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A 2 GHz FREQUENCY-DOMAIN FLUOROMETER; PICOSECOND RESOLUTION OF PROTEIN FLUORESCENCE AND ANISOTROPY DECAYS

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Abstract

We developed a frequency-domain fluorometer which operates from 4 to 2000 MHz. The modulated excitation is provided by the harmonic content of a laser pulse train (7.59 MHz, 5 psec) from a synchronously pumped and cavity dumped dye laser. The phase angle and modulation of the emission are measured with a microchannel plate photomultiplier. Cross-correlation detection is performed outside the PMT. The performance was verified by measurement of known time delays and examination of standard fluorophores. The detector displayed no detectable color effect, with the 300 to 600 nm difference being less than 5 psec. The precision of the measurements is adequate to detect differences of 20 psec for decay times of 500 ps.

This new instrument was used to examine tyrosine and tryptophan intensity and anisotropy decays from peptides and proteins. The data demonstrate that triply-exponential tyrosine intensity decays are easily recoverable, even if the mean decay time is less than 1 nsec. Importantly, the extended frequency range provides good resolution of rapid and/or multi-exponential tyrosine anisotropy decays. Correlation times as short as 15 psec have been recovered for indole, with an uncertainty of ± 3 psec. We recovered a doubly exponential anisotropy decay of oxytoxin (29 and 454 psec), which probably reflects torsional motions of the phenol ring and overall rotational diffusion. Also, the 2 GHz data reveal the time-dependent (\sqrt{t}) terms found in the presence of collisional quenching.

Introduction

Resolution of time-resolved decays of fluorescence intensity and anisotropy is of considerable interest in physics, chemistry and biochemistry. At present, most measurements are accomplished by measurements in the time-domain [1-5]. During the past three years there have been substantive advances in the alternative technique of frequency-domain fluorometry. In this method one measures the frequency response of the sample, that is, the frequency-dependent phase angle and modulation. The early phase-modulation fluorometers operated at only one to three fixed frequencies [6-10], and these limited data were not adequate to recover multi-exponential decays of intensity or anisotropy [11]. During the intervening years several reports appeared describing phase-fluorometers which operated over a wide range of frequencies [10,12-14]. However, relatively little data has appeared from these instruments, and it is not clear that their precision is adequate to support the analysis of complex fluorescence decays.

The first practical frequency-domain fluorometers were developed simultaneously by two separate laboratories [15-16]. These instruments have been used to recover closed spaced decay times [16-18], closely spaced rotational correlation times [16,19-20], and to construct time-resolved emission spectra [21-22]. Until now, the upper frequency limit has been about 200 MHz. This limit was due to two factors. First, the bandwidth of a fast photomultiplier (1.5 ns FWHM) is limited to about 220 MHz [23]. Secondly, it is difficult to obtain usable light modulation above 200 MHz due to the capacitance of the light modulators. Our attempts to use traveling-wave modulators were not successful [16], apparently due to their extreme sensitivity to temperature.

Our new instrument has a usable frequency response to 2 GHz. We overcome the 200 MHz limit using two modifications of the original design [16]. First, we avoided the use of light modulators by using the intrinsic high frequency harmonic content of a laser pulse train [24-25]. Our laser source provides harmonic content at every interger multiple of 7.59 MHz, which is the repetition rate of the cavity-dumped dye laser. Secondly, we used a microchannel plate (MCP) photomultiplier (Hamamatsu R1564U). The single photoelectron pulse width of these devices (about 50 ps) is 10 to 20-fold less than that of a standard PMT [26,27] and hence we expected its bandwidth to extend to 2 GHz.

Description of the Instrument

A overall schematic of the instrument is shown in Figure 1 [28]. The light source is presently a mode-locked argon ion laser. The ion laser pumps a dual jet dye laser, whose output is cavity dumped at 7.5862 MHz. The dye laser with R6G provides excitation wavelengths from 570 to 600 nm. For excitation with ultra-violet its output is frequency doubled to 285-300 nm. The average UV power near 0.5 mW, which is attenuated 50 to 100-fold prior to the sample. Alternatively, the beam diameter is expanded to about 5 cm using a negative lens. For our application the pulse width and shape is not critical, but the pulse width of the visible output of the dye laser was 5 psec or less.

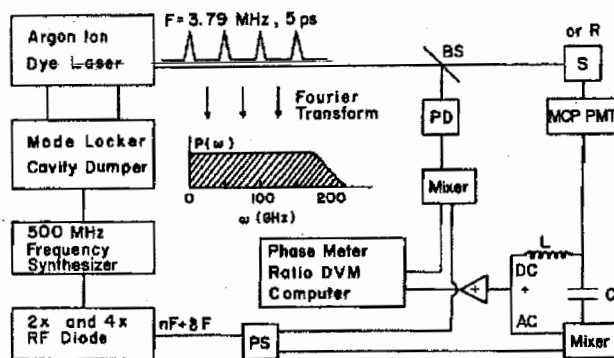


Figure 1. 2 GHz Frequency-domain fluorometer. PD - photodiode; PS - power splitter; MCP PMT - microchannel plate photomultiplier tube; BS - beam splitter; F - fundamental frequency of cavity dumped dye laser output; δF - cross correlation frequency of 25 Hz; n - number of the harmonic, S - sample; R - reference or scatter.

The pulsed laser source is advantageous in several ways. The high peak power results in rather easy and efficient frequency doubling. The UV output is essential for excitation of most fluorophores of interest, especially tyrosine and tryptophan fluorescence from proteins. The output is intrinsically modulated over a wide range of frequencies, as shown by the Fourier transform in Figure 1. Because of its intrinsic modulation the output can be used directly, rather than after passage through a modulator and associated optics. The use of an electro-optic modulator results in a considerable loss of intensity and hence decreased sensitivity. Thirdly, the output of this system is considerably more stable than that of our doubled ring dye laser. While we were able to measure protein fluorescence (285-300 nm excitation) using this source [20], its day-to-day operation was too demanding for continued measurements. Perhaps the most important feature of the pulsed laser source is that it contains harmonic output to many GHz, beyond the obtainable with any commercially available broadband modulator. Unfortunately, we are not able to use frequencies above 2 GHz because of the limitations of our detector, synthesizer and other electronic components. An interesting aspect of the harmonic method is that the Fourier components are greater than that possible with a 100% sine-modulated source [29]. Also, the dispersion of the harmonic content over a wide range of frequencies does not decrease the modulation available at each frequency. This is because all the photons in the light pulse contribute to the measurements at each frequency [30]. This may be seen by noticing that the zero frequency harmonic has the same intensity as all the higher harmonics (Figure 1). Hence, this S/N ratio is expected to be mostly independent of frequency. This is important because the data at the highest frequencies are often the most valuable in resolving rapid decays of intensity or anisotropy.

The second important feature of the instrument is the use of a MCP PMT as a detector. As presently available, the MCP PMTs do not allow internal cross-correlation. It appeared unlikely that the voltage on the intensifier plates could be rapidly varied due to their high resistance. Hence, we designed a circuit for external cross correlation. The details are provided in reference 28. We modified a 2 GHz amplifier to allow detection and amplification of the DC component of the modulated intensity, which is essential for measurement of the frequency-dependent demodulation.

The frequency range of the measurements is determined primarily by the modulation available at each frequency. The frequency-response of the entire system (laser source, amplifier and MCP-PMT) is shown in Figure 2. For phase-modulation fluorometry a modulation in excess of 20% is adequate for most measurements. The 20% limit is near 2 GHz for the MCP PMT. Modulation in excess of 50% was found to 1 GHz. The modulation values above 600 MHz appear low because of the extreme values found in the range from 20 to 600 MHz, which is due to the high amplitudes of the Fourier components in a pulse train.

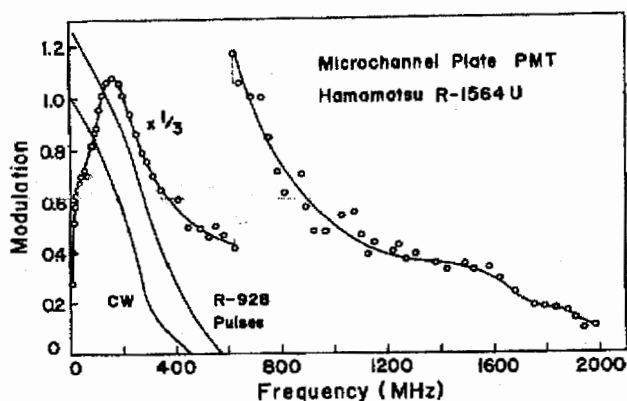


Figure 2. Frequency-dependent modulation of scattered light. The open circles are the values found for the R1564-U MCP PMT. The other lines are the approximate modulation profiles for a R928, as found for a 5 ps pulse train or a modulated CW laser beam, respectively.

Performance Testing

The performance of the instrument was evaluated in several ways. The measured time delay was linear with distance, and the measured speed of light is within 1% of the expected value. The time delays were accurate to within ± 2 ps, with only seconds of data averaging for each distance.

Our favorite test is the use of a calibrated 25 ps quartz plate from a Coherent autocorrelator (courtesy of Mr. Fred Gonzalez). The accuracy of the instrument was examined over a range of frequencies (Figure 3). The phase angles increased as expected and the measured time delays were within 2 psec of the expected value. On average, we found 27 ps for the presumed 25 ps delay. No demodulation is expected for a time delay, and no demodulation was observed to 2 GHz (Figure 3).

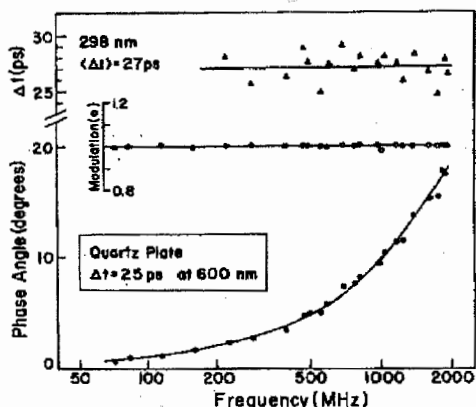


Figure 3. Time-delay measurement using a 25 psec etaton quartz plate.

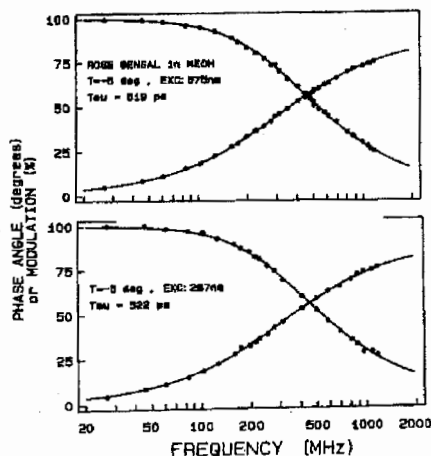


Figure 4. Frequency-response of Rose Bengal in methanol, -5°C ; Test of the Wavelength-Dependent Time Response.

Color Effects

The time-response of photomultiplier tubes can depend upon the incident wavelength [31-32]. The effect is troublesome because the decay time measurements require the comparison of scattered light at the incident wavelength with emitted light characteristic of the fluorophore. We evaluated the color effect of our instrument using rose bengal in methanol, with a decay time near 500 ps. The rose bengal was excited using either the fundamental output of our dye laser at 575 nm, or the frequency doubled output at 287 nm (Figure 4). For visible excitation the wavelength difference between incident and emitted light is near 40 nm, whereas for UV excitation this difference is near 330 nm. The measured frequency-responses (Figure 4) are superimposable, and the decay times are essentially identical (519 and 522 ps). In other experiments with rose bengal, we may have noticed a 20 ps "color effect", but this is uncertain due to variation between experiments, the possible presence of scattered light, and variability in the fits. In either event it is clear that any possible color effect is small, and probably does not limit the accuracy of the measurements. Rigler and co-workers also found no detectable color effect with the R1564U [33].

Transient Effects in Quenching

An important application of fluorescence spectroscopy in the resolution of multi-exponential decays. In Figure 5 and 6 we demonstrate the resolution of the still more complex intensity decays expected in the presence of collisional quenching. This process is expected to yield a decay with a \sqrt{E} dependence in one of the exponentials [34-35], but few observations of such decay have been reported. We examined the decay of indole in the absence and presence of 75 mM acrylamide. In the absence of acrylamide (Figure 5). The intensity decay is described by a single exponential. This is demonstrated by the small and random deviations between the data and the best single exponential fit (lower panels) and the modest value of the goodness-of-fit parameter χ_R^2 .

The frequency-response of indole in the presence of 0.075 M acrylamide shows deviations from a single exponential decay (Figure 6). This is seen from the inadequacy of the single exponential model (---), the systematic deviations from this model (o), and the 20-fold elevation in χ_R^2 to 42.4. It should be noted that the degree of quenching is rather modest (about 4-fold), and the solution is non-viscous. Because of the shorter mean decay time in the presence of quenching the frequency-response was measurable to 811 MHz. The values of χ_R^2 for the single exponential fits increases monotonically with the acrylamide concentration.

The data were then analyzed with the lower equation in Figure 6, which is the approximate form of the decay expected at low degrees of quenching. This model is consistent with the data, as seen by the good match to the data, the small deviations (Figure 6, ●), and the small values of χ_R^2 . These values of χ_R^2 near 2 were the lowest obtainable even for a double or triple exponential fits, so there is no justification to accept a decay law with more parameters than equation 5. It should be noted that the same values of R and D were recovered at several acrylamide concentrations up to 75 mM.

In further experiments we measured and analyzed data at higher acrylamide concentrations. We found that the \sqrt{E} decay law cannot explain these data. A more complete theory obtained with the radiation boundary conditions provides better fits, but even here there are deviations between the theory and the data. We believe these results illustrate the considerable resolution of complex processes which can be obtained from the frequency-domain data.

Tyrosine Fluorescence from Oxytocin

It is of interest to recover the intensity decays from proteins. Tyrosine and tryptophan are the intrinsic fluorophores of proteins. Oxytocin contains a single tyrosine residue and has the sequence Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂. Frequency-domain data for the intensity decay analysis are shown in Figure 7. The data extend from 11.38 MHz to 1.91 GHz and include 27 frequencies. The data were easily adequate to determine that at least three exponential components are needed to account for the data. The dashed line on Figure 7 (top) shows large deviations from the single exponential model. These deviations and the extreme value of $\chi_R^2 = 377$ are easily adequate to reject this model. Even the two component decay can be rejected with reasonable certainty, based on the 3-fold decrease in χ_R^2 for the three component fit and our approximate 50 degrees of freedom. The most remarkable feature of these data that the information content and signal-to-noise ratio permits facile resolution of two and three component tyrosyl decays. We know of no other multi-component tyrosyl resolution on the subnanosecond timescale.

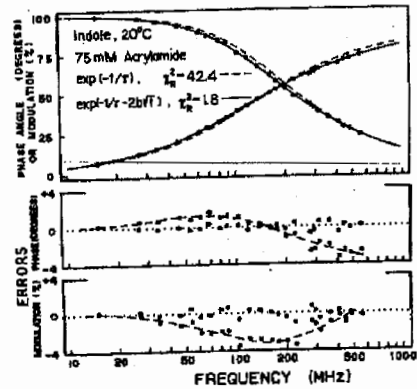
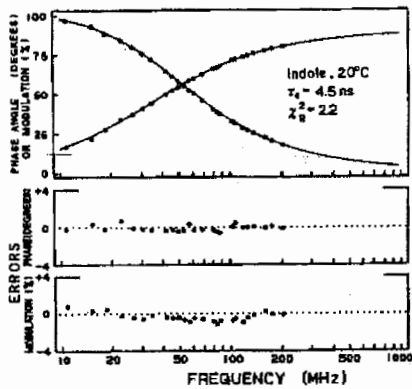


Figure 5. Frequency response of indole in the absence of quenching.

The symbols (●) indicate the data, and the solid line represents the best one decay time fit. The lower panels show the deviations between the data and the best fit.

Figure 6. Frequency response of indole in the presence of 0.075 M acrylamide.

The solid line indicates the best fit obtained using $\exp(-t/\tau - 2b\sqrt{t})$. The dashed line indicates the best fit using a single decay time ($\exp(-t/\tau)$). The lower panels show the deviations for the best single exponential fit (○) and for $\exp(-t/\tau - 2b\sqrt{t})$ (●).

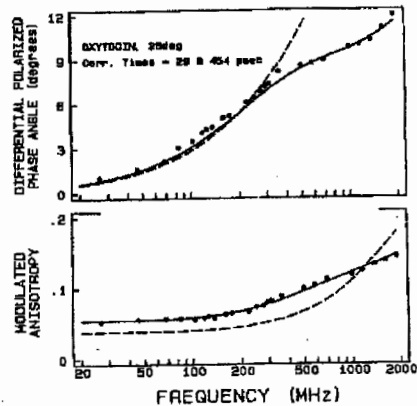
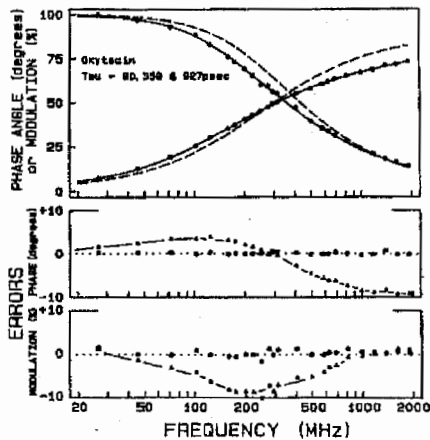


Figure 7. Frequency-domain data for the tyrosine intensity decay of oxytocin.

The upper panel shows the data (●). The solid line is the best three-exponential fit, and the dashed line the best one-exponential fit. The lower panels show the deviations between the data and the calculated values for the one (Δ) and three (●) decay time models.

Figure 8. Frequency-domain data for the tyrosine anisotropy decay of oxytocin.

The data (●) could not be fit using a single correlation time (---), but were adequately fit using two correlation times (—).

It is also of interest to determine the anisotropy decays of proteins. These decays reflect the size and shape of the proteins, and the extent of local torsional motions of the fluorescent residues. Data to recover the anisotropy decay kinetics are shown in Figure 8. These data are the phase angle difference between the polarized components of the emission (top) and the frequency-dependent anisotropy (bottom). Attempts to fit the data with a single rotational correlation time (---) results in a completely unacceptable match to the data. In contrast, a two correlation time model with values of 29 and 454 psec results in a good fit (—). The 29 psec correlation time accounts for 60% of the total anisotropy decay, and is probably due to segmental motions of the phenol ring independent of overall rotational diffusion. The 454 psec correlation time is comparable to that expected for overall rotational diffusion of oxytocin in water. It is important to note that the measurements to 2 GHz provide considerable information content above the data to the previous 200 MHz limit. Data to 200 MHz would not display the shoulder seen at 600 MHz, which represents the transition from rotational diffusion to segmental motions.

A 15 Picosecond Anisotropy Decay

We questioned the fastest rotational correlation times we could measure [36]. This test was performed using the UV output of our laser, and indole as the fluorophore, to model the emission from proteins. First, we examined indole in water at 20°C (Figure 9). The correlation time is 53 psec. At 1244 MHz the phase angle was near 12 degrees. Since this is well in excess of the degree of random error we reasoned that still shorter correlation times could be measured. Hence, we examined indole in methanol-water (75-25) at 40°C. For this sample we found a correlation time of only 15 psec. Once again, the phase at the highest frequency, over 6 degrees at 2 GHz, is well in excess of the random error in the measurements. This instrument is capable of measuring picosecond timescale rotational motions.

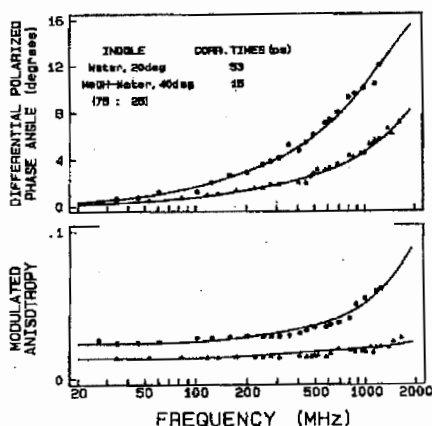


Figure 9. Anisotropy decay of indole in water at 20°C (●) and in methanol-water (75-25) at 40°C (▲). The solutions contain 0.5 M KI to decrease the decay time.

Acknowledgements

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