Measuring Fluorescence Decay Times by Phase-Shift and Modulation Techniques Using the High Harmonic Content of Pulsed Light Sources (*)

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Summary. — In the present work we discuss the possibility to measure fluorescence lifetimes by coupling pulsed excitation with phase-shift and amplitude-modulation detection techniques. We show that, because of the high harmonic content of narrow light pulses, the sample may be considered as simultaneously excited with a set of modulation frequencies up to the GHz region with noticeable power. By measuring then the phase-shift and modulation ratio of fluorescence with respect to the exciting light in the high-frequency region, impressive time resolutions may be achieved. On the other hand, since we dispose of a wide range of modulation frequencies, the problem of the multiexponential decay may be easily handled and has an exact analytical solution, provided the signal-to-noise ratio is good enough. We anticipate the possibility to introduce cross-correlation methods in order to perform the experiment in the very-low-frequency region. A short discussion about the notion of time resolution for this kind of measurement is also included.

1. — Introduction.

Atomic and molecular excited states can deactivate through a variety of relaxation channels, one of them being the radiative one. This emission of light (called in general luminescence), and particularly its time properties,

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carries valuable information about atomic and molecular dynamic properties, and it is one of the few experimental events that can provide first-hand information on these properties.

Luminescence phenomena span through an impressive number of orders of magnitude (from many seconds, as in phosphorescence from metastable excited states, to as short as $10^{-15}$ s or less, as in X-ray fluorescence), and it is easy to understand the interest to obtain radiative decay rate with great accuracy as well as to be able to measure very short fluorescence lifetimes in order to check theoretical models on molecular relaxation (photophysical and photochemical) processes.

Also the fluorescence time properties of a chromophore are in general very sensitive to its microenvironment conformational changes. By inserting suitable fluorescent probes (or with intrinsic chromophores, such as aromatic aminoacid residues) on biological macromolecules, such as proteins for example, and by monitoring fluorescence time properties as a function of relevant experimental parameters, such as temperature, pH, ionic strength, substrate concentration, etc., one can retrieve information on intraproteic dynamics related to enzymatic activity. Thus time-resolved spectrofluorometry is also becoming an important and widespread tool in biophysics and biochemistry.

Fluorescence decay measurements with nanosecond resolution are now routine operations, but in the last few years the field has been expanding and is fast moving towards the picosecond resolution mainly through the irruption into the trade of the so-called « picosecond » and « subpicosecond » pulse lasers. However, the extremely high density of photon excitation developed by these devices, when dropped inside the protein building, could induce complex relaxation phenomena from highly excited species, masking the subtle intrinsic protein relaxation patterns. Literature reports on fluorescence lifetimes of the same material system excited, on the one hand, by conventional (weak) sources and, on the other hand, by high-power lasers are often inconsistent with each other.

We feel the need for a method which, by perturbing the system under study as little as possible through a weak excitation, could nevertheless generate data on fluorescence decay times with great accuracy and high time resolution.

In the present work, after an analysis of typical methods to measure fluorescence lifetimes, we will study the properties of some kinds of pulsed light sources in the Fourier space in order to show that, by time and frequency cross-correlation procedures, it is possible to devise a new method to reach fluorescence decay times with extremely high time resolution.
2. Classical methods to measure fluorescence decay times.

Fluorescence decay data are obtained fundamentally by two distinct methods (1), conditioned as usual by technical developments.

2'1. Pulsed excitation. — Here excited states are « prepared » by an in-time (as narrow as possible) pulse of electromagnetic radiation; then the fluorescence photon emission probability is measured after \( t = 0 \) (the \( \delta \)-excitation ideal case), for every \( \Delta t \). Practically it can be done either by sampling the photocurrent of a gated photomultiplier (stroboscopic method), or by sampling the photodetector output amplitude for every \( \Delta t \) (pulse-sampling methods using fast sampling oscilloscopes or ultrafast streak cameras in conjunction with high-power pulsed lasers), or by statistical counting of individual photons (single-photon counting—SPC—method), the latter (SPC) being the most popular because of the low level of excitation needed, wide dynamical range, high sensitivity and excellent signal-to-noise ratio.

Pulsed methods, and particularly SPC, measure the fluorescent event in the real time-space

\[
I(t) = g(t) \ast F(t) + P.
\]

The (experimental) stored data, \( I(t) \), give directly the fluorescence time dispersion, \( F(t) \), obviously convoluted by the apparatus response function, \( g(t) \), on top of which parasitic contributions \( P \) pile up. It is then clear that \( F(t) \) represents the true fluorescence decay shape, free of \textit{a priori} assumption, allowing for the multicomponent decay analysis, most interesting in photochemical and photophysical processes. This is the very important advantage of the method; however, the instrumental function

\[
g(t) = I_0(t, \lambda) \ast R_{\text{pm}}(t, \lambda')
\]

is the convolution of the excitation function, the light pulse shape \( I_0 \) (wavelength dependent in general, except in the case of synchrotron radiation (2)) by the response function of the photodetector \( R_{\text{pm}} \) (also wavelength dependent (3)), but \( \lambda \neq \lambda' \) except in the obvious case of resonant fluorescence), and, even in the case of a very narrow pulse excitation, it is significantly broadened by \( R_{\text{pm}} \) of commercially available high-gain photomultipliers. The time resolution of the experiment is consequently limited to the near subnanosecond region, although important improvements have been made by introducing new kinds of pulsed excitation sources, such as synchrotron ra-

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(2) R. Lopez-Delgado: Opt. Commun., 27, 195 (1978).
(3) B. Sipp, J. A. Miehe and R. Lopez-Delgado: Opt. Commun., 16, 202 (1976).
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diation (4) and mode-locked synchronously pumped dye-lasers (5). Also, as
can be clearly seen in eq. (1), deconvolution procedures have to be applied to
experimental data in order to recover \( F(t) \). For a single exponential decay
the mathematical treatment of data might be relatively simple and straight-
forward when the rate of decay is comparable to, or longer than, \( R_{PM} \) (provided
the decay of \( I_o \) is superexponential).

However, in the multiexponential decay case (heterogeneous excited pop-
ulation decaying radiatively with more than one independent rate constant)
a far more sophisticated treatment is needed, the final result presenting a con-
siderable degree of ambiguity (strongly depending on \textit{a priori} assumptions
concerning the adopted physical model). Many interesting means of data
analysis for \( F(t) \) recovery have been discussed in the literature in the last few
years; for example, the method of moments (6), the nonlinear least-squares
treatment (7), the method of modulating functions (8), the Laplace-transform
procedure (9) or the fitting of \( I(t) \) in Fourier space (10) (with the « fast-
Fourier-transform » algorithm). Nevertheless, the ambiguity introduced by \( R_{PM} \)
(eq. (1)) will persist whatever the mathematical treatment is.

2'2. Modulated excitation. – By this method the material system under
study is excited by sinusoidally intensity-modulated light at a given frequency;
the fluorescence output will also be modulated at the same frequency, but phase-
shifted with respect to the incoming excitation wave phase, because of the in-
trinsic lifetime of the thus « prepared » excited state (11). Also a change of
the outgoing-beam modulation amplitude relative to the incoming one will
be observed, because again of the delay introduced by the excited-state lifetime.

Both phase-shift and modulation ratio are simply related to the exci-
date lifetime \( \tau \) by the expressions

\[
\begin{align*}
\text{(3)} \quad \tan \varphi &= \omega \tau , \\
\text{(4)} \quad R &= \frac{M_F}{M_R} = \frac{1}{\sqrt{1 + (\omega \tau)^2}}.
\end{align*}
\]

References:

(4) L. Lindqvist, R. Lopez-Delgado, M. M. Martin and A. Tramer: Fluorescence
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Symposium of Synchrotron Radiation, Daresbury (U. K.), January 1973, edited by
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where $\varphi$ is the angle of dephasing between the incoming and outgoing waves, $\omega$ is the angular frequency and $T_\omega$ the oscillation period of both waves; $M_e$ and $M_f$ are, respectively, the excitation and fluorescence amplitudes of modulation.

It is easy then to understand that with sufficiently high modulation frequency and accuracy on the phase measurement we may expect to reach picosecond resolution (12), even with small fluorescence intensities (13). Another important advantage of this method, as compared with SPC, is the absence of «deconvolution» problems. The fluorescence may be detected with ordinary photomultipliers, since the time-resolving power of this kind of fluorometry is no longer restricted by the pulse response capability of the detector.

However, the method has many limitations; eqs. (3) and (4) show already an intrinsic one: the assumption of a single exponential decay $\tau$ for a given modulation angular frequency $\omega$; at the same time high resolution is only achieved by very-high-frequency modulation, consequently meeting severe technical problems; also phase-shift and modulation techniques are more sensitive to signal-to-noise ratios than the pulsed-excitation methods.

Nevertheless, since the pioneering work of Gaviola (14), the technique has been significantly improved by Weber and co-workers (15) and by the later application of CW powerful lasers coupled with fast electro-optic modulators (Pockels cells). It thus became possible to analyse the case of the multiexponential decay by using a set of modulation frequencies, but up to now the problem of photon «color effect» on photomultipliers has not found a satisfactory solution because of the concomitant change in the refraction index of acousto-optic and electro-optic modulators with changing wave-lengths.

3. - The new method: coupling pulsed excitation with phase and amplitude measurements.

In what follows we will show that, by coupling narrow-pulse fast repetitive light sources with phase and amplitude detection systems, it is possible to reach even subpicosecond resolution on the fluorescence lifetime measurement in a very simple way and by using conventional photomultipliers; the method that we are going to describe here also allows for the analysis of the multi-exponential decay case. The possibility of coupling both classical methods of fluorescence time dispersion measurements has already been foreseen, in a very intuitive way, for the case of excitation with synchrotron radiation (2);

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(13) A. Muller, R. Lumry and H. Kokubun: Rev. Sci. Instrum., 36, 1214 (1965).
(14) E. Gaviola: Z. Phys., 42, 852 (1927).
(15) R. D. Spencer and G. Weber: Ann. N. Y. Acad. Sci., 158, 361 (1969).
in the present work we will try to rationalize the analysis on the basis of the source harmonic content, extending it to all kinds of fast repetitive light sources, mainly to mode-locked synchronously pumped dye-lasers and to thyratron-triggered (1) and free-running « nanosecond » flash-lamps (1).

3.1. The harmonic content of the light source. – Suppose, for the simplicity of the discussion, that our source consists of a series of equally spaced (constant repetition frequency \( v_0 = 1/T_0 \)) pulses with constant shape (let us say, nearly Gaussian), constant amplitude \( A \) and constant width \( 2\Delta \) (for instance, that is the description of synchrotron radiation out of an electron storage ring (16)), but it is easy to prove (see the next section) that one can tolerate relatively large excursions on the pulse period and width, still keeping a very good time resolution when comparing the phase of the exciting light and the fluorescence to each other; furthermore, the phase is not related to the amplitude, provided it keeps a high enough value (this is the classical problem of signal-to-noise ratio).

Now the intensity of the previously described source, or its timing function,

\[
I(t) = \begin{cases} 
A & \text{for } t \in [\Delta, 3\Delta) \\
0 & \text{otherwise}
\end{cases}
\]

Fig. 1. – a) The pulsed-source timing function; \( T_0 \): pulse period; \( 2\Delta \): pulse FWHM. b) Pulsed-source harmonic content. \( |\hat{G}(\omega)|^2 \) (source power as a function of modulation frequency \( \omega \)), \( |\hat{G}(\omega)|^2 \ast R_{PM} \) (source power convoluted by the frequency response of the photomultiplier).

\(^{16}\) R. Lopez-Delgado: Nucl. Instrum. Methods, 152, 247 (1978).
is given by the time series (see fig. 1a))

\[ f(t) = \frac{1}{\Delta \sqrt{2\pi}} \exp \left[ -\frac{1}{2} \left( \frac{t - nT_0}{\Delta} \right)^2 \right], \quad n = 0, 1, 2, \ldots, \]

whose harmonic content is given by its Fourier transform

\[ G(\omega) = \delta(\omega - n\omega_0) \exp \left[ -\frac{1}{2} (\Delta \cdot \omega)^2 \right], \quad n = 1, 2, 3, \ldots, \]

where \( \omega_0 \) is the angular fundamental frequency. The amplitude distribution as a function of frequency, or power spectrum, proportional to \( |G(\omega)|^2 \) is schematically given in fig. 1b). If \( \Delta \) is small, the harmonic content is high and consequently energy is available over a wide range of frequencies.

When now exciting the sample with such a source, the outgoing fluorescence will be modulated (eqs. (1) and (2)) at all these frequencies; therefore, information on the multiexponential decay is immediately available and, in addition, high resolution in the fluorescence lifetime measurement may be achieved by monitoring the phase shift, for the high-frequency components of the power spectrum, where noticeable intensity is still present (see table I).

Table I. - Maximum resolution for fluorescence lifetime experiment. FL: conventional flash lamp; ACO: Orsay (F) electron storage ring (ESR); SPEAR: Stanford (USA) ESR; DORIS: Hamburg (D) ESR; MLL: mode-locked laser. \( \nu_0 \): repetition (fundamental) frequency; \( 2\Delta \): pulse FWHM; \( \nu_h \): harmonic frequency whose amplitude is 10% of that of the fundamental; \( \Delta \tau_\varphi \): ultimate resolution for a 10\(^{-1}\) degree accuracy phase measurement; \( \Delta \tau_R \): ultimate time resolution for a 10\(^{-3}\) accuracy modulation ratio measurement. In the last three columns the convolution by the frequency response function of a 500 MHz cut-off photomultiplier has been taken into account.

| Source | \( \nu_0 \) (MHz) | \( 2\Delta \) (ns) | \( \nu_h \) (MHz) | \( \Delta \tau_\varphi \) (ps) | \( \Delta \tau_R \) (ps) | \( \nu_{\text{he}} \) (MHz) | \( \Delta \tau_{\varphi \text{e}} \) (ps) | \( \Delta \tau_R \) (ps) |
|--------|------------------|------------------|------------------|-------------------|-------------------|------------------|-------------------|-------------------|
| FL     | 0.04             | 1.8              | 3.8 \times 10^2 | 0.7               | 18                | \sim 3.5 \times 10^2 | 0.8               | 20                |
| ACO    | 13.7             | 1.0              | 6.8 \times 10^2 | 0.4               | 10                | \sim 6 \times 10^2  | 0.5               | 15                |
| SPEAR  | 1.3              | 0.25             | 2.7 \times 10^3 | 0.1               | 2.5               | \sim 1.5 \times 10^3 | 0.2               | 5                 |
| DORIS  | 1.0              | 0.14             | 4.9 \times 10^3 | 0.05              | 1.5               | \sim 1.5 \times 10^3 | 0.2               | 5                 |
| MLL    | (0.5 \pm 100)    | 0.01             | 6.8 \times 10^4 | 0.003             | 0.1               | \sim 1.5 \times 10^3 | 0.2               | 5                 |

The experiment can be performed with any kind of repetitive light source provided both the pulse shape and the period fulfil some requirements; the most important parameters are the pulse rise and fall times for they determine the harmonic content in the high-frequency region; the period fixes the total intensity and the spacing between the harmonics, thus governing the signal-to-noise ratios.
With this new method we show in table I that impressive time resolution may be achieved with, for example, CW mode-locked lasers and it becomes clear that at this level of resolution the optical and geometrical characteristics of the experiment have to be carefully designed, since a difference of $\sim 0.3$ mm on two light paths will already introduce a phase shift corresponding to $\sim 1$ ps.

3.2. Performing the experiment. – As stated in eqs. (3) and (4), the information on radiative decays is carried by phase and amplitude of the electric signal delivered by the photodetector. Information losses because of the frequency response function of the detector have to be taken into account and we will discuss this point below. Now, according to our new method, in principle, one has to compare the phase of the electric signal at a given frequency (for a given harmonic component) delivered by the PMT when watching the excitation source through a neutral scatter (the reference signal) as compared with the corresponding phase at the same frequency when the PMT watches the fluorescence; the experiment is then repeated for the whole set of selected frequencies.

Standard phase-sensitive detectors (vector voltmeters) need to be fed on the reference line with a sinusoidally modulated electric signal and our source produces pulsed modulation at many different frequencies; therefore, a set of very narrow bandpass electric filters is needed in order to generate reference sinusoidal waves, matching each one of the selected excitation frequencies to feed the first channel of the vector voltmeter. With ordinary readily available phase-sensitive devices phases may be measured with 0.1 degree accuracy, and in table I we show the ultimate time resolution ($\Delta \tau_c$) achieved in the fluorescence lifetime measurement with some typical pulsed light sources when the phase shift is monitored with the harmonic component whose intensity in the power spectrum is about 10% of that of the fundamental.

A far easier, although less sensitive, way to perform the experiment is by simply comparing the frequency spectra of excitation scattered light with that of fluorescence light, both directly given by the spectrum analyser (a standard electronic instrument), and finding the modulation ratio. Assuming a $10^{-3}$ accuracy on the amplitude measurement with standard equipment, we have calculated (table I) the time resolution ($\Delta \tau_R$) that may be reached by the modulation amplitude ratios (again in the 10% amplitude frequency region).

In the real experiment the quantity we measure is not the actual power spectrum $|G(\omega)|^2$, but its convolution product

$$|A(\omega)|^2 = |G(\omega)|^2 \ast R_{\text{PM}}^e$$

by $R_{\text{PM}}^e$, which is the frequency response function of the photodetector (see fig. 1b)). Its effect is to moderately decrease the signal amplitude in the low- and medium-frequency region; however, in the high-frequency region, because
of the frequency cut-off of the photodetector, the effect becomes dramatic. When $R_{PM}^P$ (by assuming a photomultiplier with $\sim 500$ MHz band width) is taken into account in the theoretical experiment (table I), the time resolution ($\Delta \tau_{nc}$, $\Delta \tau_{np}$) is still very impressive. Table I shows at the same time that beyond a certain minimum value of the light pulse width ($2\Delta t$) the harmonic content is limited by $R_{PM}^P$.

Moreover, the phototube noise is generally time uncorrelated (except for some peculiar frequencies around the 1 MHz region), then essentially flat; as the experiment is performed inside a narrow frequency band (determined by the auxiliary electric filter band width), it will result in an appreciable increase of signal-to-noise ratios.

Although the frequency analysis of synchrotron radiation, particularly from ACO (17), the Orsay electron storage ring, has shown an amazing stability in phase and amplitude, in some other pulsed sources, as in conventional flash lamps (1) for example, both pulse shape and amplitude are far from constant; but, as the harmonic content is essentially determined by the rise time of the pulse, important changes in pulse width may be tolerated without catastrophic effects on the harmonic content in the high-frequency region. Also this kind of lamp, because of the triggering system, develops relatively large excursions in the pulse period; however, on the one hand, as the reference signal is taken through a finite-width frequency filter, the «walking» period will not have an important effect on the time resolution of the experiment as long as it holds inside the filter «frequency window»; on the other hand, because the measurement is performed in a time which is very long compared with the pulse period, its «walking» is averaged and we expect the «barycenter» to keep relatively constant. Our guess is that probably period excursions as large as 20% around the barycenter could be tolerated without dramatic effects on the time resolution of the experiment.

3·3. Analysis of the multiexponential decay. – Direct measurements of the phase angle $\varphi$ and of the modulation amplitude $A$ as a function of the frequency of the harmonic component already give complete information about the heterogeneity of a fluorescent sample. In fig. 2 we show some calculations concerning two fluorophores of 1 ns and 16 ns fluorescence lifetimes, respectively, as well as a 1:1 mixture of both. However, the multiexponential problem may be analyzed in quite a different way.

Consider the addition of an arbitrary number of sinusoidally modulated components of the same frequency and variable phase and amplitude. The result will be a sinusoidal wave of equal frequency, whose phase angle $\varphi$ and amplitude $|A|^2$ are related to the phases $\varphi_i$ and amplitudes $\varepsilon_i$ of the components.

(17) M. BERGHER and R. LOPEZ-DELGADO: unpublished results.
Fig. 2. Modulation ratio calculations of fluorescence decays: a) single exponential decay, \( \tau = 16 \text{ ns} \); b) double exponential decay, \( \tau_1 = 16 \text{ ns (50\%)}, \tau_2 = 1 \text{ ns (50\%)} \); c) single exponential decay, \( \tau = 1 \text{ ns} \).

by the following expressions:

\[
\tan \phi = \frac{\sum_i \varepsilon_i \sin \varphi_i}{\sum_i \varepsilon_i \cos \varphi_i},
\]

\[
|A|^2 = \left( \sum_i \varepsilon_i \sin \varphi_i \right)^2 + \left( \sum_i \varepsilon_i \cos \varphi_i \right)^2.
\]

Suppose now that we are dealing with a set of fluorophores generating (by sinusoidal excitation) fluorescence emissions with independent exponential decays; it is well known (11) that

\[
\varepsilon_i = f_i \cos \varphi_i, \quad \tan \varphi_i = \omega \tau_i,
\]

where \( f_i \) is the contribution of the emitter \( i \) to the total fluorescence intensity and \( \tau_i \) its corresponding lifetime. By defining now the quantities

\[
N = \sum_i f_i \cos \varphi_i \sin \varphi_i,
\]

\[
G = \sum_i f_i \cos^2 \varphi_i,
\]

and introducing eq. (10) into eqs. (8) and (9), we obtain the following relation-
ships:

\begin{align}
\tan \varphi &= S/G, \\
|A|^2 &= S^2 + G^2,
\end{align}

Fig. 3. – Phase-shift measurements of a double exponential fluorescence decay. \( \tau_1 = 50 \text{ ns} \) (66%) and \( \tau_2 = 5 \text{ ns} \) (34%). • \( G(\omega) \), ○ \( S(\omega) \).

Fig. 4. – Phase-shift calculations of fluorescence decays: a) \( \tau = 1 \text{ ns} \); b) \( \tau_1 = 1 \text{ ns}, \tau_2 = 2 \text{ ns} \) (50%); c) \( \tau_1 = 1 \text{ ns}, \tau_2 = 3 \text{ ns} \) (50%); d) \( \tau_1 = 1 \text{ ns}, \tau_2 = 4 \text{ ns} \) (50%).
$q$ and $|A|^2$ are the quantities that we experimentally measure with the vector voltmeter and the spectrum analyser; therefore, we can solve the set of eqs. (13) and (14) and easily derive values for the different lifetimes $\tau_i$ and efficiencies $f_i$, since from eqs. (10), (11) and (12) it follows that

\begin{equation}
N = \sum_i f_i \frac{i\omega \tau_i}{1 + (i\omega \tau_i)^2},
\end{equation}

\begin{equation}
G = \sum_i f_i \frac{1}{1 + (i\omega \tau_i)^2}.
\end{equation}

The problem has then an exact solution since $S$ is a pure function in the case of the single exponential decay and a sum of a set of Lorentzian functions in the case of the multiexponential decay; $G$ is the imaginary part of the same function (or set of functions). This is illustrated in fig. 3 and 4 for some selected cases in different frequency spectral regions.

4. - Conclusions.

We have reviewed the two basic experimental methods to gather information on fluorescence lifetimes from excited electronic states (the pulsed excitation method, looking at the fluorescence event in the real time-space, and the modulated excitation method, watching the event in the Fourier or frequency space), showing their respective advantages and limitations. Then we have analyzed the harmonic content of a narrow-pulse fast repetitive light source in the Fourier space and show that, by coupling pulsed excitation with phase and amplitude modulation measurements, it is possible to combine the advantages of both classical techniques and to remove many of their limitations, reaching at the same time an impressive time resolution.

It should be added that one of the most interesting conclusions of this work is that, by applying the new method of fluorescence decay measurement, the mathematical treatment of experimental data produces a unique and exact solution contrary to the ambiguity of deconvolution procedures used for SPC data treatment, where the solution depends on a priori assumed physical models.

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Cross-correlation methods.

The sensitivity of our method could be substantially increased by applying classical cross-correlation methods. At the same time the measurement is rendered easier since it is now possible to work in the very-low-frequency region.

As we saw in subsect. 2.2, when a fluorescence probe is excited with sinusoidal intensity-modulated light, the outgoing fluorescence shows the same modulation, but phase-shifted. The fluorescence timing function may be written as

$$f(t) = A + B \sin (\omega_1 t + \varphi_1),$$

where $\omega_1$ is the angular modulation and $\varphi_1$ the dephasing angle.

The cross-correlation method consists of multiplying the emitted function (eq. (17)) by a sinusoidal signal, whose frequency $\omega_2$ is just slightly different from $\omega_1$. The resultant signal is the new function

$$f(t)_{cc} = [A + B \sin (\omega_1 t + \varphi_1)] [A' + B' \sin (\omega_2 t + \varphi_2)] =$$

$$= AA' + AB' \sin (\omega_2 t - \varphi_2) + A' B \sin (\omega_1 t + \varphi_1) +$$

$$+ BB' \sin (\omega_1 t + \varphi_1) \sin (\omega_2 t + \varphi_2).$$

The last term of eq. (18), the product of two sine functions, may be rewritten by using trigonometric relationships as follows:

$$BB' \sin (\omega_1 t - \varphi_1) \sin (\omega_2 t - \varphi_2) = \frac{BB'}{2} \sin [(\omega_1 t + \varphi_1) - (\omega_2 t + \varphi_2)] +$$

$$+ \frac{BB'}{2} \sin [\omega_1 t + \omega_2 t + \varphi_1 + \varphi_2] =$$

$$= \frac{BB'}{2} \sin [(\omega_1 - \omega_2) t + (\varphi_1 - \varphi_2)] + \frac{BB'}{2} \sin [(\omega_1 + \omega_2) t + (\varphi_1 + \varphi_2)].$$

If $\omega_2$ is very close to $\omega_1$, $f(t)_{cc}$ (eq. (18)) contains a constant term plus terms of frequency $\sim \omega$, plus terms of frequency $\sim 2\omega$, plus a term of frequency $\omega_1 - \omega_2$. This frequency $\Delta \omega = \omega_1 - \omega_2$ contains all the information about the phase $\varphi_1$ and the modulation amplitude $B_1$ of the fluorescence signal and its is easily filtered.

In table II we can see that, in order to achieve picosecond or subpicosecond resolution, it is necessary to use the source harmonic content in the $(10^8 - 10^9)$ Hz region. On the other side, the cross-modulation frequency $\omega_2$ may differ from $\omega_1$ by only a few hertz $(1-100)$ Hz and at this frequency it is possible to measure the phase of an electric signal with $\sim 10^{-3}$ degree accuracy.

Now, on the one hand, the cross-modulation can be obtained by using commercial mixers or by using the photomultiplier itself as a mixer; the advan-
Table II. – Time resolution as a function of the modulation frequency (ν) for a phase-shift measurement; τ ≤ 1 ns case; approximate expression.

| ν (Hz) | Resolution (ps) |
|--------|-----------------|
| 10⁶    | 280             |
| 10⁷    | 28              |
| 10⁸    | 2.8             |
| 10⁹    | 0.28            |

The resolution on the lifetime measurement.

When applying phase and modulation methods to measure fluorescence lifetimes, the resolution of the experiment is going to be in general a function of the frequency; but it will also depend on the time range and angle of dephasing. Let us illustrate the problem with a couple of examples. Suppose we are able to measure the phase with 0.1 degree accuracy at any frequency; if the decay is very short, we can use the approximate expression for the resolution

\[ Δτ \simeq \frac{\text{tg} \Delta φ}{2πν} \simeq \frac{Δφ}{ω} \]

and build Table II, where we see the time resolution increasing with frequency. However, if now the lifetime is of order of 1 ns, we have to use the exact expression for the resolution, starting from eq. (3):

\[ Δτ = \frac{Δφ}{ω \cos^2 φ}, \quad \frac{Δτ}{τ} = \frac{Δφ}{\cos φ \sin φ} \cdot \]

In this case the resolution is no longer a direct function of frequency (see Table III), but a complex trigonometric function of the dephasing angle.

The same considerations would apply for the modulation measurement. We can do a rough estimate of the resolution, for example in the case of τ ~ 1 ns; assuming ωτ ~ 1, then, from eq. (4), we have \( Δτ/τ \simeq 2(ΔR/R) \) and with ~10⁻³ accuracy in the modulation ratio the resolution will be \( Δτ \simeq 2 \text{ ps} \).
TABLE III. — *Time resolution as a function of the modulation frequency* \( (v) \) *for a phase-shift measurement; \( \tau \approx 1 \, \text{ns} \) case; exact expression.*

| \( v \, (\text{Hz}) \) | Resolution (ps) |
|-------------------------|-----------------|
| \( 10^6 \)              | 280             |
| \( 10^7 \)              | 28              |
| \( 10^8 \)              | 3.9             |
| \( 10^9 \)              | 11.3            |

Suppose again that we are able to measure \( \Delta R/R \approx 10^{-3} \) at any frequency; the resolution may be exactly calculated from eq. (4):

\[
\frac{\Delta R}{R} = \frac{\omega \tau \Delta \tau}{1 + (\omega \tau)^2} \quad \Delta \tau = \frac{[1 + (\omega \tau)^2]^3}{(\omega \tau)^2} \Delta R \tau.
\]

This equation shows that the time resolution will depend in a complex way on frequency, time and modulation. Table IV shows that, when measuring a lifetime of the order of 1 ns in the 1 MHz region, even if the modulation ratio is measured with great accuracy, the resolution is of the order of \( \pm 25 \, \text{ns} \)! One has to bear in mind these considerations when performing phase and modulation measurements.

**Table IV.** — *Time resolution as a function of the modulation frequency* \( (v) \) *for a modulation ratio experiment; \( \tau \approx 1 \, \text{ns} \) case; exact expression.*

| \( v \, (\text{Hz}) \) | Resolution (ps) |
|-------------------------|-----------------|
| \( 10^6 \)              | 25 000          |
| \( 10^7 \)              | 250             |
| \( 10^8 \)              | 4.2             |
| \( 10^9 \)              | 6.5             |

**RIASSUNTO**

Nel presente lavoro si discute la possibilità di misurare tempi di vita di stati fluorescenti eccitati da sorgenti periodiche impulsive usando misure di fase ed ampiezza nello spazio delle frequenze. Poiché un impulso molto stretto come quello che si ottiene da lampade impulsive o dalla luce emessa da un sincrotrone contiene armoniche di frequenza molto alta nella regione del gigahertz, si può pensare che il campione sia eccitato contemporaneamente da un insieme discreto di frequenze. Misurando ampiezza e fase di ciascuna armonica si può ottenere una risoluzione temporale dell'ordine del picosecondo. Inoltre il problema dell'eterogeneità del campione, cioè l'esistenza di diversi tempi di vita di fluorescenza, è facilmente risolto.

Резюме не получено.