


Toward Microbioreactor Arrays: A Slow-Responding Oxygen Sensor for Monitoring of Microbial Cultures in Standard 96-Well Plates

Journal of Laboratory Automation
2015, Vol. 20(4) 438–446
© 2015 Society for Laboratory
Automation and Screening
DOI: 10.1177/2211068215573924
jala.sagepub.com


Florian Glauche¹, Gernot T. John², Sarina Arain²,
Andreas Knepper¹, Antje Neubauer³, Detlef Goelling⁴,
Christine Lang⁴, Norman Violet⁵, Rudibert King⁵, and
Peter Neubauer¹

Abstract

In this study, a slow-responding chemo-optical sensor for dissolved oxygen (DO) integrated into a 96-well plate was developed. The slow response time ensures that the measured oxygen value does not change much during plate transport to the microplate reader. The sensor therefore permits at-line DO measurement of microbial cultures. Moreover, it eliminates the necessity of individual optical measurement systems for each culture plate, as many plates can be measured successively. Combined with the 96-well format, this increases the experimental throughput enormously. The novel sensor plate (Slow OxoPlate) consists of fluorophores suspended in a polymer matrix that were placed into u-bottom 96-well plates. Response time was measured using sodium sulfite, and a τ_{90} value of 9.7 min was recorded. For application, DO values were then measured in *Escherichia coli* and *Saccharomyces cerevisiae* cultures grown under fed-batch-like conditions. Depending on the DO sensor's response time, different information on the oxygenation state of the culture plate was obtained: a fast sensor variant detects disturbance through sampling, whereas the slow sensor indicates oxygen limitation during incubation. A combination of the commercially available OxoPlate and the Slow OxoPlate enables operators of screening facilities to validate their cultivation procedures with regard to oxygen availability.

Keywords

oxygen sensor, response time, high throughput, microbial cultures

Introduction

Dissolved oxygen (DO) is a crucial parameter for aerobic bioprocesses because the solubility of oxygen is low in aqueous solutions containing nutrients and salts, whereas exponentially growing cells have a high oxygen demand. The volumetric oxygen consumption may exceed the oxygen transfer rate, especially at high cell densities.¹ Oxygen limitation has severe effects on growth and product formation^{2,3} and is therefore monitored during production processes in bioreactors.¹ In contrast to that, the screening for microbial production strains is mostly carried out under uncontrolled conditions without any process monitoring.⁴

To overcome this lack of information, several commercial systems for online DO monitoring in shake flasks,⁵ mini-bioreactors,⁶ and microplates⁷ were developed. They all share the disadvantage of a limited number of parallel cultivations and are therefore not suitable if hundreds or thousands of putative production strains need to be screened.

Ninety-six-well plates are a common format for high-throughput screening programs. They have been characterized with regard to oxygen transfer⁸ and mixing time and have been equipped with sensors for DO,^{9,10} and pH.¹¹ In a common laboratory automation setup, numerous 96-well

¹Chair of Bioprocess Engineering, Technische Universität Berlin, Berlin, Germany

²PreSens Precision Sensing GmbH, Regensburg, Germany

³BioSilta Europe GmbH, Berlin, Germany

⁴Organobalance GmbH, Berlin, Germany

⁵Chair of Measurement and Control, Technische Universität Berlin, Berlin, Germany

Received Nov 13, 2014.

Corresponding Author:

Peter Neubauer, Chair of Bioprocess Engineering, Institute of Biotechnology, Technische Universität Berlin, Ackerstraße 76, ACK24, 13355 Berlin, Germany.

Email: peter.neubauer@tu-berlin.de

plates are incubated in a so-called plate hotel. To read out signals or take samples, the plates have to be removed from the hotel and processed. During that time, steady decrease of DO due to the cells' respiration occurs. To estimate the state of a culture during the cultivation in such a setup, the sensor needs to have a "memory" function (i.e., it should show a slow response).

Therefore, it was the aim of this study to develop an oxygen sensor for at-line measurements in 96-well plates. Because of the slow response, the oxygen value measured in the plate reader does not change much compared with the one on the shaker. With the sensor, discrimination between aerobic and anaerobic conditions should be possible. The functionality of the prototype sensor then needs to be proven by response time measurements using sodium sulfite and microbial culture broth. Then the sensor is applied to monitor growth conditions of microliter-scale fed-batch cultures.

Material and Methods

Oxygen Sensors

The DO in bacterial and yeast cultures was measured with optical oxygen sensors embedded in 96-well microplates (Microlon 600; Greiner, Frickenhausen, Germany). In addition to the sensors with slow response, commercially available microplates with an oxygen sensor with fast response (OxoPlate; PreSens, Regensburg, Germany; $t_{90} < 30$ s) were used. The response time t_{90} is the time at which oxygen has decreased to 90% of its total decrease. The sensors consist of a fluorescent, oxygen-sensitive dye and an inert reference dye. Both dyes are integrated in a polymer. Furthermore, a microsensors (NTH-PSt1-L5-TS-NS40/0.8-NOP, PreSens; $t_{90} < 2$ s) was tested to obtain the actual oxygen content within the sample for comparison with the ones detected with the sensor plates with slower response.

Manufacturing of the Sensor Plates with Slow Response

Different approaches were tested to obtain a sensor with a reproducible response time of approximately 10 min. As the original oxygen sensor of the commercial OxoPlate responds within 30 s, we tried to enlarge the response time by using different polymer layers and increasing the layer thickness. Sensor foils with different layers and thicknesses were produced using a knife coating device (ZAA 2300; Zehntner GmbH, Sissach, Switzerland) to spread the liquid sensor material with a constant thickness onto a supporting foil. The dyes were dissolved in a hydrophobic polymer. The two-layer sensor foils (SL-F2-AB, PreSens; **Fig. 1A**) consisted of a polymer layer with thicknesses between 50 and 800 μm and a polymer layer containing the fluorescent dyes

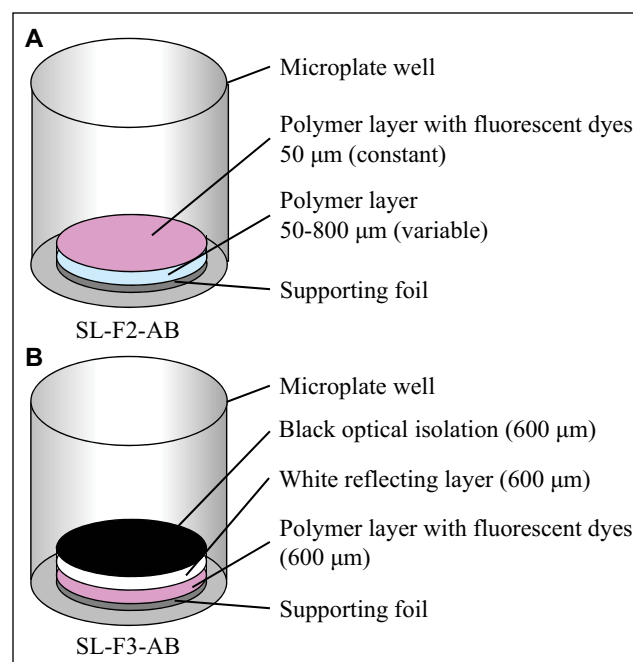


Figure 1. Schematic drawing of prototype sensor setups. **(A)** Two-layer sensor foil SL-F2-AB with variable polymer layer (50–800 μm) and constant fluorescent polymer layer (50 μm). **(B)** Three-layer sensor foil SL-F3-AB with fluorescent polymer layer, white reflecting layer, and black isolation (600 μm each). Five-millimeter spots were bonded into flat-bottom 96-well microplates.

with a constant thickness of 50 μm . The three-layer sensor foils (SL-F3-AB, PreSens; **Fig. 1B**) consisted of a layer with fluorescent dyes; a white, reflecting layer; and a black layer as optical isolation. Each of the three layers had a thickness of 600 μm . Five-millimeter spots were cut out of these sensor foils and bonded with silicone glue (No. 692-542; RS Components, Mörfelden-Walldorf, Germany) into the wells of a 96-well, flat-bottom microplate (Greiner). The supporting foil faced the bottom of the microplate.

In a third approach, liquid sensor materials with volumes between 2 and 130 μL were pipetted into round-bottom 96-well microplates (Greiner). The sensors were produced using a hydrophilic (HG-T-AB, PreSens) and a hydrophobic polymer (SL-T-AB, PreSens), respectively. The sensors were dried under atmospheric conditions for at least 24 h. After drying, the polymer layer adheres to the bottom of the microplate. For volumes of more than 50 μL , successive addition of smaller volumes was also tested, with a minimum drying time of 4 h before adding the next volume. Four wells per microplate and volume were used. For the best sensor (Slow OxoPlate), plates with 96 sensors were produced, sealed in an opaque bag, and beta irradiated. **Figure 2** shows the setup of the sensors as well as pictures of the Slow OxoPlate.

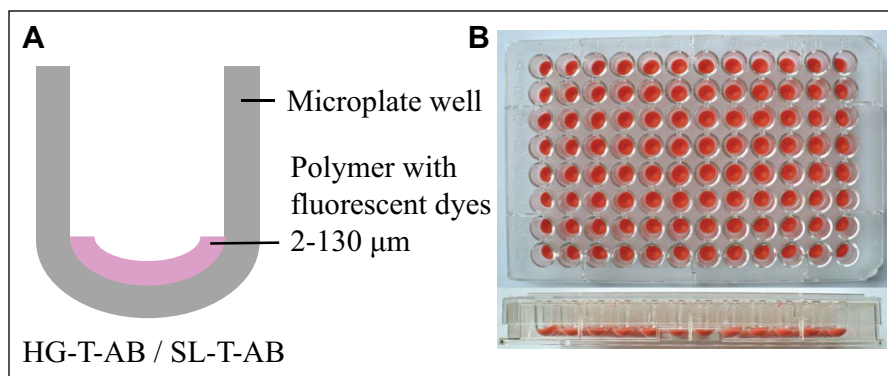


Figure 2. Third sensor setup: liquid sensor materials pipetted into round-bottom microplates. **(A)** Schematic drawing of a u-bottom well containing air-dried polymer with fluorescent dyes. **(B)** Picture of the final product (top and side view).

Sensor Characterization

For determination of the precision and resolution of the Slow OxoPlate, the sensors were calibrated using air-saturated water (cal100) and oxygen-free water (cal0). For cal100, about 20 mL of deionized water was put into a 50 mL glass vessel, closed with a screw cap, and shaken vigorously for approximately 2 min. The glass was then opened and moved gently to avoid oversaturation. For cal0, 0.2 g of sodium sulfite was dissolved in 20 mL deionized water in a closed glass vessel and moved slightly to dissolve the sulfite. Twenty-four wells of the Slow OxoPlate were filled with 200 μL of cal100 and 280 μL of cal0 for the round-bottom plates and 380 μL of cal0 for the flat-bottom plates, respectively. The wells with cal0 were closed with strips of adhesive foil (EasySeal, Greiner) to minimize oxygen ingress. The plate was incubated in a microplate reader Fluoroskan Ascent (Thermo Fisher Scientific, Dreieich, Germany) for 1 h at 37 $^{\circ}\text{C}$. The fluorescence signals were measured at 37 $^{\circ}\text{C}$ for 20 min with a measurement interval of 2 min, using the filter combinations 544/650 nm for the indicator dye and 544/590 nm for the reference dye. At these wavelengths, most fluorescent media ingredients do not display fluorescence. The calibration constants k_{100} and k_0 were calculated as the average values of the respective intensity ratios of the indicator and reference dye.

Differing from this procedure, for DO calculation of the response time measurements, k_{100} was taken from the start value and k_0 from the end value of the respective measurement.

Calculation of DO from Fluorescence Data

DO (in % air saturation [% a.s.]) was calculated from the ratios I_r of the fluorescence intensities of the indicator and reference dye and the calibration values k_{100} and k_0 using the Stern-Volmer equation:

$$DO = 100 \cdot \left(\frac{k_0}{I_r} - 1 \right) / \left(\frac{k_0}{k_{100}} - 1 \right) \quad (1)$$

Response Time Measurement

Eighty microliters of cal100 was put into the four wells containing the sensors, covering all of the sensor material. The fluorescence intensities (544/650 nm and 544/590 nm) were measured with the Fluoroskan Ascent microplate reader at room temperature with an interval of 1 min for 2 h until the signals were constant. Then a new measurement was started, measuring cal100 for 5 min with an interval of 1 min. After that, the plate was ejected and 200 μL (u-bottom plates) or 300 μL (flat-bottom plates) of cal0 containing 5×10^{-4} M of cobalt nitrate as a catalyst was added rapidly with an eight-channel pipette and carefully mixed once. The wells were covered with strips of adhesive foil (EasySeal, Greiner) and the measurement continued with an interval of 0.25 min for at least 2.5 h until the signals were constant. After converting the signals into oxygen values, the response time t_{90} was determined as the time in which the oxygen decreased to 10% of its original value of about 100 % air saturation.

Precision and Resolution

For the Slow OxoPlate, precision and resolution at 37 $^{\circ}\text{C}$ were determined. Precision was calculated as the maximum standard deviation of four sensors measured at the same time. Resolution was calculated as the maximum standard deviation of five succeeding measurement points of a kinetic of one sensor. As target values for these specifications, the ones of the standard OxoPlate (PreSens) were applied. The precision of this plate is given as $\pm 5\%$ a.s. for cal100 and $\pm 2\%$ a.s. for cal0, the resolution as $\pm 2\%$ a.s. for cal100 and 0.5% a.s. for cal0.

Strains

For bacterial cultivation experiments, *Escherichia coli* W3110 and BL21 were used. The BL21 strain was transformed with the plasmid pDgPNP, which has been constructed for heterologous expression of a purine nucleoside phosphorylase from *Deinococcus geothermalis*.¹² For yeast

cultivations, *Saccharomyces cerevisiae* AH22 harboring the plasmid pPG6 for secretion of an endopolygalacturonase from *Aspergillus niger*¹³ was used. Both strains were stored at $-80\text{ }^{\circ}\text{C}$ in media containing 20% glycerol.

Media and Cultivation Conditions

E. coli cultures were grown in EnPresso B or a prototype growth system based on EnBase technology (EnPresso B Defined), whereas baker's yeast cells were grown in EnPresso Y Defined medium (all from BioSilta Ltd., Cambridge, UK). Unless otherwise stated, cultures with EnPresso growth systems were prepared according to the manufacturer's instructions. All cultivation experiments were performed at $30\text{ }^{\circ}\text{C}$.

Microbial cultures were carried out in different systems and scales. Shake flask cultivations were performed with 25 mL EnPresso B using a 125 mL Ultra Yield Flask covered with AirOtop Enhanced Seal (Thomson Instrument Company, Oceanside, CA). For controlled glucose release, 1.5 U L^{-1} of biocatalyst (reagent A) was added, and the flasks were shaken in a Kuhner LT-X incubator (Adolf Kühner AG, Basel, Switzerland) at 200 rpm, 25 mm amplitude. Microplate cultures were carried out in two different ways. For the first experiments with optimal aeration, the sensor plates were covered with "System Duetz" sandwich covers (EnzyScreen B.V., Haarlem, the Netherlands) and shaken at 300 rpm, 50 mm amplitude.

For automated reading and sampling, experiments were performed on a robotic platform, which was previously described.¹⁴ Briefly, it consists of several instruments attached to a liquid-handling robot (Hamilton Microlab Star; Hamilton Bonaduz AG, Bonaduz, Switzerland). The system can incubate up to eight 96-well plates and analyze samples with a microplate reader and a flow cytometer. The culture plates were sealed with XPierce foil (Excel Scientific Inc., Victorville, CA) and incubated on an orbital shaker (Hamilton FAME incubator). For measurements with the microsensor, the on-deck shaker Teleshake 95 (Inheco GmbH, Munich, Germany) was used.

Prototype Sensor Tests with Bacterial Cultures

Sensor tests with microbial cultures were performed using *E. coli* W3110 cells grown in shake flasks overnight to an optical density at 600 nm (OD_{600}) of 6 to 8. From the culture, different volumes (100, 150, 200 μL) were then transferred in triplicate to the sensor plates. The plates were covered with sandwich covers and shaken for 1 h, in order to reach equilibrium conditions. Then, the plates were manually placed into a Biotek Synergy MX plate reader (Biotek Instruments Inc., Winooski, VT). The delay until measurement was less than 1 min. For both sensor plate types (Slow

OxoPlate, OxoPlate), the same reader settings were used. The excitation wavelength for both fluorophores was 540 nm. The emitted fluorescence light of the indicator was measured at 650 nm, whereas the reference fluorophore emission was measured at 590 nm. A time-resolved measurement from 0 to 500 μs was performed with a sensitivity setting of 50. Measurements were carried out every minute for 8 min. DO values were calculated from the raw data using Gen5 v1.09 (BioTek Instruments).

For comparison of the oxygen values generated with the microplates with an external reference instrument, a DO microsensor (PreSens) was applied. The Slow OxoPlate was shaken at different speeds on the Teleshake 95, which is located on the robotic platform, for 30 min. The microsensor was calibrated according to the manufacturer's instructions. A gripper transported the plate from the shaker to a position at which the microsensor DO measurement was carried out. The transport time was 12 s. Because of the rapid signal decrease, the microsensor was submerged in only one well for 5 s. Afterward, the sensor plate was transported to the microplate reader.

Cultivation on the Robotic Platform

E. coli BL21 pDgPNP glycerol stock (maintained at $-80\text{ }^{\circ}\text{C}$) was plated on LB agar containing $100\text{ }\mu\text{g mL}^{-1}$ ampicillin and incubated overnight at $30\text{ }^{\circ}\text{C}$. The grown cell material was then washed from the agar plate using 3 mL of EnPresso B Defined [Prototype]. The yeast preculture was grown as a batch culture in EnPresso Y Defined containing 20 g L^{-1} of glucose for 24 h.

The main cultures were prepared in Erlenmeyer flasks with cells from the precultures corresponding to an initial OD_{600} of 0.15. Then, 170 μL per well was distributed into the Slow OxoPlate and the OxoPlate, sealed, and placed onto the robotic platform. The liquid handler added reagent A in the predefined amounts (*E. coli*: 1 U L^{-1} , *S. cerevisiae*: 3 U L^{-1}). The plates were transported into the FAME incubator and shaken at 1000 rpm (2 mm amplitude). For DO measurement and liquid-handling steps (OD_{600} measurement, addition of reagent A), a sampling method was programmed using the Hamilton VenusOne Method Editor v4.2.1.6670. With the sampling method, the user is able to define sampling frequency and individual events using an MS Excel spreadsheet. Every 2 h, the plates were transported from the shaker to the microplate reader for the DO measurements. For OD_{600} measurements, 5 μL samples were taken after 6, 14, 23, 33, and 43 hours. This automated sampling procedure included 30-fold dilution of the samples to a total volume of 150 μL in standard, flat-bottom, 96-well plates (Greiner) and measurement at 600 nm in the microplate reader. After overnight incubation, glucose release was further increased by addition of reagent A to the cultures.

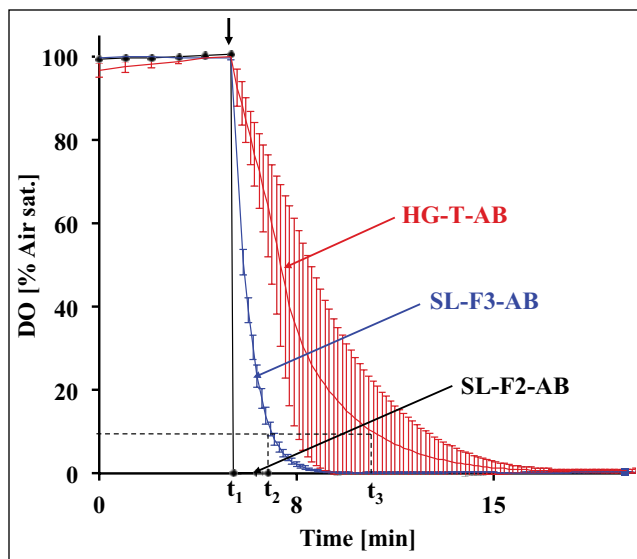


Figure 3. Response time measurements of the foil-based sensors SL-F2-AB, SL-F3-AB and the pipetted sensor HG-T-AB. After measuring oxygen at air saturation for 5 min with a measurement interval of 1 min, sulfite was added and the solution mixed. The arrow shows the time point of sulfite addition (t_1). The response times of SL-F2-AB (t_2) and SL-F3-AB (t_3) were determined.

Results

Development of a Slow OxoPlate Sensor

The robotic platform used in this study is equipped with an incubator and a spectrophotometer, which are attached to a liquid handler. To read out fluorescence signals from a sensor plate, a total transport time from the shaker to the spectrophotometer of 26 s was measured. Because larger platforms may have longer transport times, a minimum response time (t_{90}) of 10 min was defined.

The two-layer SL-F2-AB foil sensors showed inhomogeneity of the sensor foil at layer thicknesses of 200 μm or greater. For the response time measurements, the most homogeneous parts of the foil were used. The response time was <10 s for all layer thicknesses and thus much too low for the target response time of 10 min (**Fig. 3**). The three-layer SL-F3-AB foil sensor revealed excellent reproducible results, but again, the response time was only 1.0 to 1.2 min. The production of even thicker layers to enhance the response time was not possible because of the inhomogeneity of such thick sensor foils. Thus, foil sensors were not further investigated.

The pipetted sensor using a hydrophilic polymer (HG-T-AB) showed response times of 2.4 to 7.9 min for the maximum volume of 130 μL /well, which was still too low for this application. Furthermore, the reproducibility of the response times of the four different sensors was rather bad for volumes greater than 50 μL . Thus, volumes higher than 130 μL to increase the response time were not tried.

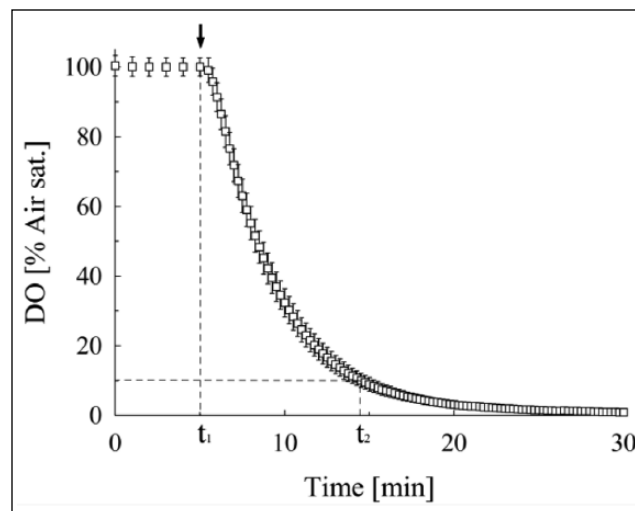


Figure 4. Response time measurements in the Slow OxoPlate with the sodium sulfite method. The arrow indicates the time point of sulfite addition (t_1). The decreasing DO was then measured with an interval of 15 s. Oxygen dropped below 10% a.s (t_2) 9.6 min after sulfite addition.

The pipetted sensor using a hydrophobic polymer (SL-T-AB) also showed a bad reproducibility for high volumes of liquid sensor material of greater than 75 μL . For these volumes, surface defects in the sensor layer were visible because of inhomogeneous drying. Thus, the sensor material was applied to the microplate well in smaller volumes step by step. After addition of the first volume of sensor material, it was dried for at least 4 h in ambient air before applying the next volume. This improved the reproducibility dramatically. A volume of 2×15 μL gave a response time of 9.7 min. This sensor was further characterized with regard to precision, resolution, and reproducibility of the response time and the oxygen values after 1 min.

The response times of 96 sensors pipetted from a different batch of liquid sensor material were determined and compared with the response times of the four sensor spots of the previous test. The results were very similar with 9.6 ± 0.8 min (**Fig. 4**) compared with 9.7 ± 0.6 min. The average oxygen values after 1 min were $91.4\% \pm 4.1\%$ a.s. compared with $90.2\% \pm 1.8\%$ a.s. from the previous test. This shows that the sensor can be produced in a very reproducible way. The slow response enables detection of the approximate oxygen value in the shaken culture after moving the microplate from the robotic system to the reader, as the measured oxygen decrease is quite small. The precision at 37 $^{\circ}\text{C}$ for this sensor was determined as $\pm 0.6\%$ a.s. (cal10) and $\pm 4.8\%$ a.s. (cal100), the resolution with $\pm 0.05\%$ a.s. (cal0) and $\pm 0.2\%$ a.s. (cal100), respectively. These values lie within the target values of the OxoPlate. The Slow OxoPlate fulfills the requirements for this application and was used for the oxygen measurements during bacterial and yeast cultivation.

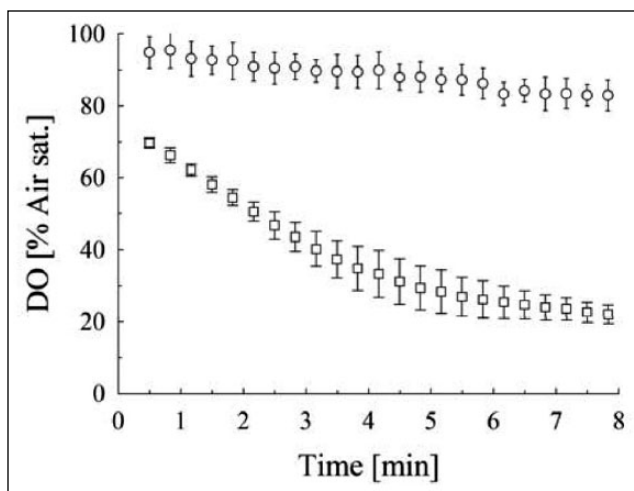


Figure 5. Triplicate dissolved oxygen measurements of *E. coli* W3110 cultures grown in EnPresso B at 30 °C. Open circles: Slow OxoPlate; open squares: OxoPlate. The cultures were grown in shake flasks overnight, and then 200 μ L per well was transferred into Slow OxoPlates and OxoPlates. After 1 h of incubation at 300 rpm (50 mm amplitude), the plates were measured in a microplate reader without additional shaking.

Proof-of-Concept for Microbial Cultivation Monitoring

To investigate the signal change during plate transport, the oxygen consumption of bacterial cultures was measured with the fast-responding OxoPlate and the slow-responding Slow OxoPlate. Cultures that were grown under glucose-limited fed-batch conditions were taken for these experiments, as they are characterized by a lower oxygen consumption rate than cells growing at their maximum growth rate. Preliminary experiments in shake flasks equipped with DO sensors were carried out to determine the time at which glucose limitation occurred. This was characterized by an increase of DO until 90% a.s. or greater, which was reached after overnight incubation.

Then, the shake flask culture was transferred into the sensor plates, which were closed with sandwich covers for well-defined aeration and shaken for 1 h. The fluorescence signals were then recorded for 8 min (**Fig. 5**) without additional shaking. The Slow OxoPlate shows an initial DO value of 95%, followed by a linear decrease. At the endpoint, a DO value of 82% was detected. In contrast to that, the signal of the OxoPlate declines from 70% to 30% within 4 min. The decline is then slowing down, reaching 21% at the endpoint.

Aerobic conditions were detectable with both sensor types, which means that both sensor types could be applied on the robotic platform for bacterial cultures under glucose limitation. However, in this experiment, cells were slow growing and the initial DO was close to 100% air saturation,

which does not resemble the actual situation during a strain screening.

Cultures with a high growth rate, which are commonly used in screening experiments under batch conditions, exhibit lower DO levels than the slow-growing cells in the first experiment. Therefore, lower initial DO levels were tested in a subsequent investigation. A DO microsensor served as a reference system for rapid at-line measurement, and an on-deck shaker was used to shorten the transport times.

At lower shaking frequencies, DO values declined (**Fig. 6**). However, even at 400 rpm, DO levels greater than 20% were detected with the Slow OxoPlate. The microsensor measurements draw a different picture. Even at delay times of 20 s from stopping the shaker until measurement, it was not possible to measure DO signals that closely resemble the sensor plate results. In addition, the microsensor was mounted above the shaken plate for online DO measurements. After stopping the shaker, a decline of DO signal from 89% to 59% in 75 s was recorded (data not shown). Because glucose-limited cultures were taken for these experiments, it can be expected that these effects are much more severe with exponentially growing microbial cultures.

Comparison of Slow and Fast-Responding Sensor Plates in an Automated Cultivation

To demonstrate the ability to detect oxygen limitation during high-throughput cultivation experiments, a reference experiment for cell line screening on a robotic platform was performed. Two culture plates were prepared for comparison of both sensor plates: the Slow OxoPlate and the OxoPlate. Both were prepared with the same layout: cultures with EnPresso B and EnPresoo Y Defined were inoculated with *E. coli* BL21 pDgPNP and *S. cerevisiae* AH22 pPG6, respectively. After an initial growth phase of 10 to 20 h, glucose release was further increased by adding reagent A. The plates were taken from the incubator every 2 h for DO measurement and combined with OD_{600} measurements at certain time points.

For both yeast and bacterial cultures, the typical DO curves of fed-batch fermentations were recorded. However, different DO profiles were obtained from the sensor types tested. For *E. coli*, the OxoPlate showed a decline followed by an increase in DO the first few hours (**Fig. 7A**). This trend is not detectable with the Slow OxoPlate. After 20 h, 12 $U L^{-1}$ reagent A was added, which provided a faster glucose release and thus a higher growth rate. Both sensor plates detect a drop in DO at that time. However, the OxoPlate indicates an oxygen limitation phase of 8 h, whereas this phase appeared to be less than 4 h when looking at the data from the Slow OxoPlate. For *S. cerevisiae*, the obtained results were similar (**Fig. 7B**). Addition of

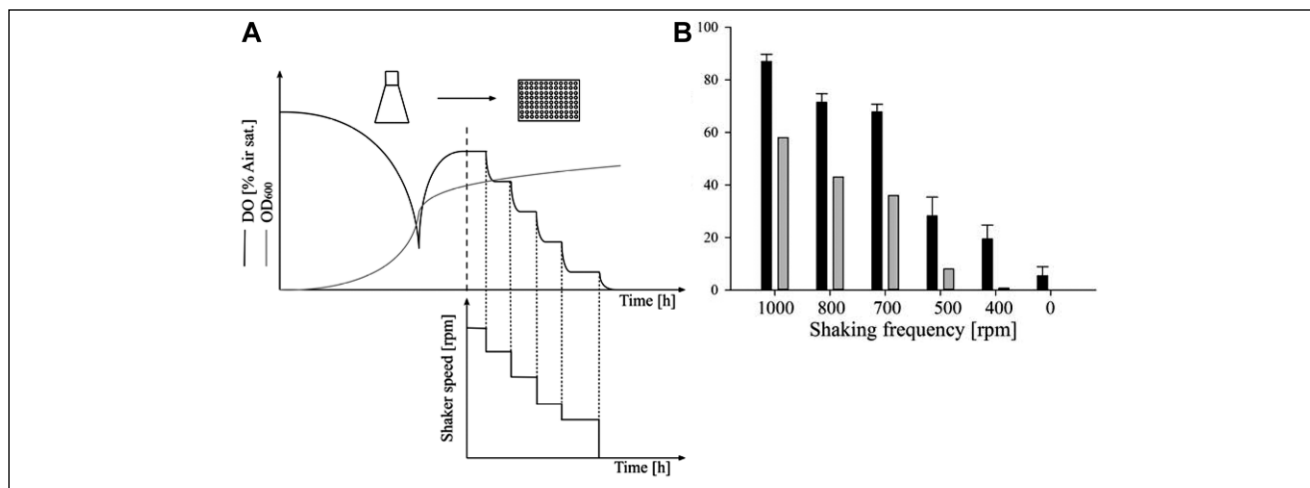


Figure 6. Comparison of at-line microsensor and Slow OxoPlate measurements of *E. coli* W3110 cells incubated at different shaking velocities. **(A)** Schematic outline of the experiment. Cells were grown overnight in a shake-flask, until glucose-limited conditions were reached. Then, the culture was transferred into a Slow OxoPlate, which was incubated at different shaking velocities. After changing the shaking speed, the plate was incubated for 30 min until the next measurement. **(B)** Dissolved oxygen levels at different shaking speeds measured at-line with the microsensor (gray) and the Slow OxoPlate (black).

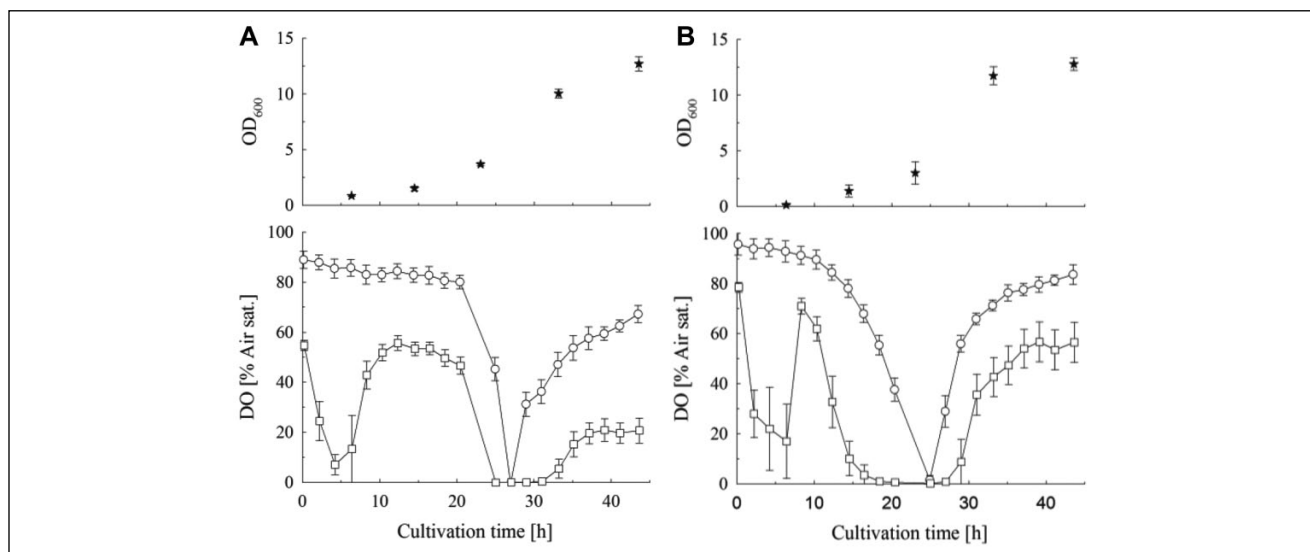


Figure 7. Application of slow- and fast-responding sensor plates for fed-batch cultures of *E. coli* BL21 pDgPNP **(A)** and *S. cerevisiae* AH22 pPG6 **(B)**. Open circles: Slow OxoPlate; open squares: OxoPlate; filled stars: OD₆₀₀. Cells were grown in EnPresso growth system for 44 h with dissolved oxygen measurement intervals of 2 h.

reagent A after 10 h resulted in a decrease of DO, indicated by both sensors. A short phase of oxygen limitation was detected with the Slow OxoPlate, whereas the OxoPlate data indicated an anaerobic phase of 10 h.

The OxoPlate was able to detect the initial exponential phase of the culture after inoculation. Because of the delay between stopping of the aeration and the measurement, the oxygen decreased further during plate transport. After the first few hours, both cultures grew under substrate limitation,

which is indicated by an increased DO level. This phase was not detectable with the Slow OxoPlate, which proves its capability of serving as an indicator for the DO concentration during shaking. After increasing the glucose-release rate, the OxoPlate indicated severe oxygen limitation in both cultures. In comparison with the data from the slow sensor, we conclude that this is mainly due to the delayed measurement. Only a short oxygen limitation phase of 4 h is observed with the Slow OxoPlate.

With the reference cultivation presented in this study, the functionality of the Slow OxoPlate was demonstrated. Oxygen limitations in microbial cultures in 96-well plates can be detected through at-line DO monitoring.

Discussion

In this study, we present the development of a new sensor type for standard 96-well plates. The targeted response time and reproducibility were achieved by sequential pipetting of $2 \times 15 \mu\text{L}$ of liquid sensor material into the plates. Sensor characterization using sodium sulfite showed a response time of 9.7 min, and further tests using microbial cultures indicated that the system can be applied for culture monitoring. Therefore, a reference experiment on a robotic platform was performed, in which DO-level monitoring helped to identify oxygen limitation phases in *E. coli* and *S. cerevisiae* cultures.

Each of the two sensor types was able to deliver information on different oxygenation states of the culture. The OxoPlate was able to detect disturbances caused by the measurements. Data on the DO level at the time of sampling may serve as a guideline for sampling frequency and time points. Signals obtained from the Slow OxoPlate closely reflected the DO curves obtained from on-line sensor systems such as the PreSens SensorDish Reader or Shake Flask Reader, which underlines that the right approach for sensor design was chosen. The importance of the adjustment of aeration conditions in small-scale cultures was pointed out by different research groups. For example, Stockmann and colleagues¹⁵ have reported a severe impact of oxygenation on the screening of phytase producing *Hansenula polymorpha* strains.

Within the study, the application of glucose-limited cultures was a good choice because their stable oxygen consumption rate over time made the adjustment of DO level by adjustment of the shaking frequency possible. Moreover, growing *E. coli* using the EnBase technology has been found to be an important factor for the scale up of recombinant protein production processes.^{3,6} In future work, we want to prove that the combination of small-scale sensor technology and high-throughput fed-batch cultivation can be seen as an enabler for process design based on QbD principles.¹⁶

In summary, the detection of oxygen limitation in aerobic microbial cultures using standard 96-well plates equipped with fluorescent sensor spots was successfully performed. By increasing the sensor's response time, at-line determination of a rapidly changing parameter such as DO was possible. With the new sensor, optimization of growth conditions on a reference plate is possible. High-throughput screening programs can be carried out afterward using standard 96-well plates. The conditions of such screening experiments are comparable with bioreactor cultivations in terms of oxygen

availability, which helps in reducing the risk of difficulties during scale up.

Acknowledgments

The authors are thankful for assistance carrying out the experimental work and programming. Michael Heiser, Susan Bigesse, and Christopher Skopnik were involved in parts of the study. Moreover, technical and material support by BioSilta Ltd. and PreSens Precision Sensing GmbH was appreciated.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Technical and material support by BioSilta Ltd. and PreSens Precision Sensing GmbH. Financial support by the German Federal Ministry of Education and Research (BMBF) within the Framework Concept "Research for Tomorrow's Production" (project No. 02PJ1150, AUTOBIO project).

References

1. Suresh, S.; Srivastava, V. C.; Mishra, I. M. Techniques for Oxygen Transfer Measurement in Bioreactors: A Review. *J. Chem. Technol. Biotechnol.* **2009**, *84*, 1091–1103.
2. Zimmermann, H. F.; Anderlei, T.; Buchs, J.; et al. Oxygen Limitation Is a Pitfall during Screening for Industrial Strains. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 1157–1160.
3. Glazyrina, J.; Krause, M.; Junne, S.; et al. Glucose-Limited High Cell Density Cultivations from Small to Pilot Plant Scale Using an Enzyme-Controlled Glucose Delivery System. *N. Biotechnol.* **2012**, *29*, 235–242.
4. Neubauer, P.; Cruz, N.; Glauche, F.; et al. Consistent Development of Bioprocesses from Microliter Cultures to the Industrial Scale. *Eng. Life Sci.* **2013**, *13*, 224–238.
5. Schneider, K.; Schuetz, V.; John, G. T.; et al. Optical Device for Parallel Online Measurement of Dissolved Oxygen and pH in Shake Flask Cultures. *Bioproc. Biosyst. Eng.* **2010**, *33*, 541–547.
6. Šiurkus, J.; Panula-Perala, J.; Horn, U.; et al. Novel Approach of High Cell Density Recombinant Bioprocess Development: Optimisation and Scale-up from Microliter to Pilot Scales while Maintaining the Fed-Batch Cultivation Mode of *E. coli* Cultures. *Microb. Cell Fact.* **2010**, *9*, 35.
7. Funke, M.; Diederichs, S.; Kensy, F.; et al. The Baffled Microtiter Plate: Increased Oxygen Transfer and Improved Online Monitoring in Small Scale Fermentations. *Biotechnol. Bioeng.* **2009**, *103*, 1118–1128.
8. Streefland, M.; Martens, D. E.; Beuvery, E. C.; et al. Process Analytical Technology (PAT) Tools for the Cultivation Step in Biopharmaceutical Production. *Eng. Life Sci.* **2013**, *13*, 212–223.
9. Arain, S.; John, G. T.; Krause, C.; et al. Characterization of Microtiterplates with Integrated Optical Sensors for Oxygen and pH, and Their Applications to Enzyme Activity

- Screening, Respirometry, and Toxicological Assays. *Sens. Actuator B Chem.* **2006**, *113*, 639–648.
10. Mercier, S. M.; Diepenbroek, B.; Wijffels, R. H.; et al. Multivariate PAT Solutions for Biopharmaceutical Cultivation: Current Progress and Limitations. *Trends Biotechnol.* **2014**, *32*, 329–336.
 11. John, G. T.; Goelling, D.; Klimant, I.; et al. PH-Sensing 96-Well Microtitre Plates for the Characterization of Acid Production by Dairy Starter Cultures. *J. Dairy Res.* **2003**, *70*, 327–333.
 12. Szeker, K.; Niemitalo, O.; Casteleijn, M. G.; et al. High-Temperature Cultivation and 5' mRNA Optimization are Key Factors for the Efficient Overexpression of Thermostable *Deinococcus geothermalis* Purine Nucleoside Phosphorylase in *Escherichia coli*. *J. Biotechnol.* **2011**, *156*, 268–274.
 13. Lang, C.; Looman, A. C. Efficient Expression and Secretion of *Aspergillus niger* RH5344 Polygalacturonase in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **1995**, *44*, 147–156.
 14. Knepper, A.; Heiser, M.; Glauche, F.; et al. Robotic Platform for Parallelized Cultivation and Monitoring of Microbial Growth Parameters in Microwell Plates. *J. Lab. Autom.* **2014**, *19*, 593–601.
 15. Stockmann, C.; Losen, M.; Dahlems, U.; et al. Effect of Oxygen Supply on Passaging, Stabilising and Screening of Recombinant *Hansenula polymorpha* Production Strains in Test Tube Cultures. *FEMS Yeast Res.* **2003**, *4*, 195–205.
 16. Long, Q.; Liu, X. X.; Yang, Y. K.; et al. The Development and Application of High Throughput Cultivation Technology in Bioprocess Development. *J. Biotechnol.* **2014**, *192*, 323–338.