Development of a Novel Fluorimeter Based on Superluminescent Light-Emitting Diodes and Acousto-Optic Tunable Filter and Its Application in the Determination of Chlorophylls *a* and *b*

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A novel, compact, inexpensive fluorimeter that has high sensitivity and no moving parts has been developed by using superluminescent (bright-blue) light-emitting diodes (SLEDs) and an acousto-optic tunable filter (AOTF). In this instrument, the recently developed gallium nitride SLEDs were used in a counterpropagating configuration to provide excitation light. These SLEDs provide not only high intensity (several milliwatts) but also wide spectral bandwidth in the blue region (from 370 to 570 nm). The AOTF can be placed before the sample to facilitate the measurements of excitation spectra or after the sample for the emission spectra measurements. This fluorimeter is suitable for the sensitive and general fluorescent analysis of a variety of compounds. It has been used, as an example, for the sensitive and simultaneous determination of chlorophylls *a* and *b*. Detection limits of 2.30×10^{-9} and 1.10×10^{-9} M have been achieved for chlorophyll *a* and chlorophyll *b*, respectively.

Index Headings: **Superluminescent light-emitting diode; Acousto-optic tunable filter; Fluorescence; Chlorophyll.**

INTRODUCTION

Lasers have been used extensively as a light source for spectroscopy. However, their applications have not been as wide as expected, considering their unique characteristics, namely, their high spatial, spectral, and temporal resolutions. A variety of reasons, including the high costs of these devices, might account for their limited use. Diode lasers, with their compact size, are low cost, are easy to use and maintain, and offer an attractive alternative to gas- and solid-state lasers. However, they usually have a narrow spectral tuning range (in the range of a few nanometers). While techniques such as an external cavity might increase the spectral tuning range of these lasers up to hundreds of nanometers, it also undesirably increases the price of the lasers to a level similar to that of the gas- and solid-state lasers. Of particular interest is the superluminescent light-emitting diodes (SLEDs). These relatively inexpensive SLEDs are potentially suitable as the light source for spectroscopy because they can provide up to milliwatts of continuous-wave (CW) light, which has a spectral bandwidth of >100 nm [conventional light-emitting diodes (LEDs) can only provide a few microwatts of power]. These SLEDs, until recently, were available only in the infrared (IR) and the red region of the visible. Recent advances on gallium nitride have made it possible to develop SLEDs for shorter wavelength regions (the blue region at ~450 nm).[1–3] Because many fluorescent compounds absorb light in the blue region, these SLEDs with their advantages (i.e., wide spectral bandwidth, high intensity, low cost) were particularly suited for the development of a novel, compact, inexpensive, and highly sensitive fluorimeter.

We have demonstrated that an acousto-optic tunable filter (AOTF) with its advantages, including being compact and all solid state and having no moving parts, fast scanning ability (μs), high resolution (angstroms), and high efficiency (>90%), can be used to develop novel instruments that otherwise are not possible with other wavelength dispersive devices.[4–14] For example, an AOTF-based rapid-scanning and multidimensional fluorimeter has been developed for the simultaneous determination of multicomponent samples. It is evidently clear that when used with SLEDs to construct a novel fluorimeter, the AOTF offers not only economy, reliability, and compactness but also high resolution, rapid scanning, and high sensitivity (by providing high throughput). Such considerations prompted us to initiate this study, which aims to synergistically use the SLEDs and the AOTF to develop a novel, compact, fast-scanning and highly sensitive fluorimeter. The instrumentation development of this fluorimeter and its application in the determination of chlorophylls *a* (Chl *a*) and *b* (Chl *b*) will be reported in this paper.

EXPERIMENTAL

Two 3.0-mm-diameter superluminescent bright-blue light-emitting diodes used in this experiment were purchased from Nichia America Corporation (Model NLPB 300A). The spectral tuning range of the SLED, as shown in Fig. 1, is from 370 to 570 nm with a peak at 448 nm and a full width at half-maximum (FWHM) of 73 nm. Its total CW output power is ~1.230 mW.

A schematic diagram of the fluorimeter is shown in Fig. 2. As illustrated, two SLEDs were used in a counterpropagating configuration to increase the intensity of the excitation light. Lenses were used to focus the blue light from the SLEDs into the sample. Fluorescence emitted from the sample was focused onto the TeO_2 noncolinear AOTF (Matsushita Electronic Components Co., Model EFL-F20) by a lens. This AOTF was placed between two cross-axis polarizers to block the transmitted light and to transmit the diffracted light. The driver for the AOTF, including the radio-frequency (rf) generator, amplifier, and modulator, is the same as that used previously.[6,7,10–14] Light diffracted from the AOTF was detected by a Hamamatsu red-sensitive detector and cooled by

Received 16 October 1996; accepted 11 April 1997.
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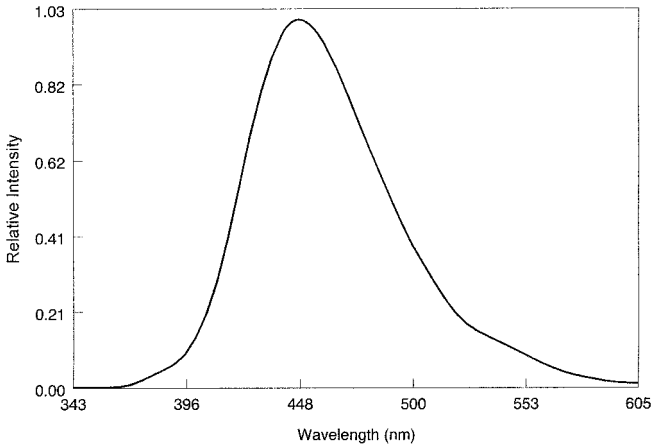

FIG. 1. Spectral output intensity of the superluminescent bright-blue light-emitting diode.

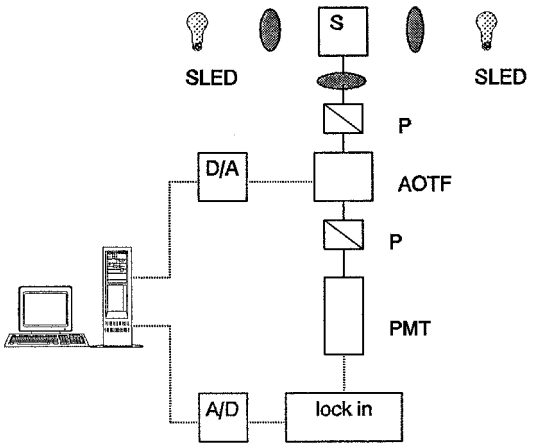

FIG. 2. Schematic diagram of the SLEDs AOTF fluorimeter: **SLED,** superluminescent light-emitting diode; **S,** sample; **L,** lens; **P,** polarizer; **AOTF,** acousto-optic tunable filter; **PMT,** photomultiplier tube; **lock in,** lock-in amplifier.

a two-stage Peltier cooler (Model R6060-02). The output signal of the photomultiplier tube (PMT), which was amplitude modulated (AM) at the same frequency as that of the rf signal (20 kHz), was amplified and demodulated by a lock-in amplifier (Princeton Applied Research Systems, Model 5207). The output of the lock-in was connected to a microcomputer by means of a 16-bit interface board. The programs written in C++, the same as those used previously,[10–14] were used to control and to drive the AOTF as well as to acquire the data.

Chlorophylls *a* and *b* were received from the Aldrich Chemical Company in ampules containing 1-mg quantities. Stock solutions, 1.10×10^{-4} M, were made by dissolving each chlorophyll in 10.0 mL of 9:1 (v/v) acetone : water solution. The respective stock solutions were refrigerated in the dark.

Sodium dodecyl sulfate (SDS) and Brij-35 were purchased from the Aldrich Chemical Company. Cetyltrimethylammonium bromide (CTAB) and cetyltrimethylammonium chloride (CTAC) were obtained from the Eastman Chemical Company. Concentrations of surfactants were adjusted to provide solutions with micellar concentrations of 1.30×10^{-3} M. Solutions of the chlorophylls, 1.10×10^{-6} M, in the respective surfactants were made by pipetting 100 μL of the 1.10×1^{-4} M stock solutions into a 10.0-mL volummetric flask and filling the remainder with the prepared surfactant solutions. The 1.10×10^{-7} M chlorophyll solutions were prepared by diluting a 1.10×10^{-6} M solution with appropriate surfactant solutions. Mixtures containing chlorophylls *a* and *b* were prepared by adding 1.0 mL of the Chl *a* surfactant solution to a standard quartz fluorescence cuvette and pipetting the required amount of the Chl *b* surfactant solution. All dilutions were conducted under red light at room temperature with the use of a microsyringe.

All fluorescence measurements were performed at 30 °C, which is above the cloud point of CTAB in order to prevent the formation of CTAB microcrystals.[15,16] Absorption spectra were taken on a Shimadzu spectrophotometer (Model 1201).

RESULTS AND DISCUSSION

Chlorophylls *a* and *b* are not soluble in water. They can be dissolved in a mixture of water and organic solvent such as 9:1 (v/v) acetone/water mixture. However, it is not possible to use this solvent mixture for their fluorescent quantitative analysis. This is because it was found that the fluorescence intensity of Chl *a* and/or Chl *b* in this solvent mixture is not linearly proportional to their concentrations. A similar observation has been previously reported and has been attributed to the aggregation of the chlorophyll molecules in this acetone/water mixture.[17–22] It is, therefore, essential to find a new medium in which the chlorophylls do not aggregate. Organized media such as micellar solutions may be able to fulfill this requirement.[15,17–20] Accordingly, effects of various surfactants, including nonionic (Brij-35), anionic (SDS), and cationic (CTAB and CTAC), were investigated. While these surfactants can effectively prevent the aggregation processes, they exert different effects on the absorption spectra of the chlorophyll. As illustrated in Figs. 3 and 4, the absorption maxima of Chl *a* and Chl *b* in acetone/water mixture are at 441 and 464 nm. The nonionic surfactant, Brij-35, shifts the absorption bands toward shorter wavelengths—specifically, to 412 and 439 nm for Chl *a* and Chl *b,* respectively. Bathochromic shifts were also observed with SDS, namely, to 414 and 441

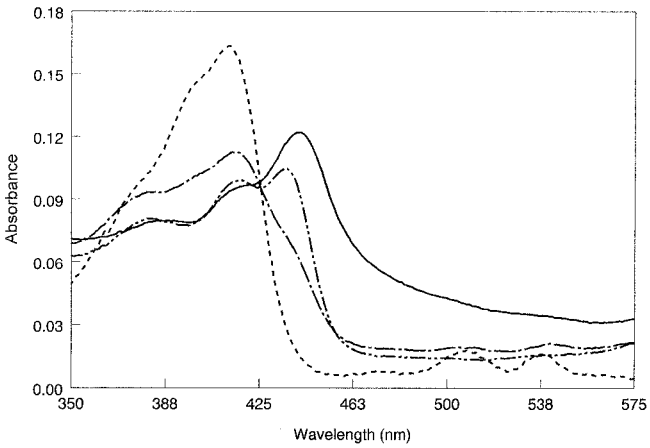

FIG. 3. Absorption spectra of 1.0×10^{-5} M Chl *a* in different media: 9:1 (v/v) acetone/water mixture (——); 5.2×10^{-2} M Brij-35 (– – –); 8.8×10^{-2}M SDS (— – —); and 8.0×10^{-2} M CTAB (— – – —).

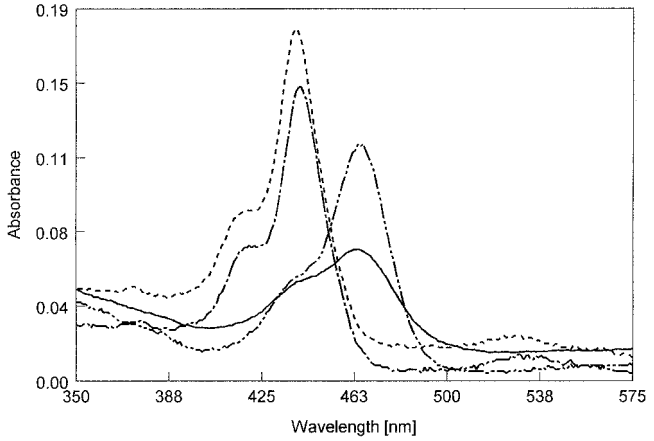

FIG. 4. Absorption spectra of 1.0×10^{-6} M Chl *b* in different media: 9:1 (v/v) acetone/water mixture (——); 5.2×10^{-2} M Brij-35 (– – –); 8.8×10^{-2} M SDS (— – —); and 8.0×10^{-2} M CTAB (— – – –).

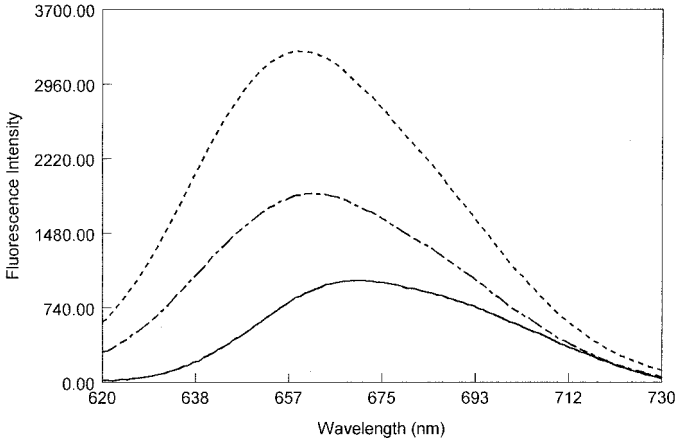

FIG. 5. Fluorescence spectra of 1.10×10^{-6} M Chl *a* (——); 1.10×10^{-6} M Chl *b* (– – –); and a mixture of 5.50×10^{-7} M Chl *a* and 5.50×10^{-7} M Chl *b* (— – –) in 8.0×10^{-2} M CTAB.

nm for Chl *a* and Chl *b,* respectively. In the cationic surfactant CTAB, the absorption maxima for Chl *a* and Chl *b* were shifted to 435 and 463 nm, respectively. Accordingly, CTAB was selected as the medium for the fluorescence determination, because the overlap between the absorption bands of Chl *a* and Chl *b* with the output of the SLEDs (Fig. 2) is maximized in this medium.

Fluorescence spectra of Chl *a,* Chl *b,* and their mixture in CTAB are shown in Fig. 5. As illustrated, Chl *a* solution exhibits a fluorescence maximum at 669 nm, whereas that of Chl *b* is at 658 nm. Depending on the relative concentrations of the chlorophylls, the fluorescence maxima of the mixture shift from 664 nm (for a mixture containing 7.33×10^{-7} M Chl *a* and 3.67×10^{-7} M Chl *b*) to 660 nm (for a mixture containing 2.75×10^{-7} M Chl *a* and 8.25×10^{-7} M Chl *b*). Spectral changes among the mixture emissions demonstrated an emission shift as well as an intensity increase as the Chl *b* concentration increased. This observation seems rather contradictory with respect to the reported study showing that the fluorescence quantum efficiency of Chl *a* is about twice that of Chl *b* (in organized media). However, it is important to realize that, with this fluorimeter, the overlap between the spectral output intensity of the SLEDs with the absorption spectrum of Chl *b* is much more than that with Chl *a.* As a consequence, relatively more Chl *b* molecules are being excited, which, in turn, produces a higher fluorescence intensity for the mixture as the concentration of Chl *b* increases.

It is possible to use this SLED AOTF-based fluorimeter for the simultaneous determination of mixtures of Chl *a* and Chl *b.* However, as described previously, not only the intensity but also the shape of fluorescence spectra of mixtures changes concomitantly with change in the relative concentrations of Chl *a* and Chl *b.* As a consequence, a multivariate calibration method, specifically the partial least-squares analysis, was needed for the simultaneous determination of Chl *a* and Chl *b.* Very good linear relationships were obtained when calculated concentrations for each chlorophyll in the mixtures were plotted against actual concentrations [correlation coefficients, SEP (standard error of prediction), and RMSD (root-mean-square deviation) values for Chl *a* and Chl *b* were found to be 0.993722, 2.9×10^{-8}, and 2.83×10^{-8}, and 0.998834, 1.6×10^{-8}, and 1.56×10^{-8}, respectively]. The limits of detection (LODs), defined as the concentration of Chl *a* and Chl *b* that yielded a signal-to-noise ratio of 2, were determined to be 7.0×10^{-9} M and 2.0×10^{-9} M for Chl *a* and Chl *b,* respectively.

As described in previous sections, the spectral-tuning range of the SLED is much wider than those of the diode lasers. The SLED used in this study can be spectrally tuned from 370 to 570 nm with an FWHM of 73 nm (Fig. 1). It is, therefore, possible to use this SLED AOTF fluorimeter for the measurements of the excitation spectra of samples. Minor modification on the optical arrangement shown in Fig. 2 was made to facilitate these measurements. Specifically, as shown in Fig. 6, rather than two SLEDs, only one SLED was used for the excitation in the modified instrument. The AOTF, placed between two cross-axis polarizers, was used to select and to scan the excitation wavelength. Blocking of the excitation light and transmitting of the fluorescence light from the sample were accomplished by means of a red cutoff filter.

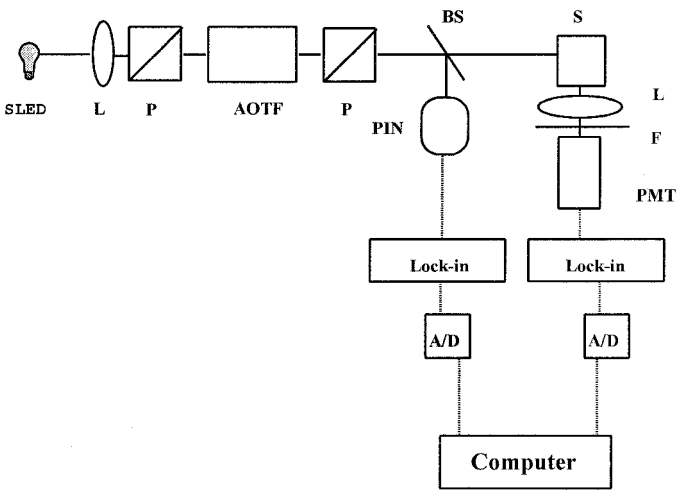

FIG. 6. Schematic diagram of the SLED AOTF fluorimeter for excitation-spectra measurements: **SLED,** superluminescent light-emitting diode; **S,** sample; **L,** lens; **P,** polarizer; **AOTF,** acousto-optic tunable filter; **PMT,** photomultiplier tube; **lock-in,** lock-in amplifier; **PIN,** photodiode.

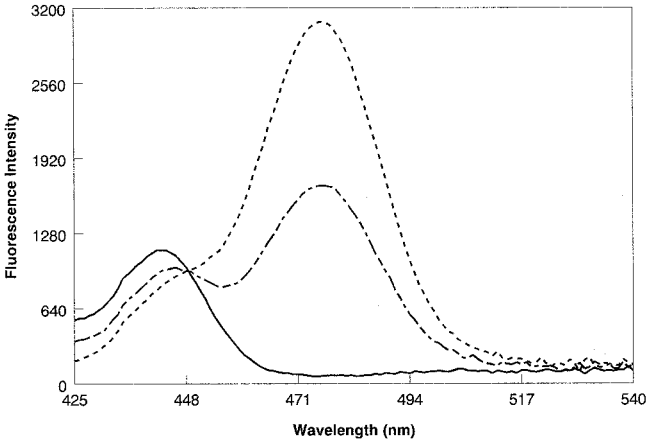

FIG. 7. Excitation spectra of 1.10×10^{-6} M Chl *a* (——); 1.10×10^{-6} M Chl *b* (– – –); and a mixture of 5.50×10^{-7} M Chl *a* and 5.50×10^{-7} M Chl *b* (— -- –) in 8.0×10^{-2} M CTAB.

The same PMT and lock-in amplifier were used to detect the fluorescence light and to demodulate the signal. In a procedure to correct for the difference in the intensity of the SLED over the tuning spectral range, a small portion of the excitation light was split by a beamsplitter, detected by a reference photodiode, and demodulated by a lock-in amplifier. The ratio of the signal (from the PMT) to the reference signal gives the corrected excitation spectrum.

The excitation spectra of 1.10×10^{-6} M Chl *a,* Chl *b,* and their 1:1 mixture in 8.0×10^{-2} M CTAB, obtained with this instrument, are shown in Fig. 7. These spectra are relatively similar to the corresponding absorption spectra shown in Figs. 3 and 4 and clearly show that the excitation spectrum of Chl *a* with its maximum at ~444 nm is much different from that of Chl *b* (with its peak at 473 nm). Because the excitation spectra of these chlorophylls are much better resolved than emission spectra, it is expected that better limits of detection may be achieved with the former. Accordingly, the partial least-squares method was used to analyze the excitation spectra for the simultaneous determination of Chl *a* and Chl *b*. Good linear relationships were obtained when calculated concentrations for each chlorophyll in the mixtures were plotted against actual concentrations. Correlation coefficients, SEP, and RMSD values for Chl *a* and Chl *b* were found to be 0.997843, 2.10×10^{-8}, and 1.88×10^{-8}, and 0.997847, 2.10×10^{-8}, and 1.88×10^{-8}, respectively. The LODs of Chl *a* and Chl *b* were determined to be 2.30×10^{-9} and 1.10×10^{-9} M, respectively. As expected, these LOD values are three and two times lower than the corresponding values of 7.0×10^{-9} and 2.0×10^{-9} obtained by using the emission spectra. This result is as expected since (1) the emission spectra of Chl *a* and Chl *b* are well overlapped, while the excitation spectra are well resolved, and (2) the excitation wavelength can be appropriately tuned (in the excitation spectra case) to the corresponding absorption peaks of each components (i.e., 444 nm for Chl *a* and 473 nm for Chl *b*) to achieve lower LODs.

The LODs obtained in this work are comparable with other LOD values obtained with conventional fluorimeters.[23,24] They are expectedly higher than the LOD value of 1.12×10^{-11} M, which was reported recently.[25] However, it is important to realize that this LOD was obtained on a fluorimeter where a high-power pulsed YAG laser was used for excitation.[25] Furthermore, the LOD values obtained in this work were for simultaneous determination of mixtures of Chl *a* and Chl *b,* whereas LOD values reported in other works were for the determination of single-component samples.

CONCLUSION

In summary, we have successfully demonstrated that a novel, compact, inexpensive fluorimeter, which has high sensitivity and no moving parts, can be developed by using SLEDs as the light source and an AOTF as a dispersive element. An example of the application of this fluorimeter, which has been demonstrated in this publication, is the sensitive and simultaneous determination of chlorophylls *a* and *b*. Because the SLEDs provide bright and broad bandwidth excitation light in the region from 370 to 570 nm, this fluorimeter is suitable for general fluorescent analysis of a large number of compounds including fluorescent labeled nucleotides. This focus is the subject of our on-going investigation.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Mauricio Baptista for his competent technical assistance. Acknowledgment is made to the National Institutes of Health, National Center for Research Resources, Biomedical Research Technology Program, for financial support of this work.

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