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Spectral Decomposition of Intracellular Complex Fluorescent Signals Using Multiwavelength Phase Modulation Lifetime Determination

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Lifetime-based spectral decomposition using a frequency-domain phase/modulation technique has been developed on our UV confocal microspectrofluorimeter prototype. Examples of such an application are first given in the case of complex mixtures of three overlapping fluorescent components in solution. Preliminary results concerning cellular potential application are also reported in the case of Snarf-1 pH sensor. Limits and perspectives of such an approach at the subcellular level are discussed in view of future improvement.

KEY WORDS: Spectral decomposition; intracellular complex fluorescence; phase modulation lifetime.

INTRODUCTION

Sensitive fluorescent indicators have been extensively developed to monitor intracellular parameters such as local pH, ionic activities, and membrane potential [1]. Unfortunately, the complexity of the intracellular medium often led to perturbation effects of the measurements, due, for example, to nonspecific interactions of the dye with the cytosolic proteins or to a nonnegligible contribution of the fluorescence background of endogenous chromophores such as NADH or flavins.

Spectroscopic discrimination is one way to solve this problem. Nevertheless, it is sometimes not efficient, especially when there is a high overlapping of the respective spectra of the various fluorescent contributions. Moreover, it is often difficult, if not impossible, to get model spectra of the different intracellular specific fluorescent species. In these cases fluorescence lifetime discrimination (using modulated excitation and phase sensitive detection) appears to be an alternative way to enhance the specific detection of the desired fluorescent contribution [2,3].

Fluorescence lifetime measurements using the multifrequency phase modulation technique has been developed on our confocal laser microspectrofluorimeter prototype (1 to 120 MHz). Measurements were performed using the frequency domain method with a modified optical multichannel detector (IRY 1024-GB; Princeton Instruments, Inc., Trenton, NJ) as originally described by Gratton *et al.* [4] (see Fig. 1). The detection window is 250 nm wide and its position is chosen in a way to detect at the same time the excitation scattered light (514.5-nm line of an argon laser) and fluorescence signal. The Phase angle of the fluorescence signal obtained on each diode is then related to those of the excitation taken as reference. A minimization procedure by "global analysis software" is then used for data processing. It enables us to determine the relative contribution of each calculated lifetime at multiple wavelengths (i.e., for several diodes). In this way the spectral shape of each lifetime contribution

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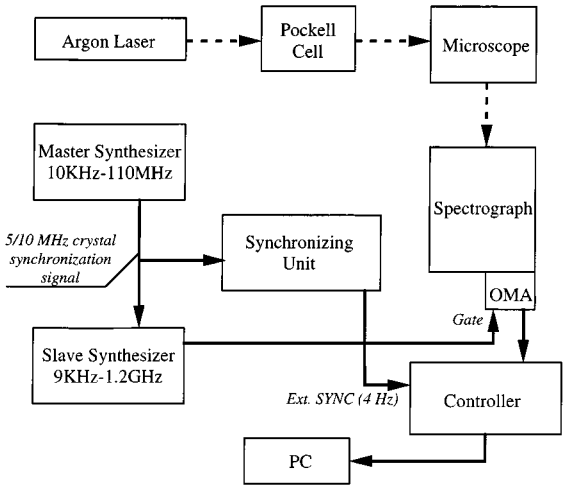

Fig. 1. Schematic block diagram of the experimental setup.

may be obtained. Thus it is possible to get the spectral decomposition of intracellular complex spectra with strongly overlapped components without getting any model spectra.

In the present study, assessment for such an application is presented in the case of a mixture of the fluorescent probe TMRM (tetramethylrhodamine ester) with one or two other fluorescent molecules (the structurally related photosensitizers hypericin and hypocrellin-A [5]). Fluorescence lifetime-based sensing has recently been proposed using frequency domain measurement. Indeed *in vitro* study has already pointed out some potential cellular applications in the case of acidic pH indicators [6]. In the present study, some preliminary results concerning absolute intracellular measurements were obtained in the case of subcellular pH measurements using the fluorescent probe SNARF-1-AM [3].

MATERIALS AND METHODS

Spectral decomposition of intracellular complex fluorescence spectra has been developed on our confocal laser microspectrofluorimeter using the frequency domain method and optical multichannel analyzer (OMA) detection (see Fig. 1). The excitation laser intensity (Model 2025; Spectra Physics, Mountain View, CA) is first modulated from 1 to 120 MHz through a Pockell cell (SLM Instruments, Urbana Champaign, IL) using the amplified output of the master frequency synthesizer (Model 6300; Adret Electronique, France) before being focused on the sample through a Zeiss UMSP 80 UV epifluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany). The excitation scattering and fluorescence signal are then "collected" on a Jobin Yvon HR 640 spectrograph (ISA, Longjumeau, France) and the output wavelength dependence emission is detected on the 1024 diodes of the OMA (250-nm coverring range). The photocathode voltage of the OMA is driven by the slave synthesizer (Model 2023 A; IFR International, Evry, France) synchronized with the master one but with 1-Hz frequency difference (1-Hz cross-correlation detection). In this way a 1-Hz modulation of the detected signal is obtained. Determination of the phase angle and modulation of the signal detected by each diodes is obtained using a home box synchronizing unit which allows triggering a 4-Hz readout of the OMA by the controller unit (Princeton Instruments Model ST121) synchronization with the master synthesizer. The relative phase angle θ and relative modulation m values of the fluorescence signal are then determined for each diode taking the excitation scattered line phase and modulation as reference ($\theta_i = \Psi_i - \Psi_{exc}$, $m_i = M_i/M_0$).

The acquisition procedure and data processing were done using ISS software (kindly provided by E. Gratton).

RESULTS AND DISCUSSION

The spectral contribution of TMRM mixed either with hypericin (Fig. 2A) or with both hypericin and hypocrellin-A (Fig. 2B) in DMSO solution were first obtained in this way. Each contribution is characterized by a specific fluorescent lifetime (i.e., 2.9, 5.4, and 1.3 ns, respectively) and actually corresponds to previously reported model spectra of these compounds [1,5].

Cellular experiments were then performed on single mouse fibroblasts (3T3 cell line) incubated with the fluorescent pH probe Snarf-1 AM [1]. Cell staining procedures were the same as those reported previously [7]. As shown in Table I for the signal detected on diode 483, minimization of the phase and modulation data of Snarf-1 fluorescence is better resolved by three distinct lifetime components (i.e., 0.66, 1.22, and 4.2 ns) than only two. A parallel minimization procedure performed on several diodes leads to the same lifetimes and allows determination of the spectral shape of each component. These fluorescent lifetimes are similar to those described previously in the literature for the protonated free form, the unprotonated free form, and the intracellular bound form of Snarf-

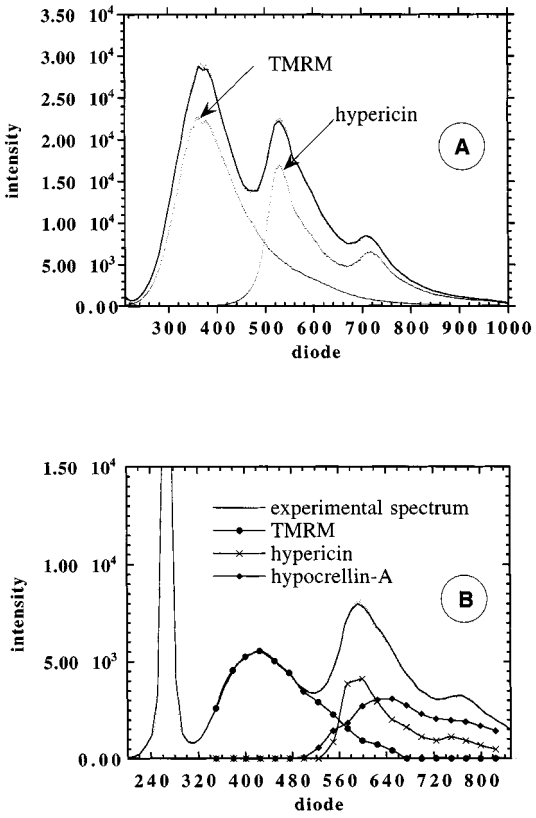

Fig. 2. Fluorescence spectral decompositions based on lifetime contibutions were obtained on TMRM solutions mixed with either (A) hypericin or (B) hypericin and hypopcrellin-A.

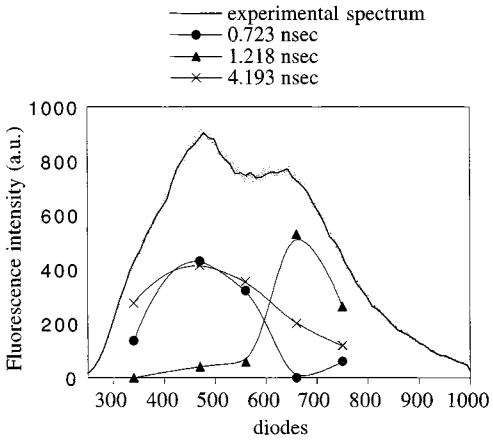

Fig. 3. Spectral decomposition of intracellular Snarf-1 using multiwavelength lifetime determination. The 0.723-ns component corresponds to the free protonated form of Snarf-l, the 1.218-ns component to the unprotonated form of Snarf-1, and the 4.193-ns component to the intracellular bound species of Snarf-1.

1 (Fig. 3) [8,9]. Actually the third component, due to some intracellular binding, is responsible for the discrepancies between conventional and intracellular data [10]. In this way time-resolved fluorescence microscopy could correct probe binding while estimating the absolute intracellular pH from the free protonated and unprotonated contribution of the probe as reported previously [8]. These preliminary results give clear evidence of the capacity of the multiwavelength phase modulation lifetime determination method at the cellular level.

Finally, it should be noted that the sensitivity of the method was determined experimentally to be only one order lower than that of steady-state detection on our microspectrofluorimeter but that the spatial resolution is not affected and can reach 0.5 μm in the confocal mode [11]. However, one important limitation of the methods is due to the absolute necessity for stability of the cellular sample during phase modulation detection from one frequency to another to get proper results, especially on cellular samples. Further development of the system can be achieved (i) by replacement of the actual master synthesizer (10 kHz–120 MHz) to reach higher modulation frequencies (up to gigahertz) and (ii) by the use of square or triangular modulation waves as excitation to get multifrequency analysis at the same time using the Fourier transform method. In this way problems due to nonstability of the biological samples (i.e., single cells) and/or of the fluorescent probes during measurements can easily be circumvented.

Table I. Results Obtained from the Global Analysis Minimization Procedure Applied to Phase and Modulation Data Recorded on Diode 483 with Either One, Two, or Three Components[a]

		1-component fit		2-component fit				3-component fit					
Sample	Diode No.	τ	χR^2	τ_1	τ_2	$f_1{}^b$	χR^2	τ_1	τ_2	τ_3	$f_1{}^b$	$f_2{}^b$	χR^2
Snarf-1	483	1.22	16.17	2.21	0.11	0.56	1.76	0.66	1.2	4.2	0.64	0.16	0.99

[a] τ_i are given as ns.
[b] $f_1 + f_2 + f_3 = 1$.

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