COMMUNICATION TO THE EDITOR

All Solid-State GFP Sensor

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Abstract: An all-solid-state green fluorescent protein (GFP) sensor for GFP measurement was developed. It is immune to interference from ambient light and works with standard flow-through cuvettes. The sensor is practically insensitive to the scattered excitation light encountered in microbial suspensions. It has a range of 0.0002–1 g/L (7.4×10^{-9} – 3.7×10^{-5} M) with limit of detection 0.00019 g/L (7.0×10^{-9} M). The sensor could be used with a UV or blue light emitting diode (LED) as a light source, depending on required sensitivity, selectivity, and background levels. Its very low cost makes it useful in a variety of applications. This article describes the construction and validation of the sensor both off- and on-line in fermentation processes. *Biotechnol Bioeng* **70**: 473–477, 2000.
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INTRODUCTION

GFP has attracted much scientific interest since its discovery by Shimomura and colleagues in 1962. Because of the self-contained fluorescence mechanism and lack of interference when fused to various proteins, GFP is widely used as a marker for protein expression and has been used as a reporter in a variety of prokaryotic and eukaryotic cells (Chalfie and Kain, 1998; Sternberg et al., 1999; Natarajan et al., 1998). The possibility to measure GFP on-line in real time allows for its use for control and optimization of bioprocesses (Albano et al., 1998; DeLisa et al., 1999; Zhao et al., 1999) and the study of growth phase-regulated genes (Cote et al., 1997; Moede et al., 1999).

The existing devices for on-line GFP measurements suffer from specific design problems. One of them is the poor reproducibility of the absolute measurements. The problem originates from the optical path, which consists of light-proof flow-through cells and fiber optics (Randers-Eichhorn et al., 1997). They are used in order to suppress ambient light. As the intensity of the fluorescent signal strongly depends on the positioning of the optical fiber both inside the cell and on the bench, the sensor requires individual initial calibration for each run.

Another problem is the selectivity of the sensors (Knight et al., 1999). Fermentation media are usually highly fluorescent, with very broad emission spectra. However, it is difficult to use aggressive filtering to eliminate extraneous light because of the low levels of the signal. As a consequence, there is a strong background signal during the measurements which slowly changes as the nutrients are depleted.

Optical fibers strongly attenuate both the excitation light and the output fluorescence and photomultipliers are used for light detection (Craig et al., 1998; Knight et al., 1999; Korf et al., 1997; Randers-Eichhorn et al., 1997). This imposes the need for specific design that provides very good optical shielding in order to avoid damage to the photodetector. As a result, the sensor becomes a bulky and expensive device: it costs approximately $2,000 when an LED with precision current supply is used as light source, and significantly more when a laser is used.

In this work, we present an all solid-state GFP sensor with high reproducibility and selectivity. The sensor is light-tolerant and works with standard glass cuvettes under room-light illumination. It is small, highly sensitive and selective, very inexpensive, and suitable for both off-line and on-line measurements.

MATERIALS AND METHODS

Sensor Design

The sensor was designed using a standard 1 cm quartz cuvette (normal or flow-through). The cuvette is the only sterilizable part of the sensor. A front-face geometry was chosen (Fig. 1). The cuvette holder was designed to provide a rigid optical path with constant wavelength. Two excitation sources were tested: high-intensity blue LED MBB51TAH-T (Microelectronics, Santa Clara, CA) with peak wavelength at 470 nm, operated at peak current 30 mA, and UV emitting LED NSHU550E (Nichia America Corp., Lancaster, PA) with peak wavelength at 375 nm,

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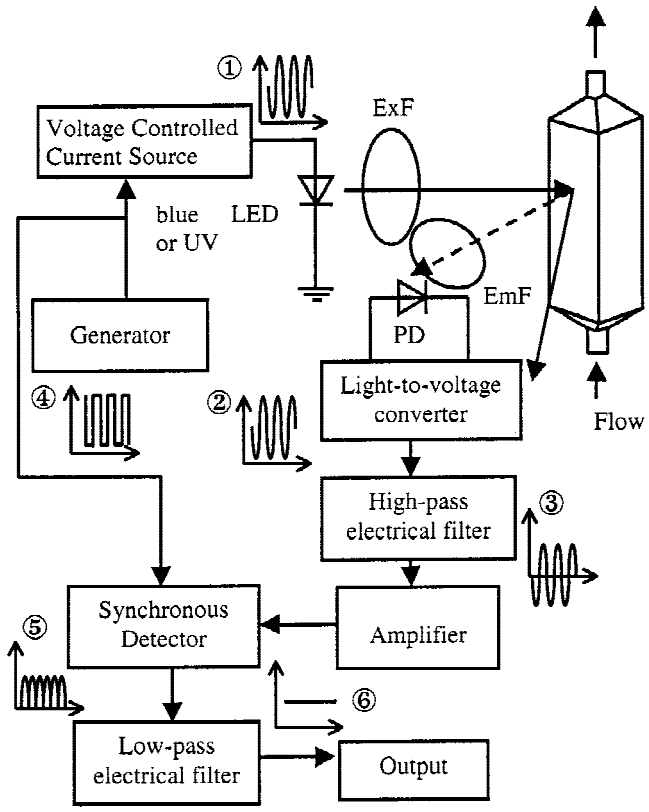

Figure 1. Diagram of the sensor. ExF, excitation filter; EmF, emission filter; PD, photodiode. Outcoming light from the cell: solid arrow, excitation light reflection; dotted arrow, fluorescence. ①, – modulation signal for LED; ② – detected fluorescence emission; ③ – signal with removed DC component; ④ – synchronization signal; ⑤ – rectified signal, ⑥ – DC component of the rectified signal. See text for details.

operated at 10 mA. As GFP possesses two maxima (major at 395 and minor at 475 nm) in its excitation spectrum, both LEDs are suitable for excitation sources.

The excitation light was electronically modulated at 1.5 kHz, using a NE 555 as a generator and voltage-controlled current source. The light from the blue LED was passed through interference bandpass 470 ± 10 nm filter with absorption >4 in the stopband. The UV LED was operated without filtering the light, as its emission spectrum is extremely narrow and possesses no "red tail." The excitation light was directed at approximately 40° toward the cuvette wall to avoid direct illumination and minimize excitation light reaching the detector. The fluorescence emission was observed through a 514 ± 10 nm filter to pick up the maximum fo the green flourescence (GFP emission maximum is at 509 nm). Both the filters were from Intor, Inc. (Socorro, NM). An additional interference filter 45-IF550 (Olympus Corporation, Lake Success, NY) with absorption 1.5 at 470 nm was used in front of the photodetector in order to increase the selectivity. The detection of the light was performed using a large active area (13 nm^2) PIN photodiode 1223-01 (Hamamatsu, Bridgewater, NJ) and light-to-voltage converter (AD745). The electrical signal was passed through first order, high-pass electrical filter with cut-off frequency 1 kHz and amplified. This removed the DC offsets and ever-present 60 Hz noise and diminished the level of the high-frequency noise. This AC signal was synchronously rectified using AD 630 (Analog Devices, Norwood, MA) and passed through integration RC chain with time constant 1 sec. The sensor (without the cuvette) costs approximately $60 to assemble.

Sensor Calibration

During the off-line calibration, the output was measured using a digital multimeter D-990 (Protec, Korea). The sensor was calibrated off-line using partially purified GFP in solution with concentration 0.78 g/L (2.9×10^{-5} M). The concentration of GFP was determined against a standard with concentration 1 g/L (3.7×10^{-5} M). The solution was consecutively diluted with deionized water in proportion 1:1 at each step. The influence of the scattering properties was evaluated using 5% (v/v) solution of colloidal silica Ludox HS30 (DuPont, Wilmington, DE) in water. All the calibration and further measurements were performed without any protection of the light detector from ambient room lights or daylight.

Fermentation

The overnight seed culture consisted of a 1% inoculum of a pBAD-GFP transformed *Escherichia coli* strain JM105 (arabinose promoter; Crameri et al., 1996) frozen stock, and 100 μM ampicillin in buffered LB media (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract, 4 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, pH 7.2) incubated at 35°C with shaking at 260 rpm (model G24, New Brunswick Scientific, Edison, NJ). A fresh seed culture consisting of a 1% overnight inoculum was started using the same media and identical culture conditions in order to begin fermentation with a mid-log phase culture. Fermentations were carried out in a New Brunswick BioFlo III fermentor inoculated with 1% of the mid-log phase culture and containing 1 L buffered LB, 100 μM ampicillin, 1 g/L glucose, and 325 μl 1% BASF Pluronic L-61 surfactant. Aeration, agitation, and temperature were controlled at 1 vvm, 300 rpm, and 35°C, respectively. Dissolved oxygen was controlled by agitation at a minimum of 6.9% relative to 100% oxygen. Culture media was continuously pumped from the vessel, through a recycle loop debubbler (Coppella and Rao, 1990), and through both the sensor described here equipped with a glass flow-through cuvette (Uvonic Cuvettes, Plainview, NY) and the previously described fiber optic bundle sensor (Randers-Eichhorn et al., 1997) before returning to the fermentor. Data of continuous readings were logged every 20 sec on a Mac II computer using a Strawberry Tree data acquisition system and Workbench software (Strawberry Tree, Sunnyvale, CA). Upon consumption of the glucose present in the media, expression of GFP was induced by the addition of 6 g/L arabinose (Sigma, St. Louis, MO).

Glucose values were measured using a YSI 2700 Bio-

chemistry analyzer (YSI, Yellow Springs, Youngstown, OH). Optical density (O.D.) values representative of the bacterial growth were measured with a Milton Roy Spectronic 401 spectrophotometer at 600 nm. Off-line fluorescence intensity measurements were made with a Perkin Elmer (Oak Brook, IL) MPF-66 spectrofluorometer with excitation at 395 nm slit width 1 nm and emission 509 nm slit width 5 nm.

High Cell Density Test

The sensor was tested off-line for an ability to follow the fluorescence changes in high cell density media. A postfermentation sample with O.D. 40 was used (46.9 g/L dry cell weight). The sample was *E. coli* W3110 [pGFPuv-CAT] with Trc-HIS plasmid (Cha et al., 2000). The sample was consecutively diluted with deionized water. The sensor was used with the blue LED as an excitation source.

RESULTS AND DISCUSSION

The sensor was designed to work with front-face geometry, as the range of the optical density of the sample could vary in a broad range. Our preliminary tests showed that in a right angle configuration the output signal is close to linear up to O.D.~10, and at O.D.~50 the output signal begins to decrease due to strong inner filter effects. In contrast, the front-face configuration exhibited an increasing signal up to O.D. 110.

The sensor was able to work under room-light illumination without use of specially designed flow cells. This was achieved using modulation of the light source and lock-in type detection of the fluorescence. The use of LED allowed light chopping without use of mechanical components. As the heat generation of the LED is minimal, it was mounted close (<1 cm) to the cuvette. The short optical path allowed coupling of almost all the excitation radiation to the sample. The enhanced signal level made possible the use of an inexpensive semiconductor photodiode as detector.

The performance of the sensor was initially evaluated using consecutive dilutions of GFP in deionized water. Calibration curves for both blue and UV LEDs as excitation sources were obtained (Fig. 2). The linearity in the both cases was excellent (correlation coefficient = 0.991 for the blue LED and 0.992 for the UV LED). The sensitivity of the instrument with the UV LED was approximately four times higher, as could be expected from the excitation spectrum of GFP. However, with UV excitation the sensor reached saturation of the output signal for GFP concentrations higher than 0.2 g/L (7.4×10^{-5} M).

The parameters of the sensor are summarized in Table I. As could be seen, the increased sensitivity leads to higher zero offset. The influence of the scattering properties of the sample on the sensor's output is negligible due to the efficient filtering employed. The increased background level did not introduce problems because of the higher selectivity of the sensor.

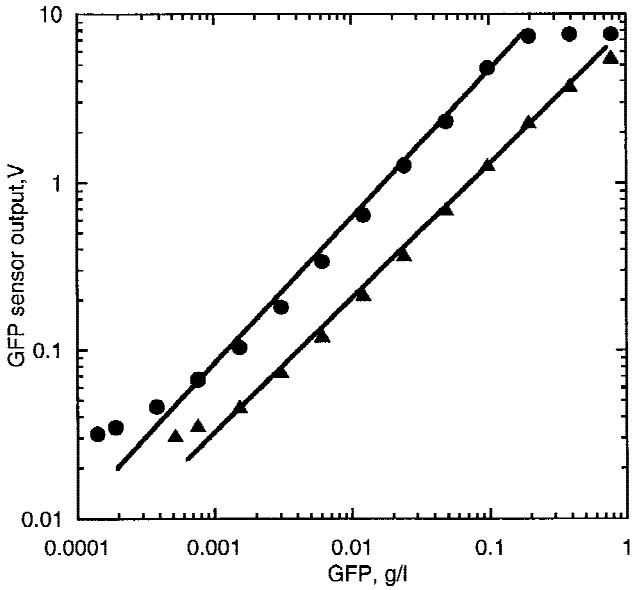

Figure 2. Calibration of the GFP sensor with different light sources. Circles = UV LED, triangles = blue LED.

Reproducibility was quite high, the standard deviation for triple measurements was less than 1%. This compares favorably with the previous fiberoptic version of the sensor, which has a standard deviation of 8%.

The sensor was validated during fermentation using *E. coli* harboring the GFP reporter gene controlled by the arabinose promoter (Crameri et al., 1996) Throughout fermentation, samples were taken for off-line measurements of glucose, OD, and fluorescence intensity. The OD at the end of the processes was 4.6 for the fermentation monitored with the UV LED and 3.5 for the fermentation, monitored with the blue LED. Figure 3 shows the on-line sensor data of the solid-state GFP sensor equipped with blue LED and compared with off-line measurements taken during fermentation. Both the runs show very high linear correlation (r^2 = 0.989 for UV LED and r^2 = 0.998 for blue LED) with the off-line spectrofluorometer measurements. The correlation between the new sensor and the previous version was higher than 0.999 (results not shown). The range of the sensor when used with the blue LED is similar to the fiber optic sensor. The use of UV LED as excitation source significantly increased the range toward the low concentrations; however, the output signal tends toward saturation at high

Table I. GFP sensor parameters with different light sources.

	Blue LED	UV LED
Sensitivity (V.1/g)	12.4	44.4
Zero value (water, V)	0.023	0.033
Scattering signal (colloidal silica), V	0.027	0.037
Background (LB media), V	0.048	0.57
Limit of detection (M),	$1.9 \cdot 10^{-8}$	$7.0 \cdot 10^{-9}$
(g/L)	0.00052	0.00019
Noise (V)	±0.002	±0.002

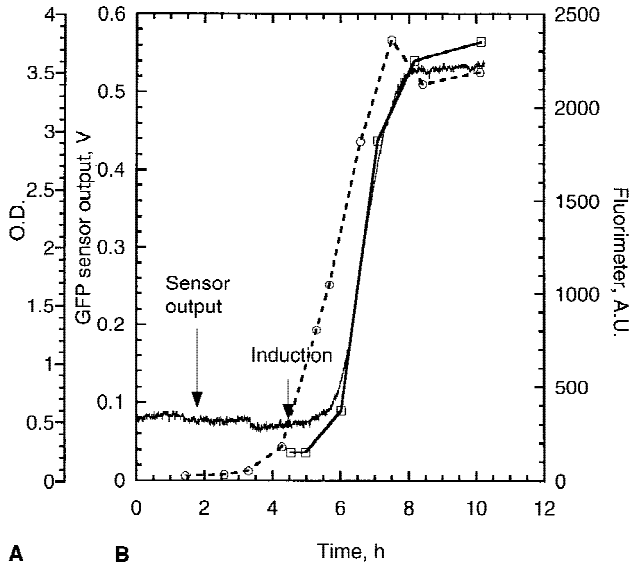

Figure 3. Comparison between the sensor's output and the off-line measurements taken on the spectrofluorometer. Blue LED used as excitation source. Circles, O.D.; squares, fluorometer data. The moment of the GFP induction is shown. **b**: Comparison between the sensor's output and the off-line measurements taken on the spectrofluorometer. UV LED used as excitation source. Circles, O.D.; squares, fluorometer data. The moment of the GFP induction is shown.

GFP concentrations, as could be expected from the calibration curve. The choice of a high (UV LED) and low (blue LED) sensitivity is an advantage as the level of GFP expression may vary widely based on the particular culture system used.

The sensor could be used also for measurements of significantly higher cell concentrations. The off-line test with a sample of O.D. 40 is presented in Figure 4. As expected, the response was close to linear to O.D. of 10; at higher optical densities the depth of penetration of the excitation light is very little, and the output result is distorted by the primary and secondary inner filter effect. However, with thorough calibration under strict experimental conditions, the sensor could be used for evaluation of GFP concentrations in samples with O.D. up to 100 (results not shown).

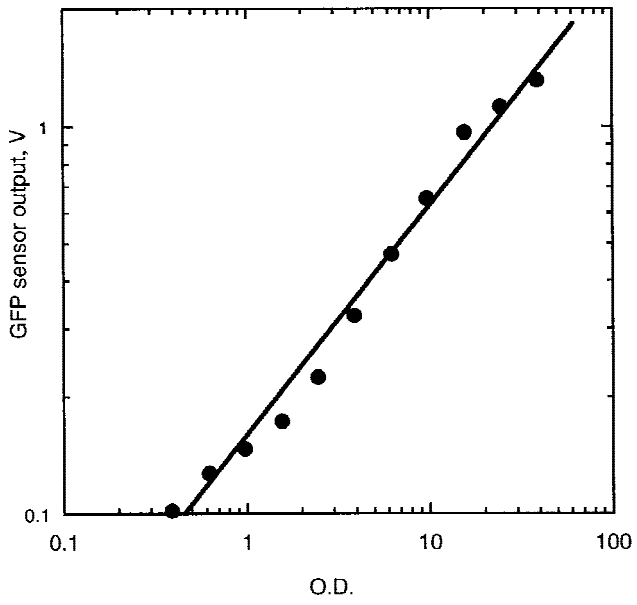

Figure 4. Off-line test of the sensor with high-cell-density sample.

CONCLUSIONS

The newest design of the GFP sensor utilizes a semiconductor photoreceiver instead of a photomultiplier tube with the added advantages that it is insensitive to room light and constructed at very low cost. It has wide dynamic range, compact design, and low detection limit. With the widespread popularity of GFP, this sensor should be indispensable for any laboratory working with GFP. We have demonstrated its abilities for either on-line or off-line measurements. Given the low cost and simple design of the sensor, the next logical step will be adaptation of the sensor for use with multiple measurements of the many available GFP-altered spectra mutants.

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