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Notes & Tips

# Frequency-domain fluorescence spectroscopy using 280-nm and 300-nm light-emitting diodes: Measurement of proteins and protein-related fluorophores

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In the past 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 achieved 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) [1–3]. In addition to the measurement of the fluorescence lifetimes, the frequency- domain method permits characterization of the rotational modes of fluorophores [3].

The choice of excitation light sources for lifetime measurements depends, of course, on the absorption properties of the target fluorophore. In fact, most of the commonly used fluorophores 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 (LEDs)[1] have been used for time-resolved measurements [4,5]. However, to study intrinsic protein fluorescence (from tryptophan or tyrosine residues), light sources in the deeper UV, specifically in the 270- to 300-nm region, are required. Often these wavelengths are obtained for frequency-domain measurements by utilizing 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, a synchrotron radiation source, or a high-power continuous wave argon–ion laser coupled with a Pockels cell.

Recently, McGuinness et al. [6] 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 LEDs as an excitation source for frequency-domain time-resolved measurements.

Fluorescence lifetime measurements were acquired using Chronos (ISS, Champaign, IL), 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. Emission spectra are acquired through the monochromator channel of the instrument.

Instrument control, data acquisition, and data analysis are performed using Vinci, Multidimensional Fluorescence Spectroscopy software (ISS). The analysis of the time-resolved fluorescence data is carried out using

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[1] *Abbreviations used:* LEDs, light-emitting diodes; NATA: *N*-acetyl-L-tryptophanamide; FWHM, full width at half-maximum.

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traditional nonlinear least squares methods (see, for example [7]).

Frequency-domain lifetime measurements were performed on *N*-acetyl-L-tryptophanamide (NATA) (Sigma–Aldrich, St. Louis, MO), L-tyrosine (Sigma–Aldrich, Steinheim, Germany), 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 at the excitation wavelength. As a lifetime reference standard, a solution of *p*-terphenyl in ethanol was utilized (decay time = 1.05 ns) [1]. Measurements were carried out at 20 °C.

The spectra of the 280-nm and 300-nm LEDs are shown in Fig. 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. [6] had a significant emission near 430 nm in addition to the primary emission at 280 nm).

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 3-dB point being at about 100 MHz). The modulation behavior was measured using Chronos; 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.

Phase and modulation data were obtained on NATA, lysozyme using the 300-nm LED and L-tyrosine using the 280-nm LED. The phase and modulation data for NATA and L-tyrosine can be fitted to single exponential decay times of 2.7 and 3.1 ns, respectively, while the lysozyme data can be best fitted with two components: $\tau_1 = 0.7$ ns, $\tau_2 = 2.58$ ns and $f_1 = 0.26$ (literature data [8]: $\tau_1 = 0.73 \pm 0.16$ ns, $\tau_2 = 2.55 \pm 0.40$ ns, and $f_1 = 0.27$). Overall, the frequency-domain intensity decay data obtained using these LEDs are in good agreement with the reported literature values for tyrosine (3.21 ns) and NATA (2.72 ns) [9].

Further frequency-domain anisotropy decays (differential phase and modulated anisotropy) were obtained for lysozyme at 20 °C in phosphate buffer, pH 7.5. Lysozyme contains six tryptophan residues and the derived rotational correlation times $\theta_1$ and $\theta_2$ for this protein are related to the overall motion of the protein ($\theta_2$) and to the segmental motions of tryptophan residues in the protein ($\theta_1$). 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 ($\theta_1 = 0.7$, $\theta_2 = 5.94$, $R_0 = 0.18$) [8]. For the calculation of these values we used the lifetime data of lysozyme (Fig. 2) that were obtained with the 300-nm LED.

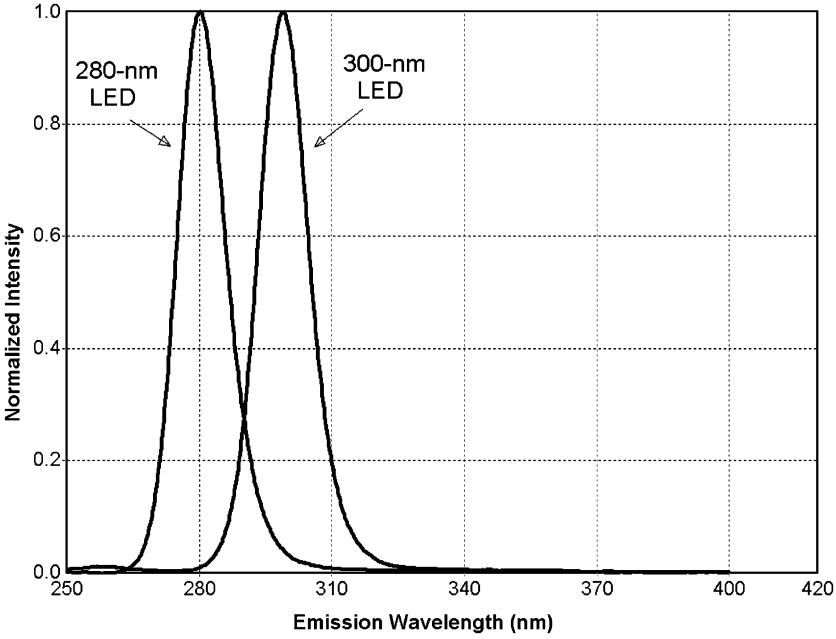


Fig. 1. Normalized spectra of the 280-nm and 300-nm LEDs. FWHM is about 10 nm for each LED. The LEDs do not show any additional extraneous emission up to 750 m.

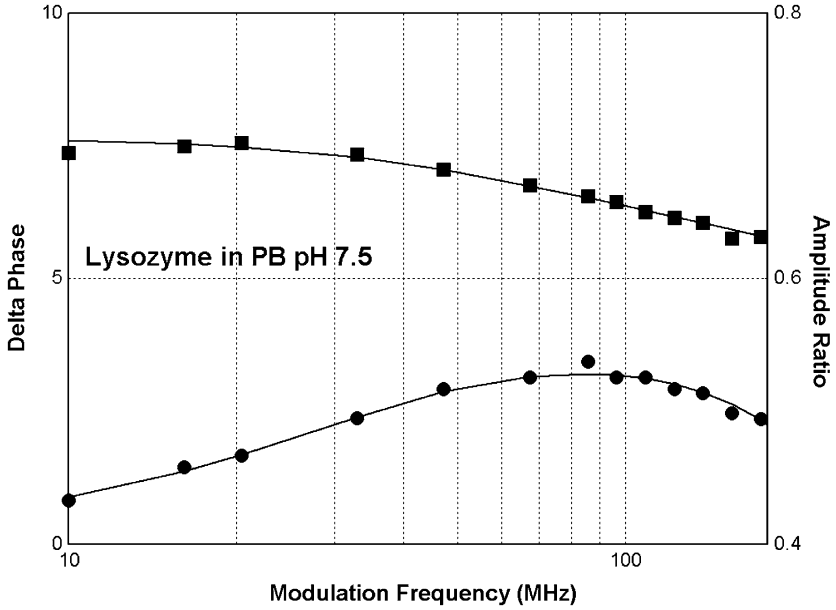


Fig. 2. Frequency-domain anisotropy decays (delta phase and amplitude ratio vs. modulation frequency) of lysozyme using a 300-nm LED as the excitation source; the emission was collected through a WG320 long-pass filter.

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.

## References

[1] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Kluwer Academic/Plenum Publishers, New York, 1999.
[2] D.M. Jameson, T.L. Hazlett, Time-resolved fluorescence measurements in biology and biochemistry, in: G. Dewey (Ed.), Biophysical and Biochemical Aspects of Fluorescence Spectroscopy, Plenum Press, New York, 1991, pp. 105–133.
[3] E. Gratton, D.M. Jameson, R.D. Hall, Multifrequency phase fluorometry, Annu. Rev. Biophys. 13 (1984) 105–124.
[4] H. Szmacinski, Q. Chang, Micro- and sub-nanosecond lifetime measurements using a uv light emitting diode, Appl. Spectrosc. 54 (2000) 106–109.
[5] P. Herman, B.P. Maliwal, H.-J. Lin, J.R. Lakowicz, Frequency-domain fluorescence microscopy with the LED as light source, J. Microsc. 203 (2001) 176–181.
[6] C.D. McGuinness, K. Sagoo, D. McLoskey, D.J.S. Birch, A new-sub-nanosecond LED at 280 nm: application to protein fluorescence, Meas. Sci. Technol. 15 (2004) L19–L22.
[7] D.M. Jameson, E. Gratton, R.D. Hall, The measurement and analysis of heterogeneous emissions by multifrequency phase and modulation fluorometry, Appl. Spectrosc. Rev. 20 (1984) 55–106 .
[8] C. Czeslik, C. Royer, T. Hazlett, W. Mantulin, Reorientational dynamics of enzymes adsorbed on quartz: a temperature-dependent time-resolved TIRF anisotropy study, Biophys. J. 84 (2003) 2533–2541.
[9] J. Wlodarczyk, B. Kierdaszuk, Interpretation of fluorescence decays using a power-like model, Biophys. J. 85 (2003) 589–598.