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Medium-throughput screen of microbially produced serotonin via a GPCR-based sensor

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Abstract

Chemical biosensors, where chemical detection triggers a fluorescent signal, have the potential to accelerate the screening of non-colorimetric chemicals produced by microbes, enabling the high-throughput engineering of enzymes and metabolic pathways. Here, we engineer a GPCR-based sensor to detect serotonin produced by a producer microbe in the producer microbe's supernatant. Detecting a chemical in the producer microbe's supernatant is non-trivial due to the number of other metabolites and proteins present that could interfere with the sensor performance. We validate the two-cell screening system for medium-throughput applications, opening the door to the rapid engineering of microbes for the increased production of serotonin. We focus on serotonin detection as serotonin levels limit the microbial production of hydroxytryptophan, a modified alkaloid that could accelerate the semi-synthesis of camptothecin-derived anticancer pharmaceuticals. This work shows the ease to generate GPCR-based chemical sensors and their ability to detect specific chemicals in complex aqueous solutions, such as microbial spent media. Further, this work sets the stage for the rapid engineering of serotonin-producing microbes.

For Table of Contents Use Only

Keywords

biosensor; GPCR; serotonin; synthetic biology; yeast

Detecting and quantifying the levels of non-colorimetric chemicals synthesized by engineered microbes relies on low-throughput chromatography-based technologies (~100

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ASSOCIATED CONTENT

Supporting Information

Materials and Methods. Table S1. Table of strains; Table S2. Table of plasmids; Table S3. Table of primers. Figure S1. Serotonin sensor controls. Figure S2. 5-HT₄-based sensor full dose response curve with serotonin. Figure S3. Serotonin sensor colony-to-colony variation when using GFP or ZsGreen. Figure S4. Serotonin sensor response to serotonin and 5-hydroxyindoleacetic acid.

samples per day), such as gas chromatography and liquid chromatography mass spectrometry (LC/MS). Chemical biosensors that convert a chemical signal into a fluorescent output enable the use of high-throughput screening technologies (up to 10^7 samples per day), such as microtiter plates or fluorescence activated cell sorting (FACS), for chemical detection. Such throughput can fast-track the engineering of microbes for the synthesis of non-colorimetric chemicals to achieve industrially relevant titers, yields, and productivities.

Previously, we engineered the yeast *Saccharomyces cerevisiae* for the production of the modified monoterpene indole alkaloid hydroxystrictosidine, a potential advanced intermediate in the semi-synthesis of the anticancer agents irinotecan and topotecan^{1,2}. Specifically, the engineered yeast converts simple sugars to serotonin, and strictosidine synthase couples serotonin with exogenously added secologanin to produce hydroxystrictosidine. As secologanin is fed to the engineered strain for the synthesis of hydroxystrictosidine, serotonin becomes the *de facto* rate limiting substrate in the process. Serotonin is not colorimetric and LC/MS was used for detection and quantification of this molecule¹.

Interested in engineering an improved serotonin-producing microbe, we sought to develop a serotonin biosensor to detect serotonin in the spent media of a serotonin-producing microbe in a medium-throughput fashion (10^3 – 10^4 samples per day) (Figure 1). We prefer to detect serotonin non-invasively in the spent media of the serotonin producer microbe as this set up allows us to decouple serotonin production from serotonin sensing. Advantages of such a system include: 1) the use of the same sensor as serotonin titers increase by simply diluting the spent media so that the serotonin concentration is in the linear range of the sensor, 2) enabling the use of random, genome wide mutagenesis strategies to engineer the producer microbe without detrimental effects on the sensor function, and 3) the future potential to high-throughput screen producer microbes by encapsulating the producer and sensor cells and using FACS.

Recently, we developed G-protein coupled receptor (GPCR)-based sensors in yeast by expressing a GPCR known to bind the chemical of interest on the yeast cell surface and coupling it to the yeast mating pathway, resulting in green fluorescent protein (GFP) expression upon chemical detection³. The proof-of-principle GPCR-based sensor detected decanoic acid using the GPCR OR1G1. We hypothesized that simply swapping OR1G1 with a GPCR known to bind serotonin, keeping the rest of the sensor cell intact, would result in a serotonin sensor. Additionally, previous GPCR-based sensors have not been shown to 1) detect specific analytes in complex media, such as microbial spent media, or 2) have the statistical parameters necessary to be used in medium-throughput screening applications. These challenges need to be addressed prior to using GPCR-based sensors for the rapid engineering of microbes for the production of chemicals, in this particular case serotonin.

Of the twelve serotonin GPCRs expressed in humans⁴, GPCRs 5-HT_{1a} and 5-HT_{1d} have been previously used to generate serotonin sensors in yeast^{5,6}. Using a yeast/human G α subunit chimera (Gpa1/G α_{i3}) and the lacZ reporter gene, the 5-HT_{1a}-based sensor had a K_D of 7 μ M while the 5-HT_{1d}-based sensor had a K_D of 2 μ M, albeit no dynamic range was

reported⁶. The 5-HT_{1a}-based and the 5-HT_{1d}-based sensors showed negligible or no response when using the native yeast Gα subunit, GPA1⁶. More recently, a 5-HT_{1a}-based sensor using Gpa1 and GFP as the reporter gene resulted in 1.25-fold increase in signal after activation with serotonin and a K_D of ~50 μM⁵. Swapping Gpa1 for Gpa1/G_{αi3} and GFP for ZsGreen resulted in a sensor with ~4-fold increase in signal after activation with serotonin and a K_D of ~20 μM⁵. Given that our previously engineered serotonin-producing strain yielded 5 mg/L of serotonin, we needed a sensor with a K_D of ~28 μM and with a significant increase in signal after activation.

Here, we engineer a GPCR-based serotonin sensor able to detect serotonin in microbial spent media. We demonstrate that, after media optimization, the sensor can be used in 96-well plate format with reliable statistical parameters that set the stage for the sensor to be used in the evolution of an improved serotonin-producing microbe. The spent media optimization and 96-well plate sensor validation workflow presented in this work is likely to mirror optimizations that would be needed to adapt other GPCR-based sensors for the detection of microbially-produced chemicals in the spent media in a medium throughput fashion.

Serotonin sensor

Previously, we engineered a GPCR-based sensor strain (W303 *far1*, *sst2*, *ste2*, pRS415-P_{Fig1}-GFP) that relies on Gpa1 to transmit signal from a GPCR on the cell surface to the yeast mating pathway, which ultimately activates expression of a reporter gene. Using this sensor strain, we screened six known human serotonin GPCRs: 5-HT_{1a}, 5-HT_{1d}, 5-HT_{2b}, 5-HT₄, 5-HT_{5a}, and 5-HT₆. GPCR 5-HT₄ has at least eleven known isoforms and all but one vary at the C-terminus⁷, which is where the GPCR interacts with the Gα subunit. We tested 5-HT₄ isoform b, the canonical isoform. GPCR 5-HT₆ has two isoforms; however only one is functional⁸, and we tested the functional isoform. All other serotonin GPCRs tested only have one isoform⁸. Without any changes to the original sensor strain, expression of 5-HT₄ isoform b in the sensor strain resulted in ~3-fold increase in signal after activation upon exogenous addition of 100 mg/L serotonin (Figure 2A). The 5-HT₄ GPCR is found in both the nervous system and the gut⁴, where 90% of the body's serotonin is found⁹. No response was seen upon expression of the reporter gene in the sensor strain in the absence of 5-HT₄, confirming that 5-HT₄ is needed for serotonin detection (Figure S1). No response was seen upon expression of 5-HT₄ in the sensor strain in the absence of the reporter gene, confirming that serotonin was not increasing the cell autofluorescence levels (Figure S1). A dose-response curve of the 5-HT₄-based serotonin sensor showed a K_D of 8.1 mg/L (46 μM) with a linear range of 0.7–10 mg/L and a maximum 2.7-fold in signal after activation (Figure 2B; Figure S2). As the titers of microbially-produced serotonin (5 mg/L) land in the middle of the linear range of the serotonin sensor, no further engineering of the linear range was required. To increase the dynamic range of the 5-HT₄-based sensor, we swapped GFP for ZsGreen. In our plasmid-based reporter system, ZsGreen showed higher colony-to-colony variability in increase in signal after serotonin activation (from 6.5- to 20-fold) than GFP (from 2- to 3-fold) (Figure S3). To make a more reliable sensor and reduce the number of false positives in our medium-throughput screen, we kept GFP as the reporter gene.

Detection of exogenously added serotonin in plain microbial spend media

As the 5-HT₄-based sensor detected exogenously added serotonin in fresh media, we determined if the sensor could also detect exogenously added serotonin in the spent media of plain yeast, i.e. yeast carrying empty plasmids rather than the serotonin production pathway (Figure 2C). Indeed, the sensor detected serotonin with a K_D of 6.1 mg/L, a linear range of 0.8–5 mg/L, and a 1.7-fold increase in signal after activation (Figure 2D). Interestingly, the linear and dynamic range of the sensor decreased when serotonin was detected in spent rather than fresh media. We speculate that the presence of yeast produced metabolites or the fact that some nutrients were consumed by the cell over time disturbed either the binding of serotonin to 5-HT₄ or the GPCR-based signaling process. To achieve a larger dynamic range to better distinguish producer microbes yielding different levels of serotonin, we examined the compositional differences between fresh and spent media to adjust the nutritional constituents of the spend media to optimize serotonin detection.

Effects of nutrient composition on serotonin sensing

Glucose concentration can affect the yeast mating pathway signaling process¹⁰. As the serotonin-producing microbe uses galactose as the carbon source¹, we determined the effect of different glucose and galactose concentrations on serotonin sensing in fresh media. The highest serotonin sensor signal was achieved in the presence of 2% glucose, the standard glucose concentration in yeast media (Figure 3A). In either the absence of glucose and presence of galactose, or the absence of both glucose and galactose, no sensor signal was observed, demonstrating the dependence of the serotonin sensor on glucose. Serotonin detection was restored upon addition of 0.4% glucose. Next, we analyzed the difference in nutrient composition between the sensor media, which lacks histidine and leucine, and the serotonin producer media, which lacks tryptophan and uracil in addition to histidine, leucine. In the absence of tryptophan and the presence of 100 mg/L of serotonin, we saw no sensor response (Figure 3B). Sensor response to serotonin was rescued upon addition of 20 mg/L of tryptophan, the standard tryptophan concentration in fresh media. Further addition of 20 mg/L of uracil, the standard uracil concentration in fresh media, had a slightly positive effect on serotonin detection. We attribute the improvement in sensor performance upon uracil addition on the reduced metabolic burden on the sensor. To ensure that the serotonin sensor responds to serotonin and not tryptophan or the pathway intermediate hydroxytryptophan, we measured the serotonin sensor response to each of these compounds in the presence and absence of tryptophan (Figure 3C). Additionally, we measured the sensor response to 5-hydroxyindoleacetic acid (5-HIAA), a product of serotonin catabolism (Figure S4). The sensor showed no response in the presence of up to 100 mg/L of tryptophan, hydroxytryptophan, or 5-HIAA. The sensor only responded to serotonin in the presence of tryptophan. We speculate that tryptophan is acting as an allosteric modulator of 5-HT₄, helping to enhance the sensor signal. Indeed, allosteric modulation of serotonin-mediated response has been seen in mammalian cells with 5-HT_{2A} in the presence of oleamide¹¹ and 5-HT_{1B/1D} desensitization in the presence of the peptide moduline¹². More generally ions, lipids, amino acids and peptides have been shown to be modulators of GPCR activity¹³. Taking these results into account, we adjusted the plain yeast spent media to 0.4% glucose and added 100 mg/L of tryptophan before serotonin detection. After adjustment, the linear

range of the sensor increased from 0.8–5 mg/L to 1–10 mg/L (Figure 3D). Although the increase in the dynamic range was not as dramatic (2-fold vs. 1.7-fold in non-adjusted spent media), the K_D stayed the same at ~6.1 mg/L. The improvement in the serotonin sensor linear range makes it suitable to screen for microbially-produced serotonin.

Detecting microbially produced serotonin in a medium-throughput assay

The serotonin sensor resulted in a 1.3-fold increase in signal after activation when detecting microbially-produced serotonin in the producer's spent media. Upon adjusting the glucose and tryptophan concentrations of the producer's spent media, we saw an additive improvement in response resulting in a 1.8-fold increase in signal after activation (Figure 4A–C). To validate the use of the serotonin sensor for medium-throughput screening applications using 96-well plates, we carried out a three day plate uniformity experiment¹⁴ in both fresh media and tryptophan and glucose adjusted producer spent media (Figure 4D–F). Using two statistical parameters for assay acceptance, Z-factor > 0.5¹⁵ and Coefficient of Variation, CV < 10%¹⁶, the assay met the criteria for use in both medias (average Z-factor = 0.7, average CV = 4.9%). We did see a slight inter-plate drift, which could be optimized by accelerating the collection time or staggering the activation times.

We have developed a serotonin sensor capable of detecting microbially-produced serotonin in the producer spent media that has been validated for use in a medium-throughput 96-well plate assay. To generate a serotonin sensor, we screened known human serotonin GPCRs by expressing them in the yeast sensor strain for serotonin detection. No other genetic modifications to the sensor strain were needed, demonstrating the ease with which GPCR-based chemical sensors can be generated. To use the serotonin sensor for the rapid engineering of chemical-producing microbes, we demonstrate that the sensor can detect serotonin in the spent media of the serotonin-producer microbe, and has the statistical parameters necessary to be used in a medium-throughput 96-well plate screen. The spent media nutritional adjustments revealed in this work will likely be needed when using other GPCR-based sensors for the detection of chemicals in microbial spent media. The 96-well plate workflow is applicable to other GPCR-based medium-throughput screens and the workflow should also be amenable to 384-well plate format pending assay validation.

When detecting microbially produced serotonin in the producer microbe's spent media, the sensor response was ~2-fold. Although the signal was statistically significant ($P=0.005$), the sensor response would benefit from improvement. Coupling of 5-HT₄ to the yeast G α subunit could be optimized by swapping 5-HT₄'s cytoplasmatic domain with that of the *S. cerevisiae* GPCR Ste2 or using Gpa1/human G α chimeras¹⁷. However, these strategies do not always result in signal improvement³. Using ZsGreen as the reporter gene and integrating it into the chromosome should also increase sensor signal⁵. Alternatively, a feed forward loop could be introduced in the sensor strain to increase signal only in the presence of serotonin. Such a loop could generally improve the signal of GPCR-based sensors. Finally, although the linear range of the 5-HT₄-based serotonin sensor is narrow, the two cell-screening system allows for the producer microbe supernatant to be diluted so that the serotonin concentration lands in the linear range of the sensor.

Validation of the sensor for two key statistical parameters, Z-factor and CV, shows that the sensor is suitable for 96-well plate screening, and it is now poised to be used in the engineering of improved serotonin-producing microbes. Given that serotonin is the limiting substrate in the production of hydroxytryptophan, a potential advanced intermediate in the semi-synthesis of the anticancer agents irinotecan and topotecan, the medium-throughput serotonin assay will likely enable the future increased production of hydroxytryptophan as well as other modified monoterpene indole alkaloids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Detecting microbially produced serotonin via G-protein coupled receptor (GPCR)-based serotonin sensor. **A.** Engineered yeast produces serotonin from simple sugars¹. **B.** Serotonin sensor. A known human serotonin GPCR (HTR) is expressed in a GPCR-based sensor cell (*W303 ste2, far1, sst2*)³. Binding of serotonin to HTR on the cell surface stimulates the yeast mating pathway (yellow), ultimately activating expression of the reporter gene, green fluorescent protein (GFP). **C.** Serotonin sensor cell detects serotonin in the spent media of the serotonin producer cell in a medium-throughput fashion (96-well plates). TPH: tryptophan hydroxylase; DDC: aromatic amino acid decarboxylase; BH_4 : tetrahydrobiopterin; $\text{BH}_4\text{-OH}$: pterin 4a-carbinolamine.

Figure 2.

Serotonin sensor engineering. **A.** Screening of serotonin GPCRs in the GPCR-based sensor strain for response to serotonin in fresh media. **B.** 5-HT₄-based serotonin sensor dose-response curve in fresh media with exogenously added serotonin. **C.** Detection of exogenously added serotonin to plain yeast spent media. The plain yeast cell carries four blank plasmids instead of the serotonin pathway. **D.** 5-HT₄-based serotonin sensor dose-response curve in plain yeast spent media with exogenously added serotonin. All experiments were done in triplicate and the error bars represent the standard deviation from the mean. In all cases, fluorescence was normalized to the sensor response in the absence of serotonin. Insets show linear range of the response.

Figure 3.

Serotonin sensor optimization. **A.** Serotonin sensor dependence on glucose. **B.** Serotonin sensor dependence on uracil and tryptophan. **C.** Sensor response to serotonin pathway intermediates tryptophan, hydroxytryptophan, and serotonin in the presence or absence of tryptophan. Trp: tryptophan; 5-HTP: hydroxytryptophan; Sero: serotonin. Experiments in panels A–C were done in fresh media. **D.** Dose-response curve of the serotonin sensor detecting exogenously added serotonin to plain yeast spent media that has been adjusted for glucose (0.4%) and tryptophan (100 mg/L) content. All experiments were done in triplicate and the error bars represent the standard deviation from the mean. In all cases, fluorescence was normalized to the response of the sensor to no serotonin or no pathway intermediates. Inset shows linear range of the response.

Figure 4.

Detection of microbially-produced serotonin. **A**. Schematic of microbially-produced serotonin detection. **B**. Serotonin sensor response when incubated with spent media from plain yeast (PPY1391) and the serotonin producer strain (PPY741). Experiments were done in triplicate and error bars represent the standard deviation from the mean. Fluorescence was normalized to the response in non-producer spent media for each condition. **C**. Representative LC/MS (multiple reaction monitoring) traces of the spent media from strains PPY1391 (grey) and PPY741 (purple). Shown: serotonin transition 176.80 → 160.00. **D**. Schematic of medium-throughput serotonin screen. Serotonin sensor response in fresh (**E**) or spend (**F**) media over a three-day experiment.