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Resolution of Complex Fluorescence Spectra Recorded on Single Unpigmented Living Cells Using a Computerised Method

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The identification and quantification of fluorescent compounds in a complex fluorescence spectra are always difficult, especially in the case of low signal:noise ratio.

We propose a computerised method that allows the resolution of low light level complex fluorescence spectra into its components.

Based on a linear combination of *N* possible characteristic fluorescence spectra, and using *N* weighting functions, this method allows the integration of fluorescence intensities over the entire fluorescence spectra and the generation of *n* equations with *N* unknowns. The compounds that participate in complex fluorescence spectra are identified and quantified. Because fluorescence intensities can be integrated we can resolve complex fluorescence spectra presenting a low signal:noise ratio. The reliability and sensitivity of our method are shown through examples of resolution of complex intracellular fluorescence of single living cells pretreated with benzo(a)pyrene. Depending on the cell type and treatment, two, four, or five components can be identified in the complex fluorescence spectra.

Key terms: Weighted linear combination, microspectrofluorometry, B(a)P metabolites

When fluorescent techniques are used to solve analytical problems in chemistry and conventional biology, judicious choices of excitation wavelengths have proved a powerful and elegant tool both for the resolution of most complex fluorescence spectra into their components and for the characterization of fluorescent chemicals (6,8,15,17). Recently, extensive use has been made the so-called Shpoll'skii spectroscopy, which takes advantage of the spectral sharpening and of the increase in vibrational structure when spectra are recorded at very low temperatures (13,14,19). The same effect is observed with excitation spectra, and tunable laser affords a simple means of selective excitation (5,20).

Unfortunately, it is not possible to use Shpoll'skii spectroscopy when fluorescent measurements are performed on single living cells. This is especially true when the goal of the experiment is to follow the intake process of a fluorescent drug into certain cells and to detect and characterize specific fluorescent metabolites. For such experiments, it is necessary first to operate at the usual temperature of the cell culture, if one intends to follow the kinetics of the phenomenon under study, and second to minimize the duration of exposure of the biological material to irradiation in order to avoid any biological damage. This generally means that excitation has to be performed at only one wavelength, which cannot be chosen so as to be the most convenient for each constituent of the complex cell fluorescent spectrum, as is the case in synchronous fluorescence analysis (8,15,17).

To overcome these problems the use of specific quenchers has been proposed. It has been demonstrated that liposoluble collisional quenchers, such as n-iodohexane and iodobenzene, selectively quench the benzo(a)pyrene fluorescence in suspended living cells previously incubated with benzo(a)pyrene and 7,8-diol-epoxide benzo(a)-pyrene [B(a)P] (4,16). Unfortunately, because of the excess of quencher that must be used in these experiments, the method cannot be extended to the study of kinetic processes on living material. Other investigators have tried qualitative comparison between the complex fluorescence spectrum and that of some possible metabolites (7).

Some years ago, taking advantage of the fact that microspectrofluorimetric techniques give a numerical distribution of the fluorescence intensity versus wavelengths, some of us proposed to tackle the problem through linear algebra (12); assuming that the fluores-

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cence spectra of L chemicals are involved in the complex fluorescence spectrum, the fluorescence intensity at each wavelength can be described a linear combination of the fluorescence intensities of each component at the same wavelength, i.e.,

$$IT_\lambda = a_1 I_{1,\lambda} + a_2 I_{2,\lambda} + \ldots \quad (1)$$

where $a_1, a_2, \ldots$ represents the respective participation coefficient of each compound 1, 2, . . . in the fluorescence; $I_{1,\lambda}, I_{2,\lambda}, \ldots$ is the fluorescence intensity of each compound at wavelength μ and IT_λ is the total fluorescence intensity at wavelength λ. Thus the use of a set of L such equations enables the determination of the respective value of the parameters $a_1, a_2, \ldots$.

This method has been used to calculate the relative participation in a cell fluorescence spectrum of free and bound NAD(P)H in various cell types and to study the variations of the bound:free NAD(P)H ratio after microinjection of glucose-6-phosphate (12). Unfortunately, because of the values of the signal:noise ratio usually associated with microfluorimetry, this method has proved inaccurate when more than two components must be taken into account.

This paper deals with a new approach to the problem. However, as in every method using fluorescence techniques, we must be especially aware of artefacts resulting from 1) inner filter effects, which could occur in pigmented cells; and 2) autoabsorption effects, which could arise when too high an intracellular concentration of probe is reached. These inconveniences have been avoided by the use of unpigmented cells and low intracellular concentrations of probes.

PRINCIPLE OF THE METHOD

The usual way of dealing with low signal:noise ratio is to perform an integration of the data over the whole spectral range. Equation 1 will then be replaced by Equation 2:

$$\sum_{\lambda=\lambda_0}^{\lambda_1} IT_\lambda = \sum_{\lambda=\lambda_0}^{\lambda_1} \sum_{i=1}^{L} a_i \cdot I_{i,\lambda} \quad (2)$$

This is by no means a good way to solve our problem, in which a set of L independent equations is required to evaluate the L unknowns. However, the needed set of equations can be generated by using L modulating functions depending on λ , $\Phi_{J,\lambda}$, with $J = 1$ to L. Then equation 2 will be replaced by the following set:

$$\begin{aligned}
\sum_{\lambda=\lambda_0}^{\lambda_1} \Phi_{1,\lambda} \cdot IT_\lambda &= \sum_{i=1}^{L} a_i \sum_{\lambda=\lambda_0}^{\lambda_1} \Phi_{1,\lambda} \cdot I_{i,\lambda} \\
\sum_{\lambda=\lambda_0}^{\lambda_1} \Phi_{J,\lambda} \cdot IT_\lambda &= \sum_{i=1}^{L} a_i \sum_{\lambda=\lambda_0}^{\lambda_1} \Phi_{J,\lambda} \cdot I_{i,\lambda} \quad (3) \\
\sum_{\lambda=\lambda_0}^{\lambda_1} \Phi_{L,\lambda} \cdot IT_\lambda &= \sum_{i=1}^{L} a_i \sum_{\lambda=\lambda_0}^{\lambda_1} \Phi_{L,\lambda} \cdot I_{i,\lambda}
\end{aligned}$$

Theoretically, any kind of modulating functions, except random ones, will be convenient. However, to 1) maximize the importance in each equation of the weight of only one component of the complex fluorescence spectrum, this function ought be proportional to $I_{J,\lambda}$ and 2) to give a higher value to the data belonging to the domain of wavelength, where the intensity of the observed complex fluorescence spectrum is significant, this function ought to be proportional to IT_λ.

The following modulating function has been considered:

$$\Phi_{J,\lambda} = IT_\lambda \cdot I_{J,\lambda} \quad (4)$$

so that the Jth equation of the previous set becomes

$$\sum_{\lambda=\lambda_0}^{\lambda_1} I_{J,\lambda} \cdot (IT_\lambda)^2 = \sum_{i=1}^{L} a_i \sum_{\lambda=\lambda_0}^{\lambda_1} I_{J,\lambda} \cdot IT_\lambda \cdot I_{i,\lambda} \quad (5)$$

If a library of the characteristic fluorescence spectrum of each component is in hand, the respective participation coefficients of each compound in the fluorescence can be evaluated through the resolution of the above set of L equations. From that information a theoretical whole-cell fluorescence spectrum can be calculated and compared with experimental data.

FITTING ESTIMATORS FOR FLUORESCENCE SPECTRA RESOLUTION

The estimation of the fit between measured and calculated fluorescence spectra will closely depend on the evaluation of the noise that overcomes photonic signals. Usually for a SIT (silicon intensified target) detector, the noise for a given channel of the detector is estimated by the relation

$$n_\lambda = \sqrt{s_\lambda + b^2} \quad (6)$$

where s_λ is the photonic signal converted in counts and b is the system noise in counts resulting from subtraction of dark currents + correction of baseline distortion = 3 counts.

However, the relation shown in equation 6 is an approximation that does not take into account two factors:

1. The gain in conversion of the photoelectrons generated by the image intensifier into counts, which modifies the expression of photonic noise.
2. The imprecision of the position of the electron beam that reads the signal stored for each channel. The noise induced by this factor will be proportional to the difference of signal between two adjacent channels and can be very important in the case of sharp fluorescence bands.

As these noises are independent, they add quadratically, and equation 6 becomes:

$$n_\lambda = \sqrt{\alpha \cdot s_\lambda + b^2 + [\gamma(s_\lambda - s_{\lambda-1})]^2} \quad (7)$$

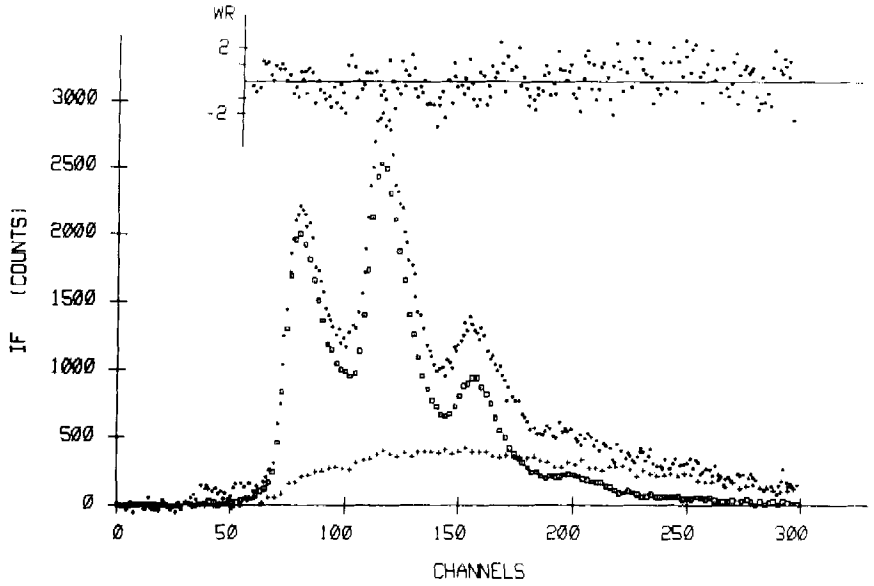

FIG. 1. Resolution of the intracellular fluorescence spectrum (●) of a fibroblast, 3T3, treated with B(a)P into: B(a)P (□), participation 69.8% intrinsic cell fluorescence (+), participation 30.2%. The top curve presents the distribution of the weighted residuals $WR = f(\text{channel})$, which gives an χ^2 value of 1.105.

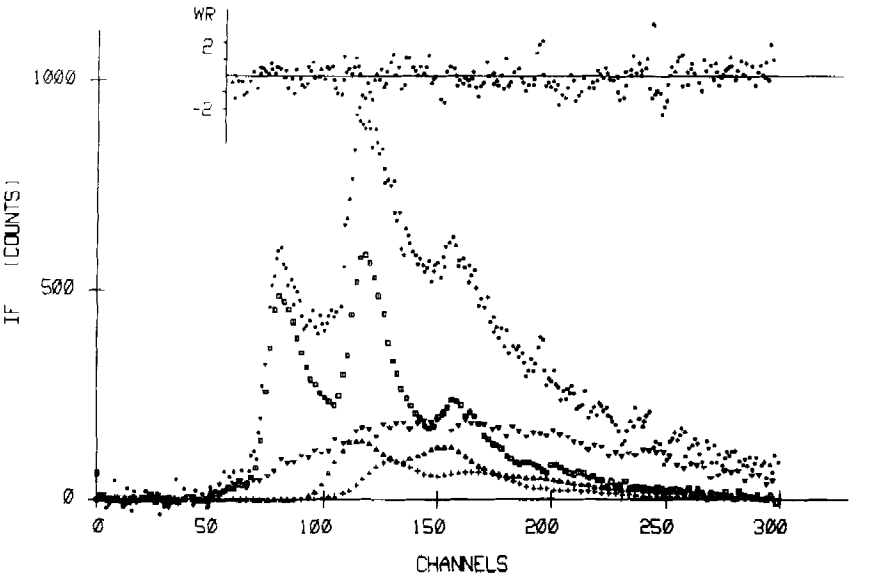

FIG. 2. Resolution of the intracellular fluorescence spectrum (●) of an RTG2 cell treated with B(a)P into: B(a)P (□); 3OH-B(a)P (+); 9OH-B(a)P (△); and intrinsic cell fluorescence (▽) with the respective participations of 45, 7, 13, and 35%. Top curve as in Figure 1, χ^2 value of 0.744.

where α is constant depending on the conversion of photoelectrons to counts and is evaluated at 0.9 and γ is constant depending on the jitter in the position of the electron beam for each channel and is evaluated at 0.24.

Accumulating K successive readouts of the TV target will convert s_λ into S_λ; n_λ into N_λ, S_λ and N_λ being defined respectively as:

$$S_\lambda = K \cdot s_\lambda$$
$$N_\lambda = \sqrt{K \alpha s_\lambda + Kb^2 + K[\gamma(s_\lambda - s_{\lambda-1})]^2} \tag{8}$$

Graphic Fit Estimation

A good way to evaluate the fit between experimental data and theoretical curve is to plot for each channel the widely used (3,9) weighted residuals (*WR*) (equation 9), defined as the ratio of the difference between the measured signal and the corresponding calculated signal versus the noise on this difference.

$$WR_\lambda = \frac{IT_\lambda - IC_\lambda}{NT_\lambda} \tag{9}$$

where IT_λ = signal measured at channel λ.

$$IC_\lambda = \sum_{i=1}^{L} a_i \cdot I_{i,\lambda}$$
$$NI_\lambda = \sqrt{(Nmes_\lambda)^2 + (Ncalc_\lambda)^2} \tag{10}$$

with $Nmes_\lambda$ evaluated according to equation 8 and $Ncalc_\lambda$ noise related to IC_λ and calculated as follows:

$$Ncalc_\lambda = \sqrt{\sum_{i=1}^{L} (a_i \cdot N_{i,\lambda})^2} \tag{11}$$

where $N_{i,\lambda}$ = noise on each characteristic fluorescence spectrum calculated according to equation (8).

On the one hand, a good fit between the experimental results and the calculated whole fluorescence spectrum will result in a graphic plot of *WR* showing a statistical distribution around zero. On the other hand, a distortion such as the one shown later, in Figure 3, will indicate to us the area of the fluorescence spectra in which we need to introduce a new characteristic fluorescence spectrum in order to account for the observed spectra.

When we must compare a large number of possible linear combinations for a given experimental spectrum, or analyse a large number of complex fluorescence spectra (sampling of cell population or kinetic studies), checking the fit of the resolution of each fluorescence spectrum with a graphic fit estimator is not possible. The use of a numeric estimator as an initial indicator of the fit would be more convenient.

Numeric Fit Estimator

We attempted to use a numeric estimator derived from the variance of the distribution of *WR*, usually called reduced chi-square (3,9):

$$\chi^2 = \sum_{\lambda=\lambda_0}^{\lambda_1} (WR_\lambda)^2 / [(\lambda_1 - \lambda_0) - L]$$

where $(\lambda_1 - \lambda_0) - L$ is the number of degrees of freedom.

In a usual case, χ^2 should be very close to 1 for a perfect fit, when the number of degrees of freedom is large enough ($\simeq$ 200).

Unfortunately, it is not possible to take advantage of the relation between the value of the chi-square and statistical probabilities. The reason for this is that the evaluation of *WR* depends on correct evaluation of the real noise NT_λ (cf. equations 10 and 11). Unfortunately, only an approximate value of NT_λ is accessible because of variations caused by flickering of excitation intensity, modification of the jitter of channel position, vibrations, etc. An overestimation of NT_λ will result in a decrease of chi-square value for 100% probability (cf. equation 9); on the other hand, underestimation of NT_λ will induce an increase of chi-square.

Consequently, when the values of chi-square were found rather different from 1, checking the fit by visual observation of *WR* remained necessary.

MATERIALS AND METHODS

Cells

RTG2 cells (rainbow trout gonad cells, American Type Culture Collection nCCL 55) were cultivated as monolayers (Falcon 25 cm^2) in minimum essential medium (MEM; Gibco) with 10% decomplemented foetal calf serum (FCS; Gibco) at 18°C. Confluent cells were resuspended after trypsine treatment (trypsine 0.25%, without Ca^{2+} or Mg^{2+}; Gibco). Sykes-Moore chambers, with the upper lamella removed, were incubated with 30,000 cells per chamber at 18°C in 5% CO_2 atmosphere. The experiments were carried out 72 h after incubation in order to eliminate the effect of trypsin.

Murine 3T3 fibroblasts (American Type Culture Collection) were cultivated as monolayers as above but at 37°C. Dispase (grade II, Boehringer) was used instead of trypsin.

Solutions

To avoid possible effects of any solvent on the characteristics of the cell membranes, ethanolic solutions of each PAH were first evaporated; resulting microcrystals were then dissolved with gentle agitation in the culture medium (10% FCS, 1% HEPES). Dissolution was obtained through interactions with proteins and lipoproteins (2). Concentrations were determined spectrophotometrically after ethyl acetate extraction. They remain in the range of 1 μM for each compound under study.

Apparatus and Fluorescence Spectra

Fluorescence spectra were recorded using a microspectrofluorimeter constructed in the laboratory (1,10,11). The detection system used in this apparatus was an optical multichannel analyser (from Princeton Applied Research Corp.) equipped with a silicon intensified target (SIT). The microspectrofluorimeter was equipped with a thermostatted stage which allowed the temperature of the cells to be controlled. Data were stored in a microcomputer (Micro II, Plessey). The fluorescence analyzed originated from a perfectly defined region of the microscopic field. The complex fluorescence spectra were resolved using a Plessey microcomputer (Micro II or 8321) running under an RT11 operating system. The resolution program was written in FORTRAN IV.

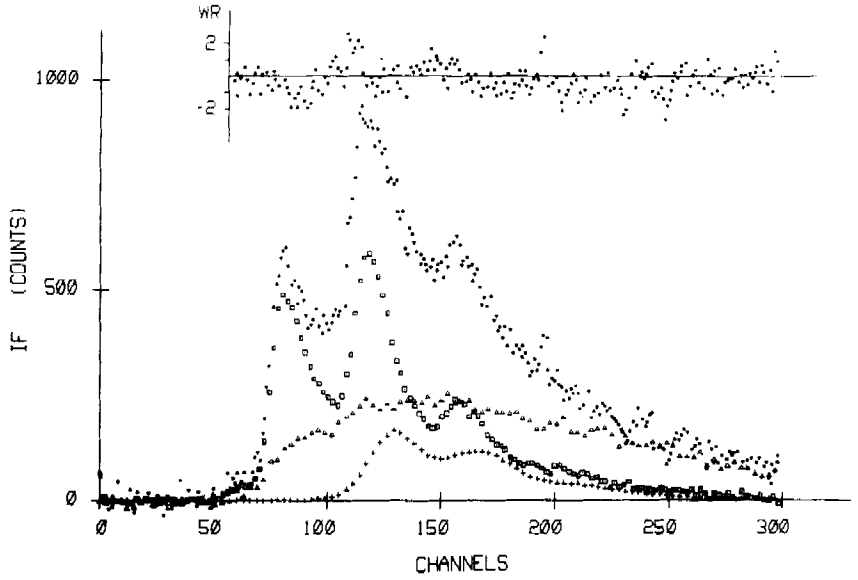

FIG. 3. Resolution of the same intracellular fluorescence spectrum (●) as in Figure 2 into: B(a)P (□); 3OH B(a)P(+); and intrinsic cell fluorescence (△) with the respective participations of 44, 12, and 44%. Top curve as in Figure 1, χ^2 value of 0.948.

In all the figures, the wavelength scale is expressed in channels, as this was the unit used in computations. Channel 50 corresponds to 386 nm and channel 300 to 545 nm: dispersion 0.64 nm/channel. Except for Figure 1, the fluorescence participation of the different compounds is rounded to the nearest 0.0 or 0.5 percent.

EXAMPLES OF RESOLUTION OF INTRACELLULAR FLUORESCENCE SPECTRA

To illustrate the possibilities of our method, we will present some examples of the resolution of intracellular fluorescence of single living cells treated by B(a)P. A first cell line—3T3 fibroblasts—has given us simple cases of complex fluorescence spectra, while more complicated ones have been obtained with a second cell line—RTG2—known for its ability to accumulate metabolites of B(a)P.

Analysis of Intracellular Fluorescence of B(a)P-Treated 3T3 Fibroblasts

Figure 1 shows the resolution of the intracellular fluorescence of a single 3T3 fibroblast incubated 30 min in B(a)P 10^{-6} M medium before being placed into fresh medium.

As we can see through the graphic plot of weighted residuals, a good fit is obtained between the experimental spectrum and the calculated one resulting from the linear combination of fluorescence due to B(a)P and intrinsic cell fluorescence. The corresponding χ^2 is evaluated at 1.105.

To show the reliability of our method, we performed a resolution of the experimental fluorescence spectrum of Figure 1 by a linear combination of three components, including B(a)P, intrinsic cell fluorescence, and 3OH-B(a)P. Respective participations of 70%, 30.4% and −0.4% were found. These results show no difference from the respective participations of B(a)P and intrinsic cell fluorescence of Figure 2, while the participation of the extra component (3OH-B(a)P) is close to zero. The distribution of the weighted residuals is similar to that shown in Figure 1, and the corresponding χ^2 is increased to 1.139.

The above data show the reliability of our method, but that our method remains efficient for the resolution of more complex fluorescence spectra remains to be proven.

Analysis of Intracellular Fluorescence of B(a)P-Treated RTG2 Cells

In this example we analysed the fluorescence spectra of the intracellular fluorescence of RTG2 cells treated with B(a)P under the same conditions as 3T3 fibroblasts. Analysing the spectrum of intracellular fluorescence of these cells as linear combinations of fluorescence characteristic of B(a)P and intrinsic cell fluorescence leads to a failure.

Using our catalog of characteristic fluorescence spectra of B(a)P metabolites, we have been able to resolve the intracellular fluorescence in four components: B(a)P, 3OH-B(a)P, 9OH-B(a)P, and intrinsic cell fluorescence spectra (Fig. 2). The results obtained when we suppressed one of the possible components or replaced the characteristic fluorescence spectra of one component (3OH-B(a)P) with another similar one [3OH-B(a)P con-

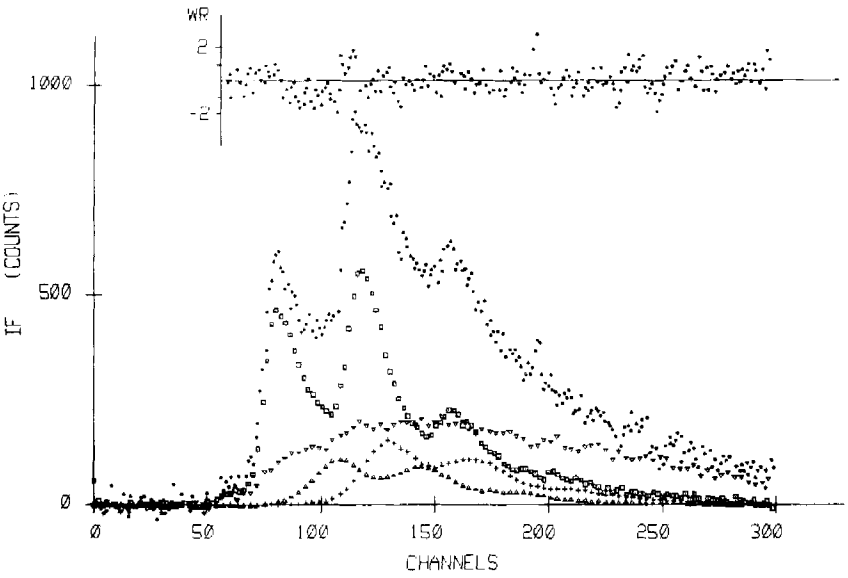

FIG. 4. Resolution of the same intracellular fluorescence spectrum (●) as in Figure 2 into: B(a)P (□); 3OH-B(a)P (+); 3OH-B(a)P conjugated to glucuronic acid (△); and intrinsic cell fluorescence (▽) with the respective participations of 43, 11, 9, and 37%. Top curve as in Figure 1, χ^2 value of 0.815.

jugated to glucuronic acid (Fig. 5)] are shown in Figures 3 and 4. They clearly show that:

1. The distribution of *WR* shows that the best fit is obtained in Figure 2.
2. The distribution of *WR* in Figure 3 exhibits two distortions, which correspond to the maximum 9OH-B(a)P characteristic fluorescence spectrum. Such distortions have been used in the search for compounds whose fluorescence participates in the intracellular fluorescence spectra.

This set of resolutions also shows that the evaluated χ^2 values are lower than 1. This has to be related to an overestimation of the noise used for the calculation of NT_λ. A correct evaluation of NT_λ should give χ^2 values greater than 1, but this did not modify the ranking of χ^2 from Figure 2 to Figures 3 and 4. Thus, for a given experimental fluorescence spectrum, the best resolution will be obtained for the lowest χ^2 value. A direct comparison of the χ^2 values obtained for fluorescence spectra recorded under different conditions would be meaningless because of the problem of the correct evaluation of the noise $Nmes_\lambda$ (cf. equation 10). These results confirm the sensitivity and reliability of our method.

Analysis of Intracellular Fluorescence of RTG2 Cells Treated Simultaneously With B(a)P and 6-Aminochrysene

To show the potential of our method, as compared with simple linear combinations, we performed the resolution of the intracellular fluorescence of an RTG2 cell treated simultaneously with B(a)P and 6-aminochrysene (6-AC), 10^{-6} M. This last fluorescing compound is usually regarded as an inhibitor of B(a)P metabolism.

As 6-AC has no known fluorescent metabolite in our experimental conditions, the resolution of the intracellular fluorescence spectra has only been performed using the following characteristic fluorescence spectra: B(a)P, 3OH-B(a)P, 9OH-B(a)P, and 6-AC intrinsic cell fluorescence (Fig. 6). The distribution of the weighted residuals indicates a good fit between experimental and calculated intracellular fluorescence spectra. The corresponding χ^2 value, 1.085, indicates a good evaluation of NT_λ.

DISCUSSION

Much research has been devoted to methods that would allow the identification of fluorescent substances in mixtures. As good as these methods may be, however, and except for the method based on the linear combination of characteristic fluorescence spectra (12), they are limited by the following factors:

1. They only give qualitative information on the mixture composition.
2. The experimental conditions used [low temperatures (5,13,14,19,20); high intracellular concentrations of quenchers (4,16); and long time necessary to record information with simultaneous scanning of emission and excitation (8,15,17)] are not suitable for studies on single living cells, either for kinetic studies or for sampling of a cell population.

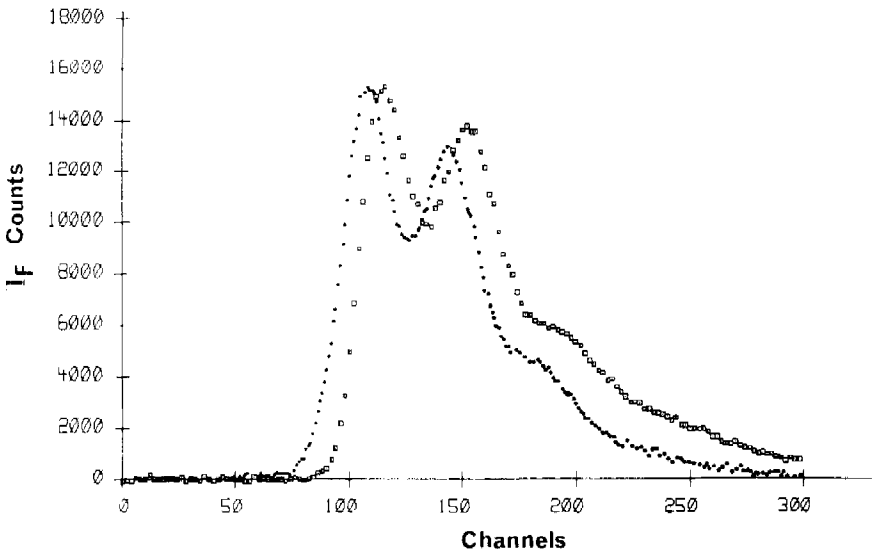

FIG. 5. Characteristic fluorescence spectrum of 9OH-B(a)P (□) and 3OH-B(a)P (●) conjugated to glucuronic acid.

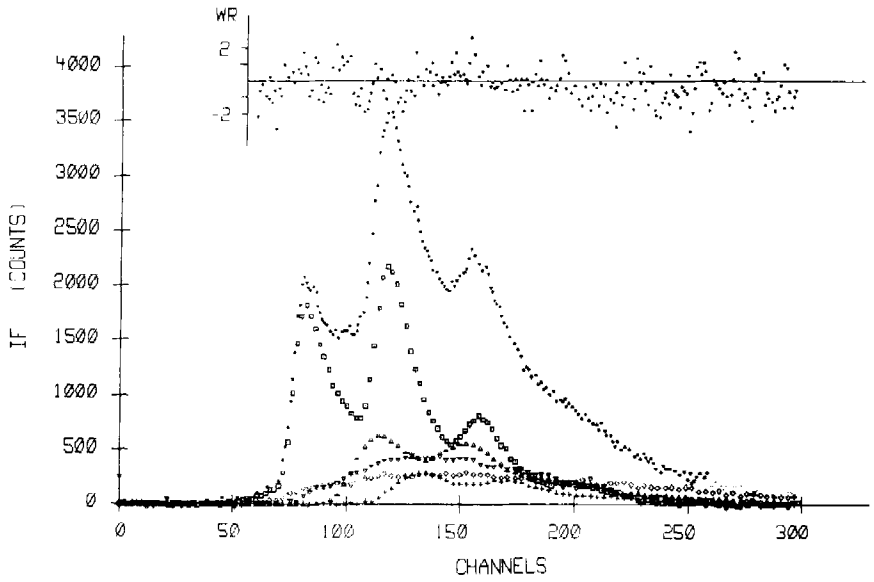

FIG. 6. Resolution of the intracellular fluorescence (●) of an RTG2 cell simultaneously pretreated with B(a)P and 6-AC into: B9A0P (□); 6-AC (◇); 3OH-B(a)P (+); 9OH-B(a)P (△); and intrinsic cell fluorescence (▽) with the respective participations of 45, 15, 7, 18, and 15%. Top curve as in Figure 1, χ^2 value of 1.085.

The only way to get quantitative information on a mixture composition by microspectrofluorimetry on single living cells, had appeared to be a method using linear combination (12). Such a method has been used with success in the resolution of absorption spectra (21,22). However, that method was no longer convenient with low-light-level complex fluorescence spectra presenting low signal:noise ratio, unless we increased the signal:noise ratio by integrating the signal over the entire fluorescence spectra. This is especially true when more than two components must be taken into account.

The method of resolution of complex fluorescence spectra that we propose—based on the use of modulating functions and integration over the entire fluorescence spectrum—shows that, once we have a library of characteristic fluorescence spectra, it is possible to identify and quantify the substances participating in a complex fluorescence spectrum. Maintaining a library of fluores-

cence spectra is not peculiar to our method alone, but is necessary for all methods whose purpose is to identify fluorescent compounds.

The results presented in this paper indicate the high specificity of our method, which allows, with a signal: noise ratio of about 30, 1) the determination and quantification of fluorescences participating in complex fluorescence spectra and 2) the discrimination between two fluorescence spectra similar in shape but shifted by 4 nm. One should be able to use such an approach for a larger number of unknown substances (as we have tried for seven different compounds). The limit to such determinations is the signal:noise ratio of the complex experimental fluorescence spectrum, which should be increased as the number of unknowns increases.

Our results also show that, among the possible linear combinations tried to account for a complex fluorescence spectrum, the best fit will be obtained with the one presenting the lowest χ^2 value. Nevertheless, the use of a graphic estimator of the fit, through *WR* distribution, remains the most valuable method.

This method may also be used not only in conventional fluorescence spectroscopy in order to identify and quantify the substances participating in a complex fluorescence spectrum, but also in the study of any spectrum, once a catalog of characteristic spectra of compounds that may participate is available.

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