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High sensitive and rapid fluorescence detection with a portable FRET analyzer

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Abstract:	Recent developments in Föerster Resonance Energy Transfer (FRET) sensors have enabled the detection of various small molecules, including ions and amino acids. However, the innate weak signal intensity of FRET sensors is a major challenge preventing the application of FRET sensors in various fields; this limitation necessitates expensive, high-end fluorometers, further limiting the potential applications of this technology. Previously we built a cost-effective, high-performance FRET analyzer which can specifically measure the ratio of two emission wavelength bands (530 and 480 nm) for the high detection sensitivity. More recently it was discovered that FRET sensors with bacterial periplasmic binding proteins has a critical temperature, 50-55 °C detecting ligands with maximum sensitivity. This report describes a protocol of the sugar content assessment including the portable FRET analyzer operation with the preparation of temperature specific FRET sensor including commercially available beverage samples. As results, additional preheating process of FRET sensor significantly increases the FRET ratio signal in measuring sugar contents using the custom made FRET device and the device with the FRET sensor was successfully applied to quantify the sugar content in commercial beverages. We anticipate that further size reduction and performance enhancement of the equipment will facilitate the practical use of hand-held analyzers in environments where high-end equipment is not available.
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2015.09.18

Avital Braiman
Editorial Director
Journal of Visualized Experiments

To the Editors: Dr. Benjamin Werth

I, along with my coauthors, would like to ask you to consider the attached manuscript entitled "**High sensitive and rapid fluorescence detection with a portable FRET analyzer**" for publication in *Journal of Visualized Experiments* as an article.

In this report, we describe a protocol in which we outline the details of biosensor preparation and the operation of the FRET analyzer for providing quantitative measurements of sugar contents in commercially available beverage samples. We anticipate that further size reduction and performance enhancement of the equipment will facilitate the practical use of hand-held analyzers in environments where high-end equipment is not available.

We believe that the findings of this study are relevant to the scope of your journal and will be of interest to its readership.

All the authors have approved the manuscript and agree with submission to your esteemed journal. There are no conflicts of interest to declare.

Thank you for your consideration. I look forward to hearing from you.

Yours sincerely,
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TITLE:
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KEYWORDS:
Fluorescence resonance energy, portable device, point-of-care testing, sugar contents, fluorometers, food assessment

SHORT ABSTRACT:
This protocol describes the rapid and highly sensitive quantification of Föerster resonance energy transfer (FRET) sensor using custom made portable FRET analyzer. FRET sensors detecting maltose were examined as examples at critical temperatures that maximize the sugar detection sensitivity, which enables practically efficient sugar content assessment.

LONG ABSTRACT:
Recent developments in Föerster Resonance Energy Transfer (FRET) sensors have enabled the detection of various small molecules, including ions and amino acids. However, the innate weak signal intensity of FRET sensors is a major challenge preventing the application of FRET sensors in various fields; this limitation necessitates expensive, high-end fluorometers, further limiting the potential applications of this technology. Previously we built a cost-effective, high-performance FRET analyzer which can specifically measure the ratio of two emission wavelength bands (530 and 480 nm) for the high detection sensitivity. More recently it was discovered that FRET sensors with bacterial periplasmic binding proteins has a critical temperature, 50-55 °C detecting ligands with maximum sensitivity. This report describes a protocol of the sugar content assessment including the portable FRET analyzer operation with the preparation of temperature specific FRET sensor including commercially available beverage samples. As results, additional preheating process of FRET sensor significantly increases the FRET ratio signal in measuring sugar contents using the custom made FRET device and the device with the FRET sensor was successfully applied to quantify the sugar content in commercial beverages. We anticipate that further size reduction and performance enhancement of the equipment will facilitate the practical use of hand-held analyzers in environments where high-end equipment is not available.

INTRODUCTION:
Föerster resonance energy transfer (FRET) has been widely used as a biometric sensor to detect small molecules, such as sugars, calcium ions, or amino acids[1-4]. FRET biosensors contain fluorescent proteins, enhanced cyan fluorescent proteins (ECFPs), and enhanced yellow fluorescent proteins (EYFPs), which are fused to both ends of periplasmic-binding proteins (PBPs). The mechanism of FRET biosensors involves the binding of sugars to PBPs located in the middle of the sensor; this causes structural changes to the sensor and subsequently alters the distance and transition dipole orientation of the two fluorescent proteins at either end of the PBPs. This change enables quantitative analysis of sugar contents by measuring the ratio of the emission wavelengths of EYFP (530 nm) and ECFP (480 nm). Due to the high sensitivity, specificity, real-time monitoring capacity, and fast response time of FRET biosensors, these sensors are widely used in environmental, industrial, and medical applications[5]. Moreover, the ratiometric measurement of FRET biosensors has important practical benefits when measuring components in complex biological samples where the sensor concentration cannot be easily controlled and background fluorescence is always present.

Despite these advantages of FRET-based sensors for quantitative visualization, the innate weak signal intensity originating from the small structural changes with incomplete domain motion transfer to the fluorescent proteins limits the application of FRET-based sensors for in vitro or in vivo analysis[6]. Consequently, most FRET biosensors require the use of expensive and highly sensitive equipment. Previously, we developed an inexpensive and portable FRET analyzer with capabilities similar to those of the existing fluorescence analyzers[7]. A recently developed, inexpensive 405-nm band ultraviolet (UV) light-emitting diode (LED) was used as the light source to replace the expensive lamp or laser for excitation of the fluorescence signal. The detection system of the analyzer efficiently focuses the dissipating fluorescence signal onto two photodetectors with a silicon photodiode. In a more recent study, we showed optimization of detection temperature (50~55°C) could significantly magnify the ratiometric FRET signal[8]. This temperature specific signal enhancement along with the custom made FRET analyzer enables one to apply FRET sensors to more general diagnostic applications with rapid and high sensitivity.

In this protocol, we evaluated the general applicability of the FRET analyzer with optimal FRET temperature by quantifying the sugar contents of commercially available beverages. This protocol includes the details of the FRET device operation with sensor and sample preparation. We anticipate that this report will promote the potential application of the portable analyzer in small-scale laboratory environments and provide an inspiration for further development of an inexpensive on-site diagnostic device with FRET based biosensors.

PROTOCOL:

1. Preparation of biosensor, CMY-BII

1.1) Construct the plasmid pET21a(+)-CFP-MBP-YFP-His6 by following the previously established protocol[2].

1.2) Inoculate 5mL of Luria broth (LB) with a single colony of an *Escherichia coli* DE3 strain and incubate at 37 °C for 16 hr with shaking.

1.3) Transfer 1mL of the overnight culture in a 500 mL flask containing 100 mL LB and incubate at 37 °C in a shaking incubator until an optical density at a wavelength of 600nm (OD_{600}) reaches 0.5 (It will take about 3 hr).

1.4) Harvest the cells in a 50-mL conical tube by centrifugation at 1,000 × g for 20 min at 4 °C.

1.5) Resuspend the pellet quickly in each tube with 50 mL ice-cold distilled water (DW) and centrifuge at 1,000 × g for 20 min at 4 °C again.

1.6) Resuspend the pellet in 50 μL of ice-cold DW with 10% (v/v) glycerol by gently swirling until the solution (electrocompetent cells) reaches an OD_{600} of 100.

1.7) Place the mixture of the electrocompetent cells (50 μL of the cells at an OD_{600} of 100)

and the plasmid pET21a(+)-CFP-MBP-YFP-His6 (10 ng) in an ice-cold electroporation cuvette in an electroporation device and electroporate (18 kV/cm, 25 μF) the mixture.

1.8) Quickly add 1 mL SOC medium to the cuvette and resuspend the cells gently, followed by recovery at 37 °C for 1 hr with gentle shaking.

1.9) Spread the cells on an LB plate containing 100 μmg/mL ampicillin and incubate at 37°C for 12 hr.

1.10) Isolate a single colony using a loop and inoculate the colony in 10 mL of LB containing 100 μg/mL ampicillin at 37 °C in a shaker for 12 hr.

1.11) Add 5 mL of the seed culture to 500 mL of LB containing 100 μg/mL ampicillin.

1.12) Add 0.5 mM isopropyl β-D-thiogalactoside(IPTG) when the OD600 reaches 0.5 and incubate the culture in a 37 °C shaking incubator for 24 hr.

1.13) Centrifuge the cells at 4,500 × *g* for 20 min (4°C) and gently remove supernatant.

1.14) Resuspend the pellet in 5 mL binding buffer (20 mM Tris-HCl, pH 8.0, 1 mM PMSF, 0.5 mM EDTA, and 1 mM DTT).

1.15) Sonicate the cells on ice with six 10-second bursts at 200–300 W and 10 sec of cooling.

1.16) Centrifuge the lysate at 10,000 × *g* for 30 min at 4 °C to pellet the cellular debris. Transfer supernatant (soluble protein) in a new collection tube.

1.17) For affinity purification of the FRET sensor proteins, load the 4 mL of cleared cell lysate on to Ni-NTA affinity column (5 mL volume) and chromatography assay using a fast protein liquid chromatography (FPLC)[18].

1.18) Wash the column once with 5 column volume the wash buffer I (50 mM phosphate buffer, 300 mM sodium chloride, 10 mM imidazole, pH 7.0).

1.19) Repeated wash step with 5 column volume the wash buffer II (50 mM phosphate buffer, 300 mM sodium chloride, 20 mM imidazole, pH 7.0).

1.20) Elute the CMY-BII sensor protein with 5 column volumes of elution buffer (50 mM phosphate buffer, 300 mM sodium chloride, 500 mM imidazole, pH 7.0).

1.21) For concentration and desalting of eluted sample, fill concentrator (membrane size of 10,000 MW) with up to the 20 mL of sample, centrifuge for 10 min at 3,000 X g. Refilled 0.8% phosphate-buffered saline (PBS) into concentrator, repeated 2 times same step.

1.22) Recover the concentrated and de-salted CMY-BII sensor, storage of purified sensor at -80°C.

2. Measurement of sugar content using the FRET analyzer

Note: The details of the FRET analyzer construction method were described in our previous work[7].

2.1) Prepare a detection solution of 0.8% phosphate-buffered saline (PBS) containing 0.2 μM CMY-BII sensor proteins.

2.2) Turn on the FRET analyzer. Press the "UP" button for 2 sec for the calibration of optimal temperature. Set the temperature to 53 °C using the "UP" and "DOWN" buttons, and press the "SET" button.

2.3) For calibration, press and hold the "UP" and "DOWN" buttons simultaneously for 2 sec. Confirm that the LED panel displays "CALIB" and press the "SET" button.

2.4) Place a 12.5 × 12.5 × 45 mm rectangular parallelepiped vessel (cuvette) containing only PBS buffer into a cuvette holder of the analyzer and press the "SET" button.

2.5) Replace with a cuvette containing only the detection solution (see 2.1) without sugar (maltose/sucrose) and press the "SET" button for baseline calibration.

2.6) Replace with a cuvette containing detection solution with 10 mM sugar and press the "SET" button.

2.7) For determination of sugar contents from beverage sample, put 1 mL beverage sample in a 1.5 mL micro centrifuge tube and centrifuge at 16,000 x g for 1 min to remove.

Note: FRET sensor based fluorescence measurement has an advantage that it does not require a special pre-treatment to the sample since only 1% (v/v) of the sample is included to the total volume. However, it is preferred to remove any material that may affect the fluorescence measurement (e.g cells, insoluble particles, lipid, fat, any material with auto fluorescence). In addition, such a high concentration of strong acid, strong base, the cleaning agent (detergent), emulsifying agent (emulsifier) which may affect the properties of the FRET biological sensor made of a protein is preferably measured after removal using an organic solvent or a neutralizer. For example, with frozen snacks subjected to elimination of dairy fat and emulsifiers, the samples are centrifuged in a microfuge tube at a speed of 15,000 rpm for 30 min, and the liquid between the bottom sediment and the top layer of dairy fat should be extracted. An equal amount of hexane is then added, followed by centrifugation at 15,000 rpm for 30 min for lipid elimination.

2.8) Take the supernatant with a 1 mL syringe and filtered it through a syringe filter (pore size 0.2 μm).

2.9) Put 0.1 mL filtered beverage sample in a 1.5 mL micro centrifuge tube containing 0.9 mL PBS and vortex gently.

Note: It is critical to dilute the beverage sample properly. In this case, 1000 fold dilution was carried out so that the sugar concentration falls within the dynamic rage of the device. It is suggested to estimate the target sugar concentration in advance by referring the sugar contents labeled on the beverage products.

2.10) Add 5 µL of the diluted beverage sample (1%, v/v) into to a cuvette containing 0.495 mL of the detection solution.

2.11) Place the cuvette into a cuvette holder of the FRET analyzer and preheat the sample solution to 53 °C.

2.12) Press the "SET" button to measure sugar contents.

Note**:** It is possible to evaluate the FRET measurement with a multilabel plate reader or a fluorescence spectrophotometer equipped with a Peltier device for temperature control by reading the ratio at 488/535 nm[7,8]. In the case of sucrose detection, one can follow the steps from 1.1 to 2.14 with CSY-LH sensor[2].

REPRESENTATIVE RESULTS:

For the quantitative analysis of sugar content using the FRET analyzer, it is necessary to build a fitted curve estimating the target sugar concentration from the observed FRET ratio. Let *r* define the ratio of the emission intensity of ECFP at 480 nm and the emission intensity of EYFP generated at 530 nm in **Equation 1**.

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

The dose-response curve of the FRET biosensor (CMY-BII at 53 °C) can be built by observing the FRET ratio, r, at different sugar concentrations. The curve can then be expressed as an S-shaped sigmoidal curve as follows:

$$\hat{r} = r_{min} + \frac{r_{max} - r_{min}}{1 + 10^{p(log_{10}x_0 - log_{10}x)}} \qquad (2)$$

where r_{max} and r_{min} represent the signal ratio with sugar concentrations of 0 and saturated (1000 µM), respectively; x_0 represents the sugar concentration at 50% response; and p represents the slope of the response, which is close to 1 or -1. In this study, r_{max}, r_{min}, x_0, and p are 2.672, 4.256, 71.779, and 1, respectively. The concentration range from 1 to 1000 µM was used in the model fitting.

Based on Equation 1 and 2, the sugar contents in commercially available beverages were quantified using the FRET analyzer. Two maltose FRET sensors were examined to test their signal, *r*, depending on various temperatures[2,8]. The first one, CMY-0, is a basic FRET based sensor consisting of CFP, MBP, and YFP with no linker peptides while the second one, CMY-BII, has a Ser-Arg linker between MBP and the two fluorescence proteins[2]. As Figure 1A shows

CMY-0 is not available in less than 50 °C of measurement temperature as there is no signal difference between 0 and 1mM maltose conditions. The signal differences of both FRET sensors were maximized between 50 and 55 °C (Figure 1)[8]. For the quantification of the sugar contents in the three types of commercially available beverages, a dose response curve of the CMY-BII sensor in 53 °C was built (Figure 2A) and maltose contents were identified in the three samples by converting the FRET ratio into maltose concentration. In Figure 2A, since sample A is made of grains such as rice and barley which are one of main maltose sources, it is reasonable the sample contained relatively high maltose content (average 11.892 g/235 mL). On the other hand, sports drink (sample C) had the lowest maltose (0.29 g/250 mL) among the three beverages. These results suggest that FRET analyzer with optimal temperature control can be used by maximizing the efficiency of sugar contents quantification without expensive high-end FRET detection devices.

FIGURE LEGENDS:

Figure 1: Measuring FRET signal difference between 0 and 1mM maltose concentrations using the FRET analyzer in various temperatures. (A) YMC-0 sensor showed no signal difference based on maltose concentration in less than 50 °C (B) YMC-BII sensor was available in a wide range of temperature as the FRET signal in 1mL maltose was distinguished from that of 0 maltose concentration. In both cases, the signal difference dramatically increases in a specific temperature range (50~ 55 °C).

Figure 2: Maltose content quantification in commercially available three beverages. (A) A dose response curve of YMC-BII. (B) Three beverage samples were examined to their quantify maltose contents. Note that Total sugar indicates the quantity of the all sugar contents (including maltose) by the beverage providers. The error bar indicates the standard deviation.

DISCUSSION:

This protocol shows the rapid and efficient quantification of the sugar contents in beverage samples using a custom made FRET analyzer[7] with optimal FRET sensor temperature control. The analyzer was designed with a recently developed, inexpensive 405-nm band UV-LED as the light source and two photodetectors with a silicon photodiode; this device is cost effective in comparison with other expensive fluorometers. For the high detection sensitivity, the device specifically measures the ratio of two emission wavelength bands (530 and 480 nm) in an optimal temperature of FRET sensors and showed better sensitivity and intensity than a fluorescence spectrophotometer device in detecting various sugars[7]. The main goal of this protocol is to support the wide applicability of the FRET based sensors with the custom made FRET analyzer. While the analyzer indirectly measures sugar contents via FRET sensors, it is clear that the device incorporates a number of benefits of FRET sensors, including widely extendable genetically engineered ligand specificity, modular design, sensor concentration-independent signals, and accurate targeting of subcellular small molecules. FRET sensors are actually used to detect a wide range of small molecules, including ions[9], heme[10], and others. Moreover, more than 20 types of FRET constructs can be easily found and ordered through the nonprofit depository, AddGene[11].

Despite the wide applicability of the FRET analyzer, there are two main issues with the operation of the device. First, since operation of the device is rather simple, sample preprocessing is the critical step for quality of detection, except in cases of device malfunctioning. In this protocol, one step (sample dilution) was sufficient for processing of liquid samples that were clearly transparent without any insoluble particles. However, it is necessary to remove any insoluble materials, such as cellular or lipid components. Any auto-fluorescent particles that can affect the FRET signal should be also removed as noted in Step 2.7. Secondly, quality control and connectivity interfacing with hospital information systems (HISs) need to be addressed, as with all types of Point-of-care testing (POCT) tools[12]. Because the signal quality of the FRET analyzer is largely dependent on the quality of the FRET sensor and the preprocessing steps, regular quality control checks are required to ensure that measurement are within the standard signal range for regular quality control data analysis. The quality control analysis includes the investigation of FRET sensor stability and storage period which are essential for further reliable applications. These can be partly achieved by well-documented guidelines and appropriate software development, which can also address the connectivity limitation. Current versions of the FRET analyzer equipped RS232 connectivity for remote command line control but wireless communication are possible in the next version of the analyzer, which should have an improved interface for the HIS. On the other hand, FRET sensors have been engineered for substrate specificity but it normally resulted in broaden the specificity[2]. Consequently, the FRET signal can be interfered by the unintended ingredients including other types of sugars in the commercial beverages. It is required further to investigate FRET sensors responding to various cases of sugar mixtures for the accurate sugar quantification. Also collaboration with companies that produce the beverages will be helpful to confirm and to adjust the calibration of the FRET analyzer.

The proposed portable FRET device with various FRET sensors is anticipated to be used to POCT applications. POCT is currently used for assessing pregnancy, blood glucose levels, biomarker proteins, infectious bacteria, and infectious viruses. POCT methods have rapid turn-around times and generally exhibit low error rates due to the reduced number of processing steps. In terms of turn-around time and errors caused by multiple processing steps, POCT is more competitive than the central laboratory testing (CLT) approach. Hand-held portable devices for POCT, such as the device described herein, have attracted increasing attention due to its potential applications in food assessment and blood sugar monitoring. In particular, glucose monitoring of blood samples in patients with diabetes requires a rapid, accurate, and cost-effective POCT method[13]. After the Ames research team developed a blood glucose detecting strip method, which contains glucose oxidase, as the first blood glucose test strip in 1965, several technologies were proposed for blood glucose monitoring purposes[12]. The FRET analyzer is also available to detect glucose in blood samples with appropriate preprocessing of blood and periplasmic glucose binding protein (MglB)[14] based FRET protein.

Importantly, simple, rapid methods for food quality assessment, particularly measurement of components of beverages, are needed; indeed, sugar-containing beverage consumption is associated with a variety of diseases and syndromes, such as increased body mass index in childhood[15], pediatric obesity[16], and risk of stroke[17], necessitating measurement of sugar

components in beverages. In this sense, glucose and fructose concentration in beverages could be a kin interest of people for the reason of healthcare.

Despite this protocol focus on the simple and high sensitive performance of the FRET analyzer with optimal temperature control, it is clear that the device with various FRET sensors detecting various small molecules including glucose and fructose[14, 15]. In terms of POCT, our portable and rechargeable device is beneficial. The battery life ranges from 10 to 40 hr depending on the heating protocol. The simple operational protocol of the analyzer will make the device easy to use, without complicated staff training requirements, as shown in the protocol session.

With technical improvements, including reduction of equipment size, minimization of pretreatment steps, and identification of practical requirements for field use, this device will promote FRET-based research development in small-scale laboratory environments.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

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Figure 1

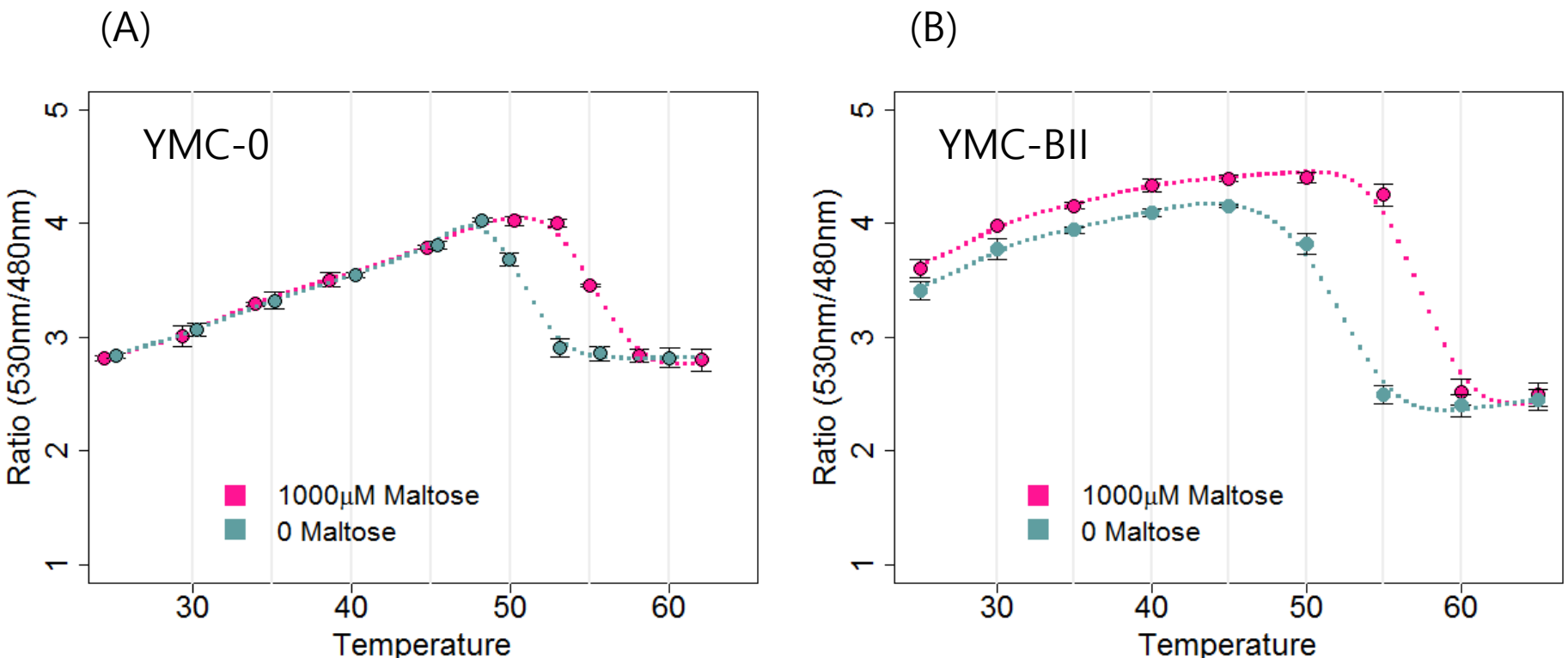
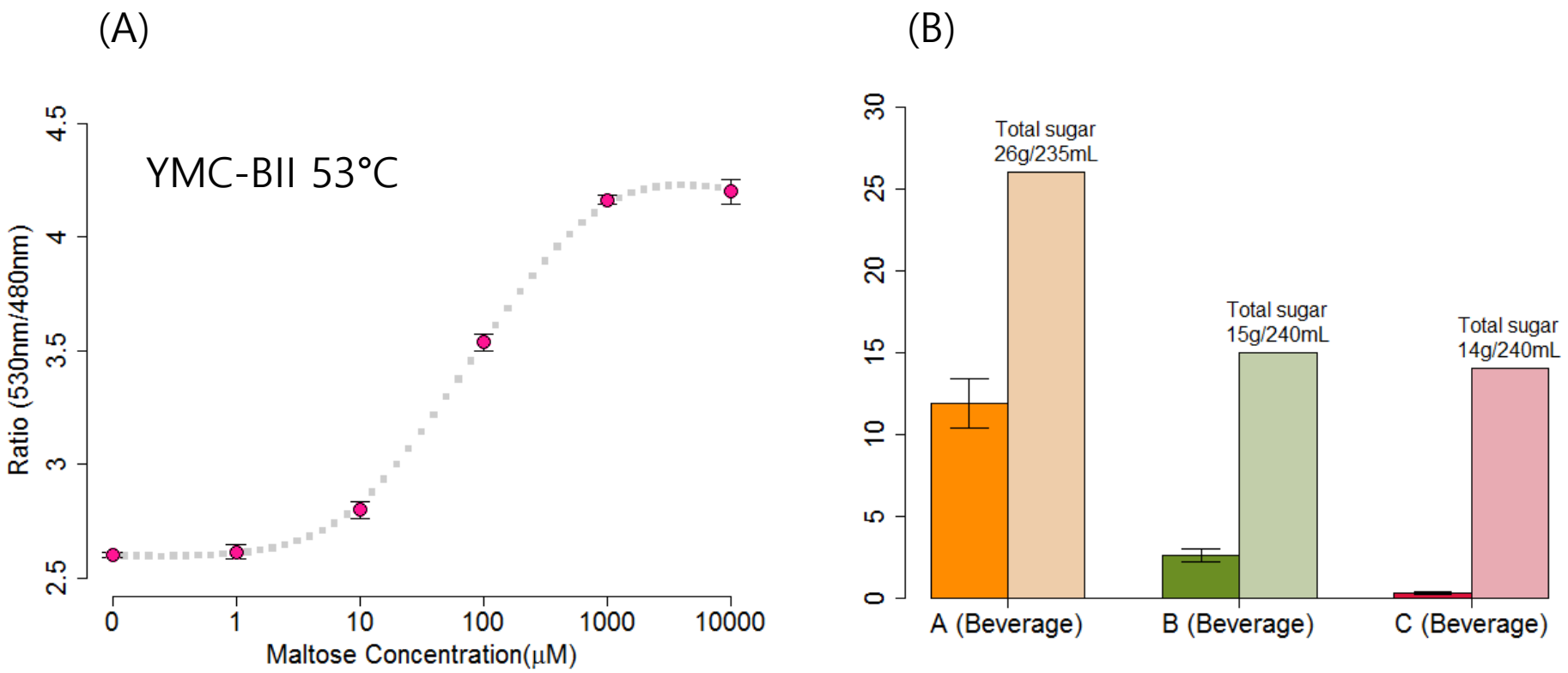


Figure 2


(A)
YMC-BII 53°C
Ratio (530nm/480nm)
2.5
3
3.5
4
4.5
0
1
10
100
1000
10000
Maltose Concentration(μM)
(B)
0
5
10
15
20
25
30
Total sugar
26g/235mL
Total sugar
15g/240mL
Total sugar
14g/240mL
A (Beverage)
B (Beverage)
C (Beverage)

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number
LB	BD	#244620
isopropyl β-D-thiogalactoside (IPTG)	Sigma	I6758
Ampicillin	Sigma	A9518
Tri-HCl	Bioneer	C-9006-1
PMSF	Sigma	78830
EDTA	Bioneer	C-9007
DTT	Sigma	D0632
NaCl	Junsei	19015-0350
phosphate-buffered saline (PBS)	Gibco	70011-044
SOC		
Resource Q	Amersham Biosciences	17-1177-01
HisTrap HP1	Amersham Biosciences	29-0510-21
Quartz cuvette	Sigma	Z802875
AKÄKTAFPLC	Amersham Biosciences	18-1900-26
Cary Eclipse	VarianInc	
VICTOR	PerkinElmer	2030-0050
E. coli JM109 (DE3)	Promega	
A (Beverage)	Korea Yakult Co. (Korea)	Birak
B (Beverage)	Lotte Foods (Korea)	Epro
C (Beverage)	Lotte Foods (Korea)	Getoray

Comments/Description

0.8% NaCl, 0.02% KCl, 0.0144% Na_2HPO_4, 0.024% KH_2OP_4, pH 7.4

2% tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MGCl2, 20 mM Glucose

6 × 30 mm anion-exchange chromatography column

a fast protein liquid chromatography (FPLC)

a fluorescence spectrophotometer

a multilabel plate reader

Electrocompetent cells

Fermented drinks

Soft drink

Sports drink

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Title of Article: Sugar content assessment using a portable FRET analyzer: a prototype of a widely applicable point-of-care testing tool

Author(s): Haseong Kim, Gui Hwan Han, Jongsik Gam, and Seung-Goo Lee

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Dear Prof. Lee,

Your manuscript JoVE54144R2 "Sugar content assessment using a portable FRET analyzer: a prototype of a widely applicable point-of-care testing tool" has been peer-reviewed and the following comments need to be addressed.

Please keep JoVE's formatting requirements and the editorial comments from previous revisions in mind as you revise the manuscript to address peer review comments. Please maintain these overall manuscript changes, e.g., if formatting or other changes were made, commercial language was removed, etc.

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Sincerely,

Jaydev Upponi, Ph.D.

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•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

•Formatting:

-Please define all abbreviations at their first use in the manuscript (IPTG).

[Step 1.12] Done

-Please include DOI information for references, if available.

•Grammar:

-1.3- “incubate at 37 °C shaker”

[Step 1.3] Done

-1.6-Please clarify the language. What constitutes the source here? The resuspension? Only the glycerol?

[Step 1.6] Revised the sentence

-1.17- “perform chromatography assay using a fast protein liquid chromatography”

[Step 1.17 – 1.22] Revised and updated the steps 1.17 ~ 1.22

•Additional detail is required:

-The protocol relies on the use of a custom made FRET analyzer; however, no step-wise details are provided regarding how to construct the analyzer. In reference 7, is there sufficient detail for viewers to build the device? If so, include a note at the beginning of Section 2 stating: Note: The FRET analyzer was constructed using the method described in…”. If not, we suggest including an additional section detailing the construction of the device.

At the end of Introduction section, a note was added for clarifying the aim of this protocol which is to promote the use of the portable FRET analyzer with FRET based sensors in lab-scale experiments.

-2.4-In what is the cuvette placed? The analyzer? Please clarify.

[Step 2.4] Changed the sentence

-2.5-What constitutes the “detection solution”? Please clarify.

[Step 2.5] The constitutes of the detection solution was described in Step 2.1. We added an indicator "(See 2.1)" in the sentence.

-2.7-Is any criteria used to choose the commercial beverage samples?

[Step 2.7] There was no specific criterion for the beverage selection. The types of beverages were indicated in the Materials/Equipment list.

-In the Materials/Equipment list, for beverages E-I, please indicate what general type of beverages these are (e.g., soda/soft drink, juice, water).

Done

•Unnecessary branding and commercial language should be removed:

-1.17-Resource Q

[Step 1.17] Removed

-Discussion-Cary Eclipse (?)

[Line 286] Changed the word (Cary Eclipse → Fluorescence Spectrophotometer)

-Please remove the commercial language from the last sentence of the Discussion: "…this device…will promote commercialization in the POCT market."

Removed

•Results: In the Figure 2 legend, please define the error bars (e.g., SD or SEM).

[Figure 2 Legend] Done

•Discussion: Please expand on the future directions of this method.

[Line 350] There is a description about the further extension of our research

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We updated the results and figures which were not used in any of previous publications

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

too little attention to accuracy, precision, error bars. Explain error bars shown in figure 2.

Updated the whole results and figures including error bars

Major Concerns: N/A

Minor Concerns:

"Silicon photodiode" is misspelled twice (silicone is not silicon)

[Line 89, 282] Corrected

Additional Comments to Authors: N/A

Reviewer #2:

Manuscript Summary:

The authors describe a method to measure the concentration of sugar using periplasmic-binding proteins (PBP) that provide a FRET signal when a conformational change due to the binding of sugars alters the distance of an enhanced cyan fluorescent protein (ECFP) from an enhanced yellow fluorescent protein (EYFP) attached to the PBP. This structural change results in a low intensity signal and in order to address this problem, the authors use the ratio of the emissions of both fluorescent proteins (ECFP and EYFP). The protocol includes the steps to the preparation of the biosensor and the steps to measure the sugar content using the FRET analyzer.

Major Concerns:

None.

Minor Concerns:

*Given that the measurement of the fluorescence ratio is an important part of the method and it is key to improve the obtained signal, I think it would be convenient to include a mention to the fluorescent ratios in the (long) abstract.

[Line 50] Updated the sentence

*Line 114. Seeing the following steps in the protocol, it seems that it is more important to allow optical density to arrive to 0,5 at 600 nm than to let the culture incubate for 3 hr. Therefore, I think that it might be clearer if it was worded saying " [...] incubate at 37ºC until an optical density at a wavelength of 600 nm (OD600) reaches 0,5 (it will take about 3 hr)."

[Step 1.3] Done

*Step 1.6.) The important point in this step seems to be to have an OD600 of 100. However, I can imagine a situation when some experimenter resuspended the pellet and it didn't yield this optical density initially. So, I think it would help if the authors stated in the text whether it would be valid to "[r]esuspend the pellet in ice-cold 10% (v/v) glycerol, which is a source of electrocompetent cells, and take the solution to an OD600 of 100)". I.e. to adjust the final OD600 to the desired concentration regardless of what the initial concentration is.

[Step 1.6] Revised the sentence

*Step 2.2.) I would appreciate if the authors stated explicitly the rationale behind using 55ºC for the FRET measurement and if they discussed how this may affect the binding of the analyte to the sensor.

[Step 2.2] There had been a major update throughout the manuscript. We mainly added descriptions about the temperature control which is exactly the same issue pointed out in this comment. Note that the optimal temperature was changed to 53 ℃. Please see the updates highlighted in red.

*Line 201 refers to values A1 and A2, but I suspect they mean rmax and rmin. In any case, A1 and A2 have not been defined earlier. So, this point should be clarified. In addition, we can imagine that x refers to the sugar concentration in the sample, but this should aslo be defined.

[Line 237, 243] Corrected the sentence. r_{max} and r_{min} were defined in line 243 and Figure 1B showed the measured values (0 and 1mM maltose concentrations) with the CMY-BII sensor at 53°C.

*Line 217 refers to a "simplified model", but this is the first mention to two models one that would be more "elaborate" or "complex model" and a "simplified" one. I would appreciate if the authors stated what they mean when they refer to the "simplified model" or to indicate which other (more complex) model they are comparing it with. If they mean that the one model that they use is a simplified version of what could more accurately account for the physicochemical processes happening in the system, the authors should state it.

[Page 7, Line 278, Figure 2 Legend] Updated the figures including the figure legends

*The authors state that ratiometric FRET measurements have advantages when the sensor concentration is not easy to control. However, beyond concentration, I think it would be good to comment on whether their method depends at any rate on the possible degradation of the sensor. In addition, I would appreciate if the authors mentioned how long the detection solution can be stored to be used in their method and whether measurements are affected by using a non-fresh detection solution.

[Line 307] We have not yet performed the analysis handling the FRET sensor degradation and storage issues. But we agreed that these issues are essential for the further reliable and stable applications of the FRET sensors and the device. A brief description was added in the Discussion section along with the quality control issue. Thank you for this comment.

*Figure 2 is missing units in the Y axis.

Figures were updated.

Additional Comments to Authors:

(Bio)chemical or other materials are often made available for other researchers to use in their research. This can be done as collaboration or under other terms agreed by the involved institutions or groups. In this case, given that the FRET measurement instrument that the authors used is a prototype (although they explain the method for other instruments), I think it would help the researcher community if the authors offered to share their prototype for research purposes. However, this is just a suggestion. If the authors have reasons not to want to share their prototype at this stage of development, I don't think they need to do it in order for this article to be published. I just think it may help others become aware of the utility of this instrument.

Thank you for your helpful comments and suggestions. In fact, we're making this protocol to promote the custom made FRET device along with optimal temperature control can be widely available in detecting various small molecules without expensive fluorescence detection devices. So any collaboration for research purposes would be more than welcome for us. As you said, this is a prototype but an upgraded FRET analyzer which is much lighter and less cost than the prototype is expected soon or later.

Reviewer #3:

Manuscript Summary:

The manuscript, titled "Sugar content assessment using a portable FRET analyzer: a prototype of a widely applicable point-of-care testing tool" describes an experimental procedure to determine sugar (or other solutes) concentrations in a complex solution using a prototype FRET analyzer developed by the authors. Closely following their previous publication, they demonstrate the procedure, using sugar content analysis in commercial beverages as an example.

Major Concerns:

The manuscript seems to have been revised twice prior to my assignment as a reviewer, but I still have a number of concerns with this manuscript. The main points that need to be addressed are followings,

1) I have doubts as to whether the procedure really benefits from publishing in a visual format. The protocol is fairly straight forward (bacterial expression followed by FPLC, then cuvette-based measurement of FRET ratio), and their previous publication describe the procedure in a sufficient detail. The most technically involving part might be the FPLC purification, but that procedure is mostly left out from this protocol (I don't even find the solutions used for this procedure).

There has been a major update to the script. This protocol mainly claims to support the wide applicability of the custom made FRET analyzer with optimal temperature control. FRET signal (ratio) intensity dramatically increases in the optimal temperature (reference 8 in the main script) and the custom made FRET analyzer with the FRET sensors can be used to quantifying target molecules in a simple manner. Please see the updates highlighted in red. The FPLC purification procedure was included in steps 1.17-1.22.

2) It is not clear to me how matrix effects are controlled. Fluorescent proteins (FPs) based sensors are innately sensitive to solutes, especially the ones that can function as quenchers such as phosphate ions (which happens to be abundant in commercial beverages). The protocol presented seems to provide a single point measurement. This would work only if other solutes contained in the beverage do not interfere with rmin and rmax. The effect on rmax can easily be assessed by adding a saturating concentration of maltose in addition to the diluted beverage. While assessing of rmin is impossible, using rmax value with the matching matrix would be better than not correcting for the matrix effect at all.

[Line 313] We agreed that the FRET signal will be interfered by the ingredients in the commercial beverage samples. Including the phosphate ions you mentioned, other types of sugars can also affect to the signal since FRET sensor engineering for substrate specificity resulted in broaden the specificity (reference 2 in the main text). So it is required further to

investigate FRET sensors responding to various cases of sugar mixtures for the sugar quantification. Also collaboration with companies that produce the beverages will be helpful to confirm and to adjust the calibration of the FRET analyzer. We added it in the Discussion section.

3) The estimated concentration would be accurate only if the substrate concentration happens to fall within the dynamic range (usually two orders of magnitude) of the particular sensor. This can be evaluated by selecting a threshold (such as 10-90% saturation). If the r value does not fall within this range a different the concentration should be measured at a different dilution.

[Line 213] We agreed that our device also only detect the FRET signal within the sugar concentration is in between 0 to 1mM of maltose. So it is critical to dilute the beverage sample properly. In our case, 1000 fold diluted for the measurement. We suggest that one can estimate the target sugar concentration approximately by referring the sugar contents labeled on the beverage products.

4) For the particular application discussed here (measuring sugar content in beverages), it seems that the end user would want to know the content of all sugars, including fructose and glucose that are often used in very high concentrations. It would be possible to measure those sugars using this system provided there is a suitable FRET sensor, but as it stands those more common sugars in beverages are not discussed. Also the authors should discuss the substrate specificities of sensors.

[Line 313, 341] The substrate specificity issues were discussed with the FRET interference on Page 8 Line 318. It is true that people nowadays have an interest of the glucose and fructose concentration in beverages for the reason of healthcare. We discussed this issue with glucose FRET sensors on Page 8 Line 341.

5) Since the main advantage of this procedure (compared to using a high-end spectrofluorometer) seems to be the affordability of the instrument, it would be nice to know the predicted price range of the instrument. Also, the authors do not discuss other technology available for determining sugar content (including the ones that do not uase FRET measurement).

Since the journal policy strictly limits the use of commercial languages, the related contents were not discussed in this protocol. But the price range was discussed in our previous paper (reference 7 in the main text). We focused on the availability of custom made FRET device with optimal temperature control of FRET sensors and sugar detection is an example. So please understand that it seems not appropriate to compare sugar detection technologies in this protocol.

Other points;

1) Figure 1 is exactly the same as a figure included in a manuscript the authors previously published (ref.7). The authors should acknowledge this and sort out the copyright issues.

2) Likewise, the data in Figure 2 seems to be identical to what has been published in ref 7.

We changed the figures.

3) Line 201- A1 and A2 are not used in the equation. Is it rmin and rmax instead? Also provide the unit for x0.

[Line 240] Revised the equations

4) The last page of the manuscript seems to have a formatting issue.

Done

I would like to see the above points revised before I recommend the manuscript for publication.

We have carried out a major update of the manuscript including the results and figures. Thank you for your helpful discussions and comments.

Minor Concerns:

- FRET is an acronym Foerster Resonance Energy Transfer to be precise.

Changed

- There are many other types of FRET sensors than what they discuss - PBP is certainly not the only type of scaffold, and there are many different types of FPs too although CFP and YFP derivatives are most common. The authors should acknowledge that.

Thank you

- The readers won't know which one of the instruments listed is the "FRET analyzer" mentioned in the protocol.

We haven’t determined the proper noun of our device yet. But we revised the text to minimize readers’ confusion.

- line 204 rate or ratio?

Ratio is correct.

Additional Comments to Authors:

N/A