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Enhancement of signal-to-noise level by synchronized dual wavelength modulation for light emitting diode fluorimetry in a liquid-core-waveguide microfluidic capillary electrophoresis system

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Abstract

The signal-to-noise level of light emitting diode (LED) fluorimetry using a liquid-core-waveguide (LCW)-based microfluidic capillary electrophoresis system was significantly enhanced using a synchronized dual wavelength modulation (SDWM) approach. A blue LED was used as excitation source and a red LED as reference source for background-noise compensation in a microfluidic capillary electrophoresis (CE) system. A Teflon AF-coated silica capillary served as both the separation channel and LCW for light transfer, and blue and red LEDs were used as excitation and reference sources, respectively, both radially illuminating the detection point of the separation channel. The two LEDs were synchronously modulated at the same frequency, but with 180°-phase shift, alternatingly driven by a same constant current source. The LCW transferred the fluorescence emission, as well as the excitation and reference lights that strayed through the optical system to a photomultiplier tube; a lock-in amplifier demodulated the combined signal, significantly reducing its noise level. To test the system, fluorescein isothiocyanate (FITC)-labeled amino acids were separated by capillary electrophoresis and detected by SDWM and single wavelength modulation, respectively. Five-fold improvement in S/N ratio was achieved by dual wavelength modulation, compared with single wavelength modulation; and over 100-fold improvement in S/N ratio was achieved compared with a similar LCW-CE system reported previously using non-modulated LED excitation. A detection limit (S/N = 3) of 10 nM FITC-labeled arginine was obtained in this work. The effects of modulation frequency on S/N level and on the rejection of noise caused by LED-driver current and detector were also studied.
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1. Introduction

The area of micro total analysis systems (μTAS) has expanded rapidly in recent years, as evidenced by the tremendous growth in related literature. The ultimate goal of μTAS is to realize complete integration of the various stages of analytical procedures on a single microdevice that is small enough to be handheld, and robust enough to be used by non-professionals. Downscaling of individual analytical functions, such as fluid manipulation, reaction, separation and detection is revolutionizing conventional chemistry to a level where their integration on a chip is becoming increasingly realistic. Nevertheless, various challenges remain to be faced to achieve that goal, among which the on-chip integration of optical detectors is one that has attracted broad interest. Miniaturization brings obvious advantages for enhancing the speed of many physical and chemical processes, while dramatically reducing sample and reagent consumption. On the other hand, at least for the most broadly applied optical detectors,

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the large reduction in the available detection volume poses difficulties for achieving concentration sensitivities comparable to those for conventional systems. Laser-induced fluorescence (LIF) is currently employed most broadly as detection system in μTAS, owing to its high sensitivity in many bio-applications when dealing with sub-nanoliter detection volumes. However, conventional lasers are relatively expensive, bulky and have rather limited lifetime. Despite its wide acceptance in μTAS, their integration on a chip-based system is out of the question. Solid-state lasers overcome some of these limitations, but at least in the foreseeable future, are still too large to be integrated on chip-based systems. In general, integration of optical components onto microfluidic chips remains to be a major challenge in the development of μTAS.

Recently, light emitting diodes (LED) are being exploited to achieve absorption and fluorescence detection in micro-analytical systems, while providing excellent prospects for detector integration and miniaturization [1–11]. As a light source, LEDs are exceptionally stable; provide high emission intensity, and a wide range of wavelengths from violet to near infrared is available. Furthermore, LEDs are small and cheap, provide long lifetime and can be operated with battery power, making them particularly attractive for portable systems.

With the rapid increase in emission intensity of LED products, interest in employing them particularly for fluorescence detection has grown significantly. Burns et al. [7] reported an integrated DNA analysis device, and more recently, Webster et al. [8] reported a monolithic capillary electrophoresis (CE) system using LED-based fluorimetric detection. Both devices employed a pulsed blue LED as excitation light source, an integrated photodiode for detecting the fluorescent signal, and a lock-in amplifier for controlling the excitation LED and for demodulating the photodiode photocurrent signal. Hillebrand et al. [9] developed a fluorescence detector for CE using a pulsed UV-LED combined with a time-discriminating and averaging acquisition system to achieve low detection limits for fluorescamine-derivatized bradykinin and lysine, respectively. Dasgupta et al. [10] and later Wang et al. [11] combined the use of a liquid-core-waveguide (LCW) technique with a LED source to produce an extremely simple optical system for LED-induced fluorescence detection in CE. For CE separation, a fused silica capillary coated with Teflon AF was used that also functioned as a LCW when filled with an aqueous solution. When being transversely illuminated at the detection point by a LED or other light source, fluorescence light excited by the light source traveled to the terminal of the capillary, where it can be collected by an optical fiber with high efficiency. Dasgupta et al. demonstrated good isolation of excitation light from the fluorescence radiation merely using broad-band filters with this LCW-CE system, in the separation of two model fluorescent dyes [10]. Later, the LCW-CE approach was applied by Wang et al. to produce a very inexpensive LED/LCW chip-based fluorimetric CE system, in which continuous automated flow-injection based sampling was coupled to LCW-CE on a chip scale interface [11]. The system achieved a throughput of up to 144 samples/h for the CE separation of a mixture of fluorescein isothiocyanate (FITC)-labeled amino acids, including arginine, phenylalanine and glycine, Despite the simplicity, compactness, low cost and high efficiency of this approach, the detection limits achieved were yet two to three orders of magnitude worse than those often reported for chip-based CE, employing more sophisticated LIF optical detection systems. An intriguing aim would be to boost the signal-to-noise (S/N) level of this system to approach those of LIF, while maintaining its merits.

Numerous approaches for enhancing the S/N ratio of LED-based CE systems have been reported, mostly exploiting the favorable property of LEDs that they can be electrically modulated with any desired pattern. In CE systems with absorption detection, enhancement of S/N ratio for LED sources have often been achieved by deriving signal and reference beams from a continuously operated LED source, and then processing the two resulting detector currents with a log-ratio amplifier [1–4]. In CE systems with LED-based fluorescence detection, a general method for enhancing S/N ratio is to modulate the LED, followed by detection of the modulated signals either employing a lock-in amplifier [7,8,12] or using a time-discriminating detection approach [9]. An improvement of over an order of magnitude S/N levels could be expected employing such techniques, but still falling short of those achievable by laser excitation sources.

In this work a novel synchronized dual wavelength modulation (SDWM) system was developed for the LED-excited fluorescence LCW-CE microdevice to approach the S/N level of LIF, while retaining its simple optical design. Two synchronously modulated LEDs with different wavelengths, alternatingly driven by the same constant current source, were employed as excitation and noise-compensation sources, respectively.

2. Experimental

2.1. Instrumentations

A microfluidic CE device based on LCW principles, similar to that described previously [11], slightly modified as shown schematically in Fig. 1, was used for the study. Briefly, a 45-mm long Teflon AF-coated silica capillary (TSU 050375, 48 μm i.d., 362 μm o.d., Polymicro Technologies, Phoenix, AZ), serving as both the separation channel and LCW for transfer of fluorescence emission light. The capillary was connected at its inlet to a 0.5 mm i.d. flow-through side arm with an opening widened to 2 mm for accommodating the capillary inlet to achieve split-flow sample introduction. The capillary outlet was connected to a 4-mm i.d. waste flow side arm, leaving an effective CE separation length of 40 mm. Platinum electrodes were inserted into the two arms and connected to a home-built high voltage supply, with the inlet electrode grounded and the outlet electrode connected to negative high voltage. The outlet of the capillary was painted

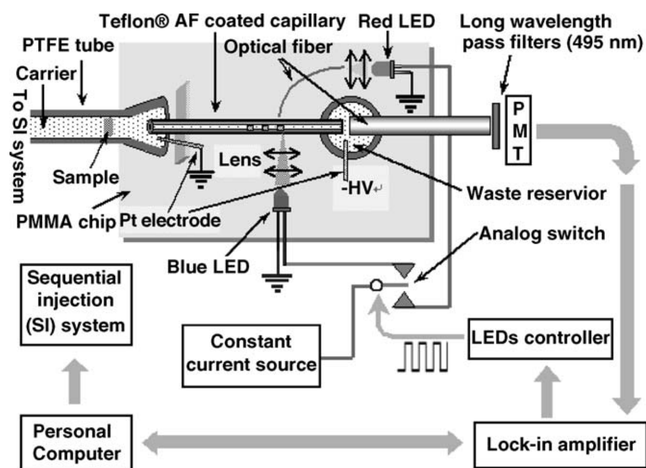

Fig. 1. Schematic diagram of the LCW-CE system with SDWM detection: −HV, negative high-voltage supply; PMT, photomultiplier tube.

black to minimize transmission of light through the capillary walls, and co-axially butted against a 600-μm diameter quartz optical fiber inserted into the waste side-arm. The other end of the optical fiber addressed the photomultiplier tube (PMT, Model CR114, Beijing Hamamatsu, Beijing). A long-wavelength pass filter with cut-on wavelength at 495 nm (Huibo Optical Co., Shenyang) was installed in front of the PMT window.

A sequential injection (SI) system was used for sample introduction. The system was comprised of a syringe pump (Model P/N 50300, Kloehn, Las Vegas, NV), equipped with a 1-mL syringe, a 2-way valve, a 6-way selector valve, driven by a valve driver module (P/N 50120, Kloehn. Las Vegas, NV), and a holding coil produced from 0.5 mm i.d., 1.5 m PTFE tubing. The SI system was controlled by a computer program written in Visual C++.

A 470-nm wavelength blue LED (Shuguang Co., Shenzhen) was used as excitation light source. The light was focused to a diameter of about 500 μm on the detection point of the capillary channel through a compound lens (focal length, 6 mm; diameter, 7.0 mm). A 645-nm wavelength red LED (Shuguang Co., Ltd., Shenzhen) was used as reference light source, the light of which was transmitted by a 400 μm diameter optical fiber to the detection point on the opposite side of the capillary. The position of the optical fiber was fixed relative to the capillary, but that of the red LED could be finely adjusted relative to the optical fiber to regulate its incidence light flux. The two LED's were driven alternatingly by a home-built constant current source and controller with a frequency of 1 kHz and an average forward direct current of 20 mA for each LED. A lock-in amplifier (SR830, SRS Inc., Sunnyvale, CA) was employed to demodulate the signal detected by the PMT, by directly connecting it to the amplifier using current input mode. The output of the lock-in amplifier was recorded through a general-purpose interface bus (GPIB) using a program written in LabVIEW 6.1 (National Instruments, Austin, TX).

2.2. Reagents

All reagents were of analytical grade, and demineralized water was used throughout. Fluorescein isothiocyanate (FITC) was obtained from Sigma Aldrich Inc. (St. Louis, MO). The carrier solution for the SI system, which also served as the working electrolyte for CE separation was 10.0 mM $Na_2B_4O_7$ buffer, adjusted to pH 9.5 using 1 M NaOH solution. A stock solution of a mixture of FITC-labeled amino acids, consisting of 5.0 μM arginine, 5.0 μM phenylalanine and 5.0 μM glycine, was prepared by reacting the dye with amino acids overnight. Working samples, consisting of FITC-labeled 100 nM arginine, 100 nM phenylalanine and 100 nM glycine, prepared by diluting the stock solution with the tetraborate buffer, were analyzed with the SI–CE system employing SDWM.

2.3. Procedures

Sample injection and separation were performed under the same field strength of $200\,V\,cm^{-1}$ in the CE system 50 μL samples were loaded into the holding coil via the selector valve, injected by the syringe valve at a flowrate of $100\,\mu L\,s^{-1}$, and carried by the working electrolyte through the split-flow interface of the SI–CE system to introduce electrokinetically a sample zone into the capillary for subsequent CE separation. A complete analytical cycle lasted 92 s.

3. Results and discussion

3.1. Working principles

The objective of the SDWM design is to improve the S/N ratio by suppressing the noise level of the detection system as far as possible. The dominating sources of noise in the present system are mainly those induced by fluctuations of the photomultiplier high voltage supply (0.05% R.S.D.) and the LED operating current (0.1% R.S.D.). Noise from the LED driving source are reflected in both the emitted fluorescence signal as well as the stray light from the excitation source reaching the PMT detector; however, the detection limit is related mainly to the noise of the stray light. The noise contributed by the latter factor can be reduced proportionally with reduction of stray light by improving the optical system, until the PMT detector shot noise becomes evident. However, no such attempts were made in this work (e.g., by using narrower band pass filters). In fact, the original stray light level of the simple optical arrangement was purposely retained to show the effectiveness of the noise rejection system studied, as well as the robustness of the system.

The experimental setup of the LCW-CE device with SDWM noise rejection function is schematically presented in Fig. 1, and the working principles of the SDWM system are shown in Fig. 2. The central feature of the system is the use of a reference LED source, with a wavelength sufficiently far

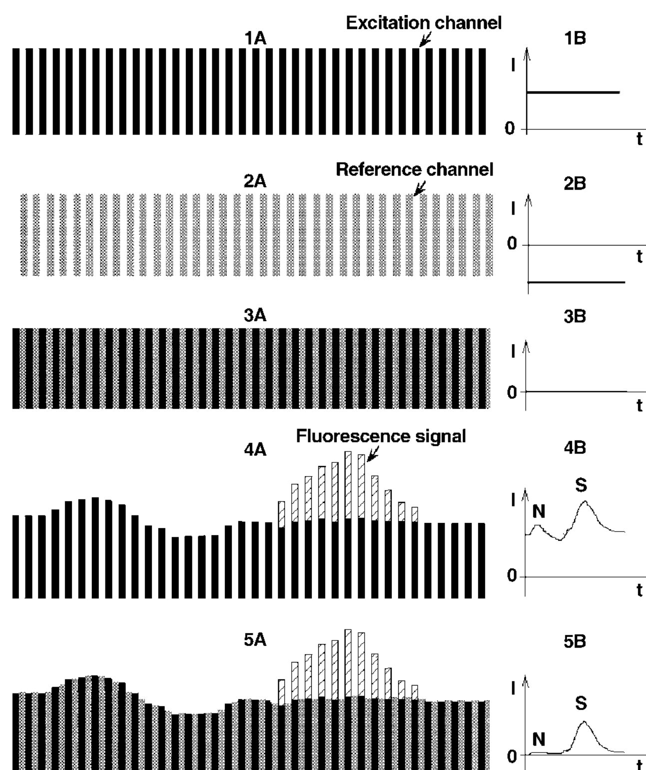

Fig. 2. Working principles of SDWM. Black, gray and striped bars represent photomultiplier tube responses for modulated excitation light, reference light (background-noise compensation) and fluorescence emission, respectively; (1–5A) and (1–5B) are the outputs of the lock-in amplifier. (1) Excitation channel works alone; (2) reference channel works alone and (3) both channels modulated synchronously, no noise considered in (1) and (3). (4 and 5) Noisy signals with fluorescence considered; (4) no compensation from reference, (5) compensated by reference; N, noise, F, fluorescence signal.

from that of the excitation source, to illuminate the LCW capillary at a position same as that for excitation. Physically, this is readily achievable because the LCW could be illuminated from all directions at a given point. The stray light from the excitation and reference sources, reaching the PMT through the LCW, are both modulated to compensate the background together with its noise through a lock-in amplifier. The position of the reference source is adjusted to obtain a signal equal to that of the strayed excitation light, and both sources are subjected to square-wave modulation, driven by the same constant current source at the same frequency, but with 180°-phase shift (1A, B and 2A, B in Fig. 2), and the signal from the strayed excitation light is compensated by that from the reference (3A and B in Fig. 2).

Since the lock-in amplifier is used as a phase-sensitive detector, only the signal and noise components having a specified frequency and phase with respect to the reference waveform of the lock-in amplifier are extracted and amplified by the latter. All other frequency and phase combinations are rejected and do not affect the measurement [13,14]. The noise contributed by the PMT detector and constant current source is the sum of a series of noises with varying frequencies, phases and amplitudes (4A and B in Fig. 2). By tuning the phase of the reference wave of lock-in amplifier to be identical with that of the modulated excitation signal, highest output could be obtained, and after demodulation and low-pass filtering by the lock-in amplifier, the background is cancelled together with the noise, while the fluorescence signal is maintained in the outputs of the lock-in amplifier (5A and B in Fig. 2).

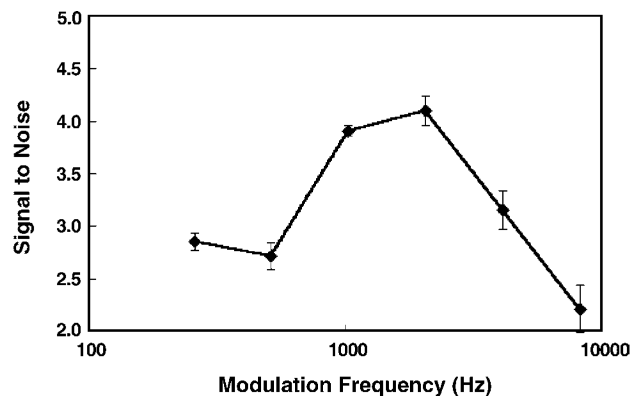

Fig. 3. Effect of modulation frequency on S/N level.

3.2. Effects of modulation frequency on S/N level

The effect of LED modulation frequencies on the S/N ratios for the SDWM system was studied (Fig. 3) within a range of 0.25–10 kHz. High S/N levels were observed when the LEDs were modulated at frequencies in the 1–2 kHz range. When the LED modulation frequencies were lower than 1 kHz, the noise was dominated by intrinsic flicker noise, often termed $1/f$ noise [13,14] that has a spectrum in which the power is approximately proportional to the reciprocal of the frequency. In this case, lower modulation frequencies resulted in worsened S/N levels. At higher modulation frequencies, the noise was subdued, but the modulated LED signals were degraded from square waveform to triangular waveform owing to deleterious effects from unidentified reactive elements in the system. The overall effect was a deterioration of the S/N level.

3.3. Performance of the SDWM system

Modulation is one of the most popular methods used for reducing noise during recovery of low-level light signals. In this work, the signal frequency is moved from the noisy $1/f$ zone at the vicinity of zero frequency to a less noisy frequency zone through modulation of the excitation source [13,14]. Synchronous modulation of two sources with different wavelengths further enhanced suppression of noise from different sources. Performance of the SDWM system was tested by deliberately creating artificial noises in the LED driver current and PMT high voltage. The capability for rejection of LED-driver current noise was studied by fluctuating the driver current at a low frequency of 0.125 Hz. The LEDs were modulated at 1 kHz and the turn-on-current was

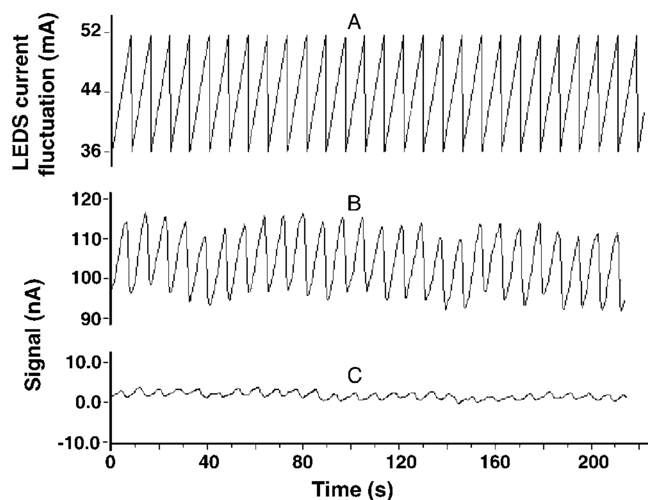

Fig. 4. Response to fluctuation of the LED-driver current in single and dual wavelength modulation mode. (A) Fluctuation of LED turn-on-current; response to fluctuation of LED-driver current in (B) single wavelength modulation mode and (C) SDWM mode.

fluctuated between 36 and 52 mA obtained by superimposing a sawtooth current upon a constant current (Fig. 4A). The outputs of the lock-in amplifier fluctuated at the same frequency with identical patterns for both LEDs, but most of the fluctuations were compensated in the final output of the SDWM system. Output signals generated by the LED-driver current fluctuations were reduced about an order of magnitude by SDWM, compared with single LED modulation (Fig. 4B and C).

The PMT negative high voltage was varied periodically from −763 to −554 V by adding a sawtooth wave to the constant negative voltage of −554 V (Fig. 5a). The recordings from the output of the lock-in amplifier again showed significant suppression of the noise level compared with single wavelength modulation (Fig. 5b and c). In principle, the

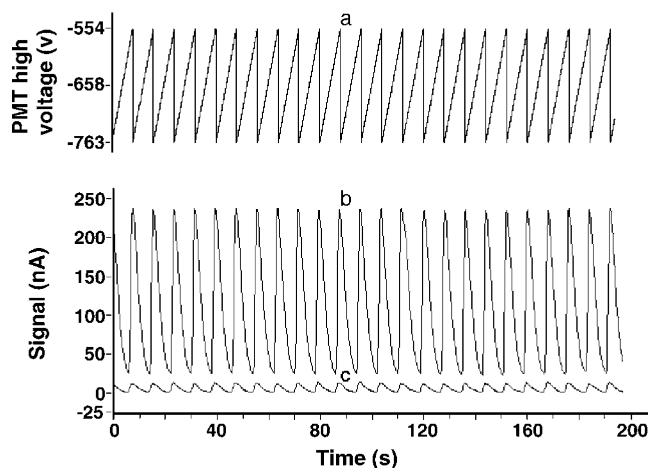

Fig. 5. Response to fluctuation of the PMT negative high voltage in single and dual wavelength modulation mode. (a) Fluctuation of PMT negative high voltage; response to fluctuation of PMT negative high voltage in (b) single wavelength modulation mode and (c) SDWM mode.

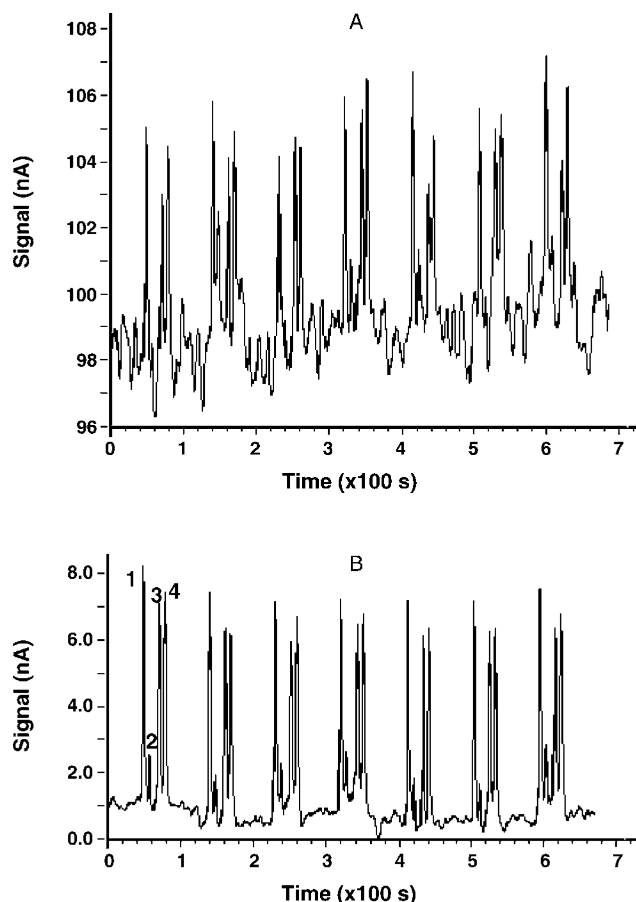

Fig. 6. Electropherograms of 100 nM FITC-labeled amino acids. (A) Single wavelength modulation detection and (B) SDWM detection; (1) arginine, (2) FITC, (3) phenylalanine and (4) glycine.

background noises caused by the PMT negative high voltage fluctuation can entirely be suppressed and be reduced to zero by the SDWM system, however, a small residual noise still remained in this study owing to difficulties in adjustment of the excitation and reference sources to obtain completely identical responses from the PMT. The differences in the observed noise pattern are the consequences of higher sensitivities at lower supporting voltages for the PMT.

The S/N ratios of SDWM and single wavelength modulation were studied under the same conditions in the separation of FITC-labeled amino acids using the LCW-CE system with LED excited fluorescence detection. Low analyte concentrations in the sub-μM range, close to the detection limit, were used in the study to demonstrate the effectiveness for suppression of noise and background signal. The S/N ratio was enhanced five-fold by SDWM, compared with single wavelength modulation (Fig. 6A and B). The signals were the same amplitude, while the peak-to-peak noise of SDWM was only one-fifth that of single wavelength modulation. The baseline signal was also reduced at least two orders of magnitude. As a result, compared with a similar LCW-CE fluorescence system reported previously without light source modulation [11], the detection limits of FITC-labeled arginine obtained with

SDWM and single wavelength modulation were improved by factors of 100 and 20, respectively. Thus, a detection limit of 10 nM FITC-labeled arginine was obtained in this work, with a detection limit and separation efficiency comparable with that obtained in reference [15] using LIF detection without light modulation.

A prerequisite of the SDWM approach to perform optimally is to equalize the stray light level of the excitation and reference light sources, which required some positional adjustments of the latter, however, this need not be practiced frequently; and in this study, a single adjustment was sufficient for prolonged working periods of several days.

4. Conclusions

The SDWM approach described in this work significantly reduced the noise level while maintaining the signal intensity in fluorimetric measurements for a miniaturized LCW-CE system with LEDs as excitation and reference sources. Application of the system to separation and determination of FITC-labeled amino acids showed detection limits approaching those of chip-based CE-LIF systems employing much more sophisticated optics. A LED-based SDWM–LCW-CE system may be developed using a dedicated lock-in amplifier, suitable for further miniaturization and production of a portable system with performance approaching that of chip-based CE–LIF systems.

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