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# Digital parallel acquisition in frequency domain fluorimetry

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The analog electronics commonly found in frequency domain fluorometers are limited to collecting only a single frequency at a time, and can be a source of systematic errors. We have developed an instrument in which most of the analog electronics are replaced with a computer-controlled digital-acquisition system. The computer is used for the direct collection of multifrequency data; filtering and calculation of the phase and modulation ratio are done by the software. From these values, fluorescence lifetimes can be determined. This new approach reduces most of the systematic errors due to the analog electronics' hardware and allows for reconfiguration of the instrument with only minor changes of the software. This digital-acquisition system is not a simple substitution of an analog element, but it provides a new function and new capabilities for frequency domain fluorometers. We have used this digital approach to build a "parallel" phase fluorometer which simultaneously collects and processes several modulation frequencies.

## INTRODUCTION

Fluorescence decay measurements can be performed in the time domain using the correlated single photon counting technique or in the frequency domain by measuring the phase shift and demodulation of the emission with respect to a sinusoidal excitation as a function of frequency. Among the advantages of frequency domain fluorimetry are high accuracy and rapid determination of fluorescence lifetimes.<sup>1-3</sup> Lifetime accuracy is ultimately limited by spurious signals which are not properly processed by the analog electronics, and thereby result in systematic errors.<sup>4</sup> The rapid determination of single- or double-exponential fluorescence decays, which can be obtained by measurements at only one or two frequencies is not possible for systems where complex fluorescence decays must be resolved. In this case, a large number of modulation frequencies is needed to obtain the full decay information.<sup>5</sup>

A block diagram of the analog electronics of a cross-correlation frequency domain fluorometer<sup>4</sup> is shown in Fig. 1. The analog electronics begin with current-to-voltage converters that change the current output from the photomultiplier tubes (PMT) to voltages. The amplified output waveform is split, with one part passing through a low-pass filter, which gives the dc value, and the other part going into an active analog band pass filter. The analog filter selects the low-frequency cross-correlated signal from the rest of the PMT output. The output of the filter is amplified, and fed into the zero-crossing detector to determine the phase and into another low-pass filter-rectifier circuit combination to determine the average ac value. Unwanted effects, not properly accounted for by the analog electronics, are caused mainly by the relatively large bandwidth and nonlinearity of the analog filters. Systematic errors can also be introduced by the peculiar noise characteristics of the zero-crossing detector used to determine the phase. The analog filters must reject harmonic frequencies by a factor of at least 200 so that

the harmonics will not interfere with the phase measurement at a given frequency.<sup>4</sup> The analog filters also suffer from center frequency drift, thermal instability of the center frequency, and amplitude dependent phase shifts. The zero-crossing detector is very sensitive to the signal noise because it uses only one point to determine the phase, and any fluctuations of this point will cause the zero-crossing detector to trigger at the wrong time, thus introducing a phase jitter. Furthermore, there is a noise associated with the indeterminate region between 0° and 360° characteristic of zero-crossing detectors.

This article describes an instrument that eliminates most of the standard analog electronics, replacing them with a digital-acquisition system. The digital-acquisition method digitizes the voltage signal immediately after the current-to-voltage converters and all signal processing is performed by software manipulation. This process removes most of the systematic errors of the standard method by improving the quality of the filters and eliminating the zero-crossing detector. The digital method also has the capability to simultaneously collect the phase and modulation data at several frequencies; resulting in a decrease in data collection time and allowing for rapid determination for complex decays. We will (1) describe the technical details of the digital acquisition and illustrate the software filtering and manipulation of the data; (2) explain the use of this system to build a parallel frequency fluorometer; and (3) present an example of a typical measurement using the parallel fluorometer.

## I. THE DIGITAL ACQUISITION SYSTEM

In the new digital-acquisition system, most of the analog electronics have been eliminated. The only analog electronic elements used are the current-to-voltage converters needed to transform the output of the photomultiplier tubes to a voltage and an amplifier to boost the signal level. The current-to-voltage converter and amplifier are built directly

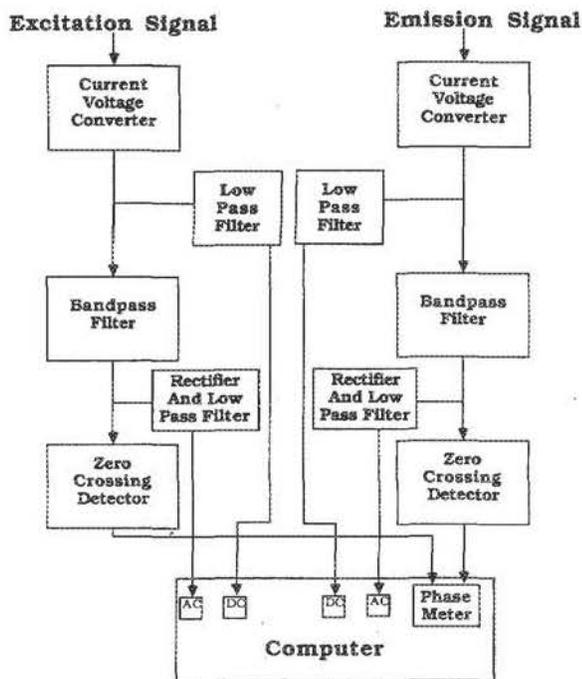


FIG. 1. Block diagram of the analog electronics of a cross-correlation frequency domain fluorometer.

into the empty slot of a commercially available data-acquisition board (Fig. 2). We have selected the MicroWay A2D-160 board because of its speed, its two collection channels, and its use of the computer's direct memory access (DMA) capabilities. This board fits into a slot of any IBM-PC compatible computer. Direct memory access relieves the central processing unit (CPU) from processing data during the acquisition period, so that data collection and storage occur in the background. Therefore, the CPU is used only for the digital filtering processes and determination of the phase and modulation values of previously acquired waveforms. The CPU is free most of the time to run normal "housekeeping" tasks, such as displaying information on the status of the instrument. The A2D-160 board has a 12-bit analog-to-digital converter with a maximum sampling rate in single channel mode of 160 kHz. In our experience, 12 bits were always sufficient to obtain good accuracy. The actual resolution is improved due to the noise level of our signal. We have estimated that in our experimental condition we have about 15- to 16-bit effective resolution. With respect to the sampling rate, we are well below the board's limits. For the measurements reported here, we have used a sampling rate of 20.48 kHz. In other measurements (not reported here), no problems were encountered up to the maximum sampling rate obtainable with the board.

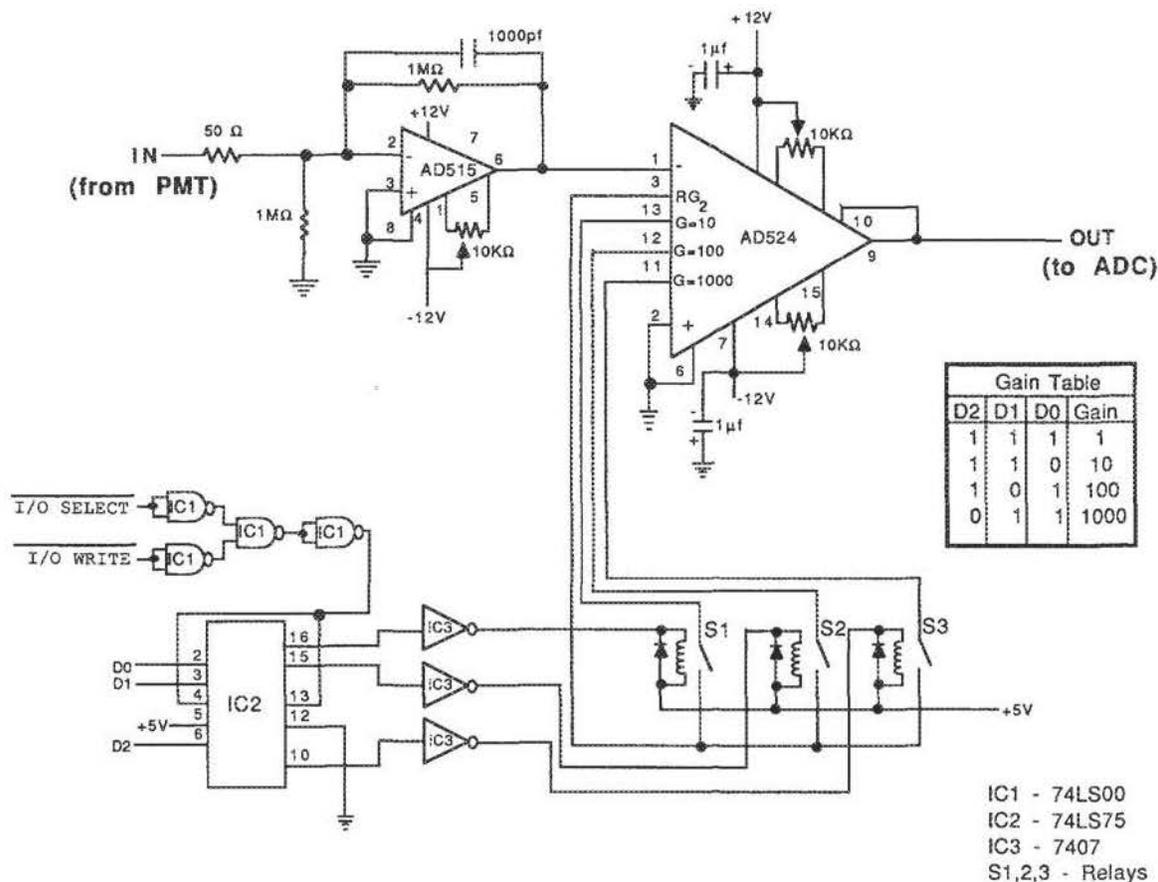


FIG. 2. Circuit addition to the A2D-160 board.

## A. Data collection and processing

In our digital-acquisition system, the most important component is the software. The main controlling program is an adaptation of the standard acquisition software used in our laboratory. The program starts by initializing the hardware and setting up data files. First, the analog-to-digital board is disabled and the on-board timer is programmed. The A2D-160 card has a 4-MHz clock which is used by an AM9513 counter chip from Advanced Microdevices. A "master reset" is issued to the AM9513, this resets and stops all counters; counter one is then loaded. This counter divides the 4-MHz clock to provide the appropriate sampling rate for the cross-correlated signal, which we have chosen to be at 40 Hz. Next, the DMA channel 1 of the IBM PC is masked, and programmed to transfer 20 480 data points from the analog-to-digital card to a storage vector in the main computer memory. The 20 480 data points represent 10 240 data points per channel, which corresponds to 256 data points per period for 40 periods. The 256 data points per period was chosen because the highest harmonic that the fast Fourier transform (FFT) algorithm, used by the filtering routine, can resolve is equal to half of the number of data points. The possibility to analyze up to the hundredth harmonic, was felt to be high enough for our application. This is not a limitation, because the number of data points per period can be increased with only a linear penalty of computation speed. Moreover, the effective number of harmonics that can be analyzed is limited by other factors. The 40-period integration range was chosen because at the cross-correlation frequency of 40 Hz used in our instrument, data are collected in 1 s, and also for the efficiency of the filtering, which will be discussed later. Channel 3 on the PC interrupt controller is masked, and a new interrupt vector, pointing to a display and save routine, is loaded. When the timer, the DMA, and the interrupt controller have been programmed, the DMA and interrupt controller are unmasked, and the timer is started. The timer is free-running, so data are collected asynchronously.

The data collection proceeds, simultaneously converting both the excitation and emission channels by using the two independent sample-and-hold circuits of the A2D-160 board. The output of the sample-and-hold circuits is sampled by the 12-bit analog-to-digital converter with a full scale range of  $-5$  to  $+5$  V. At the end of the conversion process the DMA is addressed. The DMA then transfers the output of the analog-to-digital converter into the main memory of the computer; the other sample-and-hold circuit is read, converted, and stored. The whole cycle is repeated until the 20 480 data points are collected. Once the data have been stored, the DMA generates an end-of-process which triggers the interrupt routine. The interrupt routine folds the 40 periods that arise from the 1-s integration into one, and then reduces the 256 data points into four bins, representing four phases of a period at the lowest harmonic frequency. The dc, ac, modulation, and phase of the waveform can be rapidly calculated from the values of the four bins. Those values are used only to give basic information "on the fly" about the data being collected. This information is displayed at the top line of the computer screen, and is updated every second.

This information is useful for continuous monitoring of the measuring conditions of the instrument. The interrupt routine reprograms the DMA and the interrupt controller and restarts the counter. The cycle starts again and is continuously repeated.

At the beginning of a measurement, the program sets the basic frequency of the synthesizer and asks for the reference lifetime value. A dark waveform is then digitized by repeating the interrupt cycle ten times. After the data have been collected, the averaged and folded waveform is analyzed by a FFT routine which provides additional filtering. The real and imaginary parts of the FFT are sufficient to calculate the ac, dc, phase, and modulation of up to the 128th harmonic (see the following section). These values are subtracted from the sample and reference waveforms to reduce in-phase pickup noise. After the dark waveform is measured, the sample is illuminated and the fluorescence signal is acquired. The ac, dc, phase and modulation values are determined at the same moment. The reference compound (lifetime =  $\tau_{\text{ref}}$ ) is then illuminated, and its ac, dc, phase, and modulation values are calculated. When both the sample and reference have been collected, absolute phase and modulation values are calculated using the following expressions:

$$M_{\text{corr}} = \frac{M_{\text{sam}}}{M_{\text{ref}}} (\sqrt{1 + \omega^2 \tau_{\text{ref}}^2})^{-1}$$

and

$$\phi_{\text{corr}} = \tan^{-1}(\omega \tau_{\text{ref}}) + (\phi_{\text{sam}} - \phi_{\text{ref}}).$$

The sample is again illuminated, and its modulation and phase values are determined. Absolute phase and modulation values are then calculated using the new values of the sample modulation and phase and the old reference numbers. The corrected modulation and phase numbers are averaged together, and the standard deviation is calculated. The reference sample is then illuminated and the cycle is repeated until the variance is below  $0.2^\circ$  and  $0.004$  for the phase and modulation, respectively. This entire process is automatically controlled by the on-line computer. Since the digitized waveform is stored in the computer's memory, an effective blank subtraction can be easily obtained, and it has been implemented in our system.

## B. Filtering

The digital-acquisition system excels at filtering. This operation must reject both random and harmonic noise. Simulations show that if the second harmonic has an amplitude of 0.05 of the fundamental after the filtering, and is incorrectly associated with the first harmonic, the resulting phase measurement can be off by as much as  $5^\circ$ .<sup>4</sup> This is a very large systematic error, and therefore the harmonics must be reduced to less than one part in 200 for this effect to be less than  $0.2^\circ$ . In the standard analog electronics of most commercial frequency domain fluorometers, six-pole active filters are used to perform the appropriate filtering. Precise tuning of these filters is critical to their performance, and they suffer thermal drifting problems and have amplitude-dependent phase shifts, which become a problem if the sample and reference compounds do not emit nearly equal

amounts of light. If this is the case, then the signal out of the PMT will have different amplitudes for the sample and reference cuvettes and the resulting phase shifts from the filters will introduce a systematic phase deviation. The digital-acquisition system uses a sequence of two digital filters that do not suffer from these problems.

The first digital filter is the averaging filter. Since data are collected by acquiring 40 periods in a continuous stream and folding into one period, any frequency that is not a harmonic of the fundamental will destructively interfere with itself. Also, all signals which are not synchronous with the fundamental will average out. For example, if the fundamental is at 40 Hz and a 20-Hz signal is added, then in one 40-Hz waveform there is one-half of the 20-Hz waveform and the next 40-Hz waveform will contain the opposite half of the 20-Hz waveform. When the two waveforms are folded and added, the 20-Hz signal will cancel out exactly and the 40-Hz signal will remain. The filtering action of this filter depends on the number of waveforms collected and folded. The experimental filter response of our 40 waveform-averaging filter is shown in Fig. 3(a). The points for this figure were obtained by applying a sinusoidal signal out of a HP3525 synthesizer directly to the A2D-160 board and then varying the frequency over the range specified on the figure.

An inherent property and, as we show later, an advantage of the averaging filter is that it lets the harmonics pass through. To separate the fundamental and the harmonic in-

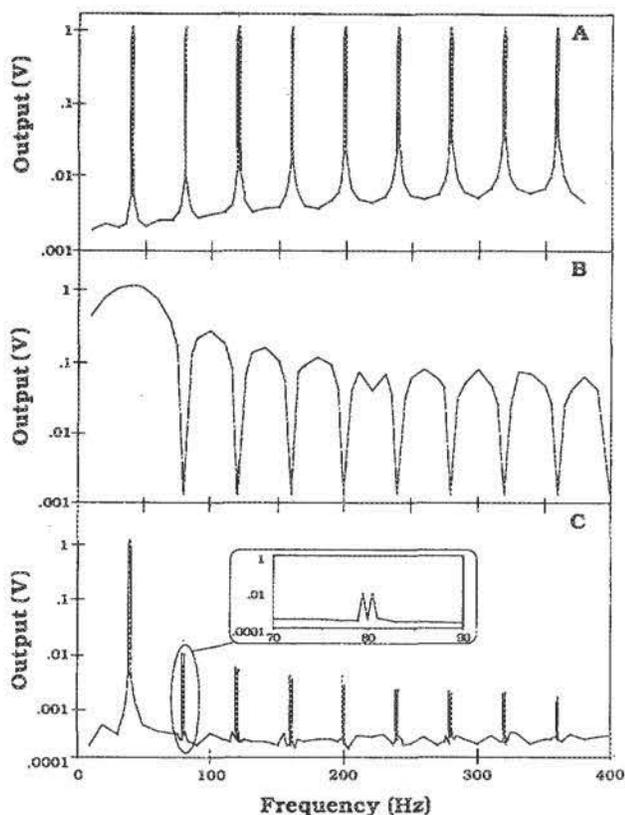


FIG. 3. (a) Filter response of the averaging filter using 10 s effective integration. (b) Filter response of the FFT routine using only the fundamental frequency. (c) Response of the two filter combined in series, using only the fundamental frequency.

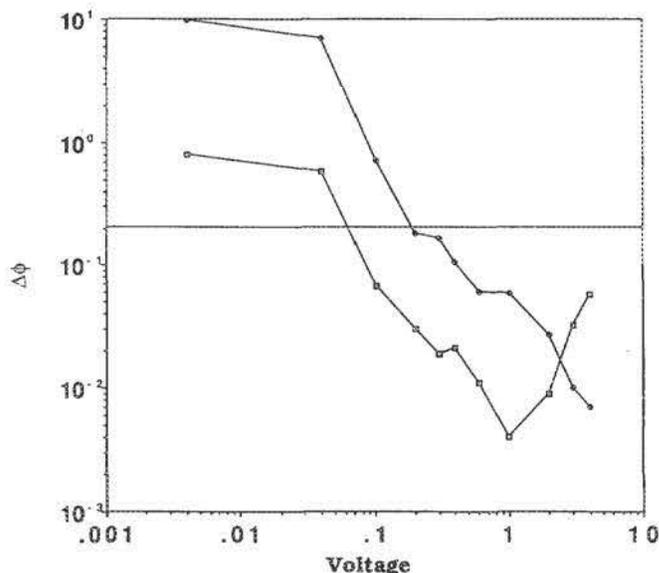


FIG. 4. Comparison of the digital (□) and analog (■) electronic acquisition. Phase errors as a function of the signal level. Noise band limited to 1 kHz of 100-mV amplitude was added to the signal.

formation, the averaging filter's output is processed by a fast Fourier transform routine. The FFT also acts as a filter, because it resolves the input waveform to a dc value, the fundamental frequency, and its harmonics. Therefore, any one of these harmonic frequencies can be rejected by simply ignoring its output from the FFT. The experimental filter response of the FFT, retaining the fundamental frequency only, is shown in Fig. 3(b) the same arrangement as in Fig. 3(a) was used to obtain the experimental points in this figure. The FFT also provides the values needed to calculate the phase and modulation of the acquired waveform. The two filters, the averaging and the FFT, are in series and the final result is the product of the two filters. The total experimental filter response, for the fundamental, is shown in Fig. 3(c). We see that the harmonics are rejected by more than a factor of 2000. This is an improvement over the current analog electronics of about a factor of 10.

### C. Reduction of systematic errors

To illustrate the advantages of the digital filter over the analog electronics, we used both methods to perform a series of measurements of phase and modulation values as a function of the amplitude of an input signal. The input signal was composed of a basic frequency of 40 Hz plus a uniform noise band limited to 1000 Hz of 100-mV amplitude. The signal at 40 Hz was varied in amplitude while the noise level was left constant. The phase of the reference with respect to the sample channel was at 180° to avoid the indeterminate region of the 0°–360° for the analog-acquisition mode, which would introduce additional phase noise. The average value and the standard deviation of ten determinations of phase are reported in Fig. 4 using the digital and the analog acquisition mode. Above 1-V signal, both methods provided adequate response: the average deviation and the standard deviation of the phase value were within 0.1°, a value commonly con-

sidered adequate for frequency domain fluorimetry. When the signal-to-noise ratio became smaller, the performance of the digital-acquisition system was clearly superior to the analog electronics (Fig. 4). The experimental conditions used in this test were typical of most of the measurements in frequency domain fluorimetry where the signal-to-noise ratio is generally about ten. The ability to accurately determine very low signals is crucial for the parallel fluorometer concept to be described in the next section.

## II. PARALLEL PHASE FLUOROMETER

The newly developed digital-acquisition system has the intrinsic capability to separate out all of the harmonic information of the cross-correlated signal. We can exploit this capability by using a light source that has high harmonic content, such as a pulsed laser system, or by pulsing the Pockel's cell modulator<sup>6</sup> used in most phase fluorometers and cross correlating with a waveform that contains harmonics. A mode-locked laser system is known to have a frequency content of several gigahertz, and pulsing the Pockel's cell or other light modulation systems can increase their frequency content. Thus, total data-acquisition time can be greatly decreased by simultaneously acquiring many frequencies in a parallel phase fluorometer.

### A. The principle of cross-correlation parallel phase fluorimetry

A single-frequency cross-correlation phase fluorometer was first described by Spencer and Weber.<sup>7</sup> In the parallel

phase fluorometer, the operating principle is the same, but it is extended to cover the harmonics of the cross-correlation signal. When a fluorophore is excited by a pulsed light source, the fluorescence has the same frequencies as the excitation, but each harmonic frequency is demodulated and phase shifted differently with respect to the exciting light.<sup>2,8-11</sup> The modulation ratio  $M$ , and the phase shift  $\phi$ , are related to the fluorescence lifetime  $\tau$ , by

$$\tan \phi = \omega\tau,$$

and

$$M = M_f/M_c = [\sqrt{1 + (\omega\tau)^2}]^{-1}$$

where  $M_f$  and  $M_c$  are the modulation of the fluorescence and the excitation, respectively. The frequency content of the fluorescence can be written as

$$F(t) = F_0 \left( 1 + \sum_{n=1}^N M_{fn} \cos(n\omega t + \phi_n) \right),$$

where  $F_0$  is the average fluorescence. The cross-correlation technique mixes the fluorescence signal with a cross-correlation signal,  $C(t)$ , which is at a slightly different base frequency,  $\omega_c$ :

$$C(t) = C_0 \left( 1 + \sum_{k=1}^K M_{ck} \cos(k\omega_c t + \varphi_k) \right).$$

The resulting signal is the product of  $V(t) = F(t)C(t)$ .

$$V(t) = F_0 C_0 \left[ \left( 1 + \sum_{n=1}^N M_{fn} \cos(n\omega t + \phi_n) + \sum_{k=1}^K M_{ck} \cos(k\omega_c t + \varphi_k) \right) \right. \\ \left. + \sum_{n=1}^N M_{fn} \cos(n\omega t + \phi_n) + \sum_{k=1}^K M_{ck} \cos(k\omega_c t + \varphi_k) \right].$$

The last term can be rewritten using trigonometric relationships as the sum and difference of the two frequencies. If we look at only the lowest frequency in this expansion, with  $n = k$ , the only term contributing to this frequency region is

$$\sum_{n=1}^K \frac{M_f M_c}{2} \cos(n\Delta\omega t + \Delta\phi),$$

where  $\Delta\omega = \omega_c - \omega$ ,  $\Delta\phi = \varphi_i - \phi_j$ . This series ends at  $n = K$  since we have assumed  $K < N$ , i.e., the cross-correlation signal has less harmonic content than the fluorescence signal. This expression contains all of the phase and modulation information of the original fluorescence signal at all the harmonic frequencies, now as harmonics of  $\Delta\omega$ , but if  $\omega_c$  is very close to  $\omega$ , then this information is at much lower frequencies that are easier to isolate and sample with our digital electronics. In the instrument described here,  $\Delta f = \Delta\omega/2\pi$  was set to 40 Hz. Figure 5 illustrates the process of frequency translation in a parallel phase fluorometer.

### B. Generation of harmonic cross correlation

For parallel phase fluorimetry, a high harmonic content in both the light modulation and in the cross-correlation signal is required. If high repetition pulsed sources such as mode-locked lasers and synchrotron radiation are used, the light modulation problem is solved, since these sources intrinsically contain a high harmonic content.<sup>10</sup> Traditionally, the cross-correlation product is obtained by applying an appropriate voltage to one of the dynodes of the photomultiplier tube. This internal mixing is quite powerful, since the PMT itself is a very good mixer. The PMT dynode chain produces good amplification with very low noise,<sup>11</sup> and it does not require any extra components. Recently, Lakowicz and coworkers have successfully implemented a different method, employing an external mixer and amplifiers.<sup>3</sup> This configuration was used in conjunction with microchannel plate detectors, but of course, it can be used with photomultipliers as well. Using the external mixer configuration, the

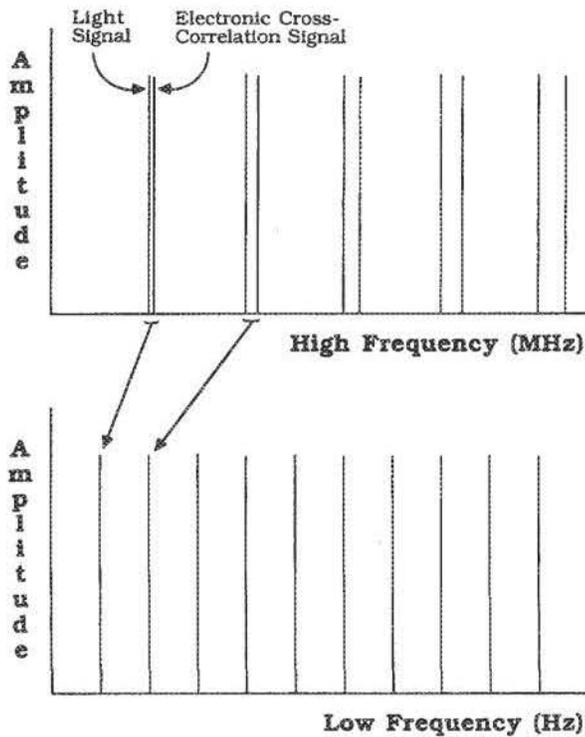


FIG. 5. Frequency translation obtained with the parallel cross-correlation method. All harmonic information is transferred to the low-frequency region.

output of the detector contains the full harmonic content of the fluorescence signal.

To analyze this signal using the parallel frequency method with cross correlation, we apply the output of a high-frequency pulser (E-H Research Labs, model G750) to one of the inputs of the mixer. The pulser is triggered by the

frequency synthesizer (Marconi, model 2022C) set at a frequency of 1 MHz + 40 Hz. The pulse width was about 2 ns. The harmonic content of the pulser, as measured using a Hewlett Packard model 8590A spectrum analyzer, is shown in Fig. 6 (for clarity in this figure the basic frequency was changed to 5 MHz). The characteristic  $\cos^2 x$  envelope seen in this figure is due to the finite width of the pulse. Of course, the overall bandwidth can be improved by using narrower pulses. The low-frequency portion of the mixer output measured using the spectrum analyzer is shown in Fig. 7. This low-frequency signal can be easily acquired and analyzed by our digital-acquisition electronics. A shortcoming of this approach is that the harmonic content density is linear in the frequency scale, while it is more appropriate to analyze frequencies on a logarithmic scale. In other words, very narrow pulses are needed to generate harmonics over a factor of 1000 in frequency. The result is that the duty cycle of the acquisition process is very low. The mixer is turned on only briefly, for a time of  $\sim 2$  ns, and then it is off for about  $1 \mu\text{s}$  at the base frequency of 1 MHz. The amplitude of the detector signal is then multiplied by  $2 \text{ ns}/1 \mu\text{s} = 2 \times 10^{-3}$ . Due to this large attenuation of the signal, relatively long integration times are required to obtain a good signal-to-noise ratio.

Instead of attempting to acquire the entire frequency range in one measurement, we have devised an approach which acquires the range from 1 to 500 MHz in three steps. First, the pulser is set at a frequency of about 1 MHz with a pulse width of 100 ns. The spectral content of this signal is shown in Fig. 8. The duty cycle becomes 1/10 with a reduction of only a factor of 5 with respect to the standard single frequency mixing (duty cycle 1/2). Using this pulsed cross-correlation signal, nine different frequencies can be collected in the range from 1 to 9 MHz. Then, the base frequency of the pulser is set at 10 MHz, with a pulse width of 10 ns so that

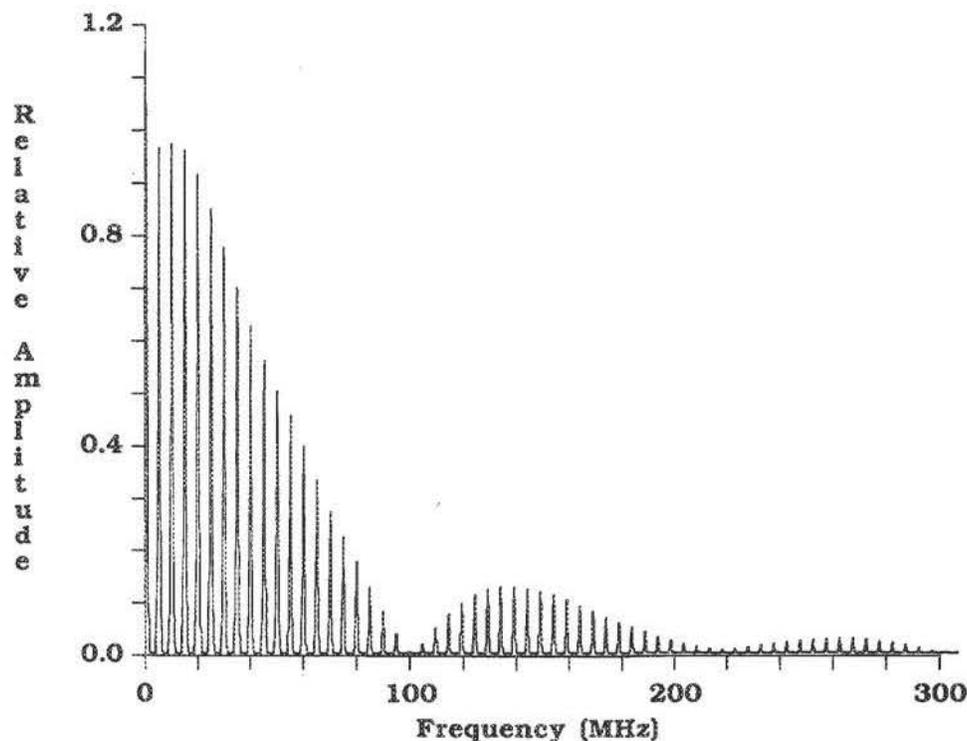


FIG. 6. Harmonic content of the cross-correlation pulse applied to the mixer using a 2-ns pulse at a repetition rate of 5 MHz.

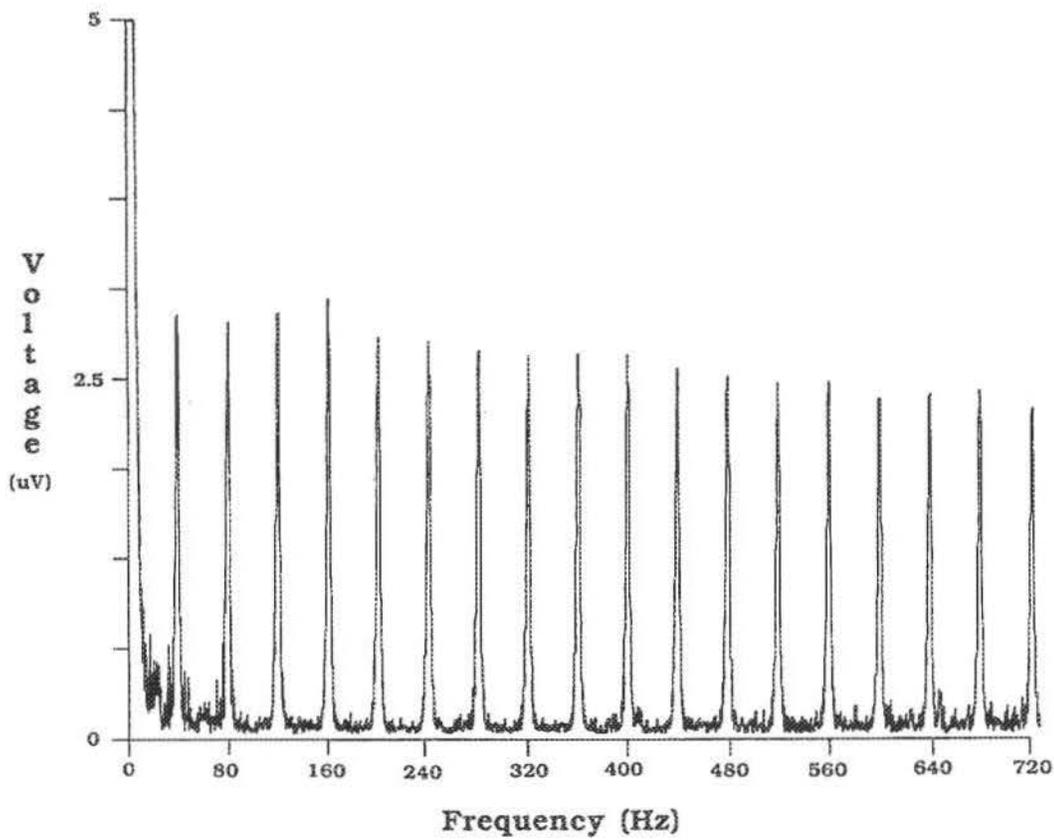


FIG. 7. Harmonic content of the laser excitation beam translated to low frequency with a cross-correlation pulse of 2-ns pulse width and a repetition rate of 1 MHz.

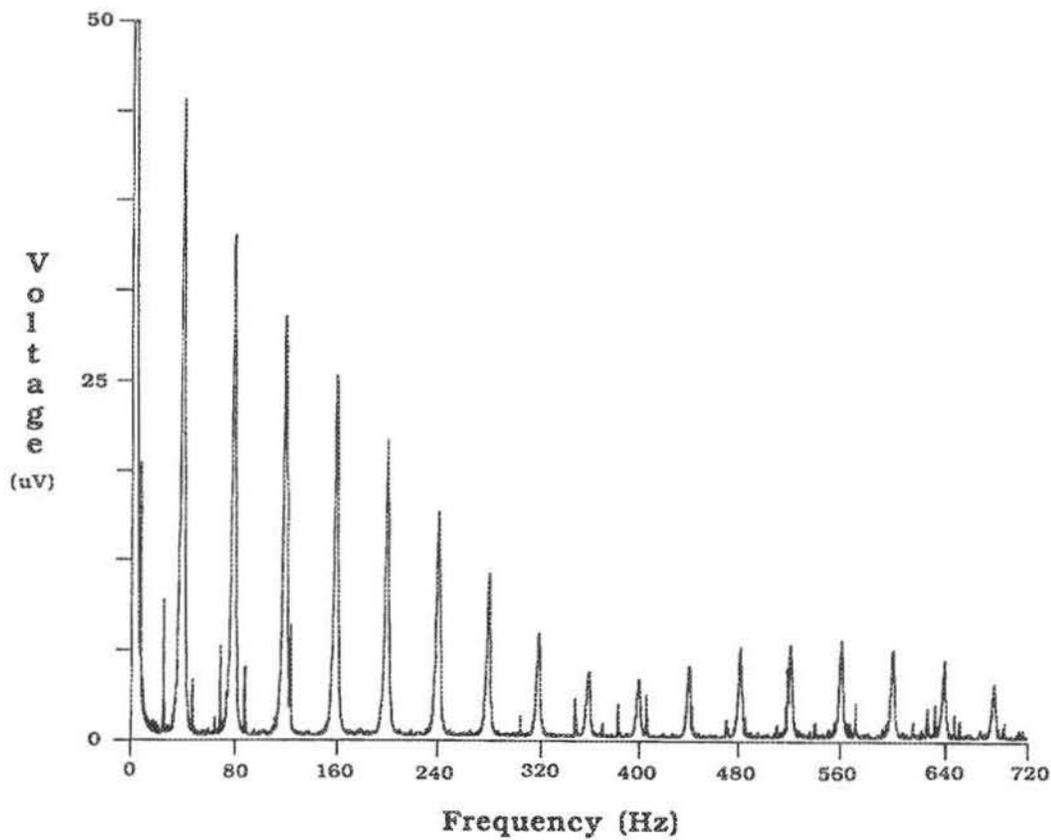


FIG. 8. Spectral content of the cross-correlated signal where the cross-correlation pulse has a duty cycle of 1/10.

the duty cycle is still 1/10. Using this pulse, nine more frequencies are collected in the range from 10 to 90 MHz. Finally, the base frequency of the pulser is set at 100 MHz and the pulse width to about 2 ns (the limit of our pulser) and frequencies are collected from 100 up to about 250 MHz. This frequency limit is imposed by our detector (Hamamatsu R928) and by the fluorescence characteristic of the emitting substance. Since this process is sequential, it can also be used with the PMT as the mixer by applying the pulsed voltage in the usual dynode gain modulation technique. At present, we have implemented this three-step acquisition, using PMT-direct mixing. Eventually, using three external mixers, this process will become fully parallel. The reduction in acquisition time with respect to the normal non-parallel mode is about a factor of 10, since about ten frequencies are collected simultaneously. A fully parallel frequency domain fluorometer with gigahertz frequency response using microchannel plate detectors can be built using state-of-the-art pulsers. Operating in parallel, the three or more external mixers of this hypothetical fluorometer would decrease the acquisition time by an additional factor of 3 or more.

### III. EXAMPLES

A typical measurement from the parallel fluorometer is shown in Fig. 9. The phase and modulation values for a solution of POPOP in alcohol are shown together with the best fit for a single exponential decay. The excitation source is a mode-locked Nd-YAG laser which synchronously pumps a dye laser (Antares model, Coherent, Palo Alto, CA). The output of the dye laser is cavity dumped and doubled to obtain ultraviolet (UV) light pulses. This pulse repetition rate is exactly 2.0 MHz. The quality of the data acquired in parallel, using a 10-s integration time for each of the three base-frequency-acquisition modes, is better than the data obtained by the standard sequential mode using the analog electronic acquisition and 10 s integration time for each point. Note that with the parallel mode the entire decay was acquired in 60 s, as compared with 540 s effective integration time needed for the normal sequential mode. The actual acquisition time in the normal sequential mode was much larg-

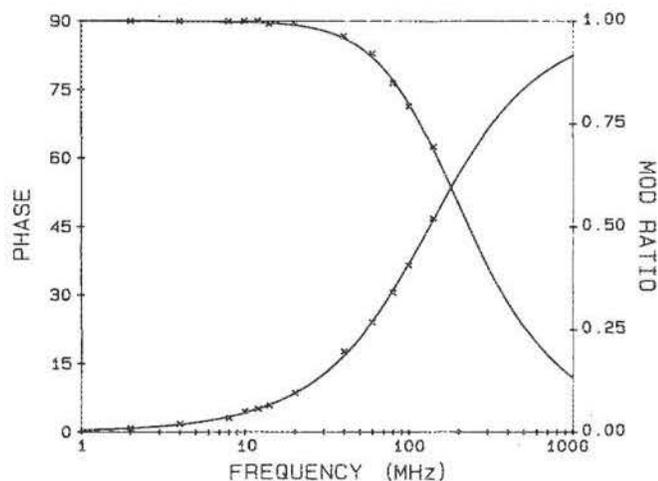


FIG. 9. Phase and modulation values of POPOP in ethanol obtained using the parallel frequency digital-acquisition method. The data are fit to a single exponential decay with a lifetime of 1.19 ns and a  $\chi^2$  of 2.6.

er (about 1000 s) due to the overhead time in manually setting the synthesizers to each new frequency and the need to acquire a dark current reading for every frequency.

### IV. DISCUSSION

The digital-acquisition method described in this article allows for much better signal filtering than the analog electronics currently used in frequency domain fluorometers and also provides for the added capability of parallel frequency acquisition. Among the advantages of the digital electronics not discussed in this article is the intrinsic capability to modify the base filter frequency by simply entering into the computer a different number for the acquisition period. Using this possibility, we have been able to determine the best cross-correlation frequency to be used on the basis of the phase noise characteristic of the synthesizer.<sup>12</sup> The cost of the digital-acquisition system is substantially reduced relative to the analog system. The A2D-160 board we have used costs about \$1000, compared to at least \$10 000 for the analog electronics found in commercial frequency domain fluorometers. The improvements given by this new digital-electronic-acquisition system can be summarized as: (1) a factor of 10 enhancement in filtering capabilities; (2) a factor of 10 reduction in acquisition time; and (3) a factor of 10 reduction in cost.

### ACKNOWLEDGMENTS

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