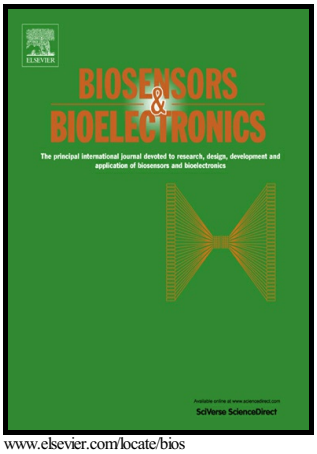
Author's Accepted Manuscript



www.elsevier.com/locate/bios

Real-time fish stress visualization came true: A novel multi-stage color-switching wireless biosensor system

Haiyun Wu, Ryosuke Shinoda, Masataka Murata, Haruto Matsumoto, Hitoshi Ohnuki, Hideaki Endo

PII: S0956-5663(18)30734-6
DOI: https://doi.org/10.1016/j.bios.2018.09.042
Reference: BIOS10782

To appear in: *Biosensors and Bioelectronic*

Received date: 12 July 2018
Revised date: 7 September 2018
Accepted date: 12 September 2018

Cite this article as: Haiyun Wu, Ryosuke Shinoda, Masataka Murata, Haruto Matsumoto, Hitoshi Ohnuki and Hideaki Endo, Real-time fish stress visualization came true: A novel multi-stage color-switching wireless biosensor system, *Biosensors and Bioelectronic,* https://doi.org/10.1016/j.bios.2018.09.042

Real-time fish stress visualization came true： a novel multi-stage color-switching wireless biosensor system

Haiyun Wu[a], Ryosuke Shinoda[a], Masataka Murata[b], Haruto Matsumoto[b], Hitoshi Ohnuki[a], Hideaki Endo[a*]

[a]Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7, Konan, Minato-ku, Tokyo 108-8477, Japan

[b]Hokkaido Industrial Technology Center, 379 Kikyo-cho, Hakodate, Hokkaido, 041-0801, Japan

[*]Corresponding author: E-mail address: endo@kaiyodai.ac.jp (H. Endo)

Abstract:

An optical communication type biosensor system has been developed which can measure blood glucose concentration, which is a stress indicator of fish, in real-time while fish swimming freely. However, this system is hard to make instant acknowledgment of fish stress level which has to contain an unavoidable delay in the judgment. In this research, we aimed to develop a novel stress visualization system which can quickly judge the levels for fish stress response instantly based on a color changeable LED while another LED was designed to send data. The present system is based on the principle of converting the output current value measured by the glucose biosensor corresponding to the stress response into a voltage value. Then, the color and

stress switching points of the LED (Red, Yellow, Green) were decided based on the voltage value gained from the biosensor which mentioned above. Furthermore, we attempted to use our biosensor system to make real-time monitoring of fish stress in vivo. As results, the proposed sensor can make real-time measurement of glucose and shows a great response to those of actual fish sample in the range from 35.36 to 300 mg dl^{-1} (R=0.9899). When the glucose concentration in the collected sample was switched to the concentration pre-sett, it was successful to switch the LED color according to the gained voltage value both in vitro and in vivo. Furthermore, when monitoring the stress responses of the fish in vivo, color switching corresponding to the sensor output current value was observed successfully.

Keywords: Biosensor; Fish; Stress; Visualization; Real-time monitoring; Physiologic responses

1. Introduction

A poor fish-breeding environment, such as overcrowding and inadequate water quality, is believed to induce stress in fish and influence their development and health (Walters and Plumb 1980, Barton 2002, Leal et al. 2018). Stress-induced fish disease has become an unavoidable and serious problem. Stressful conditions lead to the fish becoming immunocompromised (Reverter et al. 2014). Furthermore, highly infective disease can rapidly lead to mass mortality in cultured fish (Pickering and Pottinger 1989, Schwaiger et al. 1997). The fish will not appear to be stressed, however, until their natural defense mechanisms against stress have become compromised, leading to stress-related disorders. Blood components, such as cortisol and glucose, are indicators

of stress levels in fish (Barton and Iwama 1991). Among the indicators, glucose increases during stress and returns to normal values within 24 h (Nakano et al. 2014, Carmichael et al. 1984). Conventional methods for measuring blood glucose levels, however, require anesthetizing the fish and removing them from the water to draw their blood. As these procedures also induce stress, it is difficult to measure the "true" stress level in fish. Furthermore, because the stress indicator can only be measured at the time of blood sampling, it is impossible to assess continuous changes in the stress response.

To address this problem, we are counting on biosensing technology which can provide us with faster, more accurate and wider detection range which applied in many field such as health monitoring (Vásquez et al. 2017, Alam et al. 2018), toxicity detection (Shehata et al. 2016, Fekry 2017), food science(Dervisevic et al. 2015, Neethirajan et al. 2018) , etc. For fish health monitoring, a wireless biosensor system was also developed that monitors an important stress indicator (e.g., blood glucose levels) in real-time (Wu et al. 2015) in our laboratory. The system comprises a biosensor that utilizes glucose oxidase as a molecular identification element, a very small wireless potentiostat (transmitter), and a receiver. The sensor continuously, rather than statically, measures glucose levels based on the enzyme reaction, and thus monitors the stress response in real-time. The system can be used in freely swimming fish, making it possible to measure a stress response without inducing additional stress to the fish by handling.

The developed system used a wireless potentiostat that transmits the data via radio waves to a personal computer. The communication distance of radio waves in seawater, however, is limited because radio waves are markedly damped by seawater. Thus, the system is limited to use with freshwater fish. To overcome this problem, we focused on optical (visible light) communication technology, which is less damped in water

compared with radio waves. The optical system can also be used for real-time monitoring, like the radio wave system, but consumes less power, costs less, communicates faster, and has larger capacity (Shinoda et al. 2017).

Measuring the fish stress response using an optical communication system has several challenge challenging aspects. First, the current systems rely on data being transmitted to a personal computer to evaluate the level of stress, which delays the determination of the level of stress. Second, special knowledge is required for the analysis, which limits the field in which the system can be applied. Third, the simultaneous measurement of stress responses in multiple fish, e.g., such as when assessing territorial disputes, requires the use a large number of systems at the same time, making it difficult to identify the stress response of specific fish, even when using one or several personal computers(Endo et al. 2009, Yonemori et al. 2009). These problems hinder elucidation of the effects of stress factors on fish health status for physiologic studies of fish.

Overall, while real-time monitoring of the fish stress response has been conducted so far, a novel method that allows for rapid visual discrimination of changes in the stress response is required. Therefore, we aimed to develop a novel visualization system that can easily discriminate the magnitude of the fish stress response by fusing biosensor and electronic circuit technology. This system must be able to instantly discriminate the level of the stress response based on a multicolor LED while another LED sends real-time measurement data (Fig. 1). The stress visualization system is based on the principle of converting the output current value measured by the glucose biosensor corresponding to the stress response into a particular voltage value on the electronic circuit. Then, the output of the mutlicolor LED (red, yellow, or green) is

determined based on the voltage (i.e., the stress level of the fish) obtained from the biosensor.

In this research, an LED color-switching system was developed for both optical data communication and stress level visualization. At first, to confirm the validity of the system, we measured the output current and voltage of the sensor based on the blood glucose level of the fish and attempted to alter the color of the LED by changing the glucose concentration. Moreover, we examined the communication range in air, freshwater, and seawater to evaluate the practicality of data communication using optical communication technology. Furthermore, we attempted to use the proposed system to conduct real-time monitoring of fish stress levels in vivo to demonstrate the practicality of the system.

.

2. Materials and methods

2.1 Reagents and test fish

Glucose oxidase (from *Aspergillus niger*; E.C. 1.1.3.4, type VII-S; 147,000 unit g^{-1}) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Ammonia solution (25%), sodium nitrate, acetic acid, 2-phenoxy ethanol, glucose C II-Test, heparin sodium, glutaraldehyde (grade I, 25% aqueous solution), and 5% Nafion® dispersion solution were purchased from Wako Pure Chemical Industries (Tokyo, Japan). The 2-methacryloyloxyethyl phosphorylcholine polymer was purchased from NOF Corporation (Tokyo, Japan). All other reagents used for the experiments were commercial or laboratory grade.

For the test fish, we used Nile tilapia (*Oreochromis niloticus*) cultured at the Tokyo University of Marine Science and Technology. This study was carried out in

accordance with the Guide for the Care and Use of Laboratory Animals from Tokyo University of Marine Science and Technology.

2.2 Enzyme sensor preparation

The biosensors were prepared using a 15-mm length of Teflon-coated platinum iridium (Pt-Ir) wire for the working electrode and Ag/AgCl paste (BAS, Tokyo, Japan) for the counter/reference electrode. As a sensing cavity, the Teflon was stripped from one end to expose 1.0-mm of the Pt–Ir wire. Copper wire was wrapped around the Teflon-coated surface as a lead wire. The Ag/AgCl paste was applied to the Teflon wrapped around the copper wire. The sensing cavity was dipped in a 5% Nafion® dispersion solution and air-dried for 10 min. Glucose oxidase (2.5 mg) and BSA (6.0 mg) were mixed with 0.25 ml phosphate buffer (PB, 0.1 M, pH 7.8) into an enzyme solution. The Nafion-coated electrode was dipped in the enzyme solution and air-dried for 10 min. This procedure was repeated twice. The sensor was placed in a Petri dish and maintained at 35°C, and 0.05 ml glutaraldehyde (25%) was added to induce cross-linking between the glucose oxidase and BSA for 3 h. The sensor was then placed in 0.1 M PB for 1 h at 4°C. The sensing cavity was again dipped in the enzyme solution and air-dried for 10 min, and the sensor was placed in the dish for 3 h. The sensor was soaked in PB overnight at 4°C. The electrode site was dipped in 2-methacryloyloxyethyl phosphorylcholine polymer and air-dried for 10 min. This procedure was repeated three times. The sensors were soaked and stored in PB at 4°C before use. The sensor was connected to a Potentiostat (transmitter) to maintain a constant potential. A +650 mV potential (vs. Ag/AgCl) was applied to the Pt-Ir working electrode for amperometric glucose measurement.

2.3 LED color-switching system for stress visualization

2.3.1 Fabricating the LED color-switching and communication system for stress level visualization

The optical communication-type LED color-switching system for stress visualization comprised a biosensor with LED lights for optical communication and stress level visualization (hereafter referred to as the LED color-switching part) shown in Fig. 2. The light receiver comprised an LED light detector and a controller, and eventually a personal computer (PC). For data communications, the optical communicating LED (L-934SRD-G, Kingbright, Japan) of the LED color-switching part was the light source, and the light receiver received the data and converted it to an electrical signal that was transmitted every 1 second to the PC. The controller in the figure was used to adjust the communication sensitivity. In addition, a potentiostat with an applied voltage of +650 mV was incorporated in the biosensor, and operated using a 3.0 V lithium ion battery (CR 1220). On the other hand, besides the communication LED, a LED for fish stress visualization whose color switches to green, yellow and red was also fabricated on the board of the potentiostat. Two variable resistors were used to set the voltage thresholds, VcompL (for green to yellow) and VcompH value (for yellow to red). Based on these values, the multicolor LED was changed from green to yellow to red using the equation shown in Table S1. A digital multimeter (AD-5529, A&D Co., Tokyo, Japan) was used to measure Vcomp. This LED color for stress visualization was also designed and switched in real-time since the switching color was decided by the results of voltage comparison on the circuit board which always being calculated and refreshed.

2.3.2 Waterproofing the system

The system was waterproofed as shown in the underside of the Fig. 2. First, we cut off the corners at both ends of a polyethylene vinyl bag and left a ~2-mm diameter hole. A polyethylene microtube was cut into 1-cm lengths and passed through the holes. Then, we connected the sensor lead wire of the LED on one side and the glucose sensor on the other side. The space between the lead wire and the microtube was coated with Araldite® adhesive (rapid type, Huntsman Advanced Materials, Texas, U.S.A.). Next, the polyethylene vinyl bag was thermally compressed using a sealer (Shura sealer NL-101J, Ishizaki Electric Manufacturing Co., Ltd., Tokyo, Japan), which waterproofed the device. In addition, a 2-mm pitch connector (Hirose Electric Co., Tokyo, Japan) and a cap were used to connect the sensor to the potentiostat.

2.4 Setting up the LED color-switching system in vitro

We then confirmed that the LED color-switching system would properly change the color of the LED depending on the glucose concentration. In this experiment, we collected fish eye interstitial fluid (EISF) of Nile tilapia, the glucose level of which strongly correlates with blood glucose levels, and immersed the sensor in the EISF to measure glucose levels.

2.4.1 Setting up the measurement system

Approximately 1400 μl of EISF was collected from the test fish (total length: 26.6 cm, body weight: 297.0 g), and the glucose concentration was measured using a colorimetric method. Next, 600 μl of the collected EISF was dispensed into a flask and the sensor was then immersed in the EISF until the output current value become stable.

A glucose standard solution (5000 mg dl^{-1}) was added to the EISF so that the final glucose concentration was 50, 100, or 200 mg dl^{-1}, and changes in the sensor output current/voltage and LED color were recorded. In this system, when Vout equaled either of the Vcomp values, the color did not clearly change. To compensate for this, we decreased Vcomp by 0.01 V, which was the minimum unit of resolution of the digital multimeter used to obtain the measurement.

2.4.2 Confirming the color-switching value and performance

Another 600-µl aliquot of the EISF collected in **2.4.1** was placed into a flask and the sensor was immersed in it until the output current value stabilized. The Vout obtained at that time was recorded and the Vcomp value was calibrated as mentioned in **2.4.1**. In the same way as **2.4.1**, a glucose standard solution (5000 mg dl^{-1}) was added to the EISF to a final glucose concentration of 50, 100, or 200 mg dl^{-1}, and changes in the sensor output current/voltage value and color of the LED were confirmed. Also, the calibration curve for the glucose sensor was confirmed with a glucose range from 35.36 to 300 mg dl^{-1}.

2.5 Confirming the communication range of the system

We verified the range of the optical communication system over distances determined by the size of the water tank (30 × 25 × 28 cm) using three conditions: tank filled with air, fresh water, and artificial seawater (Red Sea Salt, Red Sea, TX, U.S.A.). When using water, the tank was filled to a depth of 20 cm. The transmitter was always placed at the bottom of the tank during the measurement. The receiver was positioned above the center point of the bottom of the water tank. During communication, the tip of the LED of the transmitter was always directed toward the surface of the receiver. The

distance between the transmitter and receiver ranged from 50 to 120 cm. The ability to receive the transmitted signal was evaluated.

2.6 Setting up the LED color-switching system and stress monitoring in vivo

In this experiment, we attached the system to free-swimming Nile tilapia and attempted to confirm the ability of the LED color to switch in vivo. The stress response of the test fish was measured by changing the dissolved ammonia concentration of the breeding water as a stress factor and recording the color of the LED during the monitoring.

2.6.1 Attaching the system to the fish

The transmitter [1.5×1.5×0.6 cm, 3 g (without battery)] used in this experiment was designed to attach to the fish. The transmitter was further adjusted before the experiments to maintain neutral buoyancy in the water to be more comfortable for the fish. The procedure was assumed to be relatively stress-free as the transmitter was lighter and smaller compared to the transmitters used in our previous studies.

The system was attached to either the dorsal or pectoral fins of the fish using nylon thread and located on side of the fish's body. The LED for optical communication was attached to the head of the test fish, and the LED for stress level visualization floated in the water. The biosensor was immersed into the EISF of one eye of the fish. After attaching the biosensor to the fish, the EISF on the other side of the fish was collected for setting the VcompL and VcompH as mentioned in Section 2.4. The depth of water in the water tank was 20 cm. The receiver was installed 80 cm above the tank.

2.6.2 Monitoring the stress response in test fish and confirming the color switch with a change in dissolved ammonia concentration

The design of the experiment was shown in Fig.3. Two water tanks (30 × 25 × 28 cm) were prepared, and breeding water was placed in each tank to a water depth of 20 cm. We called these water tanks A and B. Firstly, Nile tilapia (total length: 27.2 cm, body weight: 310.1 g) was placed in water tank A for at least 2 days. In addition, 25% ammonia water was added to tank B so that the final concentration of ammonia in the breeding water was 15 mg L^{-1}(Meade, 1985). Next, the sensor was implanted to the body of the fish, which was kept in water tank A overnight to allow for acclimation to the attached biosensor. Then, after operating the system according to the method described in **section 2.6.1** and confirming that the output current value of the sensor was stable, the output current/voltage value was recorded and calculated. VcompL and VcompH were calibrated as mentioned in **section 2.4.1**. After that, the measurement was started. Thirty minutes after beginning the monitoring, the fish was moved to water tank B for stress application to the fish body, and the changes in the output current/voltage of the sensor and the color of the LED of the system were assessed. As a control, several blood samples were obtained during the measurement, and they were measured by conventional methods. We also tested the rate of successful optical communication with free-swimming fish. The communication success rate (%) was calculated as the total optical communication time divided by the total test time and multiplied by 100. After the experiment, all the fish used in the experiment were healthy.

2.6.3 Calibrating the sensor

Continuous estimation of glucose levels is generally performed using either a one-point or two-point in vivo calibration method. In this paper, we used the one-point calibration method represented by the following formula:

$$G_{(t)} = I_{(t)} / S$$

$$S = I_1 / G_1$$

The one-point calibration method converts the blood glucose concentration from one blood sample, and the sensor sensitivity S (nA/mg dl^{-1}) is the glucose concentration of the blood sample obtained (G_1) and the output current value (I_1) of the sensor at that time. Then, the blood glucose level $G_{(t)}$ (mg dl^{-1}) is converted by the obtained output current value $I_{(t)}$ (nA).

3. Results and discussion

3.1 Dose-response and calibration curve of glucose sensor using the proposed system

Figure 4 (A) shows the dose-response curve when the glucose sensor was immersed in EISF and a fixed amount of glucose standard solution was added. Arrows in the figure indicate the points at which the glucose standard solution was added. From this figure, the rapid response of the sensor in response to addition of the glucose standard solution was confirmed. The calibration curve of the glucose sensor prepared based on this response curve is shown in Fig. 4 (B). The output current value of the sensor very strongly correlated ($y = 23.09 + 0.06331x$, $R = 0.9899$) with the EISF glucose concentration in the range of 35.36 to 300 mg dl^{-1}. The Error Bars were shown

in Fig. S1. Repeated 5 measurements of 70 mg dl^{-1} glucose the same biosensor had a precision (RSD) of 0.85%, with a mean current of 27.85 nA. For the sensor performance, stability and reproducibility, they were discussed in our previous paper (Endo et al. 2009, Yonemori et al. 2009). Therefore, this sensor sufficiently measures tilapia's normal blood glucose concentration, which ranges from 50 to 200 mg dl^{-1}. When stressed, however, the blood glucose level of tilapia can exceed 200 mg dl^{-1} (Rotllant and Tort 1997, Brown et al. 1992). Thus, it is possible to use this system to measure fluctuations in the blood glucose levels of tilapia.

3.2 Confirming the LED color-switching system set-up in vitro

To set the Vcomp value of the system, the glucose sensor was immersed in EISF and the glucose standard solution was added so that the glucose concentration became 50, 100, and 200 mg dl^{-1}. The resulting output current and Vout values as a reference are shown in Table S2.

The normal blood glucose level of test tilapia is ~50 mg dl^{-1} and increases to ~200 mg dl^{-1} when stress is applied (Rotllant and Tort 1997, Ellis et al. 2002). Therefore, the value of VcompL to trigger the change in color of the LED from green to yellow was set to the Vout obtained at 100 mg dl^{-1} glucose, and the value of VcompH to change the color of the LED from yellow to red was set to the Vout obtained at 200 mg dl^{-1}. When Vout equaled either of the Vcomp values, however, the color did not clearly switch. Therefore, we decreased the Vcomp value by 0.01 V, which is the minimum resolution of the digital multimeter used to measure the Vout value.

Next, to confirm that the color of the LED switches according to the Vcomp settings, the glucose concentration in EISF was changed in the same manner as

described above. The relationship between the output current, Vout, and the color of the LED is shown in Table S3, and the response curve is shown in Fig. 5.

The arrows in the figure indicate the points at which the glucose standard solution was added to the EISF. The output voltage when the glucose concentration stabilized at 50 mg dl^{-1} in Table S3 was ~0.15 V less than that shown in Table S2. Therefore, the Vcomp value for this experiment was decreased by 0.15 V. At 100 mg dl^{-1} glucose, Vout exceeded VcompL and the LED color changed from green to yellow. At 200 mg dl^{-1}, Vout exceeded VcompH, and the LED color turned from yellow to red. In addition, as can be seen in Fig. 5, the sensor responded quickly to the change in the glucose concentration, and the color changed fast (in real-time) and correctly according to the change in the glucose concentration.

On the basis of the above results, we concluded that the LED controller could change the LED color in accordance with the settings in vitro. That is, the color switching circuit of this system changed the color of the LED according to the output current/voltage of the sensor, and it was effective for visually indicating the magnitude of the stress response. Therefore, these findings suggest that the stress response of fish can be visualized using our proposed system.

3.3 Confirming the communication range of the system

We confirmed the communication range of the LED color-switching system for stress level visualization in a water tank in either air or water (fresh water or sea water). A 20-cm radius, covering the entire range within the aquarium (30 × 25 × 28 cm), could be measured at a working distance of 50 to 120 cm between the LED and the light receiver. In freshwater, the working distance between the LED and the light receiver was 60 to 120 cm. One reason for the narrower working range than in the air at a

distance of 50 cm is that the light tends to be attenuated in water compared to the air. As the communication distance increases, however, the light from the LED diffuses, so it is considered that the entire range of the aquarium could be communicated at a distance of 60 cm. On the other hand, because Nile tilapia used as a test fish in this study live in freshwater, when installing the LED switching system on tilapia, the distance between the LED and the light receiver should be set to 60 to 120 cm. A range with a 20-cm radius covering the entire tank can be measured, even in seawater. This is because we used visible light, which is not easily attenuated in water, and is thus suitable as a communication method in both freshwater and seawater. Based on the above results, the LED color-switching system for optical communication of stress level visualization can be used in both freshwater and seawater, and can potentially be adapted to all kinds of fish.

3.4 Response and calibration curve of glucose sensor using the proposed system

The LED color-switching system connected to the biosensor was attached to the test fish to measure the stress response of the fish due to changes in the dissolved ammonia concentration, and the change in the LED color of the system during the experiment was confirmed.

To set the Vcomp values, the output current value and Vout when changing the glucose concentration in the collected EISF are shown in Table S4 as a reference. The VcompL and VcompH values were obtained by subtracting 0.01 V from the Vout values obtained for 100 and 200 mg dl^{-1} glucose, respectively. Fig. 6(A) shows the sensor's Vout and blood glucose level over time when the sensor was inserted into the fish. Fig. 6 (B) shows the sensors response which was calibrated by using one-point calibration method and smoothed. The arrows in both figures indicate when the fish

were moved from the normal breeding water (water tank A) to the ammonia-containing water (water tank B). Vout of the sensor immediately before the stress load was 1.14 V (LED color: green), which was ~0.45 V less than the Vout value for 60 mg dl^{-1} glucose (1.5902 V) in Table S4. The sensor output and the blood glucose level rapidly increased after transferring the test fish to water tank B. This was considered to be due to the test fish being stressed by the increase in dissolved ammonia concentration (Knoph and Thorud 1996, Wicks and Randall 2002, Small 2004). At this time, because Vout exceeded VcompH, the color of the LED changed from green to red. After being in tank B for 40 min, the output voltage value of the sensor decreased and the color changed from red to yellow. Thus, the test fish seemed to become accustomed to the high ammonia water, leading to a decrease in the sensor output. From the above results, it was confirmed that the LED color-switching system switched color as the stress level changed, which confirmed that system can be used to visualize the fish stress response in freely swimming fish.

The communication efficiency was calculated from the total communication time and was ~85.7%. According to a previous study, data can be transmitted and received by keeping the angle between the LED and the receiver surface within ~20°. Therefore, by fixing the LED to the head of the fish body, the angle of the LED can be kept fairly constant, and the state in which the optical axis of the LED and the receiving surface of the light receiver coincide can be maintained. Thus, we considered that communication efficiency was achieved.

Immediately after obtaining the blood sample, the fish was exposed to the high-ammonia water. The glucose concentration increased drastically after ~25 min after being placed in the high-ammonia water and then decreased. High concentrations of ammonia in rivers induce stress in fish, resulting in increased gill breathing, abnormal

swimming, and increased mortality (Arillo et al. 1981, Thurston et al. 1981, Ruyet et al. 1995, Randall and Tsui 2002). The glucose concentration measured by the sensor strongly correlates with the blood glucose level measured using the conventional method. So, use of the proposed optical glucose monitoring system to visualize fish stress confirmed that the fish's stress response due to an increase in dissolved ammonia concentration can be monitored in real time.

These results indicate that it is possible to monitor the stress response and visualize the stress level in freely swimming fish in real time using the proposed biosensor system, which can help investigators evaluate fish stress responses.

4. Conclusions

In this experiment, we used the proposed optical biosensor system to monitor the stress response of freely swimming fish in real time and attempted to signal the stress level based on the color of an LED. We first confirmed that the output current value of the sensor increased as the glucose concentration increased in combination with the system and biosensor. As a result, the range of glucose concentration (35.36 to 300 mg dl^{-1}) measured sufficiently covered the blood glucose levels of the test fish. We then examined the sensor communication range in air or in water (fresh water or seawater), and it was possible to communicate within essentially the same range in air and in fresh water. In addition, this system can be used in aquatic environments including seawater. On the other hand, by confirming the change in the LED color based on the sensor output current/voltage in the in vitro state, the LED color switching circuit of this system can also visually transmit the fish stress response data to a PC. In addition, we attached this system to Nile tilapia and tried to visualize the stress response

with freely swimming fish. That is, by increasing the dissolved ammonia concentration in the breeding water and applying stress, the output current/voltage value of the sensor increased, confirming that the LED color changed based on the preset threshold values. Moreover, with a communication efficiency of ~85.7% of the total communication time, our proposed biosensor system using optical communication technology is considered suitable for real-time monitoring. Application of this system for multiple fish in the future is expected to allow for easier monitoring of fish stress responses and promote physiological research of fish, such as elucidation of the influence of an overcrowded breeding environment on fish health.

So, since the proposed biosensor system can help us to get a real-time monitoring of fish stress on both data and visualized results, it still holds some certain limitations on the way to achieving the future goal. For example, the calibration should be further simplified since it still needs to be performed on the electronic circuit. Besides, the identification of individuals cannot be performed now. It is to say, when there are a lot of test fish to measure, we cannot make instantly understanding to specific individuals or specific groups. To address these issues, our future research will try to further improve its communication and calibration methods, and hope that the control and feedback from the electronic circuit can be managed by using computer software instead of the transmitter itself through a bidirectional communication technology.

5. Acknowledgment

This research was supported in part by a Grant-in-Aid for Scientific Research B (grant No. 17H03871) from the Ministry of Education, Culture, Sports, Science and

Technology in Japan. We wish to thank Dr. Kyoko Hibi (Kokugakuin Tochigi Junior College), Prof. Pierre Morizet-Mahoudeaux (University of Technology of Compiègne, France) and Dr. Chiharu Ichioka (Ichioka Dental Office, Japan) for their helpful discussions.

References

Alam, F., RoyChoudhury, S., Jalal A.H., Umasankar, Y., Forouzanfar, S., Akter, N., Bhansali, S., Pala, N., 2018. Biosens. Bioelectron. 117, 818-829.

Arillo, A., Margiocco, C., Melodia, F., Mensi, P., Schenone, G., 1981. Ecotoxicol. Environ. Saf. 5, 316-328.

Barton, B.A., 2002. Integr. Comp. Biol. 42(3), 517-525.

Barton, B.A., Iwama G.K., 1991. Annu. Rev. Fish Dis. 1, 3-26.

Brown, G.E., Brown, J.A., Srivastava, R.K., 1992 J. Fish Biol., 41, 955-963.

Carmichael, G.J., Tomasso, J.R., Simco, B.A., Davis, K.B., 1984. Trans. Am. Fish Soc., 113, 778-785.

Dervisevic, M., Custiuc, E., Çevik, E., Şenel M., 2015. Food Chem. 181, 277-283.

Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. J. Fish Biol., 61, 493-531.

Endo, H., Yonemori, Y., Hibi, K., Ren, H., Hayashi, T., Tsugawa, W., Sode, K., 2009. Biosens. Bioelectron. 24, 1417-1423

Fekry, A.M., 2017 Biosens. Bioelectron. 87, 1065-1070.

Knoph, M.B., Thorud, K., 1996. Comp. Biochem. Physiol., Part A: Physiol. 113, 375-381.

Leal, J.F., Neves, M.G.P.M.S., Santos, E.B.H., Esteves, V.I., 2018. Rev. Fish. Sci. 10，281-295.

Neethirajan, S., Ragavan, K.V., Weng, X., 2018. Trends Food Sci. Technol. 73, 25-44.

Pickering, A.D., Pottinger, T.G., 1989. Fish Physiol. Biochem. 7, 253-258.

Randall, D.J., Tsui, T.K.N., 2002. Mar. Pollut. Bull. 45, 17-23.

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., Sasal, P., 2014. Aquaculture 433, 50-61.

Rotllant, J., Tort, L., 1997. J. Fish Biol. 51(1), 21-28.

Ruyet, J.P., Chartois, H., Quemener, L., 1995. Aquaculture 136, 181-194.

Schwaiger, J., Wanke, R., Adam, S., Pawert, M., Honnen, W., Triebskorn, R., 1997. J. Aquat. Ecosyst. Stress Recovery. 6(1), 75-86.

Shehata, M., Azab, S.M., Fekry, A.M., Ameer, M.A., 2016 Biosens. Bioelectron. 79, 589-592.

Shinoda, R., Wu, H., Murata, M., Ohnuki H., Yoshiura, Y., Endo, H., 2017. Sens. Actuators, B. 247, 765-773.

Small, B.C., 2004. Aquaculture 238, 469-481.

Thurston, R.V., Russo, R.C., Vinogradov, G. A., 1981. Environ. Sci. Technol. 15, 837-840.

Meade, J.W., 1985. Prog. Fish Cult., 47, 135-145.

Nakano, T., Kameda, M., Shoji, Y., Hayashi, S., Yamaguchi, T., Sato, M., 2014. Redox Biology, 2, 772-776.

Vásquez, G., Rey, A., Rivera, C., Iregui, C., Orozcob, J., 2017. Biosens. Bioelectron. 87, 453-458.

Walters G. R., Plumb J. A., 1980, J. Fish Biol. 17(2), 177-185.

Wicks, B.J., Randall, D.J., 2002. Aquat. Toxicol. 59, 71-82.

Wu, H., Aoki, A., Arimoto, T., Nakano, T., Ohnuki, H., Murata, M., Ren, H., Endo, H., 2015. Biosens. Bioelectron. 67, 503-510.

Yonemori, Y., Takahashi, E., Ren, H., Hayashi, T., Endo, H., 2009. Anal. Chim. Acta. 633, 90-96.

Figure captions

Fig. 1 Image of the LED color-switching system for fish stress visualization.

Proposed system realizes simultaneous data communication and stress visualization of fish. Data communication is transmitted by light, and stress visualization is reflected by the color switching of the LEDs depends on the stress level of fish.

Fig. 2 Diagram of LED color-switching system for fish stress visualization.

The system consisted of glucose biosensor, multi-functional potentiostat (stress visualization and data communication), and the data receiving part.

Fig. 3 Experiment design of stress response to ammonia concentration change. The sensor was implanted to the body of the fish, which was kept in water tank A overnight to allow for acclimation to the attached biosensor. Then, VcompL and VcompH were calibrated and the experiment started. Thirty minutes after beginning the monitoring, the fish was moved to water tank B for stress application to the fish body. As a control, several blood samples were obtained during the measurement, and they were measured by conventional methods.

Fig. 4 Response of the sensor in EISF.

(A) Typical response curve for the sensor on sequential addition of glucose solution in EISF. (B) Glucose calibration plots of the enzyme sensor (range of glucose levels 35.36 to 300 mg dl^{-1}).

Fig. 5 Evaluation of LED color-switching system performance (in vitro)

Arrows indicate the moment of standard glucose addition. I. 50 mg dl^{-1}; II. 100 mg dl^{-1}; III. 200 mg dl^{-1}.

Fig. 6 Measurement of the stress response by transferring test fish to breeding water containing dissolved ammonia.

Glucose levels in the blood plotted relative to the first sampling point were used for the one-point calibration method. The arrows indicate when the fish were moved from the normal breeding water to the ammonia-containing (15 mg L^{-1}) water. (A) Response of sensor (raw data). (B) Response of sensor (calibrated using one-point calibration method)

Figure 1

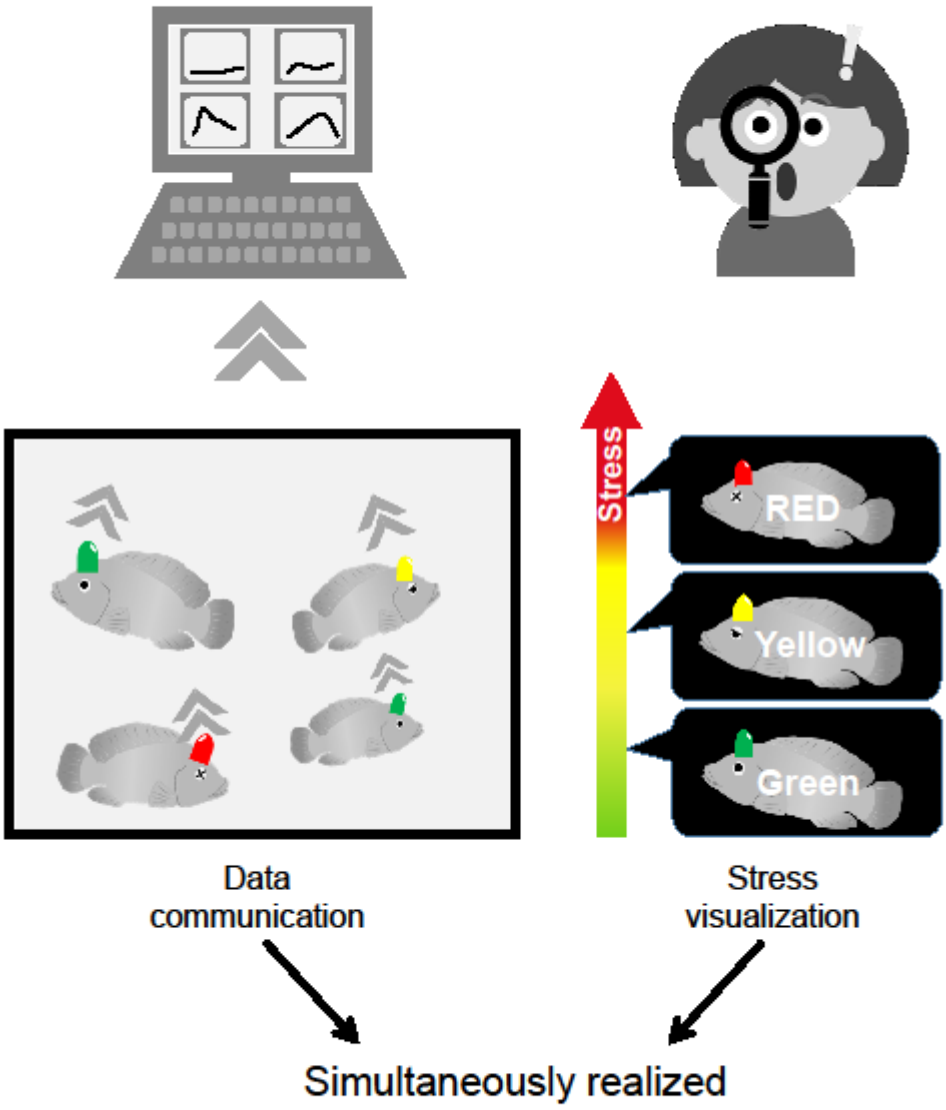

Stress
RED
Yellow
Green
Data communication
Stress visualization
Simultaneously realized

Figure 2

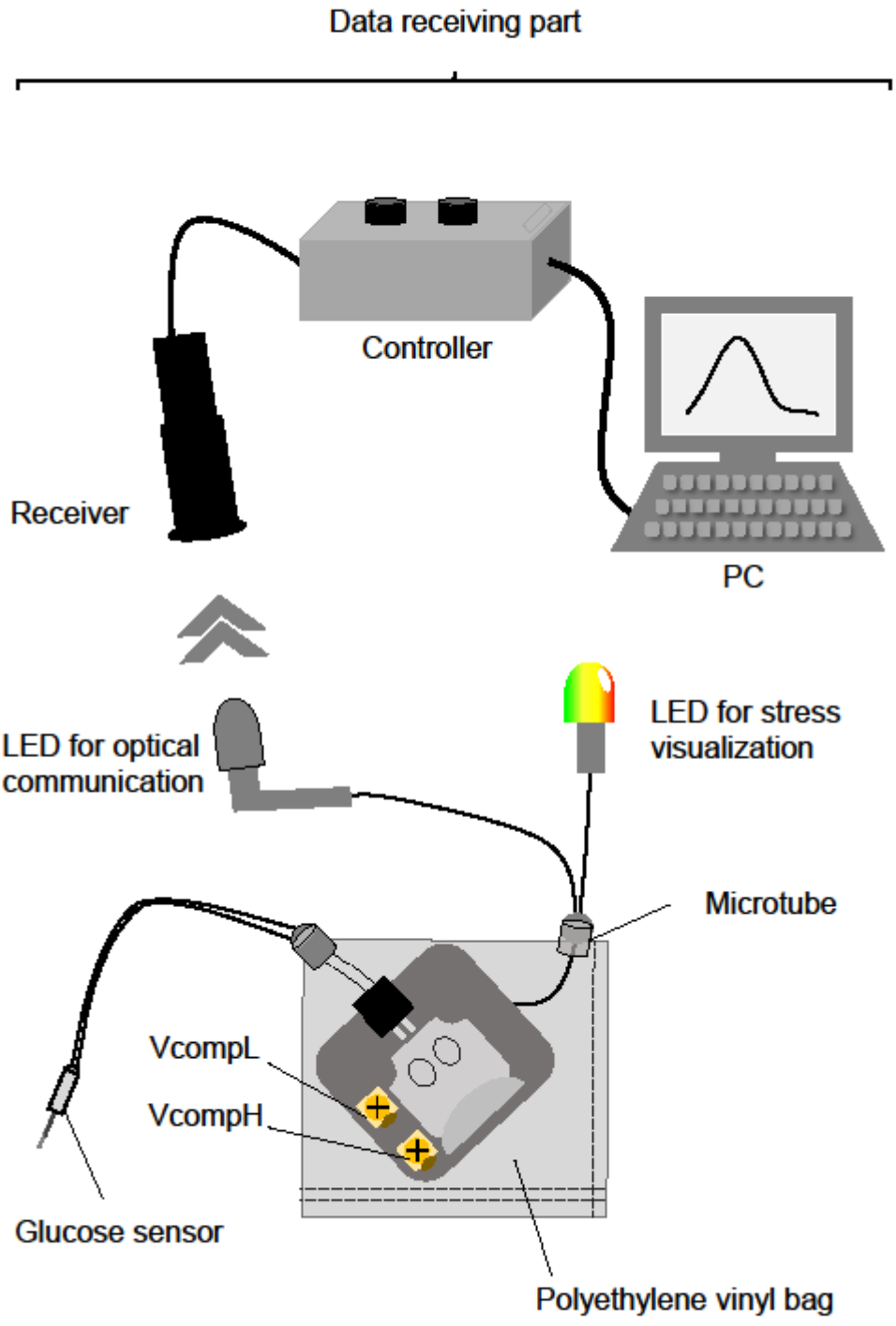

Data receiving part
Controller
Receiver
PC
LED for optical communication
LED for stress visualization
Microtube
VcompL
VcompH
Glucose sensor
Polyethylene vinyl bag

Figure 3

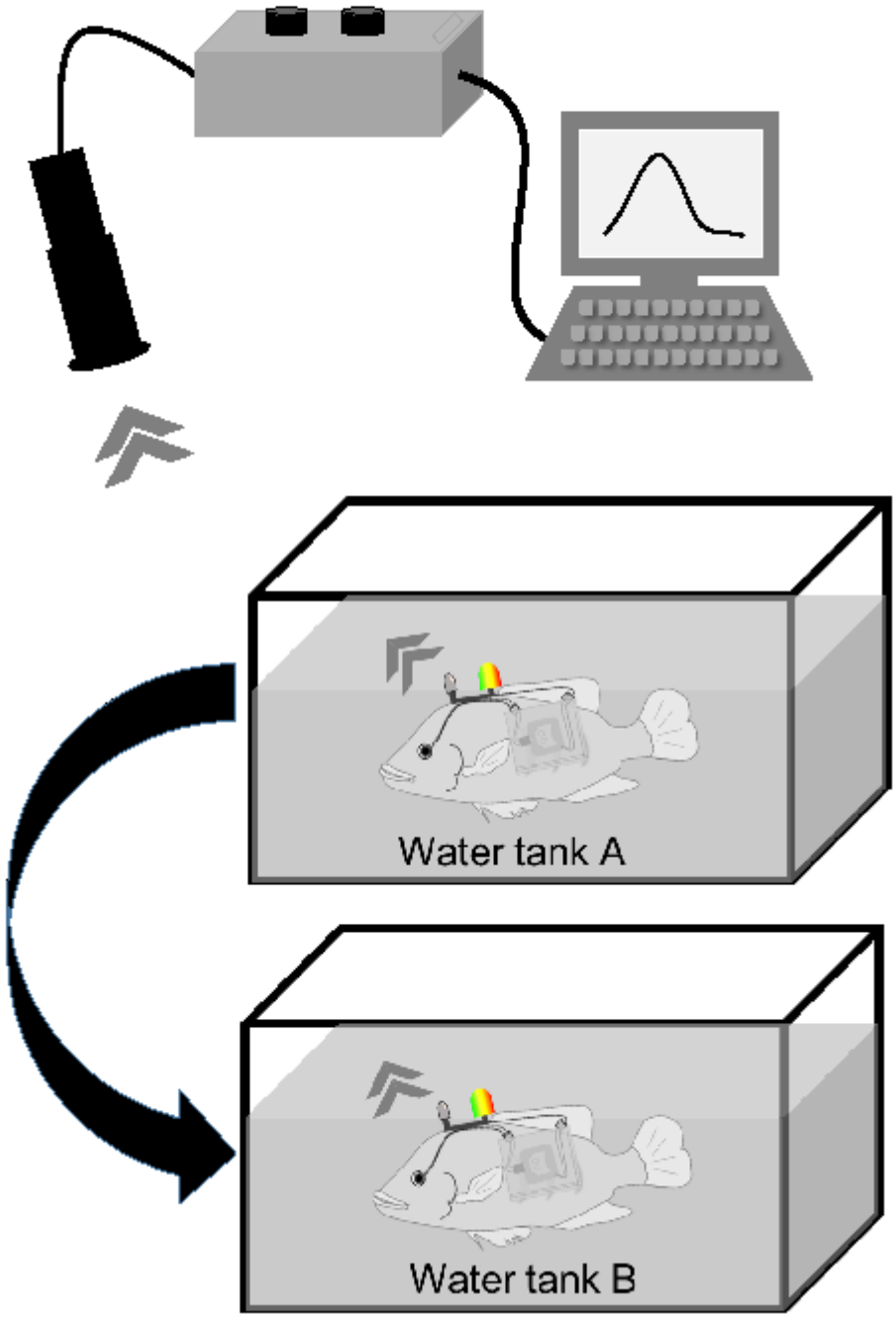


Figure 4

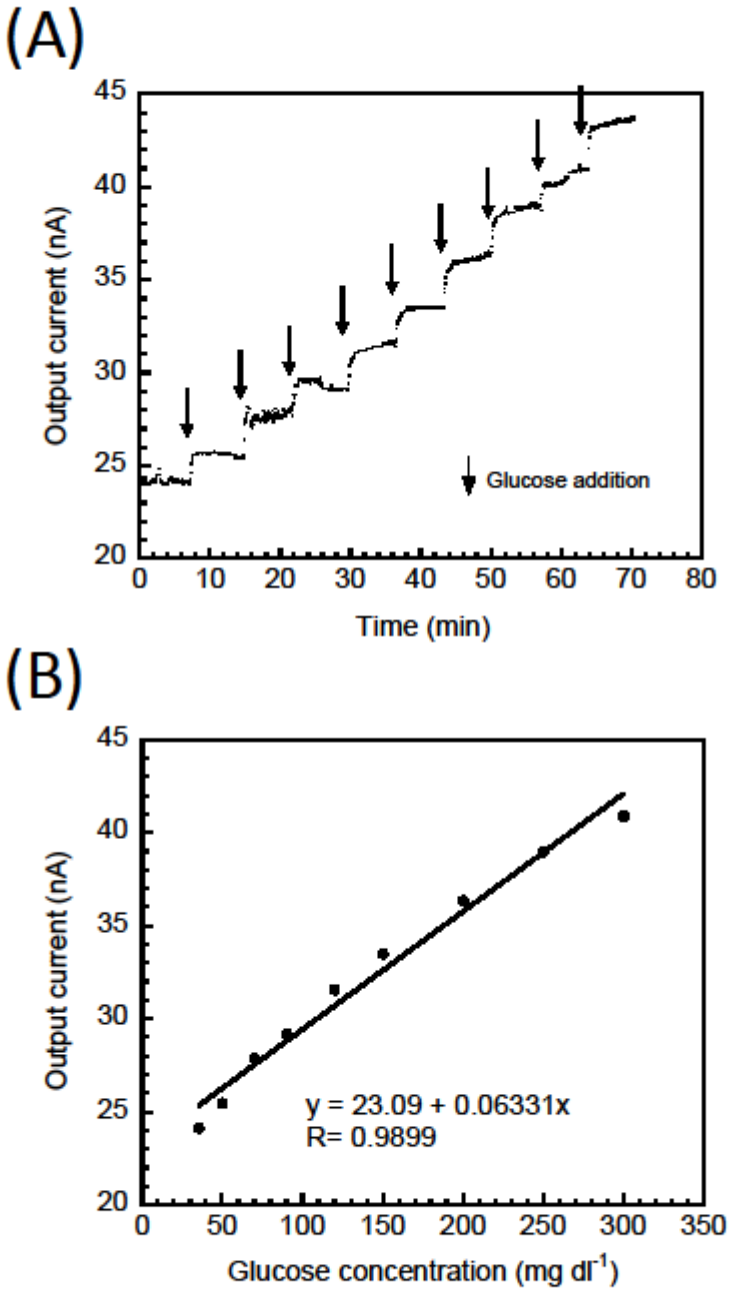

(A)
Output current (nA)
Time (min)
Glucose addition
(B)
Output current (nA)
Glucose concentration (mg dl-1)
y = 23.09 + 0.06331x
R= 0.9899

Figure 5

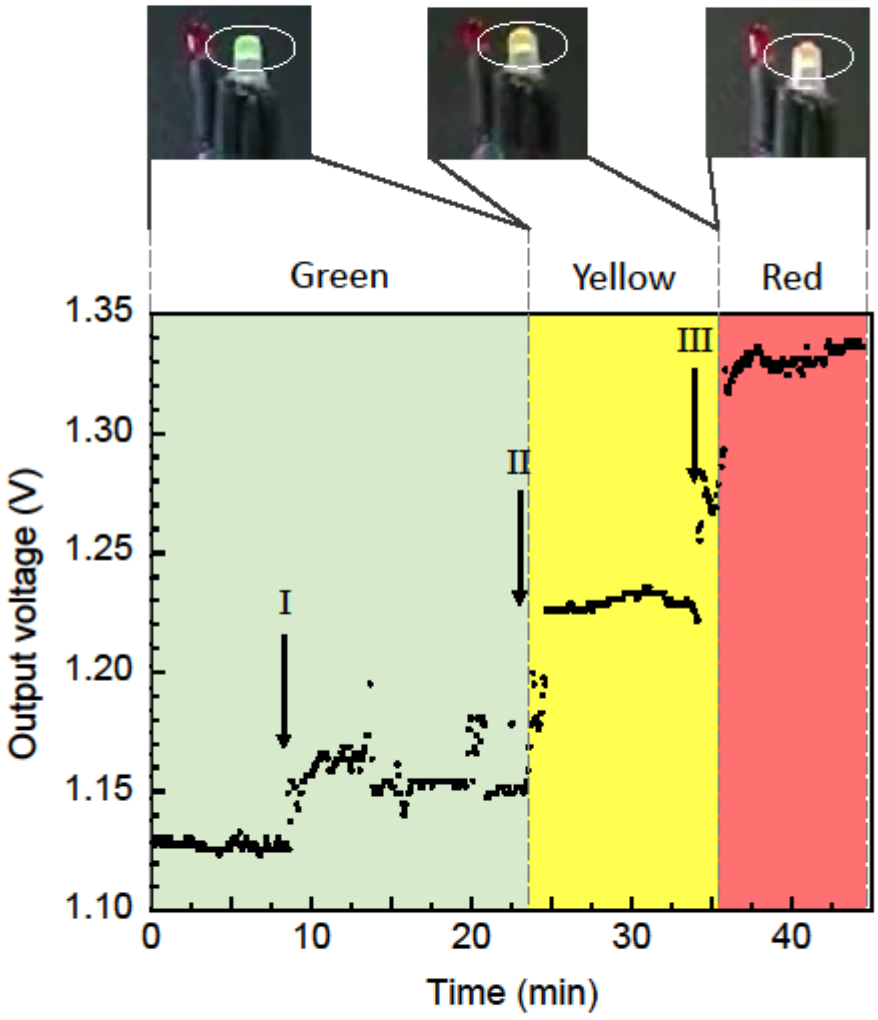
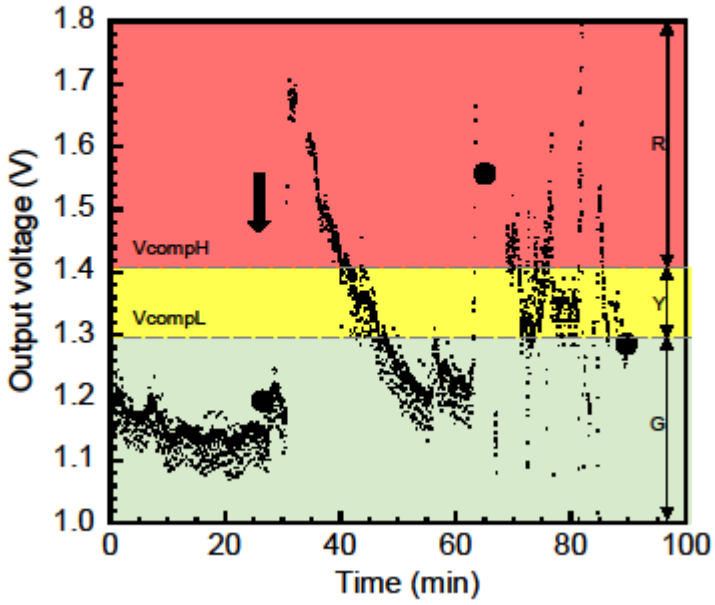
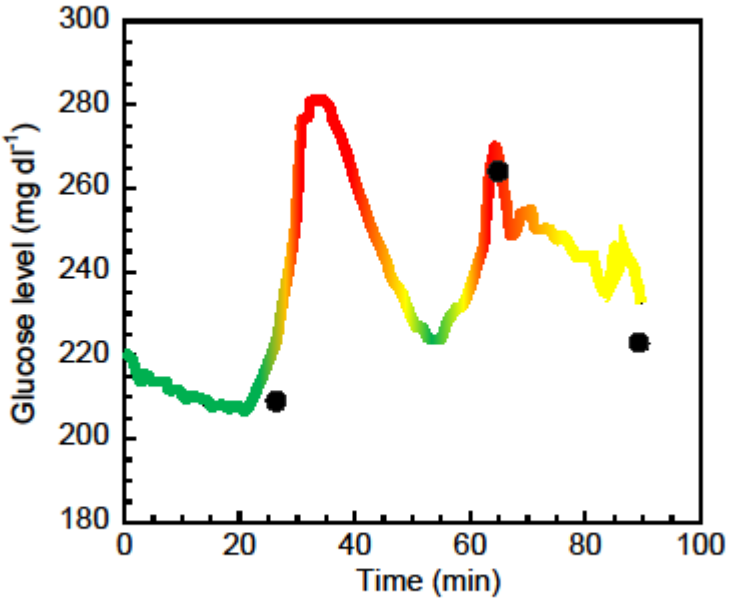

Green
Yellow
Red
I
II
III
Output voltage (V)
1.35
1.30
1.25
1.20
1.15
1.10
0
10
20
30
40
Time (min)

Figure 6

(A)


Output voltage (V)
Time (min)
VcompH
VcompL
R
Y
G
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0
20
40
60
80
100

(B)


Glucose level (mg dl-1)
Time (min)
300
280
260
240
220
200
180
0
20
40
60
80
100

Highlights

- Simultaneous optical data transmission and stress visualization when fish swim freely
- Stress response of fish finally could be observed in real-time even at a glance
- Without unnecessary stimulation, stress monitoring become more authentic and reliable