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Published in:
ACS Sensors

DOI:
10.1021/acssensors.6b00071

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Mosshammer, M., Strobl, M., Kühl, M., Klimant, I., Borisov, S. M., & Koren, K. (2016). Design and application of an optical sensor for simultaneous imaging of pH and dissolved O2 with low cross-talk. ACS Sensors, 1(6), 681-687. DOI: 10.1021/acssensors.6b00071
Design and Application of an Optical Sensor for Simultaneous Imaging of pH and Dissolved O2 with Low Cross-Talk

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ABSTRACT: Visualization and quantification of analytes such as O2 or pH is essential in biological research. Here we present the design and application of a new optical dual-analyte sensor for imaging, optimized to have low cross-sensitivity between the two analytes O2 and pH. The used indicator and reference dyes were selected to match the different channels of a commercial 2CCD (RGB + NIR) camera. A red-light emitting O2-sensitive europium complex (Eu(HPhN)3dpp) with a dynamic range of 0–20% O2 in the finished sensor was combined with a near-infrared emitting pH indicator (OHButoxy-aza-BODIPY) with a dynamic range of pH 7.2–8.8. To enable ratiometric readout, an inert reference coumarin dye (Bu3Coum) was co-immobilized with the optical indicators. In order to maximize the sensor signal, inert diamond powder was added to one sensor layer as a simple way to increase scattering of light within the sensor. Furthermore, the addition of an optical isolation layer enabled measurements in highly fluorescent samples, such as algal biofilms. The sensor was tested in a marine photosynthetic microbial mat.

KEYWORDS: dual-analyte sensor, chemical imaging, biofilm, microbial mat, sensor application

Optical sensors based on reversible changes in luminescence of specific indicators allow the measurement as well as the visualization of analyte concentrations in a variety of formats. Fiber optical based sensors enable point measurements with high spatial resolution, whereas planar sensor optodes allow the imaging and analysis of larger areas. In environmental sciences the application of planar optical sensors has, e.g., enabled new experimental studies of the composition and function of marine sediments, microbial mats, plant rhizospheres, microbial films, and photosynthetic microbes. Planar optodes can be imaged by three commonly used methods. The simplest, but at the same time most error-prone, method is intensity-based imaging, where the analyte-dependent change in sensor luminescence intensity is converted into an analytic concentration. This method is very sensitive to structural inhomogeneity, i.e., variable indicator concentrations in the planar optode or fluctuations in either the light source or detector (camera) including uneven excitation of the planar optode. In comparison, ratiometric imaging gives a more robust readout of luminescence-based sensors. In this imaging mode a second analyte-independent emission from a reference dye is measured alongside the analyte-dependent emission form the optical indicator dye. Most artifacts due to sensor heterogeneity and uneven illumination can be compensated by calculating ratios between the inert emission and the analyte-dependent emission. Even more robust measurements with planar optodes can be obtained by luminescence lifetime-based imaging, where an intrinsically referenced analyte-dependent parameter is measured. However, lifetime imaging involves the use of rather specialized and expensive imaging setups and is suitable mostly for optical oxygen and temperature probes. Lifetime-based imaging of fluorescent probes (e.g., pH sensors) showing an “on–off” characteristic is hardly possible. Ratiometric imaging, on the contrary, can be carried out for many different analytes even using relatively simple commercial color cameras, where the Bayer color filter on the camera chip enables the separation of the incident light into the three primary colors: blue, green, and red. Therefore, ratiometric images can be generated by simply comparing the images of the different color channels without the need of using multiple filters or light sources. Due to the simplicity and low cost of such RGB-camera based approaches, ratiometric, chemical imaging is currently gaining popularity within many different fields.

Received: February 1, 2016
Accepted: April 8, 2016
Published: April 8, 2016

DOI: 10.1021/acssensors.6b00071
ACS Sens. 2016, 1, 681−687
In order to enable the imaging of two or more chemical parameters simultaneously, multiple indicators can be combined within a single optode. Common RGB color cameras can in principle be used to read out two indicator dyes and one reference dye. However, such readout of dual analyte sensors with RGB color cameras is limited by the spectral overlap of the green channel with the two other channels, potentially causing cross-sensitivity between the individual sensor readouts. In addition, the choice of indicators and references is limited, as if excitations take place in the blue region and all three channels are reserved for indicators and the reference, it is very difficult to separate the emission and the excitation in the blue region. A simple way to expand the potential of ratiometric readout is to use a 2CCD camera, where the incoming light is split equally onto a normal RGB camera chip and an additional near-infrared (NIR) sensitive camera chip. This adds an extra spectral window for combining reference and indicator dyes and allows the use of NIR emitting indicators.

In this study we used a similar 2CCD imaging setup in combination with a new dual sensor system for simultaneous imaging of pH and O2 with minimal cross-sensitivity between the two analytes. In order to achieve this low cross-sensitivity an appropriate combination of indicators and sensor materials had to be found. We describe the rationale behind the sensor design, sensor manufacturing, and calibration along with a practical application for studying O2 dynamics and pH dynamics simultaneously in a complex photosynthetic microbial mat with high intrinsic fluorescence from photopigments.

## EXPERIMENTAL SECTION

### Materials.
The used indicator dyes OHButoxy-aza-BODIPY [28] and Eu(HPN)$_{2}$ [29] were synthesized according to published methods (see Figure 1 for chemical structures). Polysyrene hydrogel (Hydromed D4) was obtained from AdvanSource biomaterials (advb biomaterials.com). Polystyrene (PS, MW 250 000) was bought from ACROS Organics (acros.com). Monocrystalline diamond powder was purchased from Microdiamant (microdiamant.com). All buffer materials (acetic acid, MES, TRIS, and CAPS) as well as 2-propanol and toluene were purchased from ROTH (carlroth.com). NaCl was obtained from AnalR NORMAPUR – VWR (dvw.com/store/) chemicals, and carbon black was purchased from KREMER (kremer-pigmente.de). The PET support foil was obtained from Goodfellow (goodfellow.com). All chemicals were used as received.

### Synthesis of the Reference Dye.
Bu$_{2}$Coun was synthesized analogously to the literature procedure [30] (Supporting Information Figure S1). Briefly, 4-dibutylamino-2-hydroxybenzaldehyde prepared from 3-dibutylaminophenol [31] and 2-(6-butylbenzo[d][1h]thiazol-2-yl)acetonitrile (prepared from 6-butyl-2-benzothiazolamine via 2-amino-5-butylbenzenethiol) to give an iminocoumarin that was further purified on column chromatography (silica-gel).

### Synthesis of OHButoxy-aza-BODIPY

2-Amino-5-butylbenzenethiol. $^{1}$H NMR (CDCl$_3$, 300 MHz): 6.97 (m, 2H), 6.65 (d, 1H), 4.0–3.5 (m, 3H), 2.38 (t, 2H), 1.44 (quint, 2H), 1.28 (m, 2H), 0.88 (t, 3H).

2-(6-butylbenz[d]thiazol-2-yl)acetonitrile. $^{1}$H NMR (DMSO-d$_6$, 300 MHz): 7.47 (s, 1H), 7.40 (s, 2H), 7.24 (d, 1H), 7.03 (d, 1H), 2.58 (t, 2H), 1.55 (quint, 2H), 1.29 (m, 2H), 0.90 (t, 3H).

Bu$_{2}$Coun. $^{1}$H NMR (CDCl$_3$, 300 MHz): 8.96 (s, 1H), 7.95 (d, 1H), 7.72 (s, 1H), 7.48 (d, 1H), 7.31 (d, 1H), 6.64 (d, 1H), 6.53 (s, 1H), 3.37 (s, 4H), 2.76 (t, 2H), 1.63 (m, 6H), 1.38 (m, 6H), 0.99 (m, 9H).

### NMR spectra in Supporting Information Figures S1–S5.

### Camera Setup.
The setup used consisted of a JAI AD-080 GE 2CCD Multispectral camera (jai.com) equipped with a Schneider KREUZNACH objective (1.4/23 CCTV-LENS 400–1000 nm; schneiderkreuznach.com). A 510 nm long-pass filter (OG 510 Schott)

Figure 1. (A) Schematic drawing of the new optical O2 and pH dual sensor composed of a three layer system containing the depicted indicator and reference dyes. (B) Spectra of the dyes (absorption in dashed and emission in solid lines). The spectral sensitivity of the four channels in the dual CCD camera is also shown (filled spectra colored according to the spectral range).

was placed in front of the objective and an additional plastic filter (#10 medium yellow from leefilters.com) was placed in front of the long-pass filter to reduce the fluorescence of the glass filter and the background. The sensor can be illuminated with UV or blue LEDs (400–470 nm); we used a high power 405 nm LED (LedEngin purchased from rs-online.com) with a custom-built LED trigger (National instruments USB 6008 and an adequate power supply). Image acquisition and triggering of the LED was done with a custom-made software from Bioras ApS (bioras.com); the software is available from the authors upon request.

### Sensor Preparation.
For the O2 sensitive layer, 1 mg of the Eu-complex and 0.15 mg of the coumarin reference dye were dissolved in 1.67 g 12% w/v solution of PS (in toluene). The solution was knife coated on a dust free PET film yielding a ∼2.5-μm-thick layer after solvent evaporation.

For the pH sensitive layer, 0.05% w/v (0.5 mg) of the OHButoxy-aza-BODIPY was dissolved in 250 μL THF and added to 1 g of a 100% w/v solution of D4 (isopropanol:water, 9:1 w/v) containing 100% w/v (100 mg) diamond powder, serving as a signal enhancer. The dispersion was knife coated on top of the O2 sensitive layer yielding a ∼7.5-μm-thick pH sensitive layer after solvent evaporation.

For the optical isolation layer, 1.00% w/v (10 mg) of carbon black was dispersed in 1 g 10.0% w/v solution of D4 (isopropanol:water, 9:1 w/v) containing 10.0% w/v (100 mg) diamond powder, serving as a signal enhancer. The dispersion was knife coated on top of the pH sensitive layer yielding a ∼7.5-μm-thick optical isolation after solvent evaporation. The total thickness of the final dual-analyte sensor with optical isolation was thus <20 μm.

### Sensor Calibration and Characterization.
A dual sensor foil area of approximately 6 × 3 cm$^2$ was taped on the inside of a transparent flow chamber with a glass wall that was also used to study the microbial mat (see below) and imaging was done from the side. For calibration the pH of water in the flow chamber was adjusted using different buffers (MES, TRIS, phosphate, all at a concentration of 20 mM and a salinity of 30 PSU, equaling the used seawater). Oxygen
levels were altered with the help of compressed O2 and N2, which were mixed by a PC-controlled gas mixer (sensor-sense.nl) prior to flushing the water in the flow chamber. The calibrations and the experiment were performed at the same constant temperature (22.5 ± 0.5 °C). The chamber was sealed at both ends during calibration. Temperature and O2 levels were also monitored with a quicksilver thermometer and a fiber-optic O2 meter (PiccoloO2 equipped with a dipping probe; Pyroscience GmbH, Aachen Germany; pyro-science.com), respectively.

**Image Analysis.** The acquired images were split into the red, green, blue, and NIR channels and analyzed using the freely available software ImageJ (rsbweb.nih.gov/ij/) using the freely available plugins “Stack Sorter” and “Ratio Plus”. In order to obtain O2 and pH concentration images, the following steps were performed: For O2, the red channel (O2 sensitive emission of the Eu-complex) and green channel (emission of the coumarin reference dye) images were divided; For pH, the NIR (pH sensitive emission of the OHButoxy-aza BODIPY) and green channel (emission of the coumarin reference dye) images were divided. From these ratio images, the mean values and standard deviations were measured using ImageJ and plotted against the analyte concentration. This yielded calibration curves for O2 and pH, respectively, that were fitted with an exponential decay function (O2) or a sigmoidal function (pH) using OriginPro9.2 (originlab.com). Based on these calibrations, experimental ratio images from the biological measurements were transferred to chemical images in ImageJ using the curve fit functions; as ImageJ does not support sigmoidal fits, a linear fit to the pH-dependent ratios between pH 7 and 9 was used (Figure 2).

**Sampling of Microbial Mat and Measurement of O2 and pH Dynamics.** Sediment colonized by a dense biofilm of phototrophic microorganisms, i.e., microbial mats were collected from sheltered, sandy areas at the coast of Limfjorden, near Aggersund, Denmark (57°00’02.15N, 9°17’12.89E). The sampling site is exposed to changes in sea level resulting from winds, causing it to be exposed to air, desiccated, or flooded for time periods between hours and weeks.32,33 However, there are only minor influences due to tides.33 The intermittent desiccation inhibits grazing animals and the top layer of the sediment is densely populated by filamentous cyanobacteria34 and other microorganisms.

Sediment samples were collected in August 2015 using small (8 × 5 × 2 cm3) plexiglass boxes. The sampling boxes were cut in half and taped together prior to sampling. The samples were kept under air-saturated seawater and light exposure for at least 24 h before conducting experiments. For the dual imaging, the tape was removed and the samples cut in half and immediately pressed against the calibrated dual sensor in the flow chamber, in order to avoid oxidation of the cut surface (see Supporting Information for picture of the sampling box and the cut samples). The flow chamber was flooded with seawater (from a 10 L reservoir kept at a constant temperature 22.5 ± 0.5 °C) and the sample was illuminated vertically from above using a halogen lamp equipped with a collimating lens (KL-2500, Schott GmbH) providing an incident downwelling irradiance (400–700 nm) of 330 μmol photons m−2 s−1 for 30 min; incident light was quantified with an underwater quantum irradiance meter (ULM-500, WALZ, walz.com). Image collection started immediately after the light was switched off and pictures were taken every 30 s for 30 min. Pictures of the sample cross section were taken after the experiment under ambient and UV illumination. Those pictures were used together with the O2 images to define the sample surface.

**RESULTS AND DISCUSSION**

The aim of this study was to design an optical dual-analyte sensor with minimal cross-sensitivity between the two analytes, enabling simultaneous ratiometric imaging of O2 and pH. The idea of multianalyte imaging is not new,17,35,36 and several systems have been proposed and in part applied to measure “real world” samples.53 Nevertheless, many of these systems use expensive cameras (lifetime based imaging),27 multiple excitation sources or optical filter combinations, which results in complex imaging systems. In this study, we showed that a simple system consisting of a single camera, a single excitation source, and a single filter can be used to enable dual-analyte imaging of O2 and pH. The system takes advantage of a camera that houses 2 CCDs.17 While one of them is a “standard” color chip (RGB) the second one is solely sensitive to near-infrared radiation (NIR). The spectral sensitivities of the 2 CCDs are depicted in Figure 1B. It can be observed that the NIR channel is completely separated from the other channels. In contrast, the blue, green, and red channels show substantial overlap, a common limitation when using RGB cameras. In order to develop a sensor without cross-sensitivity those overlaps have to be considered. In the following the rationale behind the sensor design is explained.

**Dyes.** The dual sensor was optimized in order to minimize spectral overlap and subsequent cross-sensitivity. Additionally, photostability and adequate sensitivity of the indicators were important factors when designing the sensor. The chosen pH indicator OHButoxy-aza-BODIPY is highly photostable28 and its emission falls fairly well in the NIR channel of the 2CCD camera system. Preliminary investigations based on the commonly used O2 sensitive dye platinum(II)-5,10,15,20-tetrakis(2,3,4,5,6-pentafluorophenyl)-porphyrin (PtTFPP) in combination with the OHButoxy-aza-BODIPY dye showed

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*Figure 2. Calibration plots for O2 and pH for the new dual sensor. Both plots were obtained at varying concentrations of the respective other analyte. In the pH calibration, a slight cross-sensitivity to O2 can be observed at pH values < pK_a. The red line in the pH calibration represents a linear fit within the image analysis program. Points represent mean values (±SD) obtained from the entire imaged sensor.*

DOI: 10.1021/acssensors.6b00071

ACS Sens. 2016, 1, 681–687
strong cross-sensitivities in the O₂ calibration (due to an overlap of the emission of PtTFPP (λmax 650 nm) with the pH-dependent absorption of the aza-BODIPY resulting in a pH-modulated inner-filter effect) as well as in the pH calibration (due to the emission shoulder of PtTFPP in the NIR region). Spectra of the sensor containing PtTFPP as well as the respective calibration curves can be found in the Supporting Information (Figures S6 and S7). In contrast, O₂ indicators based on Eu(III) complexes did not produce cross-talk due to their very narrow emission (fwhm ≈ 4 nm) peaking at 613–617 nm. While virtually all Eu(III) complexes show insufficient sensitivity to O₂, a recently reported hydroxyphenalenone complex Eu(HPhN)₃dpp²⁹ represents a notable exception. Moreover, this complex is excitable in the blue part of the spectrum, which is particularly attractive for the design of a dual sensor system. A new Bu₃Coum dye was chosen as reference dye due to its distinct emission in the green region and high lipophilicity, which prevents its migration into the more hydrophilic hydrogel from the hydrophobic polystyrene. Spectra of all the dyes and their overlap with the 4 color channels of the 2CCD camera system can be found in Figure 1B. All the dyes used can be excited in the blue part of the spectrum (400–470 nm). A common excitation is beneficial as it minimizes the equipment needed (single LED). Additionally, in this specific dye arrangement only a single filter (long pass filter OG510) is required to block the excitation light from reaching the camera.

**Sensor System.** Besides the adjustment of the optical properties of the dyes relative to each other and the camera channel, respectively, it was also important to immobilize the selected dyes in an appropriate matrix. While O₂ indicators are commonly immobilized in hydrophobic polymers,²⁷ pH indicators need to be exposed to water and demand a hydrophilic matrix. Additional components of the sensor encompassed a signal enhancing layer³⁹ improving the optical signals due to enhanced light scattering and an optical isolation layer²⁹ for blocking background light (e.g., from chlorophyll fluorescence, a common source of fluorescence in photosynthetic systems as shown in Figure 3).

The developed sensor system consists of three layers. The layer closest to the transparent sensor support (polyethylene terephthalate) is a polystyrene (PS) layer incorporating the O₂ sensitive Eu(HPhN)dpp dye as well as the Bu₃Coum reference dye. The second layer is a D4 hydrogel containing the OHButoxy-aza-BODIPY and diamond powder. The latter layer combines pH sensing as well as signal enhancing properties due to the diamond powder. Diamond powder was chosen over the frequently used TiO₂⁵⁸ to avoid its potentially interfering photocatalytic properties. In comparison to SiO₂ (n = 1.4588), the refractive index of diamond powder (n = 2.42) is significantly higher which makes it more attractive to use for scattering enhancement of optical signals. Additionally adding diamond powder increased the adhesion between the layers and has a lower refractive index than TiO₂. A further D4 hydrogel layer consisting of the pH sensitive OHButoxy-aza-BODIPY dye and carbon black was included to eliminate background light interferences; the pH sensitive dye was added to the optical isolation layer to avoid dye migration and thus changes in the signal intensities. The optical isolation was chosen to be of the same thickness as the pH layer to ensure complete blocking of background light including potentially interfering chlorophyll fluorescence signals from the densely pigmented biofilm. Although the optical isolation layer contains the pH indicator, it has to be noted that the indicator was only added to avoid dye migration between the layers. The pH dependent emission was only detected from the underlying pH sensitive layer as the black isolation absorbs all the emission of the pH indicator within the optical isolation.

**Sensor Calibration and Evaluation of Cross-Sensitivity.** In order to evaluate potential cross-talk calibrations for O₂ and pH were conducted at varying concentrations of the other respective analyte. Stepwise changes in the O₂ concentration were conducted for two pH values: one acidic and one alkaline (Figure 2). For the other pH values, only air saturation, 100% and 0% O₂ were measured. The obtained values at intermediate pH values fell well within the obtained calibration (not shown in Figure 2). The O₂ calibration showed no cross interferences caused by changes in the pH and exhibited a dynamic range between 0% and 40% O₂, although with strongly reduced sensitivity between 20% and 40% O₂. Such low sensitivity at higher O₂ levels was expected for Eu(HPhN)dpp due to the rather long decay time (350 μs)²⁹ of this dye. As O₂ sensitivity of an optical O₂ sensor is mainly influenced by the decay time of the indicator and the O₂ permeability of the polymer, further tuning of the sensitivity can be achieved by changing the polymer matrix⁴⁰ without interfering with the spectral properties of the sensor materials.

The pH response of the dual sensor followed a sigmoidal pattern with an apparent pKₐ of around 7.96. A minor cross-sensitivity toward O₂ was observed at pH values beneath the pKₐ (Figure 2). This can be explained by the spectral overlap of the red and green channels of the camera. A decrease of O₂ results in an increase in the emission of the O₂ indicator seen predominantly in the red channel. Due to the slight spectral overlap there are subsequently minor increases in the green channel, resulting in lower NIR/G ratio values. However, for most applications, this minor cross-sensitivity between the two analytes is not problematic and lies well within the experimental error. Due to practical reasons (image analysis) the sigmoidal calibration curve was approximated using a linear fit, as the
sigmoidal curve was almost perfectly linear over a range of ±0.5 pH units around the pHₐ (Figure 2). In order to maximize the possible dynamic range of the sensor, a range of ±1 pH units around the pHₐ was chosen. Although the linear approximation deviates from the correct sigmoidal fit, especially at pH values <7.2 and >8.8, the sample used showed (Figure 4) a minimal deviation from the correct sigmoidal curve was almost perfectly linear over a range of ±0.5 pH units around the pHₐ (Figure 2). In order to maximize the possible dynamic range of the sensor, a range of ±1 pH units around the pHₐ was chosen. Although the linear approximation deviates from the correct sigmoidal fit, especially at pH values <7.2 and >8.8, the sample used showed (Figure 4) a minimal deviation from the correct sigmoidal fit. For samples covering a broader pH range, a broad range pH sensor system would be required, which consists of multiple pH indicators dyes.28 In this case a linear approximation might prove sufficient for this sample and we note that the cross-sensitivity toward O₂ has a smaller effect on accuracy of the sensor reading than the applied linear fit. For samples covering a broader pH range, a broad range pH sensor system would be required, which consists of multiple pH indicators dyes.28 In this case a linear fit would not be adequate and more complicated processing algorithms would be necessary.

Besides sensor calibration, the sensor response time is another important characteristic determining at which time scale measurements can be conducted. The response time of the dual sensor (tₚ) was <20 s for both analytes (see Figure S10). This is in good agreement with literature values for sensors of similar thickness.16 Thus, the measurements of the dynamics in the microbial mats were conducted in 30 s time steps.

Application of the Dual-Sensor for Imaging O₂ and pH Simultaneously within a Microbial Mat. In order to test the applicability of the dual sensor on a real world sample, sediment cores with a distinct microbial mat at the surface layer were collected and analyzed. A fresh cut sediment core was pressed against the dual sensor and illuminated with an external light source until steady state was reached (see setup in Figure 3). After the external illumination was switched off, images were acquired continuously for 30 min. Although it was attempted to firmly press the sample against the sensor, the rather sandy consistency of the sample complicated that. In the case of muddier sediments or samples, close contact of the sensor and the sample can be achieved more easily.

Images of the 2D O₂ and pH distribution in the microbial mat recorded directly after light exposure and after 30 min in the dark are shown in Figure 4. The surface of the microbial mat is depicted as a black line. For both analytes, distinct differences were observed between light and dark conditions. As the cyanobacteria present in the microbial mat are capable of performing photosynthesis, images recorded directly after light exposure showed increased O₂ concentration and pH values close to the surface of the mat. After light exposure, the pH around the microbial mat surface increased by up to 0.8 pH units relative to the seawater pH due to CO₂ fixation by cyanobacterial photosynthesis. In contrast, the pH image recorded after 30 min in darkness showed that pH at the microbial mat surface was similar to pH in the overlying seawater. Surprisingly, no particularly high O₂ oversaturation was observed after light exposure and this can be explained by (i) a lack in sensitivity of the O₂ sensor above air saturation and (ii) a possibly nonideal contact between the sample and the sensor. Nevertheless, this shows that it is important to adapt the used sensor to the analytical problem. The pH sensor on the other hand exhibited a perfect dynamic range for the processes observed in the microbial mat.

To analyze the pH and O₂ dynamics of the system in more detail, three different areas of interest with equal sizes were selected (see Figure 5). One was chosen to be exposed to the seawater above the microbial mat (area 1) exhibiting seawater pH and full air saturation, the second area crossed the mat–water interface (area 2), while the third area was exposed to deeper sediment layers (area 3) exhibiting approximately pH 7.8 and anoxic conditions. The changes in O₂ concentrations and pH in areas 1 and 3 are, as expected, negligible over time, while the most drastic changes occurred in area 2, i.e., around the biofilm–water interface (Figure 5C and D). Decreasing O₂ concentration and pH were observed in the latter zone immediately after the light was switched off. While the O₂ concentration reached equilibrium within the first 5 min in the dark are shown in Figure 4. The surface of the microbial mat is depicted as a black line. For both analytes, distinct differences were observed between light and dark conditions. As the cyanobacteria present in the microbial mat are capable of performing photosynthesis, images recorded directly after light exposure showed increased O₂ concentration and pH values close to the surface of the mat. After light exposure, the pH around the microbial mat surface increased by up to 0.8 pH units relative to the seawater pH due to CO₂ fixation by cyanobacterial photosynthesis. In contrast, the pH image recorded after 30 min in darkness showed that pH at the microbial mat surface was similar to pH in the overlying seawater. Surprisingly, no particularly high O₂ oversaturation was observed after light exposure and this can be explained by (i) a lack in sensitivity of the O₂ sensor above air saturation and (ii) a possibly nonideal contact between the sample and the sensor. Nevertheless, this shows that it is important to adapt the used sensor to the analytical problem. The pH sensor on the other hand exhibited a perfect dynamic range for the processes observed in the microbial mat.

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Figure 4. Calibrated false color pH (top) and O₂ (bottom) images of the studied microbial mat directly after light exposure (330 μmol photons m⁻² s⁻¹) and after 30 min in the dark. The surface of the mat is represented by the black line.

Figure 5. Extraction of time-resolved and depth-resolved dynamics from recorded O₂ and pH images. (A) The arrow indicates where vertical O₂ and pH profiles shown in panel B where extracted. Boxes visualize specific areas of interest. (B) pH (red) and O₂ (black) profiles directly after light exposure (full symbols) and after a 30 min dark period (open symbols). (C) Changes in O₂ concentration over time in the three selected areas. (D) Changes in the pH over time in the three selected areas.
darkness, the change in pH from 8.8 to 8.2, i.e., the pH of the seawater, took place much more slowly and was only completed after 20 min reflecting the balance of different microbial metabolic processes within the microbial mat.\textsuperscript{32} The new dual sensor thus allows the investigation of pH and $O_2$ dynamics of the exact same area which was subject to identical pretreatment.

Besides mapping of time dependent changes of the two analytes, concentration profiles through the sample can be extracted from the chemical images (Figure S5A,B). A set of vertical depth profiles of $O_2$ concentration and pH just after illumination and after 30 min in darkness is shown in Figure S5B. The pH profiles showed a distinct pH maximum just beneath the mat surface that disappeared during the dark period. A slight $O_2$ maximum at the mat surface was seen just after darkening, but due to the previously described sensor dynamics the measurement accuracy was restricted. Nevertheless, the $O_2$ measurement revealed that the oxic zone increased dramatically due to photosynthetic activity of the mat. Similar dynamics were observed when using commercial microsensors (see Figure S11).

\section*{CONCLUSION}
A novel dual sensor system for the simultaneous, low-cost imaging of $O_2$ concentrations and pH was developed and tested on microbial mats. The novel indicator dye was chosen due to its excitability in the blue region. The $O_2$ sensitive indicator was selected for its narrow emission peak in the red region, which minimizes cross-talk between the channels, its good $O_2$ sensitivity in comparison to other Eu(III) dyes, and its excitability in the blue region. The pH sensitive dye was chosen due to its emission in the NIR part of the spectrum and its high photostability. The system was optimized toward minimal cross-sensitivity between the two analytes, maximal signal intensity by the introduction of a signal-enhancing scattering layer utilizing diamond powder, and the blocking of background interferences by the introduction of an additional optical isolation layer. It was possible to simultaneously visualize dynamic changes in pH and $O_2$ concentration as a function of irradiance in dense microbial mats exhibiting high background fluorescence.

The sensor system is easy to use, cheap, and allows real time monitoring of dynamic processes within a pH range from 7 to 9 and $O_2$ concentration between 0% and up to 40% (albeit with limited resolution above 20%). These specific properties make this dual sensor system suitable for a broad field of environmental applications\textsuperscript{9} or process monitoring.

\section*{ASSOCIATED CONTENT}
\subsection*{Supporting Information}
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssens.6b00071.

NMR spectra of the synthesized Bu$_3$Coum, calibration plots of a sensor system using a nonideal dye combination, pictures of the experimental setup and environmental samples, and microsensor profiles measured in the microbial mat (PDF)

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\textbf{Author Contributions}

M.S. and S.M.B. synthesized the used dyes. M.M., L.K., and S.M.B. designed the dual analyte sensor. M.K. and K.K. designed the biological measurements that were performed by M.M. and K.K. The manuscript was written by M.M. with contributions from all authors. All authors have given approval to the final version of the manuscript.

\section*{Notes}
The authors declare no competing financial interest.

\section*{ACKNOWLEDGMENTS}

This study was supported by grants from the Villum Foundation (K.K., M.K.), Erasmus+ (M.M.), European Commission (M.M., S.M.B., Project SenseOcean, Grant Agreement Number 614141); MS, FP7 Project BIOINTENSE, Grant Agreement Number 312148), the Danish Research Council for Independent Research \textbar Technology and Production Sciences (K.K., M.K.), and by a Sapere-Aude Advanced grant from the Danish Council for Independent Research \textbar Natural Sciences (M.K.). We thank Sofie L. Jakobsen and Egil Nielsen for technical assistance, and Nick Blackburn (Bioras ApS) for developing the imaging software and trigger device for the new imaging system. Christoph Staudinger is thanked for helping with image analysis and for fruitful discussions.

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