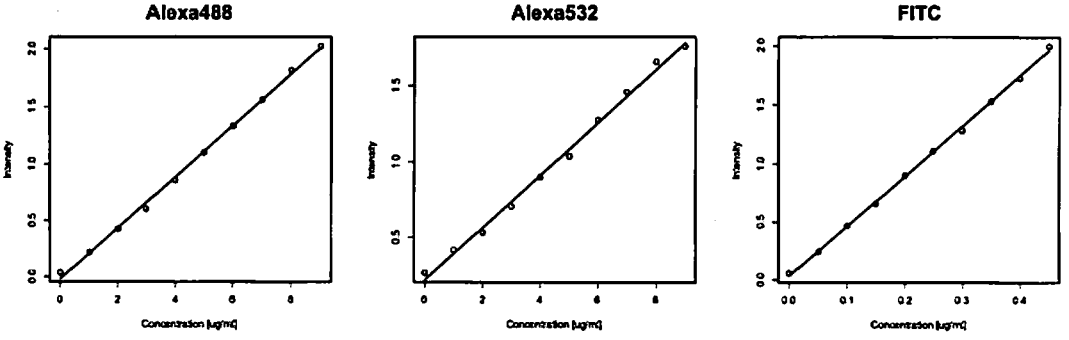

Figure 6. Linear regression results

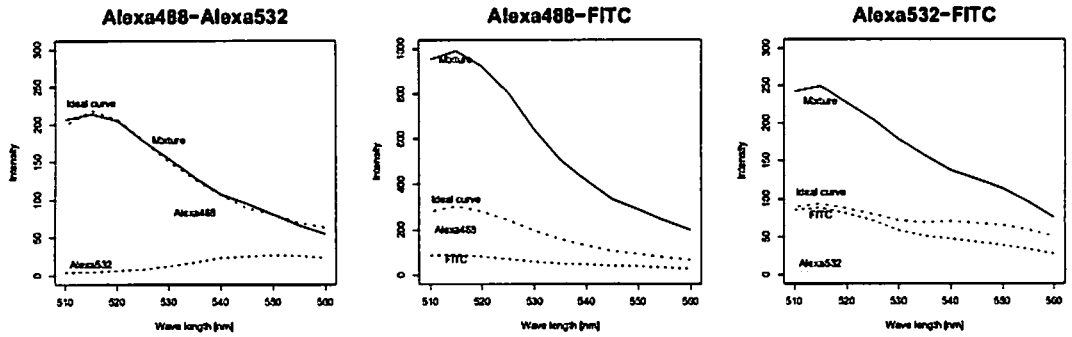

Figure 7. Spectral intensity of mixture samples and its ideal curve

For Alexa488-Alexa532, the observed spectrum fits well. We can assume that it is based on a linear combination model. Therefore, the conventional method will work properly. On the other hand, for Alexa488-FITC and Alexa532-FITC, the observed spectra differ vastly from the ideal curve. Although the reason remains unclear, apparently, the FITC fluorescence is amplified by the Alexa series. We can not assume that it is based on a linear combination model. Therefore, the conventional method will not work properly. The proposed method is required in such a case.

4.3 Estimation of concentration

To compare the conventional method with the proposed method qualitatively, we plotted the estimated concentration and correct concentration for each method.

Figure 8–13 shows the estimation result with Linear Unmixing as a conventional method and proposed method. The ordinate shows the relative dye concentration. The abscissa shows the number of samples. The estimated concentration of a sample is indicated with a circle. The correct concentration of the samples is drawn as a solid line.

4.3.1 Case in which a linear combination model is assumable

We verified the linearity of the mixture fluorescence of Alexa488-Alexa532 in Section 4.2.2. Therefore, we can regard this as an example of the case in which the linear combination model is assumable.

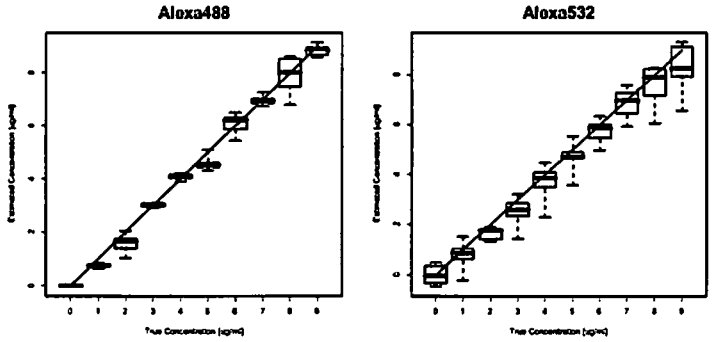

Figure 8. Estimation result for Alexa488-Alexa532 obtained using the conventional method

Figure 8 shows the result of compensation obtained using the conventional method; Fig. 9 shows that obtained using the proposed method.

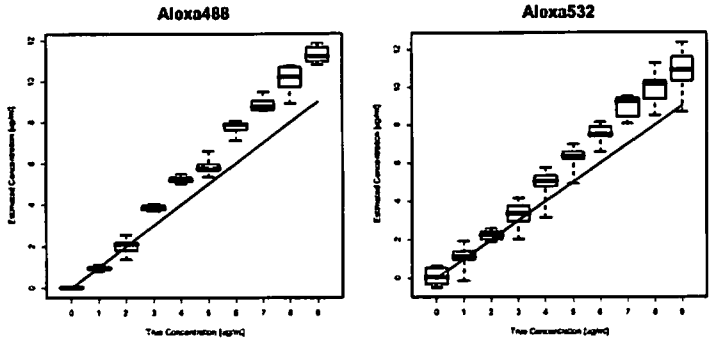

Figure 9. Estimation result for Alexa488-Alexa532 obtained using the proposed method

Because the estimated values are roughly coincident with the correct values, both are available in the case for which a linear combination model is assumable.

4.3.2 Case in which a linear combination model is not assumable

As examples of the case in which the linear combination model is not assumable, we consider the result of estimation for Alexa488-FITC and Alexa532-FITC.

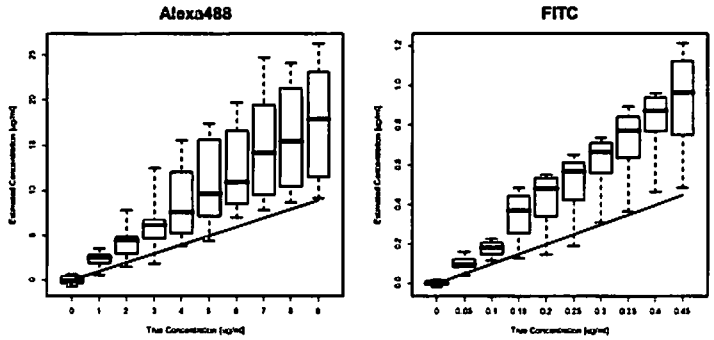

Figure 10. Estimation result for Alexa488-FITC obtained using the conventional method

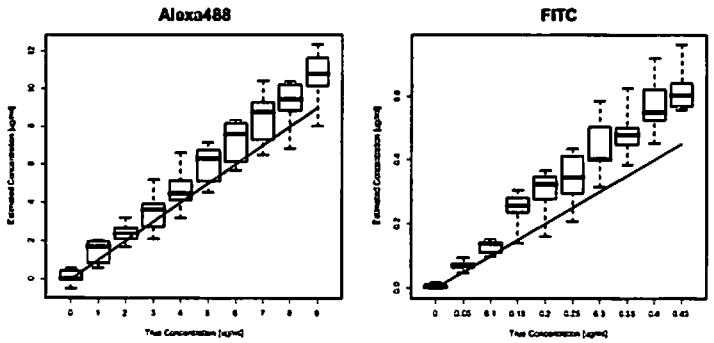

Figure 11. Estimation result for Alexa488-FITC obtained using the proposed method

Figure 10 shows the estimation result for Alexa488-FITC obtained using the conventional method. The estimated value has an inclination to be higher than the correct one. Additionally, higher concentrations of dyes produce a wider difference between estimated values and correct ones for each dye.

Figure 11 presents results of estimation for Alexa488-FITC obtained using the proposed method. As in the case of Alexa488-Alexa532, the estimated values are roughly coincident with the correct values.

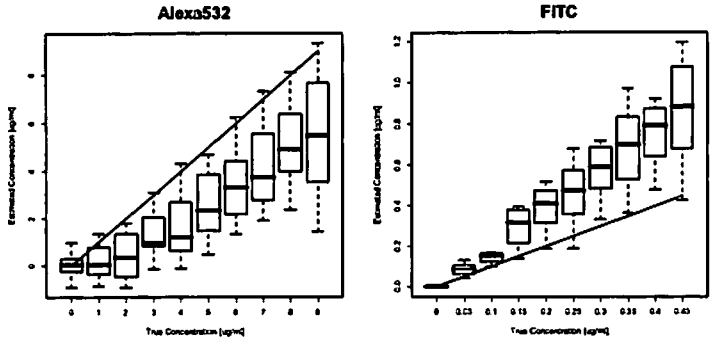

Figure 12. Results of estimation for Alexa532-FITC obtained using the conventional method

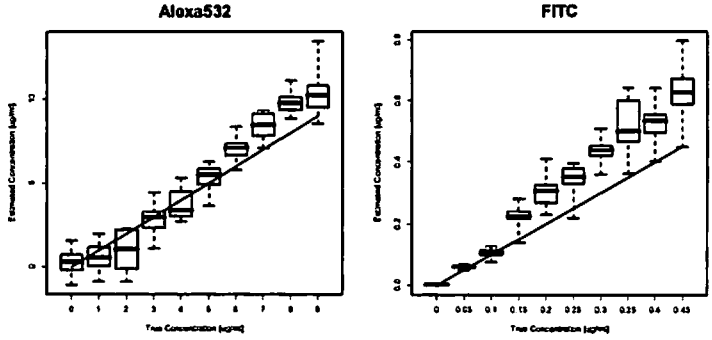

Figure 13. Results of estimation for Alexa532-FITC obtained using the proposed method

Figure 12 shows the result of estimation for Alexa532-FITC obtained using the conventional method. As in the case of Alexa488-FITC, the estimated value of FITC has an inclination to be higher than the correct one. On the other hand, the estimated value of Alexa532 has an inclination to be lower than the correct one. Higher concentrations of dyes produce a wider difference between estimated values and correct ones for each dye.

Figure 13 presents the result of estimation for Alexa532-FITC obtained using the proposed method. As in the previous case, the estimated values are roughly coincident with the correct values.

4.4 Precision of Estimates

To compare the conventional method with the proposed method quantitatively, we calculated Q-value for each method.

Table 3 is a comparative table of Q-values. The larger the Q-value is, the better the estimation. The Q-value is equal to 1 if it is estimated with no error.

For Alexa488-Alexa532, the Q-values nearly 1; the estimation is correct, with the conventional method. On the other hand, using the proposed method, both Q-values are reduced: the estimation becomes worse. However, compared to following results, it is acceptable error range.

Table 3. Comparative tables of Q-value

Dye set	Alexa488-Alexa532		Alexa488-FITC		Alexaa532-FITC	
Individual dye	Alexa488	Alexa532	Alexa488	FITC	Alexa532	FITC
Conventional	**0.987**	**0.950**	−4.51	−3.70	0.111	−2.63
Proposed	0.760	0.762	**0.772**	**0.246**	**0.785**	**0.296**

For Alexa488-FITC and Alexa532-FITC, the results obtained using respective methods differ. With the conventional method, the Q-values differ greatly from 1: the estimation is incorrect. On the other hand, using the proposed method, both Q-values are greater than ; consequently, the estimation is correct, as in the case of Alexa488-Alexa532.

4.5 Discussion

Although we achieved three results using three dye-sets, we can divide them into two cases: those in which the mixture fluorescence is and is not based on the linear combination model. For cases based on the linear combination model, both methods are available. However, the conventional method is not available for use in cases which are not based on it. In both cases, the proposed method is available for estimation. It is independent of the linearity of mixture fluorescence. We consequently conclude that the proposed method has an advantage related to dependence on linearity of mixture fluorescence.

5. CONCLUSION

We pointed out a weakness of the conventional method of fluorescence compensation: it depends on linearity of the mixture fluorescence. Although the conventional method is available for cases in which the mixture fluorescence is based on a linear combination model, it can not properly compensate in other cases. We proposed a method that is independent of the linearity of the mixture fluorescence. The concentration of each dye was estimated using the method. To verify the advantage of the presented method, we performed some experiments applying the method to cases which are based on and not based on a linear combination model. Results confirmed that the presented method is independent of the linearity of mixture fluorescence.

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