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Accepted Manuscript

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ARTICLE

Portable FRET Analyzer for Rapid Detection of Sugar Content

Cite this: DOI: 10.1039/x0xx00000x

Haseong Kim[a], Hyo Sang Kim[b], Jae-Seok Ha[c] and Seung-Goo Lee[a,d,*]

Received 00th January,
Accepted 00th January

DOI: 10.1039/x0xx00000x

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The fluorescence resonance energy transfer (FRET) is widely used as core process in biometric sensors to detect small molecules such as sugars, calcium ions, or amino acids. However, FRET based biosensors with innate weak signal intensity require the use of expensive, high-sensitive equipment. In the present study, these shortcomings were overcome with the fabrication of a sensitive, inexpensive, and portable analyzer which provides quantitative detection of small molecules in a liquid sample. The usability of the developed analyzer was successfully tested by measuring sucrose and maltose contents in commercially available beverage samples, with better performance than the conventional monochromator-type spectrofluorometer. It is anticipated that miniaturization of the equipment and improving the FRET based biosensors will contribute to practical use of this hand-held analyzer in conditions where high-end equipment is not available.

Introduction

A biosensor, consisting of a bio-receptor and a signal transducer can selectively detect substances in a liquid by transducing biological recognitions into a photonic or electrical signal, leading to the quantitation of target substances. Fluorescence resonance energy transfer (FRET)-biosensors have been developed [1] for analysis of small molecules such as sugars [2], calcium ions [3] or amino acids [4-6]. FRET biosensors contain fluorescent proteins, enhanced cyan fluorescent protein (ECFP), and enhanced yellow fluorescent protein (EYFP), which are fused to both ends of periplasmic-binding proteins (PBPs) from *Escherichia coli* [7, 8]. The mechanism of FRET biosensors is that sugars bind to PBPs located in the middle of the sensor, causing structural changes and subsequently resulting in changes in the distance and transition dipole orientation of the two fluorescent proteins at either end of the PBP. This change in FRET due to the sugar binding is measured as a ratio of emission wavelengths of EYFP and ECFP (530 and 480 nm, respectively), which allows quantitative analysis of the sugars. For instance, in the presence of maltose, fluorescence intensity of ECFP decreases and that of EYFP increases. This change of the intensity is dependent on the maltose concentration, indicating that the ratio of these two fluorescence intensity is clearly distinguished from the ratio in the absence of maltose. We previously reported a calibration curve for ratio measurements with varying sugar concentrations using a conventional monochromator-type spectrofluorometer, where an S-shaped sigmoidal curve was observed [2, 9]. We measured the sugar uptake rate by *E. coli* K12 strain MG1655 when different sugars were provided as a carbon source in minimal medium, and the amount of sugar utilized was analyzed as a comparison of wild-type *E. coli*.

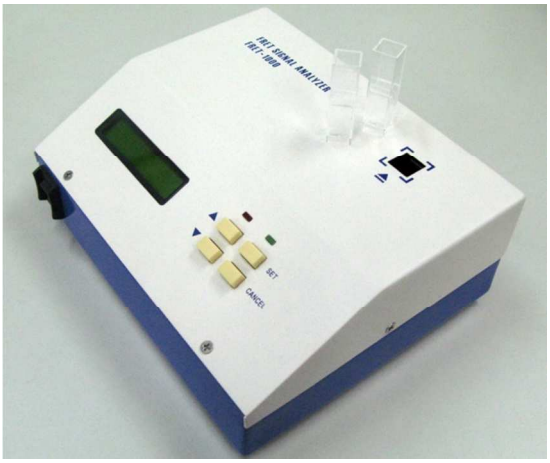

Fig 1. Fabricated portable FRET analyzer which is available for liquid samples with FRET bio-sensor in a rectangular parallelepiped vessel

However, it requires a highly sensitive and sophisticated system for FRET signal detection. The high-end fluorescence analyzers commonly used in laboratories have a high-pressure lamp or laser as a light source for sample analysis and their detectors with high sensitivity, photomultiplier tubes (PMT) or charge coupled device (CCD), are used. Despite the high sensitivity of such a system, their use in a portable and on-site practical

manner is not possible by the virtue of their large size and high cost. In the present study, an inexpensive and portable fluorescence analyzer with capabilities similar to those of the existing fluorescence analyzers was developed using the latest, high-powered LED as a replacement for the lamp or laser (Fig. 1). The developed analyzer was successfully evaluated by measuring sucrose and maltose content in beverages and frozen snacks available commercially.

Experimental

Selection of Light Source and Optical Signal Detector

In order to overcome the challenge of weak fluorescence signal intensity in a small size FRET analyzer, we devised a method to divide FRET signals into two wavelength bands and read the signals from each band. In designating the two types of wavelength bands, it is important to have the relative signal changes from each band effectively and to designate a band for the appropriate excitation wavelength while avoiding interference between the absorbance of excitation wavelength and the wavelength band of corresponding fluorescence signal. To determine these wavelength bands, overlap of ECFP absorption spectrum and FRET signal spectrum were measured in Fig. 2. As the excitation wavelength must be within 390 to 455 nm range, we tested to use different LEDs with similar excitation properties and an LED (Seoul Viosys Co., Ltd.) with 405-nm band was selected as the light source, though the absorption efficiency at 405 nm was only the half of that at 435 nm because a high-powered LED with longer band is not available.

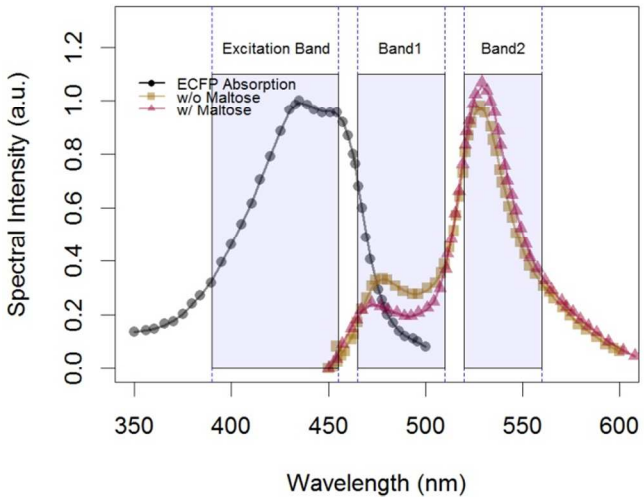

Fig 2 Absorption curve of primary fluorescence protein and primary (Band1) and secondary (Band2) FRET signal spectrum. Excitation wavelength band: 390 to 455 nm; primary fluorescence signal range: 465 to 510 nm, secondary fluorescence signal range: 520 to 560 nm

Along with determining an appropriate light source, we designed the optical signal detection system, a critical part of a hand-held FRET device. The components available for use in the detection of optical signals are PM tube, Photodiode, and Avalanche Photodiode, but based on cost and efficiency, a silicone photodiode (Si Photodiode) was selected for the detection system. Silicone photodiode is the most commonly used component for detecting light in the visible light spectrum and has response characteristics for a wide wavelength range, namely 350 to 1100 nm. The advantage is that this inexpensive component does not necessarily have high detection capabilities, but has a wide detection range. In particular, Avalanche photodiode with a detection area of 1mm diameter and Si-photodiode with 3 × 3 mm area produced similar levels of current output in our system. The current output from photodiodes is converted at a ratio of 10^7 V/A in a transimpedance amplifier (TIA), and this output passes through a 10× voltage amplifier. Then, it goes through a low-pass filter (bandwidth 1.6 kHz) and is measured as apparent diffusion coefficient (ADC).

Fabrication of Optical System

An optical system consisting of the light source and the photodiode was assembled with optical lenses and filters. The light source system for focusing the output light from the LED was constructed using two spherical lenses (Fig. 3 A) and an optical head. The fluorescence signal (green) from a FRET biosensor excited by an excitation wavelength (dark blue) are shown in Fig. 3 B. Beam distribution of source light was observed with the end of the optical head as reference and using 5-mm increments from 0 to 35 mm (Fig. 3 C). At 15 mm from the optical head, the beam size was the smallest at approximately 4 × 4 mm, and beam distribution within 5 mm from this point did not show significant changes. For the liquid sample to be used in the optical system, a 12.5 × 12.5 × 45 mm rectangular parallelepiped vessel (cuvette), commonly used in fluorescence detection devices, was used.

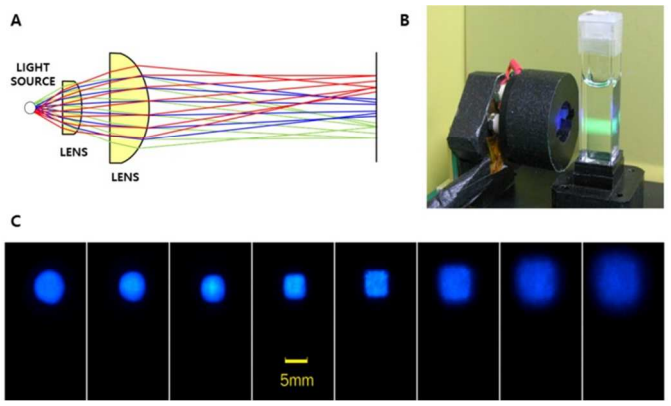

Fig 3. (A) Optical system simulation for LED optical head. (B) Fabricated optical system with LED head and green fluorescence signal of a liquid sample in a cuvette. (C) Changes in intensity distribution based on moving distance of excitation wavelength.

The detection part of the optical system consists of a lens that captures the fluorescence signal and converts it to parallel light, and a beam splitter (BS) splitting parallel light into a 50:50 ratio. Also it is equipped with band pass filters (BPFs) that allow passing of wavelength signal from each path, and two

lenses that focus each signal onto the photodiode (Fig. 4). The sensor protein in the cuvette was excited by the source light to generate fluorescence signal and this signal was split into two paths by the BS. Of the two, one path passes through the BPF that only allows the passage of primary fluorescence wavelength (465 to 510 nm), which then arrives at the photodiode to be converted to an electrical signal. The light in the second path passes through a BPF that only allows passage of secondary fluorescence signal (520 to 560 nm), which is then converted to an electrical signal different from the previous one. A simulation of fluorescence signal emitted from the sample progressing through the optical system is shown in Fig. 4 A. Fig. 4 B shows the image of detection system fabricated for this experiment. The exterior of this system is enclosed in a 55 × 65 × 55 mm vessel to prevent interference from external light and the size is approximately 95 × 95 × 60 mm, with the photodiode included. The battery and the charging circuits were internalized to allow use in the field, and additionally the facility of connecting to a computer for remote control was equipped.

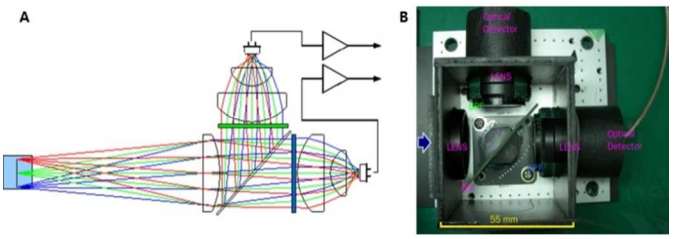

Fig 4. (A) Simulation of optical detection system for FRET signal which is split into 50:50 ratio by the beam splitter lenses. (B) Image of fabricated optical detection system.

FRET Biosensor Preparation

FRET biosensors were the same proteins that have been previously developed with allose-, arabinose-, glucose-, ribose-, and maltose-binding proteins [9]. The corresponding plasmids were transformed into *E. coli* JM109 (DE3) and cultivated in Luria-Bertani (LB) medium with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 28°C for 20 h. *E. coli* cells were harvested by centrifugation and dispersed in phosphate buffered saline (PBS, pH 7.4) containing 50% CelLytic™ B (Sigma), following which the cells were lysed using an ultrasonic treatment. The resulting solution was centrifuged, and the supernatant was injected into HisTrap™ HP column (Amersham Biosciences, Sweden) connected to a Fast-Performance Liquid Chromatography (FPLC) system. After two to three washes with a binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 1 mM PMSF, and 5 mM imidazole; pH 7.5), it was eluted by the concentration gradient slope method using a solvent (20 mM sodium phosphate, 0.5 M NaCl, 1 mM PMSF, and 0.5 M imidazole; pH7.5) containing 0.5 M imidazole. The degrees of purification in each of the eluted FRET biosensors were verified by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the sensors with confirmed degree of purification were acclimated in PBS buffer (pH 7.4) with 20% glycerol by using a HiTrap™ Desalting column (Amersham Biosciences, Sweden) connected to FPLC in order to eliminate imidazole. FRET biosensors that completed the purification process were concentrated to 5 to 10 mg/ml with 20 ml VIVASPIN (30,000 MWCO, Vivascience, Germany), they were stored in an ultra-low temperature freezer at -70°C until further analyses. The protein concentrations of each sensor were measured with protein assay reagent (Bio-Rad) using bovine serum albumin (BSA) for protein standard.

Preparation of Sugar Samples

Our quantification using FRET biosensors is a method where the one-hundredth volume of sample is transferred into a standard detection solution, 0.5 mL PBS containing 0.2 μM FRET biosensor, to read the change of FRET ratio in a fluorescence spectrometer. It is preferable to eliminate any insoluble materials in such as cellular or lipid components, and/or auto-fluorescence substances that can influence the measurement of fluorescence. In the present study, maltose and sucrose content in beer and other beverages was measured using the developed FRET analyzer. The samples were diluted 10 times in PBS and 5μL of the sample solution was taken into a micro-tube containing standard detection solution. Then, the 530/480 nm ratio was measured and the concentration of each sugar was calculated from the titration curves of maltose and sucrose.

When tested with frozen snacks, they were subjected to additional steps to remove dairy fat, emulsifiers, and food colorants. The samples were first centrifuged in a micro-tube at a speed of 15,000 rpm for 30 min and a set amount was transferred to a new micro-tube while making sure that the sediment at the bottom and the dairy fat in the top layer was excluded. Then, an equal amount of hexane was mixed and centrifuged at a speed of 15,000 rpm for 30 min. The pre-treated solution was diluted 10 times in PBS and 5μL of clear supernatant transferred to a standard detection solution following the same protocol for beverage analysis; from which 530/480 nm ratio was measured and the concentration of each sugar was calculated from the titration curves of maltose and sucrose.

Results and discussion

Dose-Response Model for FRET Signal Analysis

For fluorescence analysis, FRET ratio is defined by Equation 1 where inputs the ratio of emission intensity of ECFP at 480 nm (I_{480}nm) and emission intensity of EYFP generated at 530 nm (I_{530}nm).

$$r = \frac{I_{530nm}}{I_{480nm}} \qquad (1)$$

Then signal intensity size, an indicator of the FRET biosensor's detection capability, was calculated using Equation 2. Here, Δr is defined as the maximum difference between the ratio (r_{max})

under the condition of saturated concentration of ligand (sugar), 1 to 10 mM, being present and the ratio (r_{min}) under the condition of no ligand being present.

$$\Delta r = r_{max} - r_{min} \quad (2)$$

Note that the sugar concentration for 50% response was estimated as that for the Δr value being 1/2 for each sugar. The dose-response curve of FRET biosensor was expressed as an S-shaped sigmoidal curve by taking the measured ratio (r in Equation 3) in different sugar concentrations (x in Equation 3).

$$r = A_1 + \frac{A_2 - A_1}{1 + 10^{p(log_{10}x_0 - log_{10}x)}} \quad (3)$$

where A_1 and A_2 represent signal ratio with sugar concentration of zero and saturated, respectively; x_0 represents sugar concentration at 50% response; and p represents slope of response, which is close to 1 or -1. In the present study, the values of A_1, A_2, and x_0 were 6.150, 9.157, and $10^{1.856}$, respectively where only 1 to 1000 μM concentration ranges were used in the model fitting. The values p is derived by depending on each type of sugar being fixed at 1 or -1. Fig. 5 A shows the plot for the observed ratio and maltose concentration together with the fitted curve using SigmaPlot software. In order to evaluate the model (Equation 3), we measured the ratio and predicted maltose concentration for each point and computed Root Mean Squared (RMS) deviation which is the amount of deviation between test data and the predicted value. **Error! Reference source not found.**5 B shows the predicted concentration from Equation 3 (x axis) and the actual concentration (y axis) we used. A good linearity was seen in the 25–1000 μM range and RMS was 0.4%, which indicates our model of Equation 3 is significant.

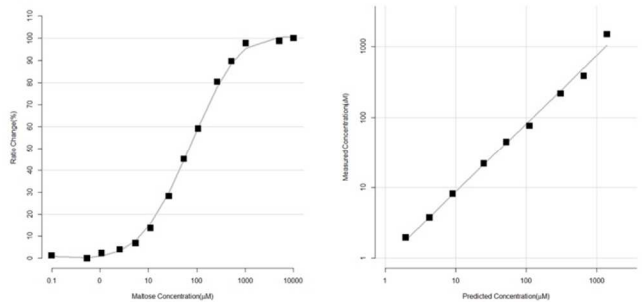

Fig. 5 (A) Ratio of secondary fluorescence signal and primary fluorescence signal based on maltose concentration. (B) Relationship between actual measured sugar concentration and predicted value from the simplified model.

In cases when the intensity of excitation wavelength is extraordinarily high, bleaching of fluorescent substance may occur. It can lead a decrease in the intensity of emission wavelength and influence the ratio of fluorescence signals. Since this phenomenon is irreversible, it permanently alters the condition of the sample and introduces an error in the result. Accordingly, it is recommended that FRET sensor system be used in ranges where bleaching of fluorescent substances is not expected. To test bleaching effect, we used two samples, one of which has ECFP/EYFP protein, at a concentration of 0.2 μM and enough maltose added in the other sample containing the sample protein to create FRET signal saturation. We confirmed the intensity ratio for each fluorescence signal was proportional to excitation wavelength intensity and any indications of bleaching were not observed. We fitted the linear line of each signal based on the excitation wavelength intensity and computed RMS, the amount of deviation of each data point from the fitted straight line (Table 1). The fluorescence signal slopes of both samples show RMS deviation of < 2%, indicating good linearity. Moreover, the ratios of both fluorescence signals remain constant (< 0.7% RMS) even with changes in excitation wavelength intensity. These results demonstrate important characteristics of the ratio of fluorescence signals not being affected by excitation wavelength and depending only on the concentration of maltose.

Table 1. Level of bleaching in signal proteins based on excitation wavelength intensity

Sample	Primary fluorescence signal slope / RMS (%)	Secondary fluorescence signal slope / RMS (%)	Ratio (Primary/Secondary) / RMS (%)
A (w/o Maltose)	7.65 / 1.5%	1.62 / 2%	4.73 / 0.65%
B (w/ Maltose)	8.94 / 1.45%	1.48 / 1.54%	5.93 / 0.52%

Performance Test of FRET Analyzer

In order to evaluate the sensitivity and accuracy of our FRET analyzer, we compared them with the results from a monochromator-type fluorescence spectrometer (Cary Eclipse, Varian, Australia). The biosensors used were allose, arabinose, glucose and maltose sensors. The monitoring parameters for the FRET analyzer are: i) temperature; ii) emission intensity at 480/10 nm; iii) emission intensity at 530/10 nm; and iv) 530/480 nm ratio. As results, clear S-shape curves were obtained for each sugar, similar to the titration curves from Cary Eclipse, as shown in Fig. 6. FRET analyzer shows more stable and clear signal intensity than that of Cary Eclipse in all cases. During fluorescence measurement, FRET biosensors experienced virtually no photo bleaching and the temperature was confirmed to have been maintained at around 23 ± 1°C. These signals from FRET analysis equipment were confirmed to have overall signal intensity two times higher than that from Cary Eclipse. Also the standard deviations of the measured values from our device were also lesser, in comparison to Cary Eclipse. Therefore, using the FRET analyzer with FRET biosensor for measuring concentrations of sugar is expected to allow more accurate analysis than the conventional monochromator-type fluorescence spectrometer.

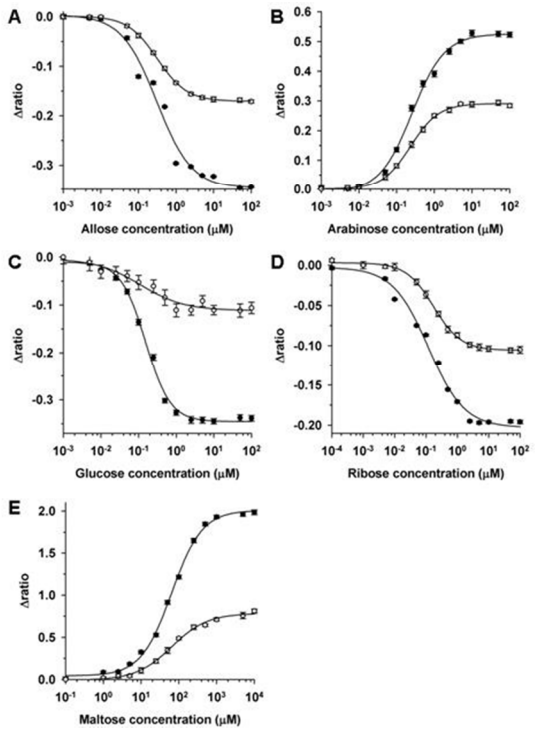

Fig. 6 Comparison of S-shaped titration curves of FRET biosensors, using FRET analyzer and Cary Eclips, for measuring (A) allose, (B) arabinose, (C) glucose, (D) ribose, and (E) maltose ●; S-shaped curve, measured by FRET analyzer, ○; S-shaped curve, measured by Cary Eclips

Table 2 Summary of sucrose and maltose contents in food ingredients measured by FRET biosensor

Product Name (Type)	Manufacturer (Country)	Shown content (g/100 ml)		Measured content (g/100 ml)	
		Carbohydrates (Content)	Sugars (Content)	Sucrose	Maltose
M** (beer)	H Corp. (Korea)	Barley	-	0.7 ± 0.06	0.5 ± 0.06
M***** (beer)	M Corp. (USA)	Malt, Corn	-	0.6 ± 0.03	0.7 ± 0.04
A**** (beer)	A Corp. (Japan)	Malt, Corn, Rice	-	0.3 ± 0.03	0.6 ± 0.05
K********* (beer)	K Corp. (Germany)	Malt	-	0.5 ± 0.05	0.7 ± 0.05
H**** (frozen snack)	H Confectioner (Korea)	24	16	21.9 ± 1.9	-
N****** (frozen snack)	H Confectioner (Korea)	23	16	22.9 ± 1.5	-
N** (frozen snack)	H Confectioner (Korea)	24	15	22.2 ± 1.5	-
B****** (frozen snack)	H Confectioner (Korea)	Citrus extract Sugar fructose syrup		24.0 ± 1.7	-
S** (frozen snack)	H Confectioner (Korea)	27	22	25.3 ± 1.4	-
S*** (frozen snack)	H Confectioner (Korea)	Citrus extract Sugar fructose syrup		11.1 ± 1.0	-
B*** (beverage)	H Corp. (Korea)	13	11	9.2 ± 0.8	3.1 ± 0.03
E*** (beverage)	E. Foods (Korea)	White rice extract (43%) Brown rice extract (33%)		0.4 ± 0.03	1.1 ± 0.05
E** (beverage)	L Beverage (Korea)	7	7	3.8 ± 0.24	-
P***** (beverage)	D Corp. (Korea)	6	6	3.3 ± 0.33	-
G***(beverage)	L Beverage (Korea)	7	7	5.9 ± 0.46	-

Sugar Content Analysis in Food, using FRET Analyzer

The FRET analyzer was used to measure the fluorescence of sugar content in food. The samples including beer, barley beverages, and frozen snacks, were treated by centrifugation to remove insoluble particles and lipid components were removed by solvent extraction, if required. Table 2 summarizes the results of sucrose content in 15 types of food items measured by purified our FRET sensor[2]. As commonly known, the amount of sucrose in most frozen snacks measured very high at 11 to 25 g / 100 mL and even in beverages, sucrose content was measured at approximately 3 to 9 g / 100 mL. In addition, sucrose content of 0.3 to 0.7 g / 100 mL was identified in beer. Maltose content was generally around 0.5 to 0.7 g / 100 mL in beer. In barley beverages, maltose content was 3.1 g / 100 mL; which showed the high maltose content among all food items (Table 2).

Conclusions

This study describes the development of a portable hand-held FRET analyzer that can efficiently quantify the sugar content in liquid samples. Weak fluorescence signals were strengthened by dividing the secondary (band2) signal with the primary (band1) signal of the FRET signal spectrum (Fig. 2). A small and inexpensive 405-nm band UV-LED was used as the light source for excitation of the fluorescence signal. In addition, the detection system was designed to effectively focus the dissipating fluorescence signal onto two photodetectors with a silicone photodiode. Since our device is specifically designed to detect the ratio of the two particular emission wavelengths, its performance with respect to its sensitivity and wavelength detection range cannot be directly compared to that of other commercially available fluorometers. However, in our experimental conditions, the proposed device shows better sensitivity and intensity in detecting various sugars than does the Cary Eclipse (Fig. 6). In addition, the small size and light weight of our device is advantages compared to the other devices. Our device measured 20cm (Width) / 15cm (Height) / 6cm (Depth) and it was weighted 1.5 kg.

Hand-held and portable devices, like the one we developed, have recently drawn much attention owing to their applications to various monitoring purposes such as food quality assessment, environmental hazard monitoring, and blood sugar monitoring. In particular, a portable FRET device can be used for point-of-care testing (POCT), which patients themselves or non-professionals can easily perform. POCT is currently used

for assessing pregnancy, blood glucose levels, biomarker proteins, infectious bacteria, and infectious viruses, and is receiving increasing attention because it can be done easily using blood or urine samples without the need for expensive medical equipment. For such on-site measurements and the diagnosis of samples, our hand-held device initially achieved successful for the quantitative analysis of maltose and sucrose contents in food items. Furthermore, it was found to have advantages with respect to its high sensitivity for signal detection, portability, and ease of use despite of low manufacturing cost (less than one thousand dollars). So our FRET analyzer is clearly beneficial for measuring small molecular contents with FRET sensors, particularly in a small-scale laboratory environment. However, this system still requires technical improvements to reduce equipment size and minimize the pre-treatment of samples. Also Identifying practical requirements for field use will contribute to future commercialization and the establishment of a potential market for FRET analyzers.

Acknowledgements

This research was supported by grants from the KRIBB Research Initiative Program, the Next-Generation Biogreen 21 Program (PJ009524), and the Intelligent Synthetic Biology Center of Global Frontier Project (2011-0031944).

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[a] Synthetic Biology & Bioengineering Research Center, Korea Research Institute of Bioscience and Biotechnology, South Korea,
[b] Fiberpro, Inc. 26-55 Gajeongbuk-ro, Yuseong-gu, Dae-Jeon, South Korea
[c] Humedix, Co. LTD, Gwanyang-dong, Dongan-gu, Anyang-si, Gyeonggi-do, South Korea
[d] Biosystems and Bioengineering Program, University of Science and Technology, South Korea
* To whom correspondence should be addressed, Email: sglee@kribb.re.kr

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