**Biosensor mixture with modular optimization enables to maximize signal amplification**

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# Abstract

It has long been studied to use bacterial biosensors for the monitoring of the harmful substances which activate a specific transcription factor followed by the expression of downstream reporter proteins. However, inherently weak signal of the biosensors consisting of heterologous genes and promoters are one of the main limitations that cannot applicable to environment monitoring. Here, we propose a new approach of biosensor mixture to enhance the performance of biosensor cells by modularly separating strains into detector and reporter, and decoupling the growth and reaction phase of the biosensors cells. The modularly optimized biosensor mixture showed maximum 10 fold increases of the fluorescence signal in comparison with the homologous whole-cell biosensor. Despite the direct use of these biosensors is limited due to the GMO issues, progressive improvement of synthetic biology based biosensor performance along with low cost measuring device could be highly useful for the general public in monitoring hazardous compounds such as agricultural chemicals and even explosives such as TNT in a large area of land.

# 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 [1–3]. 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. In particular, heterologous DNA parts such as transcription regulator and promoters in the whole cell biosensor host such as *E. coli* are not guaranteed their maximum activities in their original host strains. 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. In spite of the freeze dried technique of biosensors, activity loss is still in problematic, especially in dry step [9]. Recent development of synthetic biology significantly improves the sensitivity and availability of biosensors by introducing delicately designed genetic circuits [10, 11, 12]. Wan X et. al., showed a promising example for the applications with the ultra-high sensitive biosensors by introducing cascade regulations of transcription factors [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 [14].

Here, we report a new approach of biosensor mixture to enhance the biosensor performance by mixing the modularized biosensors each of which can be optimized based on its harboring DNA parts such as regulatory proteins and promoters. Also decoupling of the biosensor growth and reaction phases enables the heterologous biosensor mixture to be free from the growth dependent promoter activities with significantly shorten reaction time of biosensor. As a proof of concept, two different types of biosensor cells were used for the biosensor mixture. One type of the biosensors directly detects target substrates along with the production of high affinity cell-cell communication molecules while the other type of cells was designed to receive the communication signal which induces strong expression of reporter genes.

# Results

## Biosensor pair construction with cell-cell communication genetic circuit

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 [15]. 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 1M 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 biosensor mixture

The rational of signal amplification of the paired microbial biosensor is based on the much smaller low detection limit (LOD) of LuxR regulator in reporter cells than the phenol responsive DmpR in detectors. Figure 2A shows DmpR triggers red fluorescence above 5M phenol while the LOD of reporter cells is approximately 0.05M (Figure 2B) which is hundred times lower than the DmpR case. The paired biosensor with equal seed portion of detector and reporter cells clearly higher specific fluorescence than the whole cell biosensor of our previous study [6]. In order to investigate optimal portion of detector and reporter cells, the inoculation ratio for each cell type was divided into 0, 5, 10, 15, 20 μL and added to the culture medium to make up the final 40 L of detector and reporter cell mixture volume in a 1ml LB mediam. The cells were treated with the final concentration of 100 μM phenol followed by the 15-hour culture at 30°C. Fluorescence strength in the nine different cases were measured and the highest expression of green fluorescence was exhibited by the ratio of 15 μL detector and 25 μL reporter. However, the green fluorescence intensity is not strong enough as we initially expected in comparison with the 1:1 ratio case. Considering the red fluorescence from detector cells along with green signal in each cultured mixture, the total signal strength of 15:25 might not be the strongest anymore. We discussed this is possibly because the activation time imbalance between DmpR and LuxR of detector and reporter cells, respectively. DmpR protein is the sigma-54 factor dependent transcription factor whose activity is elevated upon nitrogen deficiency [16]. So, in the presence of inducing molecules, the fluorescence signal of detector is expected to increase starting from at the end of exponential phase of cell growth while the LuxR transcription factor in reporter cells is based on sigma-70 which triggers the fluorescence protein expression from at the early stage of cell growth.

## Freeze based decoupling of biosensor growth and reaction

In order to investigate the transcription factor activation phase difference, biosensor mixture with 1:1 portion of detector and reporter cells were cultured with final concentration of 0, 10, and 100 M phenol followed by time-course measurement of the red and green fluorescence. In figure 3A, red fluorescence from detector cells starts to be shown approximately 10 hours after the main cell culture starts while green fluorescence triggered by LuxR in reporter cells were shown approximately 4~5 hours ahead of the red fluorescence of detector cells as we assumed. So our strategy was to freeze the biosensor cells which are harvested in the exponential phase, right before the DmpR in detector cells is activated, so that the thawed biosensor mixture directly reacted with substrates without cell growth. To evaluate the freeze approach, the DmpR based whole cell biosensor, GESSv4 [6] were collected in their healthiest state and prior to sigma 54 expression during exponential phase. After centrifugation of the cells, they were suspended with 1/10 culture volume of fresh LB and glycerol before its storage at -70°C. For the reaction with phenol, the thawed cells were mixed with 9/10 volume of minimal media with 10% acetate. Our approach (LB-LB-MM) showed approximately 2.5 fold higher fluorescence level responding to phenol than the normal LB condition without freeze step at 19 hours (Figure 3C). This high fluorescence might be mainly due to the supplement of glycerol and fresh LB effects along with freezing stress. One of the advantages of this approach is that the separately stored freezing biosensors enable to control the portion of the detector and reporter cells in the biosensor mixture. We diluted the stocked cells to minimal media 10% acetate in the portion ranging from 1/100 to 1/2 and measured time course fluorescence and OD to compare their reaction efficiency. Despite the maximum fluorescence was observed in the case of 1/100 dilution at the final concentration of 100M phenol in Figure 4A, optimal condition was chosen with 1/10 diluted condition that optical density directly increases without lag phase of growth along with the fluorescence, which is exactly we expected from the freeze biosensors.

## Optimal ratio and durability of the freeze based biosensor mixture

With the selected freeze conditions of the biosensors, we searched for the optimal portion of the freeze detector and reporter cells maximizing the fluorescence signal. Note that to reduce the signal loss coming from the different fluorescence protein in the mixture, the egfp gene in the reporter cell was substituted by the rfp gene so that all the cells in the mixture show red fluorescence responding to phenol. By using the proposed freeze method, the detector and reporter cells with rfp gens were stocked at -70°C. In the following day, ten samples of different portion of detector and reporter cell stocks (10:0, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10) were tested in responding 0, 5, and 50M of final phenol concentration (Figure 5C top). In contrast to the previous growth based portion, 1:9 ratio of detector and reporter ratio showed approximately twice stronger signal than the detector alone (10:0 case) at 50M phenol. At 5 M phenol, the fold change is even better approximately 10 times than the case of detector alone. In order to investigate the durability of the freeze stocked biosensors, the same freeze cells above were tested after 1 year with the same conditions (Figure 5C bottom). Despite overall fluorescence decreases approximately 80% comparing to the result before 1 year, the trend of detector and reporter ratio is still similar, and the maximum fluorescence was shown in 3:7 and 2:8 cases. The main reason of the detector portion increased comparing one year before is possibly the leaky expression of DmpR promoter in the freeze stock. The DmpR mediated biosensor is known that the LB media causes higher background fluorescence signal than that of minimal media [6]. Therefore, one can further expect the performance improvement of the biosensor mixture by optimizing stock media components with more delicate freeze condition.

## Hetero-strain mixture and bead application

One of the advantages of the proposed mixture system is that the performance of each system can be maximized separately. For example, the *Pseudomonas putida* KT2440 strain that origin of DmpR protein has the optimum growth temperature of 30°C and the optimum temperature of AHL production by LuxI is also 30°C. However, in the case of *E. coli*, the host strain of the DmpR and LuxI/R system, the optimum growth temperature is 37°C so the detecting reaction may be impeded due to the differences in microbial growth and protein activation conditions. The method suggested in this study involves culturing the detector and reporter cells under their individual optimum conditions so that detecting reactions can be carried out at 30°C regardless of their growth, which minimizes the functional impediment under each condition. In addition, it was anticipated that the expression and activation of DmpR protein originated from *Pseudomonas putida* would be maximized when *Pseudomonas* was used as the detector host. So *P. putida* detector was constructed by inserting the dmpR-luxI-rfp circuit into the pSEVA vector [17], whose performance was measured alongside the *E. coli* reporter. The resulting GFP signal was shown to have been strengthened by approx. 5 times when compared to the *E. coli* sender (Figure 6A).

Lastly, we investigate the compatibility of the freeze based biosensor mixture with alginate bead [18]. 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 6??). 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 ??).

# Conclusion

>> 여기서부터 다시 검토

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. Also, in the case of previous whole cell biosensors, at least 16 hours are required for the seed and main culture before the hazardous detection. Despite freeze dried biosensor significantly extends durability and availability of the biosensors, activity loss is inevitable [?]. The present study sought ways to maximize the signaling amplitude through the detector-reporter coupled biosensor based on cell-cell communication genetic circuit to tackle the inherent weak signal. In addition, we 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 without signal loss. But its application is still limited because of the GMO problem which could be circumvented by investigating a novel method with cell free systems. One of the good examples is shown by Wan, X. et. al. They developed *in-vitro* based genetic circuit that enables to make low cost detection kit for viruses without the time consuming preprocessing steps [13]. However, the applicability of biosensors, especially whole cell biosensors, are still in question as not many applications have been successfully applied in solving real world hazardous monitoring problems. However, significant improvement of biosensor performance has been achieved in the basis of the synthetic biology []. Along with the high-performance biosensors, 3D printing based fluorescence detection device that can be manufactured for less than 100 dollars is likely to be highly useful in detecting explosive materials in Africa or harmful substances in a wide area of agricultural lands.

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>]

# Materials and Methods

## Detector and reporter cell construction

To construct the detector 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.

For red receiver reporter, the pR-luxR-rfp, the pS-dmpR-luxI-rfp plasmid constructed as above was amplified through PCR with primer RFP\_receiver\_BF and primer RFP\_receiver\_BR, and the resulting Insert DNA was amplified with primer RFP\_receiver\_IF and primer RFP\_receiver\_IR using pR-luxR-egfp as the template, to create the backbone, the pR-luxR-rfp plasmid was constructed after the Gibson Assembly, and the plasmid so obtained was transformed in the *E.coli* DH5ɑ strain to be used as the receiver.

RFP\_receiver\_BR gctcgctcatatgtatatctcctttttattcgactataacaaaccattttcttg

RFP\_receiver\_IF agatatacatatgagcgagctgatcaagg

RFP\_receiver\_IR aacagaagcttagccatggctg

RFP\_receiver\_BF catggctaagcttctgttttggcggatgagag

## 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.

Freeze stock의 효과를 확인하기 위해 아래의 실험을 진행.

1% seed 접종하여 OD 0.4~0.6 사이의 cell을 1) 다른 처리없이 기질 처리한 군 (LB-non freeze), 2) 50% glycerol과 7:3 의 비율로 섞은 후 deep-freezer 에서 하루 보관 후 녹인 후 기질 처리한 군(LB-freeze) 3) 3000 rpm에서 10분 원심분리 한 뒤 상층액을 버리고 0.1% M9 acetate를 배지 볼륨과 같은 양의 0.1% M9 acetate 배지를 넣어 준 후 기질 처리한 군(LB-M9-non freeze) 4) 3000 rpm에 10분 원심 분리 시킨 후 상층액을 버리고 배지 볼륨의 1/10의 fresh한 LB를 넣고 resuspension 후 50% glycerol 과 7:3의 비율로 섞은 뒤 24시간 이상 deep-freezer에 보관된 샘플을 녹여 0.1% M9 acetate 배지에 10% 접종한 후 기질 처리한 샘플(LB-LB-M9-Freeze) 4개를 96-well plate에 200 µl 접종 후 1% 비율로 phenol을 처리하여 600 rpm에서 37도에서 반응 시키며 0, 3, 6, 9 시간에 multi-plate reader로 형광과 OD를 측정하였다.

## 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.

# Conflicts of interest

The authors declare that there are no conflicts of interest.

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