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Wide-band acousto-optic light modulator for frequency domain fluorometry and phosphorimetry

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Multifrequency-phase and modulation fluorometry allows for accurate analysis of fluorescence decay in the frequency domain. Essential to these frequency domain methods is a high-frequency modulation of the light source. Techniques for generating wide-band modulation of light are currently limited to the use of Pockel's cells and intrinsically modulated sources such as mode-locked lasers and synchrotron radiation. We present a method that employs two acousto-optic modulators in series for use with cw light sources. This modulator system gives two orders of magnitude more intensity output than the Pockel's cell modulator and requires less than one-tenth of the rf driving power. In addition, the Pockel's cell system is limited to modulation frequencies less than 250 MHz, whereas the particular implementation discussed here gives a quasicontinuous distribution of modulation frequencies from dc to 320 MHz. To obtain this range of frequencies, acoustic standing waves are set up simultaneously in each modulator, and the desired modulation frequency is achieved by choosing the proper combination of the two standing-wave frequencies. Light modulation is obtained at twice each of the individual standing-wave frequencies and at the sum and difference of twice the two acoustic frequencies. Data are presented to illustrate the use of this system for the measurement of phosphorescence as well as fluorescence decay.

INTRODUCTION

Until recently, multifrequency-phase fluorometry has been limited to a few selected modulation frequencies in the narrow range of 6–30 MHz.[1] During the last 5 years, the frequency range has been increased by several orders of magnitude to encompass dc to several Gigahertz.[2–5] In addition, the availability of low modulation frequencies has opened up the possibility of frequency domain phosphorimetry.[6] The measurement of phase and modulation data over this wide frequency range allows for analysis of the complex emission decays usually found in biological systems.[7,8] Due to the increased analytical capabilities of this method, a surge of interest in multifrequency-phase fluorometry has developed among many researchers.[9,10] One of the critical elements of a multifrequency-phase fluorometer is the modulated light source. Ideally, this source should be stable, provide good intensity throughout the ultraviolet and visible spectra, and have continuously variable modulation frequencies from dc to several GHz, at which point the light detector becomes the limiting factor. These criteria have so far been most closely met by intrinsically modulated sources of light such as mode-locked lasers and synchrotron radiation.[2,5] Both of these sources can provide excellent intensity and wide-band modulation. However, these sources are expensive, may not be generally available, and do not always provide the stability needed for lifetime measurements. The most commonly used technique for obtaining wide-band modulation (up to 250 MHz) from a cw light source is the Pockel's cell.[4] This electro-optical method requires a well-collimated input beam, high voltages, and at least 10 W of rf driving power for efficient modulation. In addition, due to the relatively low voltages used in the wide-band modulation scheme, the Pockel's cell attenuates the light beam considerably and does not allow sufficient intensity to be transmitted in the far ultraviolet (below 250 nm).

We report here the use of two standing-wave acousto-optic modulators to achieve wide-band modulation. These standing-wave devices differ from "normal" acousto-optic modulators, which use a traveling-wave carrier frequency that is amplitude modulated. Light passing through these traveling-wave modulators is intensity modulated at the amplitude-modulation frequency. The original modulator used in a cross-correlation frequency domain fluorometer was the Debye–Sears tank, which is a standing-wave acousto-optic modulator that uses a liquid medium for the acoustic-wave propagation.[1] More recently, standing-wave acousto-optic modulators have been used for frequency domain measurements to amplitude modulate the output of a mode-locked laser[5] and to obtain high (40–320 MHz) and very low (dc to 10 MHz) modulation frequencies.[11] In principle, using acousto-optic modulators, it should be possible to use uncollimated cw sources, such as high-pressure xenon arc lamps, which can provide good intensity over a wide spectral range. Our new standing-wave acousto-optic modulator system gives quasicontinuous modulation frequencies from dc to 320 MHz using a laser light source. Each modulator typically requires about 1 W of rf power for efficient modulation, and since the modulating medium is quartz, the modulators provide good transmission throughout both the ultraviolet and visible ranges. With temperature control of the modulators, the modulation frequencies are found to be stable and reproducible over a period of several months. In this article, our new dual-modulator system will be described and the

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basic theory of its operation outlined. Applications of the new modulator for the measurement of fluorescence and phosphorescence lifetimes will be shown.

I. APPARATUS

Two automated devices built simultaneously in Urbana and Göttingen, which differ slightly in construction, but whose operation and performance are identical, are shown schematically in Fig. 1. For the data presented in this article we use the 325-nm line from a Liconix 4240NB HeCd laser (in Urbana) and various lines from a Spectra-Physics 2035 argon-ion laser (in Göttingen), although the light source can be any cw laser with output above the quartz cutoff wavelength. The laser light passes through the two modulators in series, described in detail later. The modulated output of the second modulator is channeled (either directly or via a quartz fiber optic) to the entrance slit of an optical module designed in house. The light is then passed through a beam splitter which diverts part of the light to the excitation reference photomultiplier. The remaining light passes through a Glan–Thomson polarizer oriented at the magic angle (54.7° from vertical) to eliminate Brownian rotational effects on intensity decay measurements, is focused on the sample, and the resulting luminescence is observed at a right angle from the excitation. For fluorescence depolarization measurements an additional polarizer is placed in the emision light path. The photomultiplier housings, cross-correlation and data-acquisition circuitry, and ADC interface card for a PC-compatible computer were obtained from ISS Inc. (Urbana, IL). The second dynodes of the two photomultipliers (Hamamatsu R928) are modulated at the excitation-modulation frequency plus a cross-correlation frequency of 40 Hz. This signal is generated by a frequency synthesizer (Marconi model 2022C), which is phase locked to the acousto-optic modulator frequency, and is amplified by an ENI 403LA rf power amplifier for the high-frequency (fluorescence) measurements or a stereo amplifier for low-frequency (phosphorescence) measurements. The electronics for data acquisition operate at the cross-correlation frequency, since all the phase and demodulation information from the sample emission is preserved in the beat frequency.[1]

The key elements of our systems are the acousto-optic modulators. The modulators are custom made by IntraAction Corp. (Belwood, IL). Each consists of a fused quartz bar, approximately 1 cm × 1cm × 5 cm, whose faces are all optically flat and parallel. Along one side of the bar, lithium-niobate transducers are attached (see Fig. 2). This transducer interface is the main restriction on the high-frequency response of a modulator due to impedance-matching considerations. The transducers produce acoustic vibrations in the quartz bar, and at certain frequencies the normal modes of the quartz bar are excited and acoustic standing waves are generated. Under these conditions, the quartz bar acts as a diffraction grating which turns on and off at twice the acoustic frequency. That is, an 80-MHz standing wave in the medium produces a beam of light modulated at 160 MHz. To achieve light modulation we must have a standing wave, and consequently only a quasicontinuous distribution of frequencies is available. The standing-wave resonances in our modulators are located approximately 330 kHz apart, and generally we obtain sufficient modulation within 25 kHz of the resonance. The position of the resonance frequencies can be shifted due to the thermal expansion of the quartz bar, and we find the change to be roughly -10 kHz/°C over our frequency range. In general if the modulators are kept between 30 and 40 °C, we observe better stability of the modulated light.

The theory of standing-wave acousto-optic modulators was first described by Raman and Nagandra-Nath[12] and is described in depth in the literature.[13] The light output from a modulator consists of a zero-order or undiffracted line and several higher-order lines. The zero-order line consists of an unmodulated component which results from the light that is not diffracted even when the acousto-optic "grating" is on and of a modulated part corresponding to the light that reaches the central line when the grating is off. In our systems, the central line of the output of the first crystal is selected with an iris and becomes the input of the second crystal. With a second iris, only the zero-order line from the second modulator is chosen to be focused on the fiber-optic cable or sent directly to the sample chamber. The distance between each modulator and the following iris is approximately 1 m. This distance is required in order to separate the central line from the higher-order lines if no lenses are used to diverge the diffraction pattern.

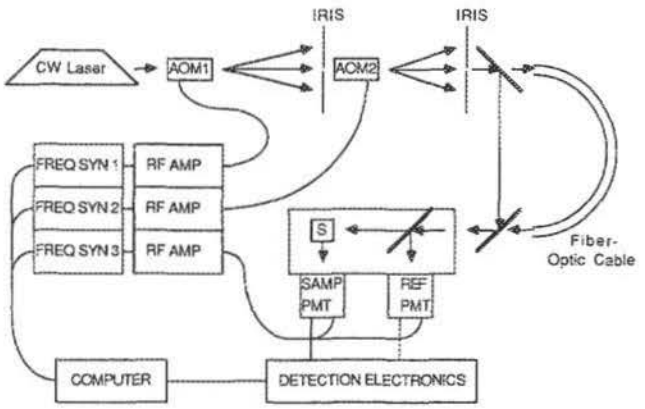

FIG. 1. Schematic layout of the instrument. The light passes from the second iris to the sample compartment either directly or via fiber-optic cable. AOM: standing-wave acousto-optic modulator; S: sample location; FREQ SYN: phase-locked frequency synthesizers; PMT: photomultiplier tubes; RF AMP: radio frequency or stereo (for low frequencies) amplifiers.

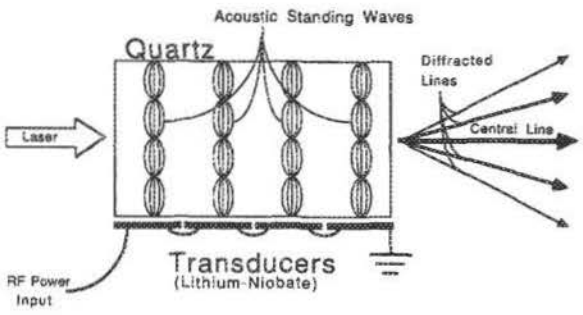

FIG. 2. Schematic drawing of one of the standing-wave acousto-optic modulators.

II. OPERATION OF THE MODULATOR

To obtain wide-band modulation, we use three different modes of the modulator series combination which are selected automatically by a computer program. As stated above, the four modulation frequencies generated at the output of the modulator series are twice each of the individual acoustic frequencies ($2f_1$,$2f_2$) and also twice the sum and difference of the two modulators acoustic frequencies $[2(f_2+f_1),2(f_2-f_1)]$. To cover the wide-band frequency range, we use three of these four frequencies as follows. For modulation frequencies below 30 MHz, we use $2(f_2-f_1)$ in what is called the difference mode; for frequencies between 30 and 180 MHz, we use the $2f_2$ component of the modulation, called the direct mode; and for higher frequencies, we use $2(f_2+f_1)$, called the sum mode.

To illustrate the generation of wide-band modulation, we examine the modulators one at a time. We define the acoustic standing-wave frequency in the first modulator to be f_1, and for the second modulator f_2. For a single modulator with incoming light of intensity I_0 and assuming that there is no light absorbed by the quartz or lost in reflections, the theoretical intensity output of the zero order line is

$$I_1 = (I_0/2)[1+\cos(2\omega_1 t)], \tag{1}$$

where $\omega_1 = 2\pi f_1$. This represents a case where, at maximum modulation, the zero-order line is completely extinguished. In practice, we find with our modulators that the maximum amount of light diverted from the zero-order line is about 80%, and so the effective modulation we obtain is

$$I_1 = (I_0/10)[6+4\cos(2\omega_1 t)]. \tag{2}$$

The central line output of the first modulator consists of light that is unmodulated and of light which is modulated at $2f_1$. The zero-order line from a first modulator is now used as the input of a second modulator. When this light passes through the second modulator, with acoustic standing waves at f_2 (we will assume $f_2 > f_1$ for simplicity of derivation), both parts interact with the diffraction grating of the second modulator, the output of which becomes

$$I_2 = (I_0/100)[6+4\cos(_2\omega_1 t)][6+4\cos(2\omega_2 t)]. \tag{3}$$

We can consider the parts of this product separately to determine the final modulation frequencies. Part of the unmodulated light remains unmodulated, while the rest of this light is modulated at $2f_2$. Likewise, part of the light which is modulated by the first crystal at $2f_1$ is unchanged by the second modulator and remains modulated at $2f_1$. By carrying through the multiplication and using the identity

$$\cos\alpha\cos\beta = \tfrac{1}{2}[\cos(\alpha+\beta)+\cos(\alpha-\beta)], \tag{4}$$

we find that the fraction of light modulated by the first modulator that is modulated again by the second modulator will contain frequencies of $2(f_2-f_1)$ and $2(f_2+f_1)$. The total intensity output of the second modulator is represented by the expression

$$I_2 = (I_0/100)\{36+24\cos(2\omega_1 t)+24\cos(2\omega_2 t) + 8\cos[2(\omega_1+\omega_2)t]+8\cos[2(\omega_1-\omega_2)t]\}. \tag{5}$$

The frequencies available at the output of the second modulator are the sum and difference of twice the two modulators' acoustic frequencies and also twice each of the individual acoustic frequencies (see Fig. 3). We can quantify the degree of intensity modulation at each frequency by its ac/dc ratio. The dc component is about 36% of the incoming cw intensity. The ac/dc ratio for $2f_2$ is $\frac{2}{3}$ or 67% modulated, and for the sum and difference modes this ratio is $\frac{2}{9}$ or about 25% modulated. In our system, the frequency of the first modulator f_1 is fixed at a standing-wave resonance of 40.04 MHz, or for modulation frequencies over 260 MHz, at 80.198 MHz. Both modulators are temperature controlled such that the resonance frequencies do not change from day to day. A standing wave is maintained in the first modulator regard-

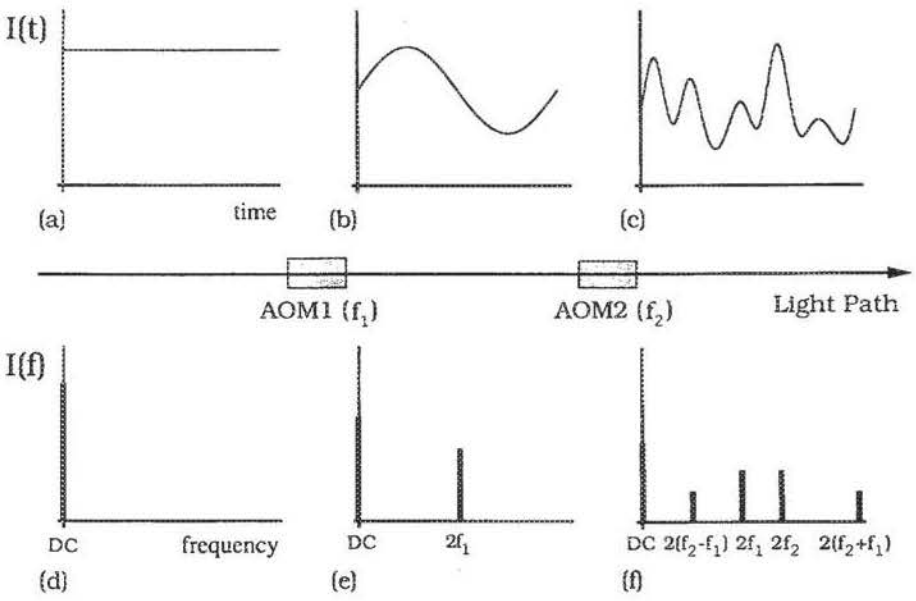

FIG. 3. Intensity profile of the light beam as it passes through the modulator series. Graphs (a), (b), and (c) show the time profile, while graphs (d), (e), and (f) show the corresponding frequency domain profiles. Graphs (a) and (d) show the profiles of the cw laser beam, (b) and (e) are the profiles after passing through one modulator, and (c) and (f) represent the output after passing through the second modulator.

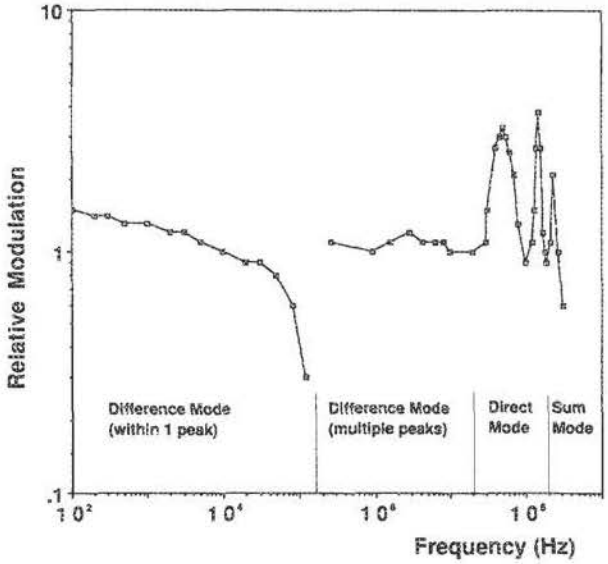

FIG. 4. Frequencies available from the acousto-optic modulator systems and their relative modulation.

less of the mode of operation so that the dc output intensity of the modulator series remains relatively constant over the entire frequency range. Using the difference mode, we obtain continuous modulation frequencies up to 100 kHz by varying the acoustic frequency of the second crystal within the resonance peak. By moving to subsequent resonances, one can produce frequencies 330 kHz apart throughout the range of 100 kHz to 30 MHz. The direct-mode modulation has a frequency of twice that of the acoustic wave in the second quartz bar. The frequency range of the direct mode is limited by the impedance matching between the transducers and the quartz bar. Since in the second modulator we can set up acoustic resonances from 15 to 90 MHz, the direct mode can be used to obtain modulation from 30 to 180 MHz. The sum mode is used to obtain frequencies above 180 MHz. For frequencies between 180 and 260 MHz, f_1 is kept at 40.04 MHz. To cover even higher frequencies (260–320 MHz), we must change f_1 to 80.198 MHz. The available frequency range and the modes of operation are illustrated in Fig. 4. The effective modulation is not constant across the frequency range due to the resonances of the transducers. During a

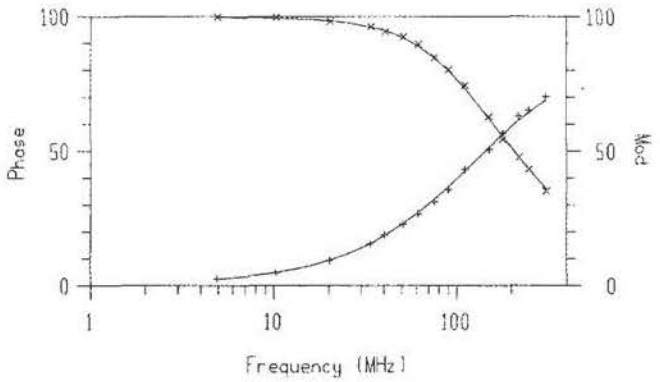

FIG. 5. Data from POPOP in Ethanol. This measurement is fit to a single exponential component with a lifetime of 1.33 ns.

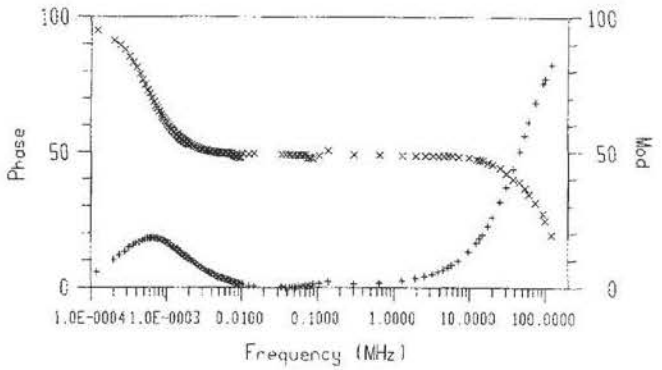

FIG. 6. Data from Eu complex and rhodamine B in ethanol that shows both fluorescence and phosphorescence in one measurement. This is a complex system that exhibits excited-state reaction behavior, but shows the ability of our instrument to acquire data in a single run from 100 Hz to over 100 MHz.

measurement, the modulation frequencies used are chosen from a look-up table by the computer and loaded into the frequency synthesizers through a GPIB interface.

III. DATA AND APPLICATIONS

To show the applicability of this new instrument, we have chosen several luminescent systems: POPOP in ethanol (Fig. 5), a tris (thienyl trifluoroacetone)-bathophenanthroline, disulfanate europium complex (Eu Complex), and rhodamine B in ethanol (Fig. 6), and a single tryptophan protein $P1$, a contractile protein of the intestinal brush border cells (Fig. 7). These data sets together demonstrate the frequency range from dc to 320 MHz and excitation wavelengths from 290 to 351 nm.

IV. FUTURE PERSPECTIVES AND CONCLUSIONS

All of the data we have presented in this article has been gathered using a laser as the excitation source; yet one of the advantages of standing-wave modulators is their large aperture, which naturally lends itself to use with lamp sources. In order to use a lamp with this acousto-optic modulation scheme, it would be desirable to use only a single modulator with both frequencies resonating in the same medium. This design has been realized using a single transducer by Piston

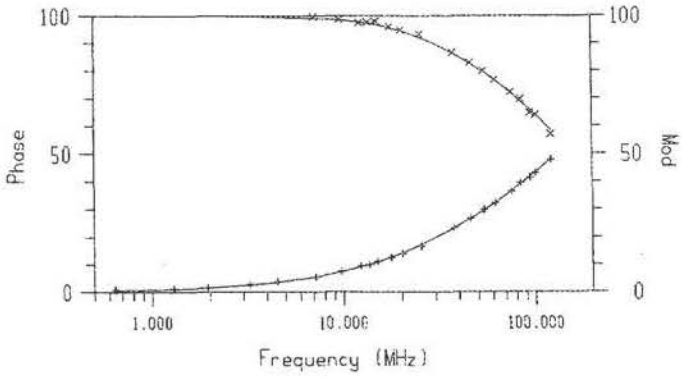

FIG. 7. Protein $P1$ data with excitation at 290 nm and emission collected through a WG320 filter. The data can be fit to a double exponential decay with lifetime values and fractions: $\tau_1 = 1.265$ ns, $f_1 = 0.705$ and $\tau_2 = 4.233, f_2 = 0.295$.

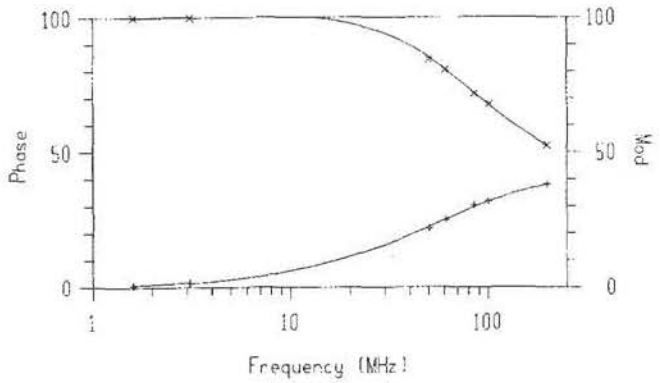

FIG. 8. Lysozyme data obtained with xenon arc lamp as excitation source, and with two standing waves mixed externally and applied to a single crystal to achieve low-frequency modulation. This data is fit to a double exponential decay with lifetime values and fractions: $\tau_1 = 2.304$ ns, $f_1 = 0.604$ and $\tau_2 = 0.311$, $f_2 = 0.396$.

and Gratton,[11] but the stability was dubious due to heating effects. We show in Fig. 8 data from lysozyme taken with the two frequencies mixed externally and applied to a single transducer, and using a xenon arc lamp as the excitation source. We have found several problems with this single-transducer method. One problem is that quartz is the only material currently used in commercially available modulators that transmits in the UV. However, the separation of the diffracted lines output from a quartz modulator is poor due to the high sound velocity and low index of refraction of quartz. The less the separation of diffracted lines, the larger the distance (or the more complicated the optics) required to isolate the central light beam. There are also heating problems where the power absorbed in the quartz from one standing wave affects the second resonant frequency and vice versa, thus making tuning difficult and introducing modulation drifts. To avoid these heating problems, one could have two transducers, one on the side and the other on the top of the crystal, which would operate independently. Also, new acousto-optic materials which provide lower sound velocities and higher indices of refraction, therefore giving greater separation of the diffraction pattern, could simplify the use of this method with lamp sources. Another future improvement may come with the development of new transducer materials. New piezoelectric polymers that often have response in the GHz range should increase the upper frequency limit of standing-wave acousto-optic modulators.

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