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Complete List of Authors:	Miller, Rachel; University of Notre Dame, Chemistry and Biochemistry Brown, Galen; University of Notre Dame, Chemistry and Biochemistry Barron, Elsa; University of Notre Dame, Chemistry and Biochemistry Luther, Jamie; University of Notre Dame, Chemistry and Biochemistry Lieberman, Marya; University of Notre Dame, Department of Chemistry and Biochemistry Goodson, Holly; University of Notre Dame, Chemistry and Biochemistry

Development of a paper-immobilized yeast biosensor for the detection of physiological concentrations of doxycycline in technology-limited settings

Rachel A. Miller[1], Galen Brown[1], Elsa Barron[1], Jamie Luther, Marya Lieberman[1,*], Holly Goodson[1,*]

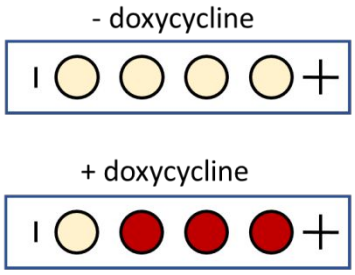
Affiliations:

[1]Department of Chemistry and Biochemistry, University of Notre Dame, IN 46566

*Co-corresponding authors

Abstract

To combat pharmaceutical counterfeiting and antibiotic resistance in low- and middle-income countries (LMIC), there is a need for improved low-cost, portable methods that monitor pharmaceutical concentrations relevant to dosage forms and physiological fluids. To address this problem, we turned to paper analytical devices (PADs), and have recently extended this technology by incorporating whole cell yeast biosensors into PADs to create biological paper analytical devices (bioPADs). The goals of the work presented here were to build on our initial bioPAD technology by showing that fluorescence can be used as a read-out and that the yeast-based system can function in complex matrices. More specifically, we embedded the bioengineered yeast on a paper test strip and showed that this simple biosensor can detect physiologically relevant concentrations of doxycycline in both human urine and raw bovine serum. In this work we also manufactured an inexpensive and portable device capable of reading the fluorescent signal of the bioPAD. This work demonstrates the untapped potential of fluorescent yeast biosensors for use in LMICs.



Introduction:

There is a need for improved analytical tools suitable for detecting pharmaceuticals in technology-limited settings. The primary need for such tools is to monitor the quality of pharmaceutical dosage forms, as it is estimated that at least 10% of all medical products sold in low- and middle-income countries (LMICs) are substandard and/or falsified.[1] Giving pharmacists and customs workers a tool to test incoming batches of drugs could prevent substandard or falsified pharmaceuticals from making their way to patients. There are additional needs to detect physiological concentrations of drugs in order to monitor drug regimen adherence, examine the efficacy of drugs, and to test food and agricultural products, such as animal feed, for pharmaceutical contamination or intentional dosing.[2-4] However, the low concentrations of pharmaceuticals in physiological fluids such as urine or blood and the complex nature of these matrices present challenges to their detection.

The gold standard method for detecting many pharmaceuticals is high performance liquid chromatography (HPLC).[5] However, the initial cost of equipment, price of consumables, and lack of trained personnel make this technology out of reach for many areas. A device that is inexpensive, robust, user-friendly, stable, and portable would be ideal for monitoring pharmaceuticals in LMICs. Progress has been made towards developing inexpensive and portable devices to monitor pharmaceutical quality in LMICs, including chemical based paper analytical devices.[6-8] However, these devices are not selective enough to discriminate between certain closely related classes of drugs, and they do not necessarily respond to biologically relevant species of pharmaceuticals. We have turned to developing yeast whole cell biosensors in order to address these needs because yeast are hardy, inexpensive, and capable of expressing a variety of highly selective sensing circuits.[9,10]

Our group developed the first whole cell yeast biosensor that was incorporated into a portable paper substrate.[11] This biological paper analytical device (bioPAD) detected the antibiotic doxycycline, which is a drug that is widely used in LMICs and has been reported as substandard.[1,12] While the bioPAD had some desirable characteristics (e.g. it was stable at 37°C for >200 days and capable of detecting 30-10,000 µg/mL doxycycline), some aspects of the device were not user-friendly. For example, the original reporter system in the bioPAD was based on the lacZ gene, whose gene product cleaves the modified sugar X-gal to produce a blue color. Development of this reporter in yeast requires cell lysis with liquid nitrogen as well as an additional 4 hr. incubation after an overnight incubation. Moreover, the X-gal solution used for color development is not shelf stable. In addition, it failed to reach the sensitivity needed to monitor physiological concentrations of doxycycline in serum.

In order to make the bioPAD more user-friendly, we sought to integrate a fluorescent reporter into our bioPAD. Fluorescent reporters do not require cell lysis or additional reagents for signal development unlike other reporters that require expensive, unstable, and/or toxic reagents (e.g. luciferase, horseradish peroxidase, and beta galactosidase). Additionally, the fluorescent signal accumulates relatively quickly in yeast cells. Moreover, advances have been made towards the development of portable fluorescence detectors.[15-19] These characteristics make fluorescent reporters good candidates for use in technology-limited settings. We also

sought to improve the sensitivity of the bioPAD device in order to measure levels of doxycycline found in urine and serum, ~30 µg/mL and 1 µg/mL respectively.[3,21] This goal has practical significance because in addition to its function as an antibiotic, doxycycline is also used as a malaria prophylactic. Close adherence to a daily regimen of 100 mg of doxycycline is needed in order to confer protection against the malaria parasite.[4] Better methods are needed in order to monitor regimen adherence (e.g. direct testing of biological fluids) in military and aid workers and to study doxycycline resistance of malaria in LMICs.[2,4,5]

In this manuscript, we report the results of these efforts. We describe an inexpensive and user-friendly bioPAD technology capable of detecting doxycycline at concentrations as low as 0.3 µg/mL, which is a relevant concentration found in human serum. The bioPAD is compatible with complex substrates including serum and urine, making it suitable for monitoring physiological concentrations of doxycycline. In addition, we describe a portable fluorescence reader that would allow for monitoring the concentration of doxycycline in LMICs.

Experimental

Materials

Yeast nitrogen base without amino acids was purchased from BioWorld (Cat# 30626020). Amino acid dropout media was purchased from U.S. Biological (Salem, MA). Trehalose dehydrate was purchased from Calbiochem (Darmstadt, Germany). Calcium chloride, kanamycin, and ampicillin were purchased from Fisher Scientific (Waltham, MA). Ahlstrom 319 cellulose sheets were purchased from Midland Scientific (Chicago, IL). Sodium alginate-medium viscosity was purchased from Sigma-Aldrich (St. Louis, MO). Doxycycline was purchased from DOT Scientific (Burton, MI). Raw bovine serum was purchased from Equitech Bio (Kerrville, TX). Pooled human urine was purchased from Innovative Research (Novi, MI). Materials used for the fluorescent light box are listed in Supplemental Information.

Molecular Biology of Yeast

Tetracycline receptors were first discovered in bacteria and were later adapted for use in eukaryotes.[22] The response mechanism of the yeast biosensors described here utilizes the reverse tetracycline transactivator (rtTA).[23] In the presence of doxycycline, rtTA will turn on the transcription of genes that are downstream of the TetO promoter. In our system, the TetO promoter is placed upstream of a fluorescent reporter gene, yEmRFP (Fig. 1). The Herrero lab developed the yeast tetracycline responsive plasmid pCM176, which was used in the original bioPAD.[11,23] pCM176 is a low copy plasmid that contains rtTA and a β-galactosidase reporter downstream of the tetracycline response element. We cloned fluorescent protein yEmRFP into plasmid pCM176, replacing the lacZ gene using the BamHI and PstI sites, forming plasmid pCM176-yEmRFP. pCM176-yEmRFP was transformed into *S. cerevisiae* strain CML282 using a lithium acetate technique. Strain CML282 constitutively expresses the tetracycline repressor protein, tetR, which inhibits expression of proteins under control of the TetO promoter in the absence of doxycycline. The parent strain CML282 and plasmid pCM176 were generously provided by the Herrero Lab.[23] The sequence for the sensitivity-enhanced and low-noise rtTA (rtTA-SELN) was kindly provided by Kaern lab[20] and we had rtTA-SELN synthesized by Genscript.[20] We cloned rtTA-SELN into pCM176-yEmRFP, replacing the original rtTA, forming the plasmid pRM02. This plasmid was transformed into parent strains CML282 and psy580a.

psy580a was generously provided by the Silver lab.[24] See Table 1 for a detailed description of yeast strains and plasmids used in this study.

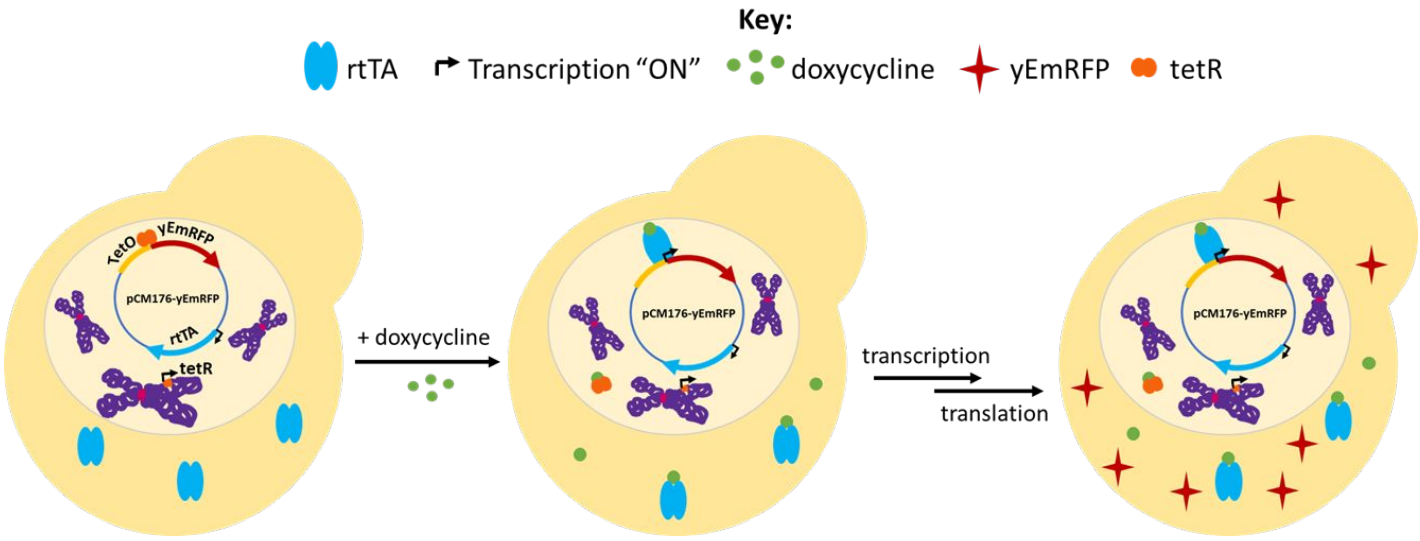


Figure 1. Biosensor Schematic

The doxycycline sensing plasmid, pCM176-yEmRFP is pictured above inside the nucleus of a yeast cell, parent strain CML282 (not to scale). This biosensor has a fluorescent reporter that is induced by the addition of doxycycline and is repressed in the absence of doxycycline. CML282 constitutively expresses the tetracycline repressor, tetR, from the LEU2 locus in the yeast chromosome and the reverse tetracycline transactivator (rtTA) is constitutively expressed from pCM176-yEmRFP. In the absence of doxycycline, TetR inhibits transcription of the reporter circuit and rtTA is in an inactive state. In the presence of doxycycline, tetR binds to doxycycline, causing it to release inhibition of the reporter circuit and rtTA binds to doxycycline, becoming active. Active rtTA induces transcription of the reporter gene, in this case the fluorescent protein yEmRFP. When enough yEmRFP protein accumulates, the fluorescent signal can be recorded.

Strain or plasmid	Phenotype, genotype, and/or description	Source or Reference
S. cerevisiae		
CML282	MATa; ura3-1, ade2-1, CMVp(tetR-SSN6*)::LEU2, 112 his3-11, 15 trp1Δ2, can1-100	[15]
Psy580a	Mat a; ura3Δ52, trp1Δ63, leu2Δ1, Gal2+	[16]
Tetracycline response plasmids		
pCM176	Ap^r; constitutively expressed rtTA and a lacZ reporter downstream of TetO promoter	[15]
pCM176-yEmRFP	Ap^r yEmRFP replaces lacZ in pCM176	This study
pRM02	Ap^r ; Sensitivity enhanced rtTA with low noise mutation G72P; yEmRFP under control of the TetO promoter	[12] ; This study
pRM03	Ap^r ; Sensitivity enhanced rtTA (P72G); yEmRFP under control of the TetO promoter	This study

S. cerevisiae strains and plasmids from this study. See Supplemental Information for detailed plasmid maps (SFig. 7).
*tetR-SSN6 encodes a protein which represses transcription of genes downstream of the TetO promoter in the absence of doxycycline.

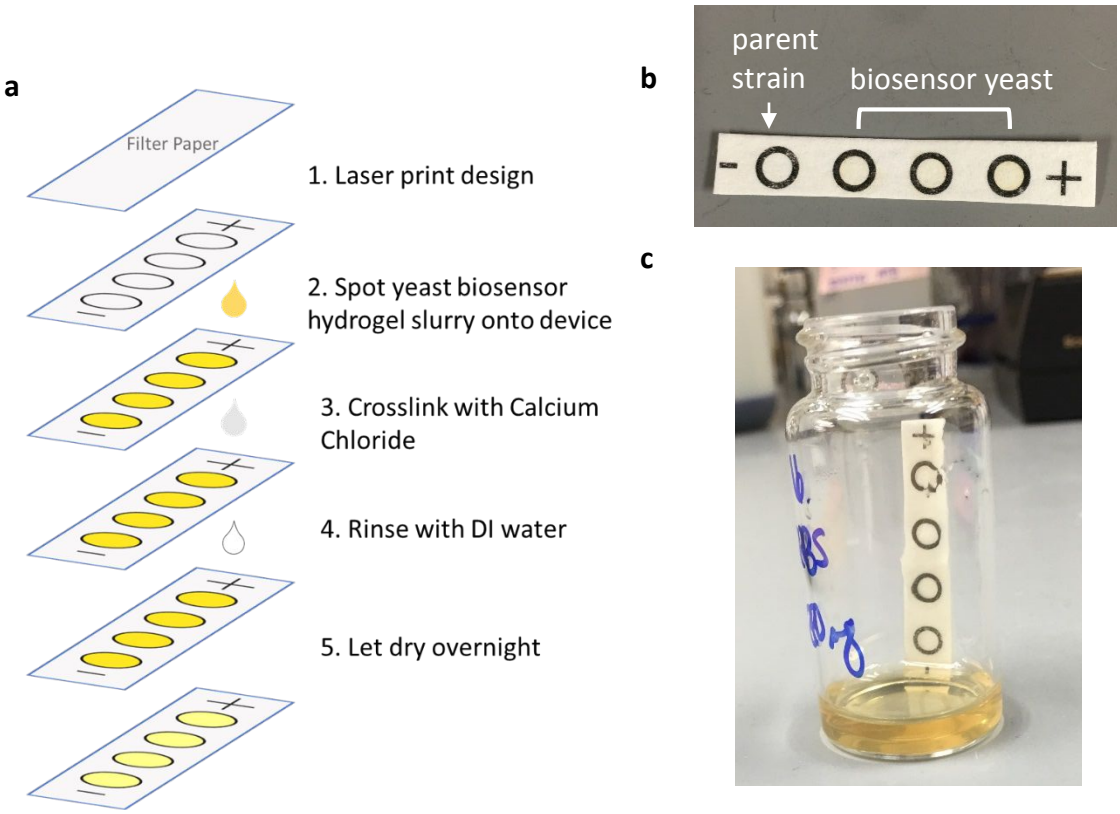


Figure 2. Fabrication of 4-spot bioPAD strips for the detection of doxycycline
a) The fabrication protocol for the bioPAD strip 1) The bioPAD design is laser printed onto filter paper. 2) Yeast biosensors are grown, spun down, and mixed with sodium alginate hydrogel before being spotted onto the printed bioPADs. 3) The bioPADs are then submerged into $CaCl_2$ solution in order to crosslink the hydrogel, entrapping the yeast and causing them to adhere to the filter paper. 4) The bioPADs are then rinsed with deionized water to remove excess calcium ions and 5) allowed to dry at room temperature overnight. b) finished bioPAD test strip. The 3 closest circles to the "+" sign contain biosensing yeast and the last circle contains the parent strain of yeast, psy580a (without the biosensing plasmid) and serves as a negative control. c) bioPAD strip being treated with a solution containing doxycycline in a scintillation vial. Doxycycline in solution travels up the paper strip via capillary action and interacts with biosensors within the ink circles.

Fabrication of bioPADs

The bioPAD is composed of 4 components: filter paper, ink, yeast biosensors, and hydrogel. Fabrication of 12-lane bioPADs was reported in Weaver et al. 2015.[11] We observed heterogeneity in the yeast spots in these 12-lane devices and sought to address this variation of yeast spot size in our 4-spot bioPAD test strip fabrication protocol. We attributed the majority of this heterogeneity to random fluctuations in how much yeast was deposited onto the filter paper. Because the yeast slurry is very viscous, it makes pipetting small volumes of the slurry difficult. Also, variations in the time that yeast were allowed to soak into the filter paper was also observed to affect yeast spot formation. We addressed these issues in the 4-spot fabrication protocol by 1) outlining the area that the yeast are deposited in laser-ink in order to control how far they spread 2) using snipped pipette tips for the yeast slurry in order to prevent "sieving" of the yeast which can cause build-up of yeast in the pipette tip that randomly

dispenses 3) letting the yeast spots sit for a standard amount of time before the bioPADs are crosslinked. The fabrication of the 4-circle bioPAD test strip described in this manuscript is outlined in Figure 2. The printed design was drawn in Inkscape (SFig. 8). This design was printed onto Ahlstrom 319 filter paper using a HP Color LaserJet CP3525 laser printer. The yeast biosensors were prepared as previously described.[11] Briefly, yeast were grown to optical density 1 (OD; measured at 600 nm and 1 cm pathlength) in selective media (synthetic complete media minus tryptophan to maintain the plasmid), spun down and resuspended at 1/200x in a solution of 2% sodium alginate and 5% trehalose to give a final concentration of 200 OD units per mL. After vortexing vigorously, the yeast-hydrogel slurry was spotted onto the filter paper using 10 µL slurry for each spot with a multichannel pipettor. The pipettor tips were snipped in order to accommodate the viscous hydrogel slurry. The bioPADs were fabricated under non-sterile conditions after initial growth of the biosensors. After addition of the yeast-alginate slurry, the bioPADs sat for 4 min to allow the slurry to soak into the filter paper. These bioPADs were then submerged in 4% calcium chloride for 10 min in order to crosslink the hydrogel, entrapping the yeast biosensors. The bioPADs were then submerged in deionized water for 1 min in order to remove excess calcium ions and laid out on paper towels to dry overnight at room temperature before use. The bioPADs were wrapped in aluminum foil and sealed in plastic zipper-sealed bags with ~6 g Drierite desiccant and stored at 4°C unless noted otherwise.

Response Generation and Development of bioPADs

BioPADs were placed in contact with a doxycycline solution and allowed to incubate for 16 hrs to generate biosensor response before being imaged. This treatment process will be referred to as bioPAD development in this paper. The 12-lane bioPADs were developed in 600 mL beakers to which 50 mL of synthetic complete (SC) media had been added, with or without doxycycline (Fig. 3a). BioPADs were positioned so that the media reached the printed blue line on the bioPADs. The bioPADs were attached to the side of the beaker with a large paperclip. These beakers were sealed with parafilm and developed for 16 hrs at 30°C unless noted otherwise. The bioPADs were developed in non-sterile conditions to reflect real-world conditions of the endpoint users. The 4-circle bioPAD test strips were developed in capped scintillation vials with 2 mL of SC media and measured after a 16 hr incubation at 30°C unless described otherwise (Fig. 2c). When indicated, the media for the 4 spot bioPADs were supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin in order to control off-target microbe growth.

Microplate Yeast Assays

A single colony of yeast was selected from an agar plate to inoculate an overnight culture in selective media. All liquid cultures were grown at 30°C and with shaking (250 rpm). From this overnight culture, a new outgrowth culture was started at 0.1 OD. Once yeast had reached mid-log phase (0.3-0.8), they were diluted to an OD of 0.05 in selective media, and 198 µL aliquots were added to the 96-well plate after 2µL of doxycycline stock. Every condition was tested in triplicate in each plate. The plates were then incubated at 30°C with shaking on a Synergy H1 plate reader (BioTek). Fluorescence and OD600 were measured every hour for 25 hrs. Fluorescence measurements were taken with the diffraction grating set to 580/610 nm and

gain set to 100. Unless specified otherwise, fluorescence was normalized by subtracting the fluorescent signal of media blanks from the fluorescence of samples and then dividing by blanked OD_{600}: [(Fluorescence$_{test}$ – Fluorescence$_{media}$)/(OD_{test} – OD_{media})].

BioPAD Imaging

BioPADs illuminated with an X-Cite 120PCQ lamp and were imaged on an Olympus SZX16 dissecting microscope set to 0.7x magnification with the Olympus RFP1 filter. Images captured by an Olympus DP72 color camera were downloaded from the dissecting microscope and analyzed in ImageJ. A region of interest (ROI) was drawn within yeast spots and the average intensity of the pixels inside this region was measured in the red channel. This measured signal was background subtracted by subtracting the average intensity of pixels from a ROI drawn directly beneath the fluorescent yeast spot. A similar process was also used to measure the mean intensity of yeast spots on the bioPAD reader. Signal from bioPAD strips was background subtracted by subtracting the mean pixel intensity within the parent yeast strain spot (for details of this reader, see below and Supplementary Information).

BioPAD Reader

The fluorescent lightbox used to image fluorescent bioPAD strips contains a Raspberry Pi processor and camera within a light-weight plywood housing (Fig. 5a). Samples are illuminated by rows of LEDs, and excitation light is filtered out before reaching the camera with layers of gel filter sheets. BioPADs are read by placing the bioPAD into the fluorescent reader and clicking the “capture image” icon on the PAD reader program. A sliding door was incorporated into the box design in order to decrease background signal from ambient light. A full description of the construction and assembly of the fluorescent lightbox and guidelines for installing its free image analysis software can be found in the Supplemental Information. The specifications provided have been optimized for measuring signal from red fluorescent protein, but the lightbox could easily be adapted for imaging other fluorophores.

Results and Discussion

Given that the original bioPAD system was already successful at detecting doxycycline levels that could be found in pharmaceutical dosage forms, we focused on expanding bioPAD technology in order to monitor physiological levels of doxycycline in technology-limited settings. To meet this goal, we needed to improve the sensitivity of the biosensing yeast 30-fold and make the bioPAD device more compatible with technology-limited settings. As shown below, by replacing the beta-galactosidase reporter with a fluorescent one, we were able to eliminate the need for additional reagents and cut the protocol by 4 hrs. We increased the sensitivity of the biosensors further by using a “sensitivity enhanced” version of the receptor. BioPAD strips respond well to doxycycline in up to 50% human urine or bovine serum. These results indicate that bioPADs can detect doxycycline in physiological fluids.

Fluorescent Reporter is Compatible with bioPAD Technology

In order to simplify the use of the bioPAD (Fig. 3a), a fluorescent reporter was integrated into our yeast biosensor. Initially, a green fluorescent protein (GFP) was used to replace the lacZ

reporter gene in our biosensor because GFP is well-characterized as a reporter in cell biology and it has been shown that yeast can produce amounts of GFP that are measurable with a fluorescent plate reader in as little as 6 hrs.[20] However, we found that the GFP fluorescence was subject to interference by the fluorescent emission of doxycycline as well as background fluorescence from the filter paper used in the biosensor. Because of these observations, we replaced the GFP reporter with a yeast-optimized red fluorescent protein,[25] which greatly decreased background fluorescence in our device. A gene encoding the red fluorescent protein, yEmRFP, was incorporated into our biosensing plasmid, pCM176, using standard biochemical techniques.[11,25] The resulting yeast biosensor was incorporated onto a paper substrate using our previously published methods.[11] We then used a dissecting microscope to monitor over time the fluorescent signal of bioPADs treated with 100 µg/mL doxycycline (Fig. 3b).

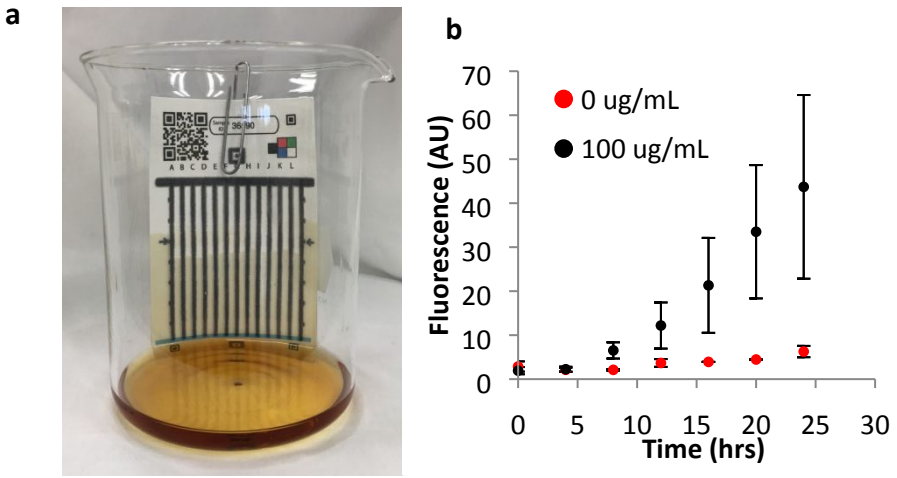


Figure 3. Fluorescent bioPAD
a) A developing bioPAD. Yeast is spotted in a line indicated by the arrows. The bioPAD is placed in a reservoir of yeast media that has been treated with doxycycline. b) Timescale. BioPADs were developed in media with 100ug/mL or without doxycycline. Error bars = S.D. of at least 6 spots of yeast. This experiment is a composite of two replicates.

In initial experiments, we found that the level of fluorescence in treated bioPADs rises above untreated controls after 6 hrs of incubation and continues to increase until at least 24 hrs; a 16 hr incubation provides strong signal when visualized by eye (Fig. 3b) and represents a reasonable time frame for test development, as it is the length of a typical overnight timespan. For these reasons, a 16 hr incubation was chosen as the standard development time for fluorescent bioPADs. It should be noted that the development protocol for the fluorescent bioPAD is shorter than the previous bioPAD protocol by 4 hrs. Unfortunately, it is unlikely that we will be able to significantly decrease this incubation time using the current model of our assay because it relies on the yeast to produce reporter protein and this takes time. One way to speed up signal accumulation is to include an efficient enzymatic reporter, which would allow many reporter molecules to be produced per protein made in response to analyte. However, enzymatic reporters that are currently available require the addition of expensive, unstable, and/or toxic developing reagents (Luciferase, Horseradish Peroxidase, and Beta-Galactosidase). Enzyme-based colorimetric reporters that do not require reagents (e.g. violacein and other

chromoproteins) usually take at least 24 hrs for signal to develop[13,14] An overnight incubation, while not ideal, is reasonable given that this assay has a faster turnaround time than many drug testing services in the U.S. and can be performed on site. We have shown that the development protocol for the fluorescent bioPAD is more user-friendly than its previous version as it does not require additional reagents or manipulations and can be read directly after incubation.

After establishing that the fluorescent reporter improved the usability of bioPAD technology, the sensitivity of the fluorescent bioPAD was determined. BioPADs were developed for 16 hrs in various concentrations of doxycycline, and fluorescent signal was measured on a dissecting microscope (Fig. 4a). Signal was statistically different from untreated bioPADs at 3 µg/mL and above (Student's t test; $p < 0.05$). These results indicate that the fluorescent bioPAD device is 10-fold more sensitive than the original version (Fig. 4a), for which the lowest detectable concentration of doxycycline was 30 µg/mL.[11] However, this fluorescent bioPAD was still not sensitive enough to detect physiological levels of doxycycline in serum, which are on the order of 1 µg/mL doxycycline.[21] In order to reach the more stringent detection goals for physiological fluids, we sought to increase the sensitivity of the doxycycline-sensing machinery in the yeast biosensor.

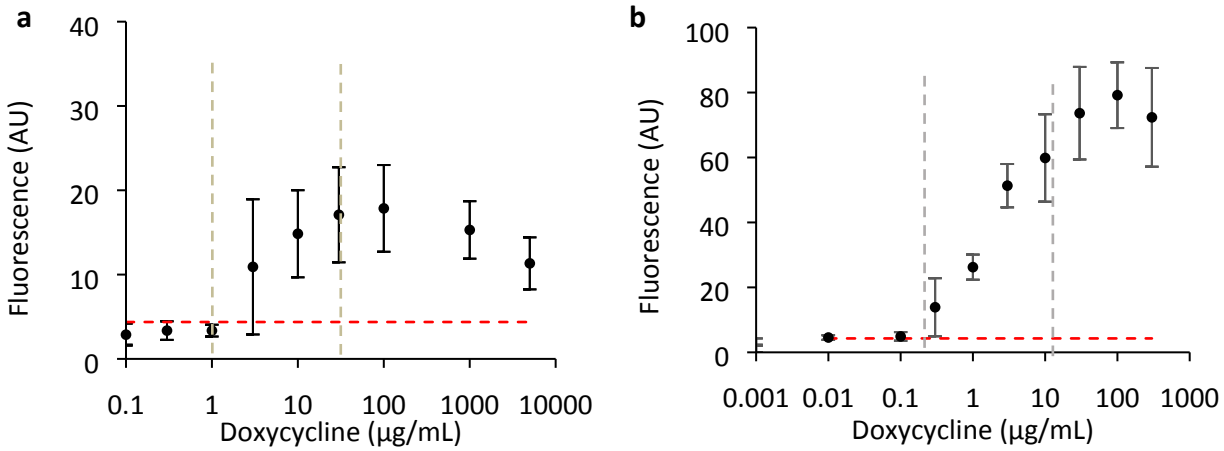


Figure 4. Fluorescent bioPAD dose response

The red line indicates the level of signal in untreated bioPADs + 1 S.D. Vertical dashed lines indicate detection goals for physiological concentrations of doxycycline in serum and urine, 1 and 30 µg/mL, respectively. Error bars = S.D. of 18 spots. N = 3 sets of 6 spots. a) bioPADs made from fluorescent biosensing yeast with repressor and original rtTA b) Sensitivity enhanced bioPADs made with biosensor psy580a and plasmid pRM02. This biosensor contains plasmid pRM02 in the yeast strain psy580a, which does not contain the tetracycline repressor. PRM02 has the sensitivity enhanced, low-noise (SELN) variant of rtTA described in the text.

The bioPAD can Detect Physiological Levels of Doxycycline with Sensitivity-Enhanced rtTA

To understand how one might improve the sensitivity of the biosensor, it is necessary to understand in more detail how it works. The bioPAD sensing machinery utilizes the reverse tetracycline transactivator (rtTA) to "turn on" transcription of reporter genes in the yeast biosensors in the presence of doxycycline (see Methods — yeast molecular biology for more information).[26] The system used for the bioPAD described above includes a repressor protein that suppresses expression of reporter genes in the absence of doxycycline (in addition to the

rtTA). The stated reason for including this repressor is to minimize background expression of reporter genes,[23] but one could also imagine that it might reduce the intensity of the final signal.

To improve the performance of the biosensor, we took two approaches. First, we replaced the rtTA in the fluorescent biosensor with a sensitivity enhanced (SE), low noise (LN) variant that was described in Roney et al. 2016, which will be referred to as rtTA-SELN in this paper.[20] Second, we moved the rtTA-SELN sensing plasmid (pRM02) into another yeast strain (psy580a) that both lacks the repressor protein mentioned above and is ADE+. The ADE+ background was chosen to minimize background signal from the ade- mutation in the CML282 yeast, which naturally produces red-colored products over time, turning yeast pink. In preliminary experiments we observed that there was an increase in signal in psy580a biosensors compared to the original CML282 background regardless of the rtTA used (SFig. 1). This is likely due to the loss of the repressor protein that was present in the original sensor yeast. The biosensors with rtTA-SELN also had higher signal than those with the original rtTA (SFig. 1). For these reasons, we moved forward with the rtTA-SELN in the psy580a strain.

The sensitivity enhanced psy580a-pRM02 biosensors were incorporated into the 12-lane bioPADs and developed with doxycycline. The signal of this sensitivity enhanced bioPAD is higher than the original fluorescent bioPAD and has a linear range that extends into the lower physiological levels of doxycycline (Fig. 4b). Signal from 0.3 μg/mL doxycycline and above is significantly different from untreated samples (Student's t, $p < 0.05$). These bioPADs reach half of their max fluorescence at a doxycycline concentration ~1 μg/mL, which is the level of doxycycline that can be found in the serum of individuals taking 100 mg dosage forms of doxycycline, the recommended dose for malaria prophylaxis.[3] Together, these findings show that the biosensing yeast are well-suited to detect physiological concentrations of doxycycline. Due to the success of the improvements made to the biosensing yeast, including increased sensitivity and improved signal output, our next goals were to adapt the bioPAD technology to be read in the field and to test it with physiological matrices.

The bioPAD can be read with a low-tech device

Because dissecting microscopes like the one used to collect bioPAD fluorescent data are unlikely to be available in the field, we developed an inexpensive device to read the fluorescent bioPAD signal. The basic components necessary for fluorescence detection, a light source and appropriate filter, were incorporated into a lightweight portable platform capable of on-board quantitation (Fig. 5a). In this fluorescence lightbox, a Raspberry Pi processor drives a camera, touchscreen, and LED strips all housed within a simple plywood box. Specifications for construction of the lightbox and setting up the Raspberry Pi are available in the Supplemental Information; the software code is publicly available on GitHub (https://github.com/PaperAnalyticalDeviceND/Litebox). The lightbox currently costs $130 to produce, can be used long-term without consumables, and packs flat for assembly on site, which is ideal for use in LMICs.

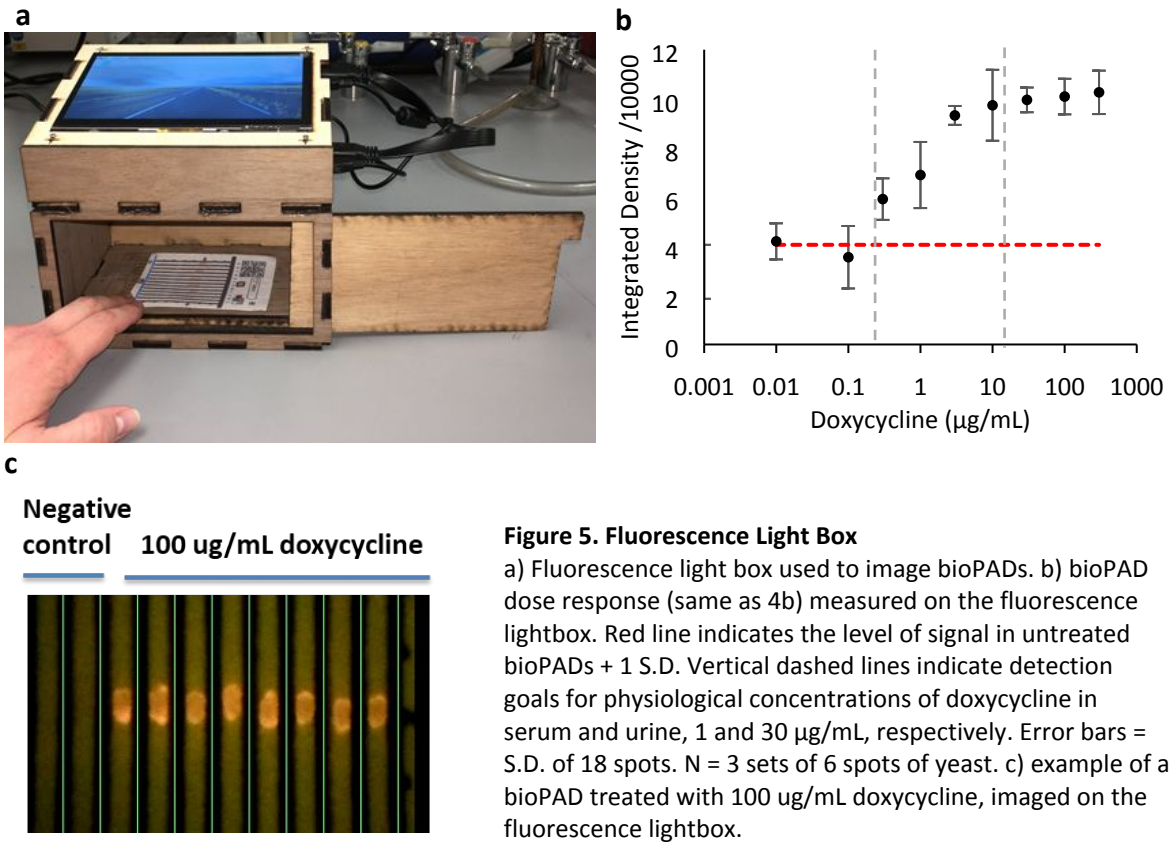


Figure 5. Fluorescence Light Box
a) Fluorescence light box used to image bioPADs. b) bioPAD dose response (same as 4b) measured on the fluorescence lightbox. Red line indicates the level of signal in untreated bioPADs + 1 S.D. Vertical dashed lines indicate detection goals for physiological concentrations of doxycycline in serum and urine, 1 and 30 µg/mL, respectively. Error bars = S.D. of 18 spots. N = 3 sets of 6 spots of yeast. c) example of a bioPAD treated with 100 ug/mL doxycycline, imaged on the fluorescence lightbox.

To use this device, developed bioPADs are placed directly into the lightbox and imaged by tapping an icon on the touchscreen. Currently, the images are downloaded onto a personal computer and analyzed in ImageJ. The dose response curve from images captured on the fluorescence lightbox is similar to the dose response curve measured on the dissecting microscope (Fig. 4b and 5b). The bioPAD reader was able to capture the signal difference in bioPADs treated with 0.3 µg/mL doxycycline or greater even though signal:noise is lower in images taken on the fluorescence lightbox (Fig. 5b). This decrease in signal:noise is likely caused by excitation light that makes it to the camera in the lightbox due to its less powerful filter.

However, the inexpensive filter sheet was kept in the lightbox design because the signal:noise is sufficient for the intended application.

The bioPAD can be Developed in Physiological Fluids

One of the desired (yet previously untested) applications for the bioPAD device is to measure physiological levels of pharmaceuticals in biological fluids. It would be ideal for a field-friendly device to be able to measure physiological analytes directly in common biofluids, without extensive sample preparation or concentration. Doxycycline has been measured in human serum and urine using HPLC,[21] but this method of detection is not accessible to many areas of the world. Much work has been done to develop biosensors for use in point-of-care (POC) diagnostics and other physiological applications including some previous work with whole cell biosensors in physiological fluids.[27,28,29] However, we are unaware of inexpensive devices that can be used to detect doxycycline in physiological fluids in a low resource setting.

In order to determine the utility of bioPADs in physiological fluids, bioPADs were cut into strips and treated with synthetic urine or raw bovine serum mixed with media. We used serum in these experiments instead of whole blood in order to avoid fluorescent interference from blood cells, which contain hemoglobin. It is unlikely for the color of urine to influence the result of the fluorescence detection because the autofluorescence of urine is outside of the emission spectrum of yEmRFP.[30] Preliminary results from these tests showed that bioPADs produced fluorescent signal in response to doxycycline when developed in up to 50% synthetic urine or raw bovine serum (SFig. 5). This observation was surprising because our preliminary tests with biosensors performed with the microplate assay showed that biosensors were negatively impacted by treatment with as little as 1% raw bovine serum (SFig. 4). The improved performance of the bioPADs relative to the microplate assay with bovine serum might be due to latent immune response in this sample type. We speculate that liquid-suspended biosensors suffered from some immune response in the microplate assays and that the hydrogel-encased yeast biosensors are protected from this response.

Because our tests with the bioPAD strips looked promising, we worked to engineer a test strip that could be used in the field, requires less sample, and is made of minimal materials. These bioPAD strips contain four spots of yeast: three spots of psy580a-pRM02 and one of the parent strain, psy580a, which acts as a negative control (Fig. 2b). Early tests showed that biosensor signal in response to doxycycline depended on the ratio of filter paper surface area to the volume of the development solution (SFig. 5). By reducing the surface area of the paper substrate, the test can be run with less sample volume: 2 mL total fluid (in our tests, 1 mL media + 1 mL test matrix was used) instead of 50 mL like the earlier 12-lane version. By streamlining the design, we have reduced the cost of materials per bioPAD from $0.41 to $0.13 per device.

In order to assess the ability of the bioPAD test strips to detect doxycycline residues from prophylactic doses of this antimalarial, we developed them in media supplemented with pooled human urine or raw bovine serum and then spiked with doxycycline to mimic levels expected to be found in human serum and urine, 1 and 30 μg/mL respectively. The bioPAD signal from the 30 μg/mL treatment level was consistently different from untreated bioPADs, but not at the 1 μg/mL level, likely due to the variation seen in these samples (Supplemental Table 1).

To address the variability problem, we took two approaches. First, we tested heat treatment of bovine serum to inactivate the innate immune response. However, this had no detectable effect on signal of bioPAD strips in response to doxycycline (Fig. 6). Second, we controlled microbe growth by the addition of non-tetracycline antibiotics (ampicillin and kanamycin) to the development solutions. We observed that these antibiotics do not negatively impact biosensor performance, and in combination with heat treatment they help to decrease variation between replicate tests (Fig. 6). With the combination of heat treatment of the serum and antibiotics to knock down microbial growth, bioPADs dosed with doxycycline at levels down to those found in serum (1 μg/mL) had fluorescent signal that was statistically significant from samples not treated with doxycycline (Supplemental Table 1; Fig. 6). BioPAD test strips developed in 50% pooled human urine have similar responses to those developed in media alone (Fig. 6). This finding suggests that bioPAD strips will be able to monitor doxycycline excreted through urine.

It should be noted that this experiment provides proof of principle for the use of bioPAD test strips in physiological matrices, but it does not take into account the dilution effect on the concentration of doxycycline that would be found in these matrices. BioPADs are not inhibited by 50% final concentrations of human urine or raw bovine serum, indicating that the bioPAD may perform similarly in pure physiological matrices. Additionally, it is possible that whole blood could be used in this assay, which would remove an additional sample preparation step. Other groups have published methods for removing red blood cells from whole blood samples using paper for analytical purposes.[31] This technology could be integrated into the bioPAD design.

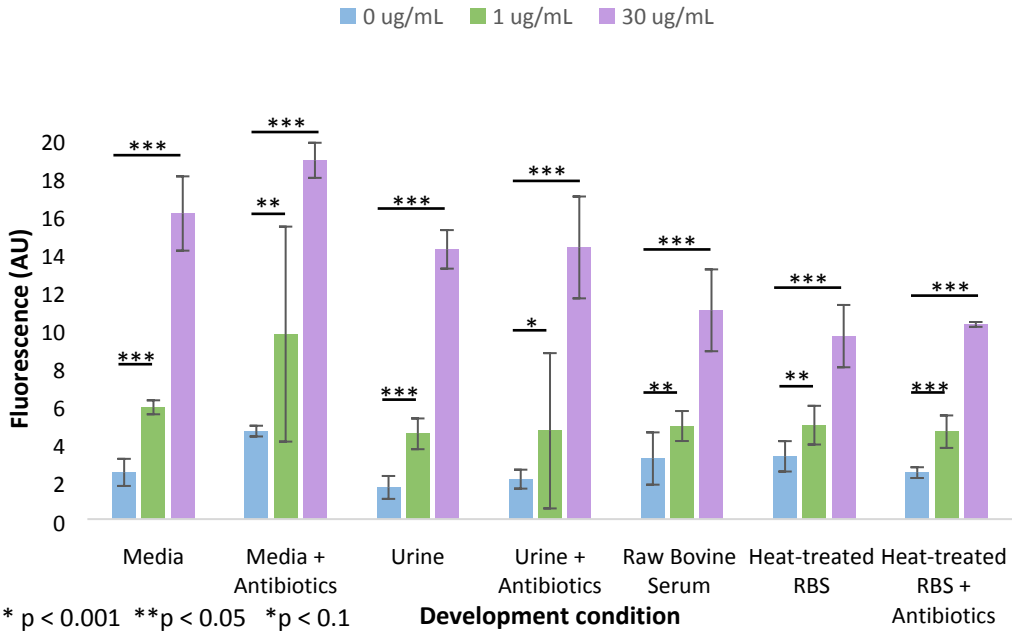


Figure 6. Activity of bioPAD test strips in physiological matrices

4-circle bioPAD test strips were developed in 2 mL of solution (1 mL media + 1 mL matrix) and then treated with doxycycline to reach a final concentration relevant for analysis of doxycycline in serum or urine, 1 or 30 µg/mL doxycycline respectively. Additional non-doxycycline antibiotics were added to some samples to control unwanted microbe growth. After incubation, bioPAD test strips were imaged on the fluorescent light box. Note that the imaging conditions used for this experiment were optimized for bioPAD test strips and differ from the settings used in Fig. 5. N = 3 replicates, with 3 spots of biosensor yeast. Error bars = S.D. of 9 spots of yeast. Signal in treated bioPADs were compared to their counterparts that were developed in media only. Samples treated with 30 µg/mL doxycycline in media alone had higher signal than those treated with 30 µg/mL doxycycline and developed in any type of bovine serum ($p < 0.05$).

Conclusions

In this work, we have described a fluorescence-based whole cell yeast biosensor for use in technology-limited settings. Fluorescent reporters are widely available and easily manipulated in a lab setting and we have now shown that they can be adapted for use in technology-limited settings by incorporating a fluorescent, doxycycline yeast-biosensor onto a portable paper substrate and showing that this biosensor can be imaged by a portable, inexpensive device. The bioPAD is also sensitive enough to detect doxycycline in physiological fluids and is compatible with complex physiological matrices including bovine serum and human urine. We are optimistic that fluorescent whole cell yeast biosensors can be utilized for other medical diagnostics and are encouraged by the findings of others that show whole cell yeast biosensors can be used to detect pathogenic fungi in physiological fluids.[29] We are currently investigating the utility of a bioPAD to detect the hormone estrogen, which is a desirable target for both its clinical relevance and because hormone pharmaceuticals have been found to be substandard in LMICs.[1,32] Others have successfully made use of the human estrogen receptor in yeast-based biosensors to detect estrogenic activity in samples (known as yeast estrogen screens).[10,33-35] We plan to use a similar strategy to make the bioPAD sensitive to estrogen. The bioPAD could be expanded to detect other analytes for which there are available inducible promoters. Possibilities include detection systems for metal contamination like copper and arsenic as well as phosphate.[36] We expect that this work could open up the biosensor community to incorporate user-friendly fluorescent reporters into whole cell biosensors for use in technology-limited settings.

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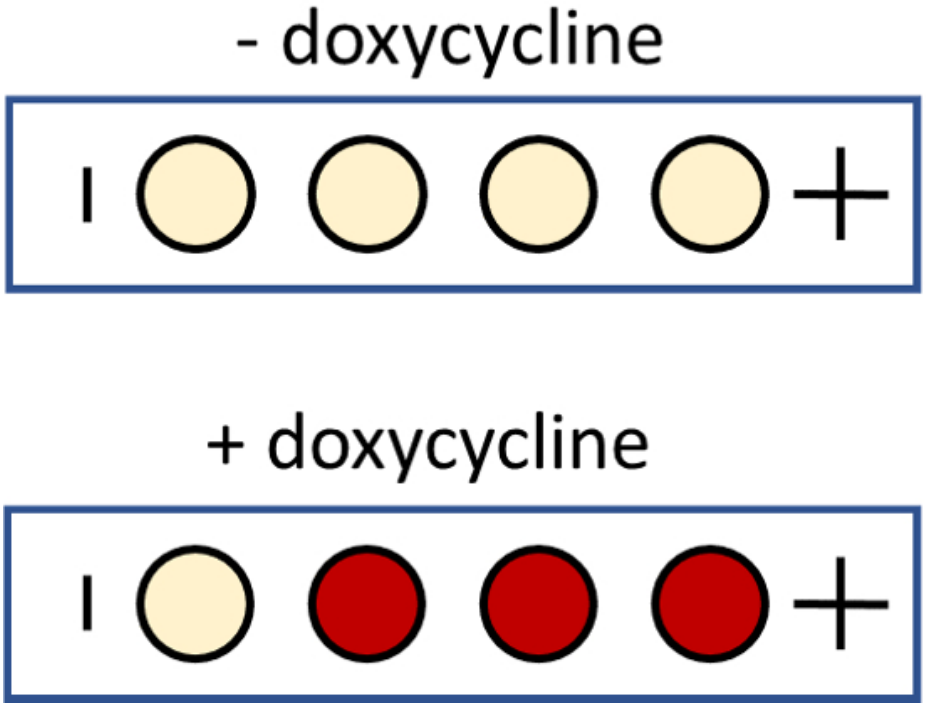

This work describes the first fluorescent whole cell yeast biosensor adapted for use in technology limited settings to detect doxycycline, which could pave the way for use of this technology in the biosensor community.

101x75mm (149 x 149 DPI)