

Frequency-Domain Spectroscopy Using 280 nm and 300 nm LEDs

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Introduction

In the last few decades, fluorescence methods have become extremely important in many areas of science and technology including analytical chemistry, biophysics, cell biology, clinical chemistry and biotechnology, to name but a few. Determinations of the excited state lifetimes of fluorescent molecules are often critical for quantitative evaluation of the processes or reactions of interest. Fluorescence lifetime measurements have traditionally been realized using either the *impulse response* method (in which excitation is by a very brief pulse of light after which the direct decay of the fluorescence is observed) or the *frequency domain* method (in which the intensity of the excitation light is modulated sinusoidally and the phase shift of the fluorescence, relative to the excitation, is determined). These methods have been reviewed numerous times (see, for example, [1-4]). The frequency domain approach has also been utilized with high-repetition rate pulsed sources [5,6].

In addition to fluorescence lifetime determinations, the frequency domain method permits characterization of the rotational modes of fluorophores [1-4, 7]. In the time domain, such information is obtained from anisotropy decay data; the frequency domain equivalent of these data is obtained by differential polarized phase fluorimetry (also known as dynamic polarization). In this approach, the sample is illuminated by parallel, polarized light, the intensity of which is modulated at high frequencies. The measured phase delay, ($\Delta\Phi$), between the parallel and perpendicular components of the emission, for a single, spherical rotator is related to the rotational parameters of the system. Analysis of the phase delay, and/or modulation ratio, versus frequency curves permits one to characterize the fluorophore's rotational parameters.

The choice of excitation light sources for time-resolved measurements depends, of course, on the absorption properties of the target fluorophore. In fact, most of the fluorophores commonly used absorb light at wavelengths in the mid-UV or longer, i.e., above 350 nm. For these probes, lasers such as argon-ion, krypton-ion, helium-cadmium and helium-neon have been widely utilized [1-3]. More recently, light emitting diodes have been used for time-resolved measurements [8, 9]. However, in order to study intrinsic protein fluorescence (principally from tryptophan or tyrosine residues), light sources in the deeper UV, specifically in the 270 - 300-nm region, are required. One way to achieve these wavelengths in frequency domain measurements has been to utilize a xenon-arc lamp coupled with a monochromator (for wavelength isolation) and a Pockels cell (for light modulation). Alternatively, this goal has been realized using the harmonic content of a mode-locked laser [6] or synchrotron radiation source [7] or by utilizing a high power CW Argon-Ion laser coupled with a Pockels cell.

Recently, McGuinness et al. [11] described the use of a new class of LEDs that emit near 280-nm as the excitation source for time-domain fluorescence lifetime measurements on proteins. In this report we evaluate the use of this class of LED as an excitation source for frequency domain time-resolved measurements.

Materials and Methods

Measurements were acquired using ChronosFD (ISS, Champaign, Illinois), a frequency-domain lifetime spectrometer designed for using light emitting diodes, laser diodes and other lasers (continuous wave, mode-locked); the instrument was also utilized for characterization of the LEDs. Two synthesizers (Model IFR2023) provide modulation frequencies in the range from 9 KHz to 1.2 GHz; for modulation in the frequency range from 90 Hz up to 1.5 MHz (required when measuring decay times in the range from one millisecond to one microsecond) Low Frequency Modulator can be utilized. One synthesizer drives the LEDs directly at a frequency f . The second synthesizer drives the gain of the light detector at a frequency $(f + \Delta f)$ where the quantity Δf , called the cross-correlation frequency, is of the order of 1 KHz. At the exit of the light detector, we record signals at the sum (that is $2f + \Delta f$) and at the difference (that is Δf) of the two frequencies; the low frequency component Δf is utilized to determine the DC, AC and phase components of the signal.

Emission spectra are acquired through the monochromator channel of the instrument. The monochromator is equipped with a 100 mm focal length, 1200 lines/mm, holographic grating (resulting in a 8.3 nm/mm linear dispersion). The light detector is a photomultiplier tube (Model R928 by Hamamatsu); the PMT and the circuitry have a frequency response up to 600 MHz. The novel LEDs are based on AlGaIn/GaN chip/chip array technology; the chip is encapsulated into a metal-glass TO-39 package with UV transparent optical window and mounted into a housing that includes the electronics providing the DC biasing and the RF signal. These LEDs require a DC forward current of about 30 mA and reverse voltage of 6 Volt; for modulation, a RF signal of +13 dBm is used.

Instrument control, data acquisition and data analysis are performed using Vinci - Multidimensional Fluorescence Spectroscopy software (ISS, Champaign, Illinois). The analysis of the time-resolved fluorescence data is carried out using traditional non-linear least squares methods (see, for example, [12-14]). These methods evaluate how close a model (decay time or rotational correlation time) selected by the user matches the data acquired with the instrument: a Marquardt-Levenberg algorithm is utilized for the minimization routine of the χ^2 -function that compares the selected model with the experimental data. The analysis includes fitting routines for the determination of multiple decay times (exponential, non-exponential, lifetime distributions); for the determination of rotational correlation times (isotropic, anisotropic and hindered rotators); for the display of time-resolved spectra and the resolution of spectra using phase and modulation.

Frequency-domain lifetime measurements were performed on N-Acetyl-L-trypto-phanamide (NATA) (Sigma-Aldrich, St. Louis, MO); lysozyme (Sigma-Aldrich, St. Louis, MO). Solutions were prepared in 0.1 M phosphate buffer, pH 7.5 and all optical densities, measured on a Perkin-Elmer Lambda 40 UV/Vis spectrophotometer, were below 0.1. As a lifetime reference standard, a solution of p-terphenyl in ethanol was utilized (decay time = 1.05 ns). Measurements were carried out at 20 °C.

Results and Discussion

LED Characterization

The spectra of the 280-nm and 300-nm LEDs are shown in Figure 1. The full width at half-maximum (FWHM) is about 10-nm in each case. No additional emission bands are observed at wavelengths up to 750-nm (we note that the LED utilized by McGuinness et al (11) had a significant emission near 430-nm in addition to the primary emission at 280-nm).

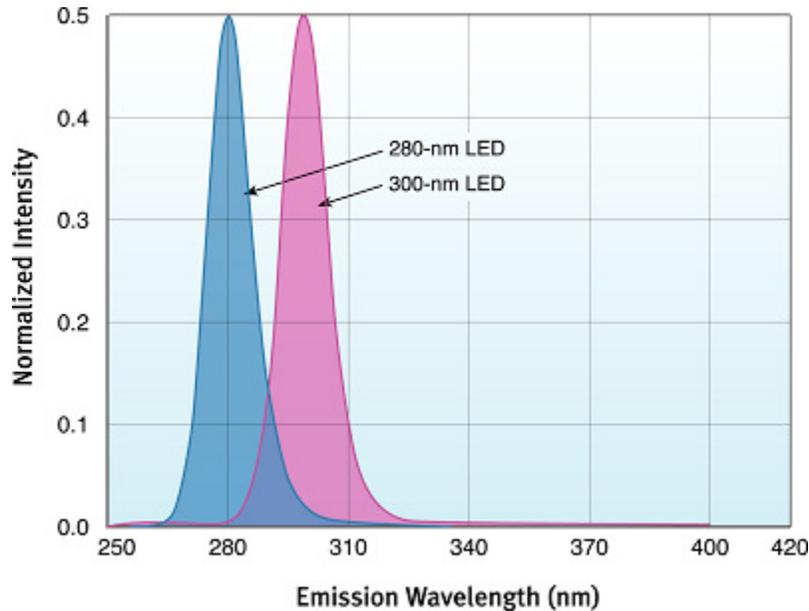


Figure 1. Normalized spectra of the 280-nm and 300-nm LEDs. FWHM is about 12-nm for each LED. The LEDs do not show any additional extraneous emission up to 750-nm: is not detectable in this wavelength range.

The modulation-depth for each LED for the frequency range up to 500 MHz is given in Table I.

$$m = \frac{AC/2}{\langle DC \rangle}$$

The modulation is defined as: $m = \frac{AC/2}{\langle DC \rangle}$, where $AC/2$ is half the value of the average alternate component of the signal—that is half the value peak-to-valley of the signal at the cross-correlation frequency—and $\langle DC \rangle$ is the average value of the direct component. For both LEDs, good modulation was achieved up to 400 MHz (the 3dB point being at about 100 MHz). The modulation behavior was measured using ChronosFD™; that it is the result of the frequency response of the radio frequency amplifier used to amplify the signal at frequency $(f + \Delta f)$ sent to the light detectors of the instrument; and it is a function of the frequency response of the light detectors electronic circuitry. To achieve satisfactory lifetime measurements, a modulation of 5% is sufficient.

Frequency [MHz]	Modulation Depth[%]	
	280-nm LED	300-nm LED
1	52	44
10	61	55
50	100	100
100	65	75
150	23	31
200	19	25
250	16	20
300	11	19
350	10	12
400	9	9
450	9	12
500	5	7

Table 1. Modulation Depths of the 280-nm and 300-nm LEDs for modulation frequencies from 1 – 500 MHz.

Time-resolved Measurements

Phase and modulation data were obtained on NATA (Figure 2), lysozyme (Figure 3) using the 300-nm LED and L-tyrosine using the 280-nm LED (Figure 2). The phase and modulation data for NATA and L-tyrosine can be fitted to single exponential decay times of 2.7 ns and 3.67 ns, respectively, while the lysozyme data can be best fitted with two components: $\tau_1 = 0.7$, $\tau_2 = 2.58$

and $f_1 = 0.26$ (Figure 3). Overall the frequency domain intensity decay data obtained using these LEDs are in good agreement with the reported literature values [2, 10].

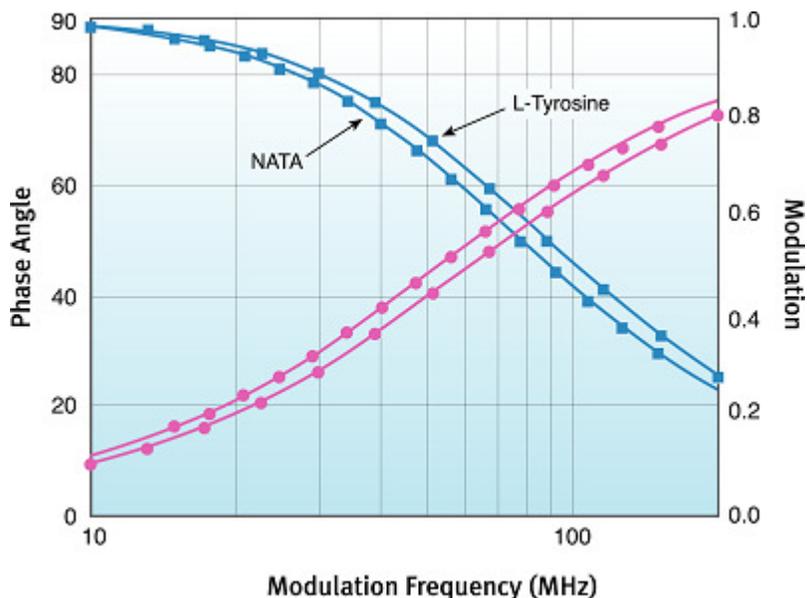


Figure 2. Frequency responses for L-tyrosine (excitation at 280-nm) and NATA (excitation at 300-nm). For both samples, the emission was collected through a WG320 long-pass filter. The single-exponential decay time for tyrosine was determined to be 2.7 ns and for NATA a single-exponential decay time of 3.1 ns was measured.

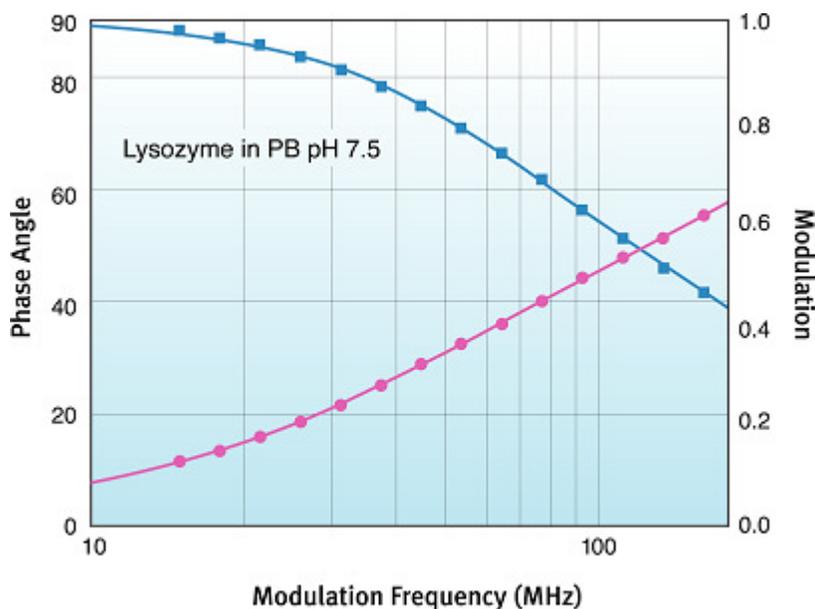


Figure 3. Frequency response (phase angle – purple, modulation – blue) of lysozyme. The excitation source was a 300-nm LED; emission was collected through a WG320 high-pass filter.

Further frequency domain anisotropy decays (differential phase and modulated anisotropy) were obtained for lysozyme at 20 °C in phosphate buffer pH 7.5 (Figure 5). Lysozyme contains six tryptophan-residues and the derived rotational correlation times θ_1 and θ_2 for this protein are related to the overall motion of the protein (θ_2) and to the segmental motions of tryptophan-residues in the protein (θ_1). Also the calculated values for the rotational correlation times ($\theta_1 = 0.8$ and $\theta_2 = 6.08$) and the R_0 of 0.17 are in good agreement with the reported literature data [10]. For

the calculation of these values we used the lifetime data of lysozyme (Figure 3) that were obtained with the 300-nm LED.

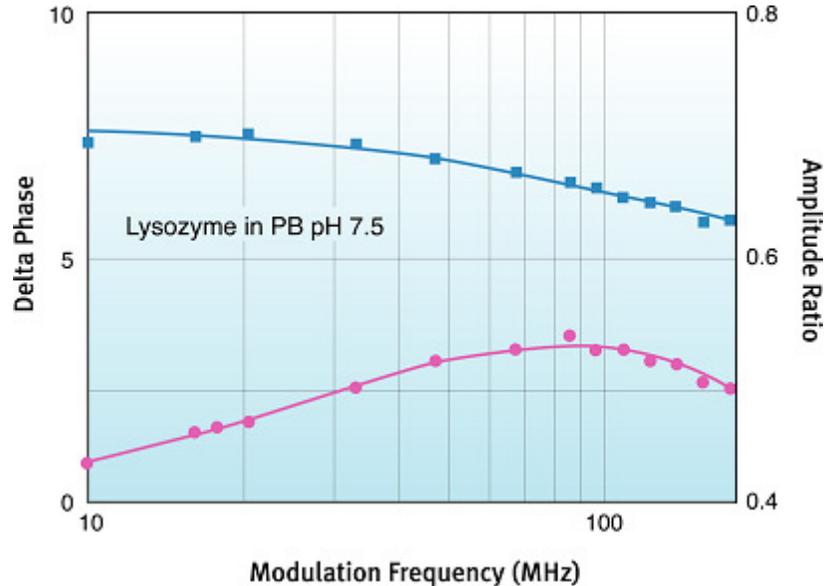


Figure 4. Frequency domain anisotropy decays (differential phase (purple) and modulation (blue) vs. modulation frequency) of lysozyme using a 300-nm LED as the excitation source; the emission was collected through a WG320 long-pass filter.

Conclusion

These results demonstrate that this class of LEDs can be effectively utilized for frequency domain measurements up to 400 MHz and hence provide an inexpensive alternative to laser sources to study the intrinsic fluorescence of proteins.

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