**Mixed microbial biosensors for enhanced visualization**

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**Abstract**

Global environmental pollution problems caused by transformation into high-consumption society and industrialization, especially the soil and groundwater pollution, are one of the major environmental issues that are recognized as a threat to human health.

**Introduction**

In mass-consumption society and industry, people are constantly at risk of being exposed to the leakage of harmful substances, there has been a need for an efficient hazardous material monitoring technique with high practicality. Whole cell biosensors have had obtained great interest as an alternative of chemical base methods which are open expensive and requires complex protocols [1, 2, 3]. In particular, since the discovery of various microorganisms like *Pseudomonas* that have adapted to toxic substances including non-biodegradable aromatic compounds [4] and acquired the genes for enzymes to decompose them, research has found a way to develop whole cell biosensors that can fluently detect the harmful substances by combining those genes and fluorescent proteins such as GFP [5, 6]. The best known example is the biosensor that can detect TNT using *E. coli* [7]. In general, these microbial biosensors rely on a light or fluorescence reporter that efficiently informs the observer of the minute changes in the environment detected by the microorganism [8]. However, the inherently weak signal of whole cell biosensors limits the visualization efficiency, while the use of fluorescence requires expensive high-capacity laser and filter devices. An additional obstacle to the commercialization of microbial biosensors is the prolonged time required by the preprocessing steps such as the cell culture and reaction processes.

Recent development of synthetic biology significantly improves the sensitivity and availability of biosensors by introducing delicately designed genetic circuits [9, 10, 11]. Wan X et. al., showed a promising example for the applications with the ultra-high sensitive biosensors by introducing cascade regulations of transcription factors [12]. Also *in-vitro* based genetic circuit development enables to make low cost detection kit for viruses without the time consuming preprocessing steps [13]. However, in the design point of view, the expression of a sensing regulator gene would be inevitably compromised by the expression of reporter genes in biosensor cells due to the limited energy sources such as ATP and NADH in a cell []. Here, we report mixed microbial biosensor that can significantly increase the fluorescence signal by mixing two different types of biosensor cells. One group directly detects target molecules along with the production of high affinity cell-cell communication molecules while the other group was designed to receive the communication signal which induces strong expression of reporter genes. The high-affinity inducer and its binding regulator pair is able to trigger an expression system with less copy number of the binding regulator protein so that the cell spends more resources on expressing reporter genes.

**Results**

**Cell-cell communication based mixed microbial biosensor construction**

The biosensor used in this study is based on the whole cell biosensor consisting of phenol triggered DmpR and its downstream reporter proteins [6]. The activated DmpR switches on the expression of the reporter gene which fluorescence can be quantified for the phenol concentration. Quorum-sensing molecules such as Acyl homoserine lactone (AHL) have been widely investigated for diverse cell-cell communication based genetic circuit system [14]. This study employs *luxI*/*luxR* gene pair generating and recognizing AHL to construct a bacterial communication module which is coupled to the *dmpR* based biosensor (Figure 1A). We constructed two types of biosensors one of which, called “Detector”, responses to target molecules by the DmpR regulator which induces the expression of downstream *luxI* and *turboRFP* reporter genes (suppl. Table 1 primers). The other type of biosensor, called “Reporter” which consists of AHL inducible *luxR* and its downstream enhanced green fluorescence protein (EGFP) gene. Once the detector cells are triggered by hazardous compounds such as phenol, they show red fluorescence and generate AHL that induces the green fluorescence expression of the reporter cells. In order to confirm the two different biosensors are properly behaved in responding to phenol, detector and reporter cells were co-cultured in a LB medium with the same seed ratio and measured their single cell level fluorescence with flow cytometry (Figure 1B). When there is no phenol in the culture broth, neither green nor red fluorescence were not observed. But as the phenol concentration increases ranging from 1uM to 1mM, the cell population was clearly separated into two groups of green and red fluorescence, which means both the detector and reporter cells properly produces red and green fluorescence proteins, respectively, mediated by AHL as a response of the phenol molecules.

**Co-culture based mixed microbial biosensor optimization**

For the co-culture of the sender and the receiver cells, the inoculation ratio for each cell type was divided into 0, 5, 10, 15, 20 μL and added to the 1 mL culture medium to make up the final 40 mL, after which the fluorescence strength in nine different cases were compared. The cells were treated with the final concentration of 100 μM phenol with the seed. Following the 15-hour culture at 30°C, the fluorescence was measured, and the highest expression of green fluorescence was exhibited by the ratio of 15 μL sender and 25 μL receiver. Next, the signal strength of the whole cell system composed solely of the sender-receiver system and the dmpR and gfp genes, was measured. When the sender-receiver system under the finally selected optimum condition and the single whole cell sensor under the same condition were each made to react with the phenol compounds, the sender-receiver system produced approx. twice stronger signal than the single whole cell system (Figure 1 C).

The result comes from the difference between the phenol sensitivity of DmpR and the AHL sensitivity of LuxR (Figure 1 D), which led to high level DmpR expression induced by a known concentration of phenol through the AHL production by LuxL, and the subsequent high level expression of gfp, indicating the enhanced visualization of biohazard detection.

In addition, the advantage of the system having separate modules of sender and receiver, is that the performance of each module can be maximized. For example, the Pseudomonas putida KT2440 strain that provides DmpR protein in the sender has the optimum growth temperature of 30°C while the optimum temperature of AHL production by LuxL is also 30°C. However, in the case of *E. coli*, the host, the optimum growth temperature is 37°C so that the visualization of biohazard detection may be impeded due to the differences in microbial growth and protein activation conditions. The method suggested in this study involves culturing the sender and receiver cells under their individual optimum conditions so that biohazard reactions can be carried out at 30°C regardless of their growth, which minimizes the functional impediment under each condition. In particular, it was anticipated that the DmpR protein expression and activation would be maximized when Pseudomonas was used as the sender host; thus, the *P. putida* sender was constructed by inserting the dmpR-gfp-luxI circuit into the pSEVA vector, whose performance was measured alongside the *E. coli* receiver. The resulting GFP signal was shown to have been strengthened by approx. 5 times when compared to the *E. coli* sender (Figure 1 E).

**Freeze stock based reaction ready biosensors**

In the case of previous whole cell biosensor, at least 16 hours are required for the seed culture and the main culture before the microbial growth and the reaction with harmful substances, in addition to the time required for the preparation of culture solution and samples. However, the limitation of such protocol for detecting harmful substances from various samples has long been pointed out. This study designed a novel protocol in which the reaction ready biosensor is stored in a stable state for a long time; i.e. the microbes for the sensor are cultured and frozen for storage in optimum state so that their reaction with harmful substances can be readily estimated when required, as rapidly as less than four hours. The DmpR-based biosensor used in this study has the sigma-54 transcription factor whose activity is elevated upon N deficiency. Its signal displays a steadily increasing pattern following the exponential phase, so that when the cells are harvested after the exponential phase and transferred to PBS for the reaction with harmful substances, the signal from the reaction increases rapidly, thereby decreasing the reaction time (Figure 2A). The results indicate that the time taken for biohazard detection would be greatly reduced through the use of biosensors if the sensor cell culture solution is stored under appropriate conditions right before activation so that it can be readily used for analyzing the harmful substances after PBS dilution. For this, it was suggested that the sensor cells in their healthiest state and prior to sigma 54 expression be collected during exponential phase, and mixed with fresh LB and glycerol before its storage at -70°C. The stocked sensor cells are then PBS diluted and their reaction with harmful substances are measured. The GFP fluorescence was stronger in the sample where fresh LB had been provided than in the sample with glycerol alone (Figure 2B). Also, when the stocked cells were 1/10 or 1/5 PBS diluted and their reaction with harmful substances were compared, the 1/10 diluted sample showed continuously increasing OD and fluorescence although the increased OD following the reaction may have been a false positive and had prolonged reaction time (Figure 2C). On the other hand, the 1/5 diluted sample showed the OD and fluorescence that stopped increasing after approx. four hours, allowing precise quantification based on concentration as well as reducing the 16-hour reaction time by approx. 1/4. Additionally, to achieve even higher visualization signaling effects, the rfp gene was substituted by the sfgfp gene in the sender that was designed for comparing the fluorescence of the sender-receiver system, which enhanced the signal strength and the usability of the system. The sender-receiver system with the substitution exhibited approx. 3 times stronger signal strength than the gfp fluorescence of the receiver alone (Figure 2D).

**One year after remain the signal**

The limitation of the previously constructed sender-receiver system lies in that it is a GMO that cannot be applied to the actual natural environment. Thus, in this study, the sender-receiver cells were mobilized in the form of beads and placed in a transparent reactor with a known amount of samples taken from the environment, in order to use the system as a detection agent for determining the presence of harmful substances in the samples. The detection of harmful substances can be done by estimating the GFP fluorescence produced by the bead sensors inside the reactor, and the details regarding the remote fluorescence detection device and specific detection protocol will be explained in the next section. In this section, how the mobilized beads were created and the characteristics of signal transformation and amplification by the beads based sender-receiver system will be described. First, to create the beads of approx. 2 mm diameter for the mobilized bead sensor, PBS diluted freeze stock solution and sodium alginate solution (2% w/v) were mixed, and dropped onto the 0.2 mM CaCl2 solution using a syringe pump. The biosensor beads were then spread evenly on 90 mm plate, at the center of which 10 L of 2 mM phenol was applied. The result showed that, the rfp signal of phenol detecting sender was observed within approx. 1 cm radius area; while the gfp fluorescence in the receiver was observed within approx. 2 cm radius area, which is twice the area of the sender (Figure 3A). Such expansion of the detection area indicates that the detection activity for phenol that could not be detected solely by signal amplification, has been transformed to the AHL signal with high sensitivity. In addition, when the beads were created from the freeze stock sender reporter cells carrying the rfp to gfp substitution and placed in a 20 mL flask with the final 10 M phenol sample for the four-hour reaction, the fluorescence monitoring of the beads showed stronger fluorescence in the phenol sample as with the culture solution (Figure 3B).

**Conclusion**

Since around 20 years ago, there has been ongoing research on biosensors based on the use of microorganisms. In particular, the research on microbial biosensors for detecting harmful substances or monitoring the human intestinal microflora has attracted much attention along with heightened interest in environmental pollution and health as well as synthetic biology. Generally, light or fluorescence are used as the most efficient visualizing medium that delivers to the observer the information detected by microorganisms regarding minute changes in the environment. However, such optical methods are limited as the biological signals are inherently weak and the use of fluorescence requires expensive high-capacity laser and filter devices. To overcome the disadvantages, the present study sought ways to maximize the signaling amplitude through the sender-receiver coupled biosensor based on cell-cell communication and to allow rapid access to the sensor cells through the freeze stock protocol. Furthermore, the study validated the use of low-cost remote fluorescence detector composed of a smart phone connected to the 3D printer and Arduino, which can replace the expensive fluorescence monitoring device by being able to detect as little as 1 M concentration of harmful substances contained in a given sample. The present study also opened up a possibility of resolving social issues such as the monitoring of soil, water, or air pollution, by converging synthetic biology research and maker technology. The novel method is expected to realize the use of cell free systems that will solve the GMO problem, while the direct use of the bead based sensors in the environment will enable real-time monitoring, especially in detecting explosive materials in Africa or harmful substances in a wide area of agricultural lands. The biohazard detection device that can be manufactured for less than 100 dollars is likely to be highly useful for farmers as well as the general public as it can be distributed without the cost burden. The device will also be useful in remote areas as its components will be readily available through 3D printing.

Rapid construction of biosensors [Pardee, K., Green, A. A., Takahashi, M. K., Braff, D., Lambert, G., Lee, J. W., … Collins, J. J. (2016). Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. Cell, 165(5), 1255–1266. <https://doi.org/10.1016/j.cell.2016.04.059>]

Applicability [George church]

**Materials and Methods**

**Sender/Receiver cell construction**

To construct the sender cells, the pGESSv4 plasmid (pGESSv4, ACS Synth. Biol., 3: 163~171, 2014) that was isolated from *P. putida* as it contains the dmpR gene, was put through PCR amplification, and luxL gene (GenBank: Acc.No. CP000021.2) originating from Vibrio fischeri was synthesized and amplified through PCR. Each PCR product was fed into gel-purification and Gibson Assembly (Master Mix Assembly Master Mix, NEB, USA), followed by ligation. To construct the receiver cells, luxR gene (GenBank: Acc.No. CP000021.2) was synthesized at Bioneer and amplified through PCR, while the pGESSv4 (pGESSv4, ACS Synth. Biol., 3:163~171, 2014) plasmid containing eGFP gene was also amplified through PCR as the template DNA. Each PCR product was gel-purified before Gibson Assembly (Master Mix Assembly Master Mix, NEB, USA) and ligation, to create pGESSv4-LuxR. V. The site of Lux operon whose expression is regulated by the LuxR transcription factor originating from V. fischeri, i.e. the DNA sequence containing the lux box and the promotor (GenBank Acc.No. CP001133.1), was synthesized at Bioneer (pGENB1-E.LuxBOX), and the DNA fragments containing the sequence were amplified through PCR. Next, the pGESSv4-LuxR containing the luxR gene was put through PCR amplification, and each of the PCR products was fed into gel-purification, Gibson Assembly (Master Mix Assembly Master Mix, NEB, USA), and ligation, to generate the sender/receiver plasmids: pS-dmpR-luxI-rfp and pR-luxR-egfp. Each plasmid was transformed in the *E. coli* Dh5a strain to complete the sender/receiver cell construction. For the *P. putida* sender, the pS-dmpR-luxI-rfp plasmid constructed as above was amplified through PCR with primer11f and primer11r, and the resulting Insert DNA was amplified with primer12f and primer12r using pBBRBB-eGFP (Addgene Catalog #32549) as the template, to create the backbone. The pBBRBB-dmpR-luxI-rfp plasmid was constructed after the Gibson Assembly, and the plasmid so obtained was transformed in the *P. putida* KT2440 strain to be used as the sender.

**Sender/Receiver cell-based biosensor assay**

100 mg/mL ampicillin was added to a 14 mL round bottom tube containing 1 mL LB broth medium. A single colony of the sender or the receiver cell was transferred using a loop from the plate to the tube. The *E. coli* and *P. putida* transformants were cultured at 37°C and 30°C, respectively, in a 200rpm shaking incubator overnight, before they can be used as the seeds. For the main culture, 100 g/mL ampicillin was added to each of the 14 mL round bottom tube containing 1 mL LB broth. An adequate amount of seeds were taken from the sender or the receiver cell for the final 4% inoculation. They were cultured at 37°C in a 200rpm shaking incubator until the OD600 (optical density 600nm) was approx. 0.5. Next, the seeds were treated with the substrate (phenol) and cultured at 30°C for 15 hours in a 200rpm shaking incubator. After the cultivation, each sample was thoroughly mixed and 200 l was loaded onto the 96 well plate. The OD600, GFP (ex: 485nm; em: 535nm) and RFP (ex: 531nm; em: 595nm) were measured using the multi-plate reader (VICTOR).

**Freeze stock protocol**

A single colony of the sender or the receiver cell was inoculated into the 14 mL round bottom tube containing 1 mL LB broth and 100 g/mL ampicillin, which was cultured at 37°C in a 200rpm shaking incubator overnight. 1% inoculation was carried out using the 125 mL baffled flask containing 20 mL LB broth and 100 g/ml ampicillin, which was cultured at 37°C in a 200rpm shaking incubator until the OD600 reached approx. 0.5. Next, the culture solution was centrifuged at 4°C in 3000 rpm centrifuge that allows temperature control. After 10 minutes, the supernatant was removed, and fresh LB broth of one tenth the volume of culture solution was added to the pellet before cell suspension via tapping. The resulting solution was mixed with 50% glycerol solution in 7:3 ratio, and rapidly cooled using liquid nitrogen, prior to its storage at -80°C for generating the freeze stock.

● 특히 본 연구에서 사용한 페놀 감지 유전자회로는 Pseudomonas putida 유래의 전사조절단백질(dmpR), 프로모터, 리포터(형광단백질)로 구성되어 있으며 1μM ~ 100μM의 페놀 농도 감지 범위를 갖고 있음 (SL. Choi et al., ACS Synthetic Biology 2013). 위 유전자회로를 탑제한 대장균을 감지 센서로 활용하기 위하여 유전자회로의 신호를 극대화 할 수 있는 조건에서 장시간 보관 및 필요시 신속하게 꺼내어 바로 사용가능한 조건을 탐색함. 이를 위해 유전자회로 탑제 pUCB19 플라스미드를 대장균 DH5alpha에 형질전환 후 ampicillin이 50 ㎍/ml 첨가된 LB 고체 배지에 도말한 후 37℃에서 12시간 배양함. 배양 후 단일 콜로니를 골라 다시 ampicillin이 50 ㎍/ml 첨가된 LB 고체 배지에 루프(loop)를 이용하여 도말한 뒤 37℃에서 12시간 배양함. 이 후 단일 콜로니를 선별하여 ampicillin이 50 ㎍/ml 첨가된 1mL LB 액체 배지가 든 14ml 튜브에 접종 한 뒤 37℃도 200rpm에서 12시간 진탕배양 하여 전배양액으로 사용함. ampicillin이 50 ㎍/ml 첨가된 LB 액체배지 20ml에 1%(v/v)로 접종한 후 OD600nm 값이 0.5가 될 때까지 37도 200rpm에서 배양함. 그 후 위 배양액을 50ml 튜브에 옮기고 4℃ 1977g에서 10분간 원심분리를 수행한 후 상층액을 분리한 뒤 ampicillin이 50 ㎍/ml 첨가된 LB 액체 배지를 2ml 넣고 세포를 풀어줌. 대장균이 균일하게 풀어진 것을 확인 한 후, 멸균된 50% Glycerol을 1.5ml EP 튜브에 0.3ml 세포배양액을 0.7ml 넣고 액체 질소에 급속 냉각 시킨 뒤 -70 ℃에 넣어 보관함. 위와 같은 방법으로 stock된 세포를 꺼내어 37℃에서 해동한 뒤 ampicillin이 50 ㎍/ml 첨가된 Minimal media (0.1% Acetate) 9ml에 넣어 10배 희석하여 바이오센서 혼합용액으로 사용함. 그 결과 기존 전배양 본배양에 걸친 방법에 비해 높은 신호를 얻을 수 있었으며 이는 sigma 54기반의 DmpR 단백질 활성으로 인한 영향으로 보임 (그림 71). 이러한 방법으로 센서혼합액의 저장성을 개선하여 가용성을 높이고 준비 기간 및 신호 편차를 크게 줄이는 효과를 얻을 수 있음. 유전자 회로 프린팅을 위한 바이오잉크 개발: 유전자 회로의 프린팅을 위해서는 기존 cell free 시스템에서 사용되는 solution들을 scale up하여 프린팅 장비와 연계할 필요가 있음. 본 연구에서는 cell free 시스템을 위한 S12 lysate와 in vitro translation, transcription에 필요한 아미노산과 에너지 source 등이 함유된 Master mix를 제조하여 프린팅을 위한 바이오잉크의 재료로 사용함.

**Alginate bead based bacterial sensor**

Two different solutions were prepared: a mixture containing 6 g sodium alginate dissolved in 200 ml DW and a mixture containing 2 g calcium chloride dissolved in 100 ml DW. An adequate amount of the sender or the receiver cell was taken from the freeze stock and mixed in an appropriate ratio, so that they could be added to the 200 ml sodium alginate solution until the OD600 reached approx. 2. A pump was connected to a tube of approx. 2 mm diameter, through which the alginate solution containing the cells was dropped onto 100 ml calcium chloride solution, to create the alginate bead of approx. 2 mm diameter. The bead was left to stand in CaCl2 solution for approx. 30 minutes for stabilization, and the CaCl2 was removed by PBS washing.

**Remote fluorescence detection device**

For the frame design required by the device housing, Fusion 360 software of Autodesk was used. The design comprised 64 components, and for printing each component, 3D printer (Ultimaker2+TM) with Fused Filament Fabrication technique was used. Polylactic acid (PLA) was the base material.

For operating the device, two 42 steps two-phase four-wire step motors (5.5kg/cm torque, 1.8degree/step, 4.2v, 1.5A, 42BYGH2637A-C, Coact Moter) were used, and the resolution of the motor driver was set at 16 (SBC-10, Moterbank). For fluorescence excitation, a green laser module of 532 nm wavelength was used, and Arduino (Intel® Edison kit for Arduino) was used for controlling the driver and the two step motors. The device control code was developed using Open-source Arduino Software (IDE) (refer to the supplementary information for details regarding the code). The smart phone used to capture the images was Samsung Note 5, and the software was SelfiShop Camera application that could transmit the signals from Arduino by TRRS audio jack for controlling the smart phone (refer to the supplementary information for circuit design and software). The image recording of the smart phone was carried out by the 570 nm band path filter. For the device to monitor the scattered beads of the sample solution, it has to remember the position of each bead and turn the camera at fixed intervals for rotation. This required the smart phone and the control computer to be connected to Arduino via WiFi, and the Sidesync software allowed the monitoring of the smart phone screen through the computer screen so that the motor connected to Arduino could be controlled by the computer.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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