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Encapsulating genetically modified *Saccharomyces cerevisiae* cells in a flow-through device towards the detection of diclofenac in wastewater

C. Schirmer[a,*,1], J. Posseckardt[a,1], A. Kick[a], K. Rebatschek[a], W. Fichtner[a], K. Ostermann[b], A. Schuller[b], G. Rödel[b], M. Mertig[a,c]

[a] Kurt-Schwabe-Institut Meinsberg, Kurt-Schwabe-Str.4, 04736 Waldheim, Germany
[b] Institut für Genetik, Technische Universität Dresden, 01062 Dresden, Germany
[c] Professur für Physikalische Chemie, Mess-und Sensortechnik, Technische Universität Dresden, 01062 Dresden, Germany

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ABSTRACT

Recently it has been proposed to use sensors based on genetically engineered reporter cells to perform continuous online water monitoring. Here we describe the design, assembly and performance of a novel flow-through device with immobilized genetically modified yeast cells that produce a fluorescent protein upon stimulation with diclofenac whose intensity is then detected by fluorescence microscopy. Although other devices employing immobilized cells for the detection of various analytes have already been described before, as novelty our system allows safe enclosure of the sensor cells, and thus, to obtain fluorescent signals that are not falsified by a loss of cells. Furthermore, the yeast cells are prevented from being released into the environment. Despite the safe containment, the immobilized reporter cells are accessible to nutrients and analytes. They thus have both the ability to grow and respond to the analyte. Both in cell culture medium and standardized synthetic wastewater, we are able to differentiate between diclofenac concentrations in a range from 10 to 100 µM. As particularly interesting feature, we show that only the biologically active fraction of diclofenac is detected. Nowadays, contamination of wastewater with diclofenac and other pharmaceutical residues is becoming a severe problem. Our investigations may pave the way for an easy-to-use and cost-efficient wastewater monitoring method.

1. Introduction

Environmental monitoring of chemicals can be achieved by traditional laboratory analytical methods. While these methods are very precise and sensitive, they require special laboratories, transport of the samples into the laboratory and especially trained staff to perform the analysis. Therefore, such an analysis is usually very expensive, time consuming and has the risk of transport-mediated damage. Whole-cell biosensors employing living, genetically engineered microbial cells (bioreporters) as recognition element could be used to monitor chemicals directly in the environment (Belkin, 2003; Close et al., 2009; Daunert et al., 2000; He et al., 2016; Park et al., 2013; van der Meer and Belkin, 2010). Compared to enzymes, antibodies, receptor proteins and nucleic acids as recognition elements, microbial cells are easy to manipulate and can be cost efficiently produced in large quantities (Park et al., 2013). Furthermore, they offer a higher stability compared to enzymes. Most importantly, genetically engineered microbial whole-cell biosensors allow to indicate the bioavailability of the analyte (Close et al., 2009).

The concept of whole-cell arrays for parallel detection of a variety of different analytes was introduced by van Dyk et al. (2001) and is reviewed by Elad et al. (2008). So far, most of the presented studies in the field of genetically engineered whole-cell biosensors are laboratory proof-of-principle assays, where the whole-cell bioreporters are incubated with the target chemical and the expression of the quantifiable reporter protein is detected by standard laboratory equipment. Yet, only a few platforms provide a solid support for the bioreporter containment, offer the possibility to supply nutrients and analytes to the

* Corresponding author.
E-mail addresses: christine.schirmer@ksi-meinsberg.de (C. Schirmer), juliane.posseckardt@ksi-meinsberg.de (J. Posseckardt), alfred.kick@ksi-meinsberg.de (A. Kick), katrin.rebatschek@ksi-meinsberg.de (K. Rebatschek), wolfgang.fichtner@ksi-meinsberg.de (W. Fichtner), kai.ostermann@tu-dresden.de (K. Ostermann), astrid.schuller@tu-dresden.de (A. Schuller), gerhard.roedel@mailbox.tu-dresden.de (G. Rödel), michael.mertig@ksi-meinsberg.de (M. Mertig).
[1] Authors contributed equally to the results.

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bioreporters by means of microfluidics and include a sensing device (Roggo and van der Meer, 2017). Elad et al. described a flow-through biosensor for online continuous water toxicity monitoring based on poly(dimethylsiloxane) (PDMS) microfluidics incorporating agar-immobilized bioluminescent recombinant reporter bacteria (Elad et al., 2011). Yagur-Kroll et al. presented a flow-cell biosensor for online continuous water monitoring based on a disposable porous aluminum oxide chip (Yagur-Kroll et al., 2015). Buffi et al. trapped agarose beads containing genetically engineered microorganism in a microfluidic PDMS device (Buffi et al., 2011). Cheol Gil and coworkers developed a toxicity sensor based on agarose immobilized *Escherichia coli* (Cheol Gil et al., 2000). In all of these studies, an escape of genetically engineered microorganisms from the microfluidic device cannot be excluded and potentially provokes harmful consequences for the environment. Furthermore, a variation of signal intensity due to a loss of reporter organisms may occur. Hence, the goal of the present work was to design a microfluidic device based on commercially available microfluidic components that (i) serves as a compartment for the genetically engineered bioreporters, (ii) offers microfluidic supply of the bioreporter cells with nutrients and analyte, and (iii) provides the possibility of signal readout by fluorescence microscopy.

In our studies, diclofenac serves as the analyte to be detected by genetically modified yeast cells. The nonsteroidal anti-inflammatory drug (NSAID) diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid, $C_{14}H_{11}C_{12}NO_2$) is administered as oral tablets or as topical gel. Upon oral administration between 60% and 70% of the dose are excreted in the urine and 20–30% in feces as the parent drug or as metabolites (Vieno and Sillanpää, 2014). Measured mean concentrations in municipal waste water vary between 0.11 µg/l and 2.3 µg/l, but can rise up to 203 µg/l in pharmaceutical manufacturer's waste water in South Korea (Vieno and Sillanpää, 2014). Harmful effects to different organisms have been demonstrated at relevant environmental concentration levels (Stülten et al., 2008), for example renal lesions and alteration of the gills in fish at 5 µg/l diclofenac (Pérez-Estrada et al., 2005). Thus, presence of diclofenac in municipal wastewater treatment plants is a severe problem. To date, for the detection of diclofenac concentration different methods have been described. Besides classical analytical methods, such as UV–vis spectrophotometry (Agatonović-Kuštrin et al., 1991), fluorimetry (Damiani et al., 1999), high performance liquid chromatography (Sane et al., 1987), liquid chromatography (Beaulieu et al., 1990), capillary electrophoresis (Jin and Zhang, 2000), LC–MS (Abdel-Hamid et al., 2001), differential scanning calorimetry (Bucci et al., 2000) and nuclear magnetic resonance spectroscopy (Fattah et al., 1988), different electrochemical sensors (Arvand et al., 2012) have been exploited for the determination of diclofenac. Recently, Schuller et al. reported the engineering of *Saccharomyces* (*S.*) *cerevisiae* yeast cells which produce a fluorescent protein upon exposition to diclofenac (Schuller et al., 2017). Here we describe the design of a microfluidic flow-through device with entrapped reporter yeast cells allowing fluorescence protein detection upon addition of an analyte. The device exhibits two separate chambers, enabling simultaneous analysis of analyte and reference, thus offering the possibility to control for matrix effects. The special design of the device includes a cell-impermeable membrane, which allows employing genetically modified reporter cells that should not escape from the sensor into the environment. Furthermore, we demonstrate that loss of reporter cells would lead to diminished fluorescent signals, thus leading to misinterpretation of the analyte concentration. To prove functionality of the flow-through device, the genetically modified diclofenac-sensitive yeast cells designed by Schuller et al. are used (Schuller et al., 2017). We show that the yeast cells cannot escape from the microfluidic device - they are literally inclosed, but can still be supplied with nutrients through a porous membrane. Additionally, we prove that the analyte passes to the yeast cells and induces the production of a fluorescent reporter protein. The development of the relative fluorescence intensity, detected for the cells in the microfluidic device by fluorescence microscopy, is in line with the results of control experiments using flow cytometry.

2. Material and methods

2.1. Growth media, chemicals and genetically modified yeast strains

Transformation of *S. cerevisiae* strain BY4741 [*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*] was carried out as described previously (Schuller et al., 2017). Resulting transformants were: (1) *S.c.* BY4741 p426PDR5::TGFP(1000), which produces turbo green fluorescent protein (TGFP) in the presence of diclofenac, (2) *S.c.* BY4741 p426GPD::TGFP, constitutively expressing TGFP in the presence of glucose, was used as a positive control, and (3) *S.c.* BY4741 p426GPD, which is not able to produce TGFP, served as negative control. Both diclofenac-sensitive and control cells were pre-cultured in selective synthetic complete medium without uracil (SC-ura), containing 6.9 g/l yeast nitrogen base with ammonium sulfate (Formedium, UK), 20 g/l D(+)-glucose (Roth, Germany) and 1.962 g/l Kaiser mix SC complete drop-out: -ura (Formedium, UK). For plating of yeast cells, 2.0% (w/v) agar (Fluka, Germany) has been added to the selective synthetic complete medium. Diclofenac stimulation in the microfluidic device occurred in synthetic minimal medium (MM), containing 6.9 g/l yeast nitrogen base with ammonium sulfate, 20 g/l D(+)-glucose, 60 mg/l L-histidine, 80 mg/l L-leucine and 20 mg/l L-methionine (Roth, Germany). Agarose with low melting gelling temperature (Plaque agarose) was purchased from Biozym (Germany). Ultra-pure water with 0.055 µS/cm (Micro Pure UV/UF, TKA, Germany) was used for the preparation of all solutions. Diclofenac sodium salt (Sigma, Germany) was used for preparation of a 20 mM diclofenac stock solution in absolute ethanol (VWR, France). Dilutions of the diclofenac stock solution were made with absolute ethanol. Synthetic wastewater was prepared on the basis of DIN 38412. For preparation of twofold concentrated synthetic wastewater, 500 mg skimmed milk powder (Gabler-Saliter, Germany), 100 mg peptone (Roth, Germany), 54 mg Na_2HPO_4 (Riedel-de-Häen, Germany), 178.3 mg NH_4Cl (Roth, Germany), 41.7 mg $C_2H_3NaO_2$ (Roth, Germany), 58.4 mg NaCl (VWR, Germany), 14.7 mg $CaCl_2{\cdot}2H_20$ (Fluka, Germany), 20.3 mg $MgCl_2{\cdot}6H_20$ (Roth, Germany), 7.4 mg KCl (Fluka, Germany) were filled to 500 ml with ddH_2O and autoclaved.

2.2. Design and manufacturing of the microfluidic flow-through device

The design of the device is based on a commercially available microscopy cell (MicCell™; GeSiM, Germany) (Gast et al., 2006) with an additional subjacent PDMS layer and an intermediate porous membrane (Fig. 1), as described in the following. A glass coverslip constitutes the bottom of the device, on which a 500 µm thick PDMS-layer frame forming two rectangular chambers (17 mm × 5 mm × 0.5 mm) is placed (Fig. 1a). These "cell" chambers contain the genetically modified yeast cells (Fig. 1b) which are immobilized in agarose. One of the chambers is used for TGFP expressing cells, while the other harbours non-fluorescent control cells. A hydrophilic track-etched membrane with a pore diameter of 1 µm and a thickness of 17 ± 3 µm (Oxyphen, Switzerland) is used to cover the two lower chambers (Fig. 1c). The membrane with an exactly defined pore size and a pore orientation in vertical direction to the membrane surface is permeable for nutrients and the analyte, but impermeable for the yeast cells (average diameter 5 µm). Thus, the genetically modified yeast cells are enclosed in all three dimensions: at the bottom by the glass coverslip, at the sides by the PDMS frame and at the top by the Oxyphen membrane. Supply with medium and analyte is performed from two upper PDMS "supply" chambers with a height of 500 µm, which are placed congruently on the lower chambers (Figs. 1d and SI-1).Fluidic in- and outlets are realized as in the commercially available MicCell™-System.

The masters for manufacturing of both the lower and the upper PDMS chambers were milled from poly(methyl methacrylate) (PMMA). The negative master for molding the upper PDMS chambers can directly

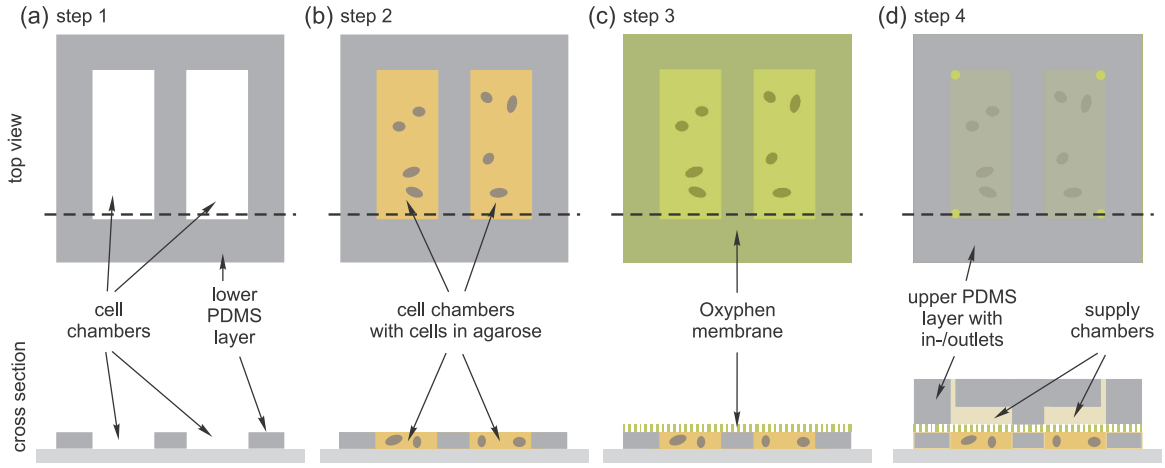

Fig. 1. Schematic graph of the microfluidic device setup for the safe enclosure of genetically modified yeast cells in top view and cross section with the dashed line indicating the cutting edge (dimensions are not in scale and given in the text). A glass slide with a PDMS frame (a) allows the formation of two lower chambers - a measurement chamber and a reference chamber (b) in which the yeast cells are immobilized in agarose. The chambers are covered with an Oxyphen membrane (c). On top of the Oxyphen membrane, a second, upper PDMS frame (d) is positioned congruently with the lower chambers, leading to the formation of two upper chambers for the supply with nutrients and analyte. The upper PDMS frame is supported by an acrylic carrier for the fluidic interconnections (displayed in Fig. SI-1).

be placed into the casting station delivered by GeSiM, while molding is carried out according to the manufacturer´s instructions. In brief, ten parts of Sylgard 184 silicone elastomer were mixed with one part curing agent (Dow Corning, USA), degassed and injected into the assembled casting station including the PMMA master. All inlet and outlet holes were thereby kept open with channel spacers. After overnight curing, the PMMA lid with the attached upper PDMS frame was removed from the casting station. The PMMA master for the lower PDMS frame is a positive master. Firstly, a negative PDMS master was molded from the PMMA master by pouring the PDMS directly into the PMMA master, degassing and curing for 24 h. Then, the resulting negative master was treated with oxygen plasma (Nano-RF-PC, diener electronic) for 1 min (100% O_2, 60 W) and silanized with Trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (Sigma-Aldrich, Germany) from the gaseous phase in a desiccator under lowered pressure for 1 h. The so prepared PDMS master was placed onto an aluminum support, filled with PDMS, degassed and carefully covered with a PMMA lid. The whole arrangement was fixed with screws and cured for 24 h. The curing of the lower PDMS layer in this self-made casting station leads to very planar surfaces of the lower PDMS-layer frame on both sides, ensuring tightness of the assembled flow-through device.

2.3. Assembling of the microfluidic device, culturing of cells within the sensor and diclofenac stimulation

Firstly, PDMS channels, fittings, tubes and syringes were cleaned with ethanol and purified water, and then dried with a stream of synthetic air. Surfaces of both the lower and upper PDMS chambers (Fig. 1a, d) were rendered hydrophilic by treating with oxygen plasma for 1 min (60 W). Diclofenac-sensitive cells and control cells were precultured in SC-ura at 30 °C in a shaker at 180 rpm for 18 h. Cell density was determined by measuring the optical density at 600 nm (OD_{600}) using a NanoDrop spectrophotometer (ThermoScientific, USA). The desired cell number was pelletized (3000 g, 7 min), washed twice with fresh MM without glucose and adjusted to an OD_{600} of 2.0 in twofold concentrated MM (2x MM) without glucose. Cell suspensions were mixed with 1.5% low melt agarose in purified water leading to an OD_{600} of 1.0 in single MM without glucose. This condition prevented the cells from proliferation during the device assembly, thus leading to a well-defined start of the stimulation experiment. Each of the lower chambers of the device was filled with 40 µl of the respective liquid cell-agarose-suspension (Fig. 1a). After solidification, the Oxyphen membrane was placed onto the lower chambers, covered with the upper PDMS chambers, and the whole arrangement was fixed in the MicCell™ support in order to tighten the device. Then it was mounted on an inverted fluorescence microscope (Axio observer.Z1, Carl Zeiss Microimaging, Germany). Standard fittings were placed into the fluidic inlets for connection of the tubes (Upchurch scientific, USA) to the syringe pump (Harvard Apparatus, USA). The tubes and the upper PDMS chambers were filled with MM with glucose. While pumping MM through both upper chambers with a rate of 20 µl/min, cells were allowed to equilibrate in the glucose containing medium for 1 h. Diclofenac stimulation was started by change of the medium pumped through the supply chambers. To this end, different diclofenac concentrations were prepared in either MM or a mixture of 2× MM with 2× synthetic wastewater (leading to a final concentration of 1× MM and 1× synthetic wastewater). The analyte solutions were pumped through the upper chambers with a rate of 20 µl/min for 12 h. Photographs of the device setting mounted on the fluorescence microscope are shown in Fig. SI-1.

2.4. Readout and analysis of optical response

Due to immobilization of the genetically engineered yeasts with agarose, sedimentation of the cells in the device chambers is prevented. In fact, during the whole stimulation experiment, cells were fixed in different z-positions. Since the *PDR5* promoter is leaky, resulting in the production of low TGFP levels even in absence of diclofenac (see Supporting Information, Fig. SI-2), the agarose-embedded yeast cells can be localized at the beginning of the stimulation experiment in the lower chambers by fluorescence microscopy using evolve®–EM 512 camera (Photometrics, USA) mounted on Axio observer-Z1 microscope (Carl Zeiss Microimaging, Germany). Middle z-position was chosen for analysis of TGFP fluorescence intensity during diclofenac stimulation. In each of the two chambers with immobilized yeasts, four different xy-positions were analyzed. Microscopy images of all eight chosen positions were taken every 10 min with 200× magnification and an exposure time of 30 ms using Zeiss optical filter set 10 (Carl Zeiss Microimaging, Germany). Time-series started with the pumping of glucose containing medium and ended 12 h after diclofenac addition. Quantification of fluorescence intensity was carried out with the open source image processing software Image J (Schneider et al., 2012) with the bio-formats plugin (Linkert et al., 2010). Because of the immobilization of the cells in agarose, the number of cells cannot be extracted by a segmentation based algorithm during the entire duration of the experiment. Therefore, the total fluorescence of the recorded

images was analysed. The first five intensity values in time after diclofenac addition were averaged (i_0). The normed fluorescence intensity (i) was calculated as the difference of the measured fluorescence intensity (i_t) at every time point and i_0: $i = i_t - i_0$. Error bars in the diagrams represent the standard deviations of the fluorescence intensities of the four xy-positions in each chamber. For more clearness of the figures, only every third error bar is shown.

2.5. Stimulation experiment in suspension flasks and flow cytometric analysis

After overnight culture in SC-ura at 180 rpm at 30 °C, cell density was determined by measuring OD_{600} and the desired cell number was pelletized (3000 g, 7 min), washed once with fresh MM and adjusted to an OD_{600} of 1.0 in either MM or a mixture of 2× MM with 2× synthetic wastewater (leading to a final concentration of 1× MM and 1× synthetic wastewater) spiked with different diclofenac concentrations. Suspension flasks were cultured at room temperature without shaking in order to have similar conditions like in the microfluidic device arrangement. Every hour, a sample of each cell suspension was taken and TGFP fluorescence (excitation 488 nm; emission filter 525/40 nm) was analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Germany).

In order to determine the number of dead cells, cells were stained with propidium iodide (PI; Sigma-Aldrich, Germany) prior to flow cytometric analysis. Dead cells exhibit reduced membrane integrity allowing PI to penetrate into the cell and intercalate into the DNA, thus showing a red fluorescence (excitation 488 nm; emission filter 690/50 nm). Data are displayed as percentage of TGFP-positive and PI-positive cells, respectively. The mean of three flow cytometric measurements was calculated with error bars representing the respective standard deviation.

3. Results and discussion

3.1. Characterization of nutrient supply and tightness of the flow-through device chambers

A characteristic for the presented device layout is the encapsulation of the bioreporter cells in two lower chambers (Fig. 1c) to ensure that genetically modified yeast cells cannot be released from the flow-through device into the environment. We suppose that loss of yeast cells during the experiment would lead to a diminished fluorescence signal, and thus to a misinterpretation of the analyte (i.e. diclofenac) concentration. Simultaneously, in order to allow transport of nutrients and analyte to the cells, supply chambers are located above the cell chambers (Fig. 1d). Separation of upper and lower chambers is achieved by a membrane which is impermeable for the yeast cells. We first investigated the nutrient transport from the supply chambers to the cells through the track-etched membrane. The simulation of glucose diffusion through the Oxyphen membrane showed that nutrient supply of the sensing cells is sufficient (see Supporting Information, Fig. SI-3). In order to prove this hypothesis experimentally, we followed growth of constitutively fluorescing control cells (*S.c.* BY4741 p426GPD::TGFP) within the device arrangement. Since the cells were seeded in an agarose-medium-mixture without glucose, proliferation can only occur in the presence of glucose which must be supplied from the medium flowing through the upper supply chambers. During budding of the yeast cells, both the mother and the offspring cell fluoresce due to the presence of the plasmid containing the *GPD::TGFP* construct. Indeed, the number of fluorescent yeast cells increased, when glucose containing medium was flowing above the membrane (Fig. 2a upper row). In contrast, no increase in number of fluorescent cells was observed under glucose free conditions (Fig. 2a lower row). Quantification of the development of the fluorescence intensity over time strongly supports these results (Fig. 2b blue and red graphs).

We next investigated whether there is a need for pumping the medium through the upper chambers or if it is sufficient to fill them with glucose containing medium once. In the case of pumping a continuous increase of the TGFP fluorescence intensity is observed. In contrast, there is a reduced rise of fluorescence intensity if the medium is not continuously renewed by pumping (Fig. 2b). Altogether, these results demonstrate that growth of the embedded yeast cells within the device chambers takes place if there is an adequate nutrient delivery through a track-etched membrane by constant renewal of medium in the supply chambers.

In order to prove that the embedded yeast cells do not escape from the cell chambers, we analyzed the liquid which has been pumped through the supply chambers by plating it on an agar plate. For comparison the membrane was not implemented on purpose, i.e. agarose-immobilized cells were not separated from the medium. In the presence of the membrane no yeast colonies could be detected on the agar plates (Fig. 3a) demonstrating that the cells could not escape from the cell chamber. In contrast, a remarkable growth of yeast cells on the agar plates was observed in the absence of the membrane, when the medium was flowing directly over the agarose-immobilized cells (Fig. 3b). Further analysis of the surface of the agarose-immobilized cells by electron microscopy showed that a portion of the immobilized cells is located directly at the surface of the gel layer (Fig. 3c). Upon proliferation of the cells during the experiment, the number of cells at the gel surface increases whereby the cells are growing in groups due to the constraints of the gel (Fig. 3d). Overflowing medium will exert shear stress to the surface-exposed cells and thereby remove some of them from the gel. The membrane between the upper and the lower chambers impedes this cell loss. As expected, the uncontrolled loss of cells leads to a diminished fluorescence signal. Diclofenac-sensitive cells were overflown with medium spiked with diclofenac and fluorescence intensity was analyzed, comparing the normalized fluorescence intensity measured in the setups with (Fig. 3, red line) and without (Fig. 3e, black line) the membrane. In this experiment we noticed a marked decrease of the fluorescence intensity in the chamber lacking the membrane (Fig. 3e). Thus, the safe enclosure of bioreporters in our microfluidic device prevents them from reaching the environment and ensures that the fluorescent signals are not reduced.

3.2. Measurement of sensor activity

To investigate the dependency of fluorescence intensity on diclofenac concentrations, diclofenac-sensitive yeast cells were overflown with medium spiked with different diclofenac concentrations. For comparison, non-fluorescent control cells were examined. The microscopy images showed a slight increase of fluorescing cells over time for unstimulated diclofenac-sensitive cells, whereas a strong increase was seen upon addition of 100 µM diclofenac (Fig. 4a, b). As expected, no increase of the fluorescence intensity was detected in the negative control (Fig. 4c). Since the *PDR5* promoter allows a low basal TGFP production in the absence of diclofenac (see Supporting Information, Fig. SI-2), also unstimulated cells show an increase of fluorescence intensity over time, which is probably due to the increase of the cell number (Fig. 4a). Quantifying the fluorescence intensity, we could observe that the fluorescence intensity was rising with increasing time as well as with the diclofenac concentration (Fig. 4d). This holds true for diclofenac concentrations up to 75 µM, whereas at a concentration of 100 µM, the fluorescence intensity was diminished compared to the intensity at 75 µM (Fig. 4d, e). Van Leeuwen and coworkers showed that high diclofenac concentrations such as 100 µM have an influence on the mitochondrial respiratory chain and inhibit the growth of yeast (van Leeuwen et al., 2011). Therefore, the diminished fluorescence intensity with 100 µM diclofenac may be caused by a reduced cell growth.

In order to verify the described results of the microfluidic sensor experiments, we repeated cell stimulation by diclofenac in suspension

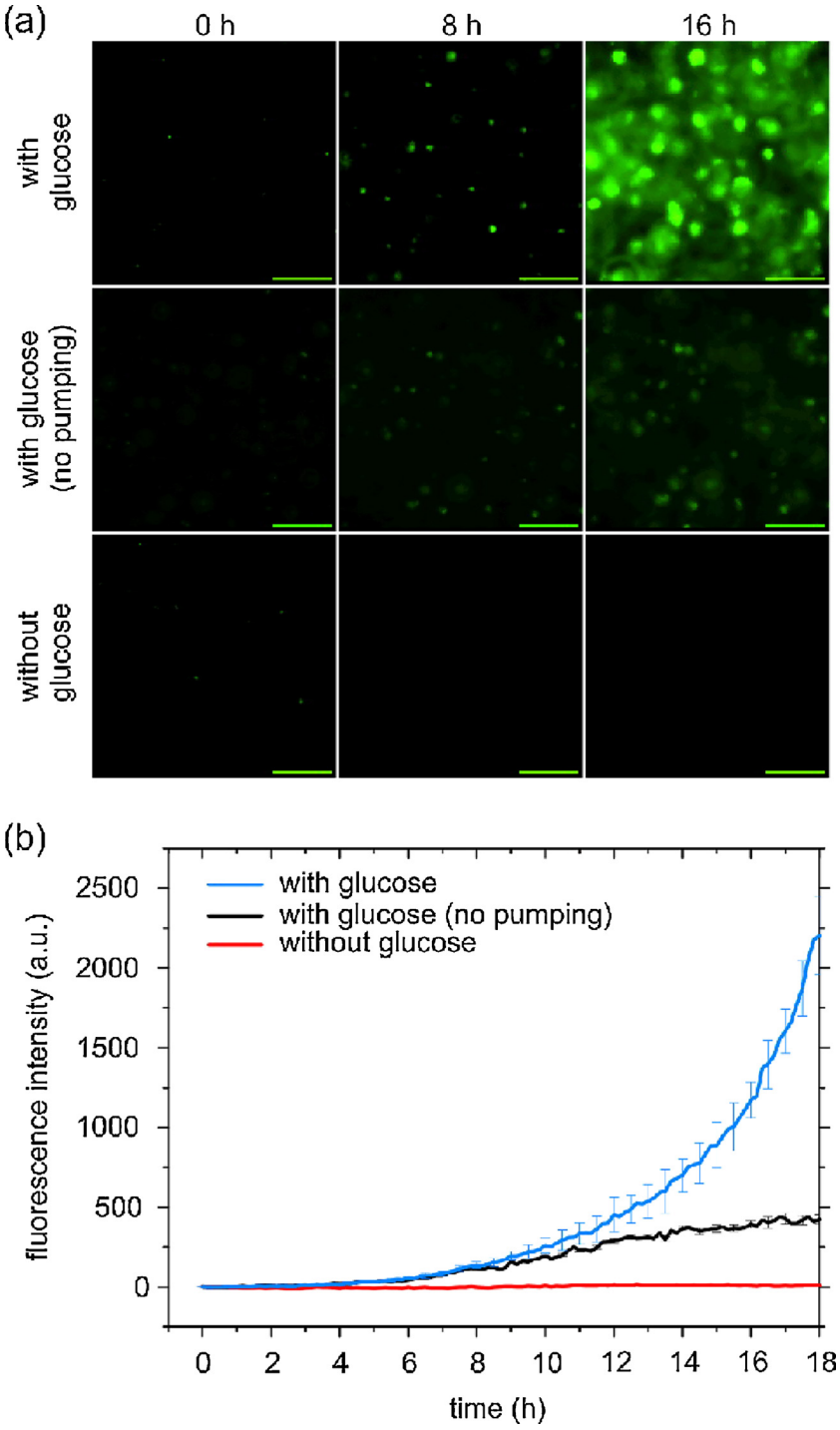

Fig. 2. Nutrient supply of the yeast cells embedded in the sensor. Constitutively fluorescing yeast cells (*S.c.* BY4741 p426GPD::TGFP) were seeded in the lower sensor chambers under glucose free conditions and overflowed with glucose containing (–) or glucose free medium (–). As a control, the upper chamber was filled with glucose containing medium, but not renewed by constant pumping (—). Growth of cells was followed by (a) microscopy images (scale bar = 100 µm) and (b) subsequent analysis of fluorescence intensity. (For interpretation of the references to colour in text, the reader is referred to the web version of this article).

flasks under the same conditions (room temperature, no shaking) and determined the percentage of fluorescing cells by flow cytometry (Fig. 4f, g). Although we surveyed percentage of fluorescing cells rather than the overall fluorescence intensity, we basically observed the same qualitative behavior as obtained in the microfluidic system. The percentage of fluorescing cells increased with increasing diclofenac concentration and time. The only difference is that the percentage of fluorescent cells does only saturate at diclofenac concentration above 50 µM (Fig. 4g), whereas the fluorescence intensity in the microfluidic cell shows a maximum at 75 µM diclofenac (Fig. 4e). We suppose that this observation is caused by the fact that we do not see growth inhibitory effects by flow cytometry. Flow cytometric analyses provide statistics on the portion of fluorescing cells and their respective intensity, but do not allow an accurate counting of the cell number. To obtain results that are completely comparable to those of the microfluidic experiment, one would need to multiply the mean of the fluorescence intensity per cell by the cell number in a defined sample volume.

3.3. Synthetic wastewater

Since a system working on basis of living microorganisms can be disturbed by components of the samples other than the analyte, we studied the microfluidic diclofenac sensor under conditions which are comparable to real wastewater. First we investigated if the yeast cells are perturbed in presence of wastewater samples. In order to have

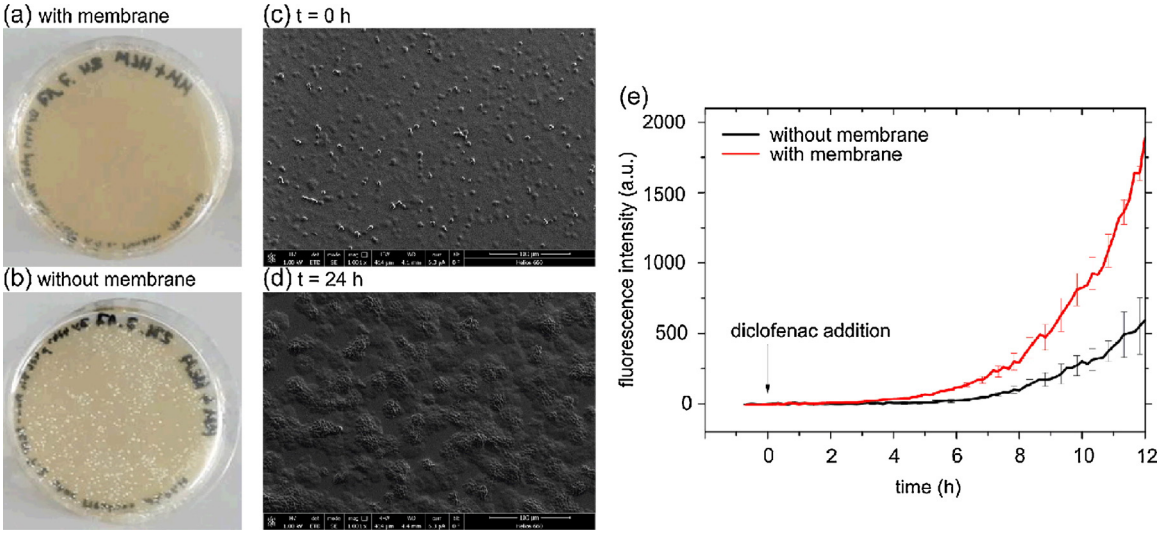

Fig. 3. Enclosure of the cells in the sensor assembly. Diclofenac-sensitive yeast cells (*S.c.* BY4741 p426PDR5::TGFP(1000)) were cultivated in the microfluidic device for 24 h with continuous flow of medium spiked with 100 µM diclofenac through the supply chambers. (a, b) 200 µl of the medium having passed the membrane were plated on an agar plate containing selective synthetic complete medium without uracil. Setups with (a) and without (b) membrane are compared. (c, d) SEM micrographs of the surface of the agarose-immobilized yeast cells before start of medium flow (c; t = 0 h) and after 24 h of overflowing medium (d; t = 24 h). (e) Temporal development of fluorescence intensity in the cell chamber with (–) and without (−) mounted membrane. (For interpretation of the references to colour in text, the reader is referred to the web version of this article).

standardized conditions, we used synthetic wastewater. We prepared synthetic wastewater, mixed it with medium and evaluated the growth of cells and the number of dead cells compared to culture in pure medium. The concentration of all medium components was the same in both preparations. However, some of the ingredients of the synthetic wastewater may serve as additional nutrients to the yeast cells. We could see that the yeast cells grow and survive in presence of synthetic wastewater within the experimental time. Nevertheless, in presence of synthetic wastewater, the cells grow a bit faster and exhibit few more dead cells compared to growth in pure medium (see Supporting Information, Fig. SI-4). We assume that the faster growth in the medium-wastewater-mixture is due to additional nutrients present in the wastewater.

In the microfluidic device we observed that synthetic wastewater spiked with diclofenac led to an increase of the fluorescence intensity, augmenting the diclofenac concentration resulted in a higher fluorescence intensity at the earliest 6 h after start of stimulation (Fig. 5a). Interestingly, compared to the experiments performed in pure medium, a continuous rise of fluorescence intensity with increasing diclofenac concentration could be seen with a maximum at 100 µM diclofenac, the highest concentration investigated (Fig. 5b). We also verified the stimulation of TGFP production by culturing of the cells in suspension flasks in presence of synthetic wastewater and analyzing percentage of fluorescing cells by means of flow cytometry (Fig. 5c, d). High diclofenac concentrations did not lead to a decrease or stagnation of the percentage of fluorescent cells, neither in immobilized cells in the device chambers nor in suspension. We assume that diclofenac binds to components present in synthetic wastewater, e.g. albumin, which is part of the milk powder used for the production of synthetic wastewater. Indeed, it was shown that pharmaceutical agents like diclofenac are easily bound to proteins (Borgå and Borgå, 1997). One can assume that the uptake of protein-bound diclofenac into the cells is hampered, and thus, the biological activity of diclofenac is reduced. Apparently, the yeast cells only recognize the biologically active fraction of diclofenac, which is lower in presence of synthetic wastewater compared to medium, and hence does not inhibit growth of the yeast cells at higher concentrations.

It has to be pointed out that this conclusion is based on the time and concentration dependence of the individual experiments, because comparison of the absolute values between the experiments performed in pure medium (Fig. 4) and in wastewater (Fig. 5) is currently not possible. This is due to the fact that the cells originate from different yeast transformations. The number of plasmids within the cells may vary, and therefore the absolute values of fluorescence may vary as well. This may explain the observation that the absolute values measured for 0 µM diclofenac in medium and wastewater differ (e.g. after 12 h: 28 a.u. in pure medium, and 111 a.u. in wastewater). In an advanced version of the flow-through device, a stable gene number will be achieved by genomic integration of the *PDR5-TGFP*-construct.

Currently our microfluidic device in combination with a fluorescence microscope allows differentiating between diclofenac concentrations in synthetic wastewater in the range from 10 to 100 µM. We were able to detect a minimum of 10 µM diclofenac both in cell culture medium as well as in synthetic wastewater. However, hitherto existing diclofenac sensors possess detection limits as low as 3.4 fM (Azadbakht and Beirnvand, 2017), which is far less than what can be achieved with the genetically engineered yeast cells at the moment. Yet, it was not the aim of our study to engineer a sensor that undercuts that detection limit for diclofenac, but rather develop a microfluidic setup which allows detection of fluorescent proteins expressed by genetically modified yeasts. To our knowledge the here presented microfluidic setup is the first system which allows detection of the bioactive fraction of diclofenac with living microorganisms. In further steps, the scope of application of our microfluidic device can be easily enlarged by substituting the diclofenac-sensitive yeast cells by genetically modified cells that are sensitive to other agents or more sensitive to diclofenac. Moreover, proliferation of constitutively fluorescing cells can also be monitored with this device. The special construction of the microfluidic device with the porous membrane between upper and lower chambers even allows the monitoring of cell suspensions instead of agarose-immobilized cells. Further research is ongoing in order to lower the diclofenac detection limit within this microfluidic setup, e.g. by engineering of the *PDR5* promoter region of the yeast cells (Schuller et al., 2017) or by amplifying the signal via a sensor-actor-system using the yeast pheromone system (Gross et al., 2011).

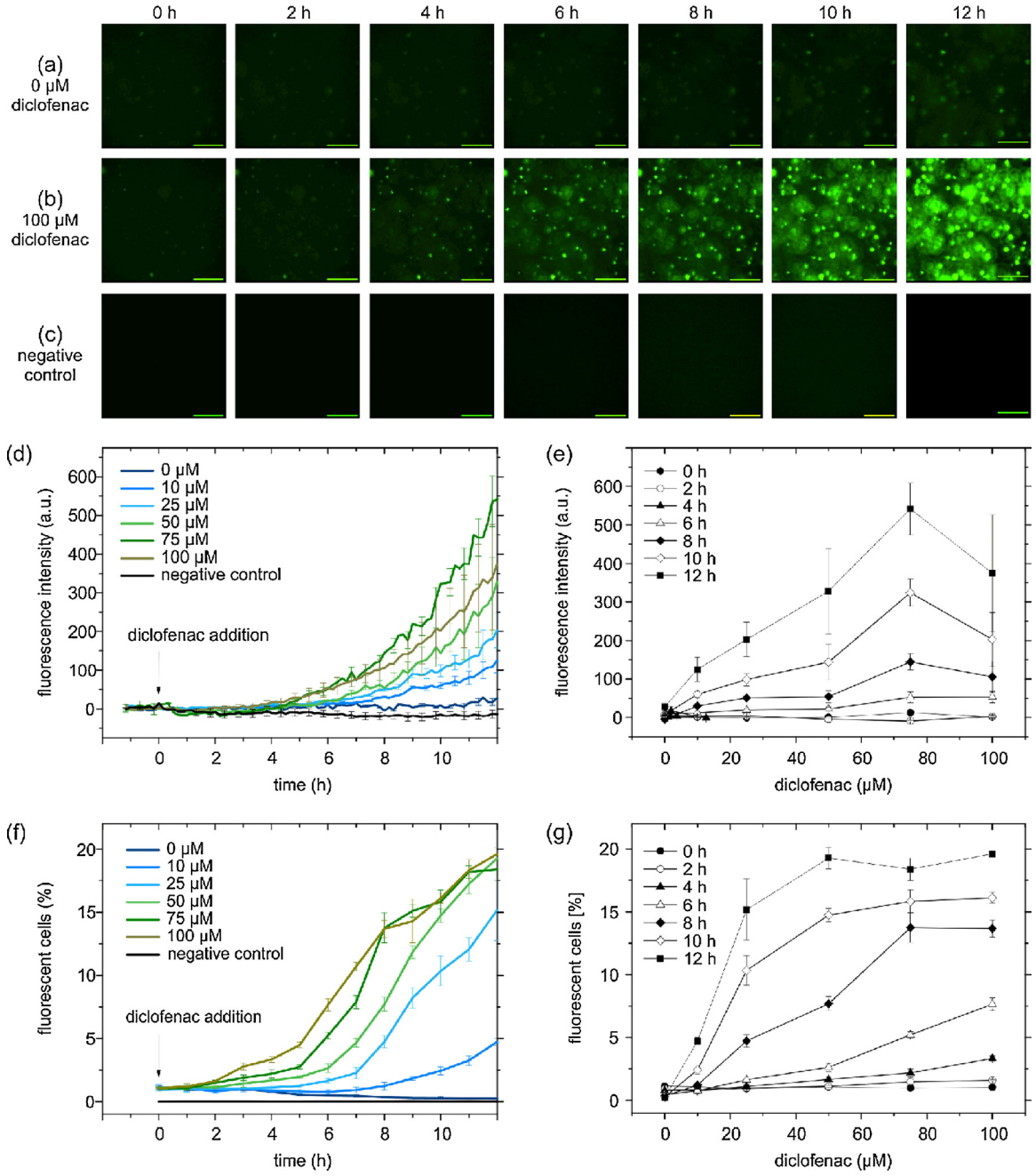

Fig. 4. (a–c) Diclofenac stimulation. Diclofenac-sensitive yeast cells (a, b) and non-fluorescing control cells (c) were immobilized in the lower sensor chambers. Media without diclofenac (a) and with 100 µM diclofenac (b, c) were pumped through the supply chambers for 12 h. Development of fluorescence was followed by microscopy images. Scale bar = 100 µm. (d, e) Change of fluorescence intensity as a function of time and diclofenac concentration. (d, e) Non-fluorescing control cells and diclofenac-sensitive yeast cells were immobilized in the lower sensor chambers. Media spiked with different diclofenac concentrations were pumped through the upper chambers for 12 h. Change of fluorescence intensity is analyzed as a function of time. (e) To verify the stimulation of TGFP production, respective cells were cultured in suspension with different diclofenac concentrations and analyzed by flow cytometry.

4. Conclusions

Several examples for sensors hosting reporter microorganisms for the detection of diverse classes of chemicals have been reported. For a better manageability, the living microorganisms are often immobilized in gel-like matrices (Buffi et al., 2011; Charrier et al., 2011; Elad et al., 2011; Lee et al., 2005; Tani et al., 2004). However, none of the hitherto presented devices allows the safe enclosure of the immobilized microorganisms. Fluid media or samples that overflow the cell-gel-mixture can elute cells out of the measuring chamber, leading to signal loss and misinterpretation of the analyte concentration. In contrast, in the microfluidic device presented here, the embedded cells are protected from shear stress by the introduction of a membrane. It creates a compartment for the genetically engineered bioreporter cells which allows supply with nutrients and analyte coupled with simultaneous signal readout by fluorescence microscopy.

Based on the commercially available MicCell™ system, we developed a microfluidic device with inclosed genetically modified yeast cells, enabling the detection of diclofenac both in cell culture medium and synthetic wastewater. To our knowledge, this microfluidic setup is the first to detect diclofenac with genetically engineered sensing cells. Although lots of analytical methods for the quantification of

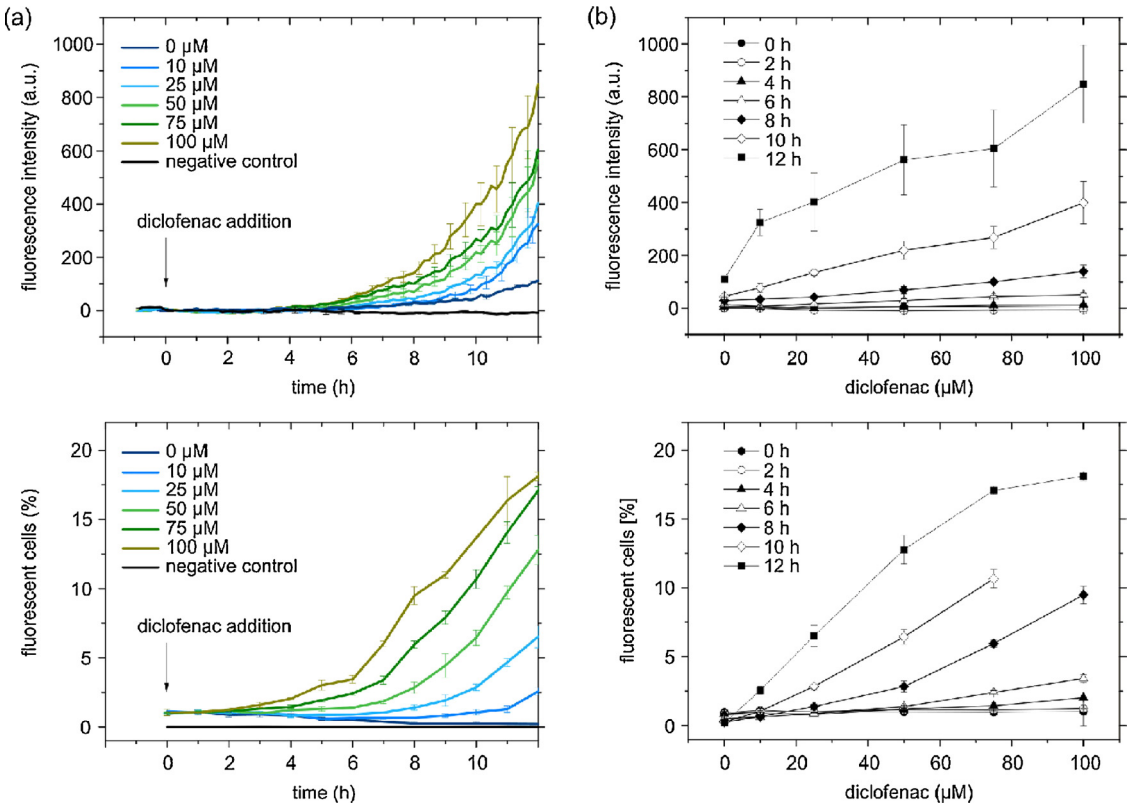

Fig. 5. Detection of diclofenac in synthetic wastewater. (a, b) Non-fluorescing control cells and diclofenac-sensitive yeast cells were immobilized in the lower sensor chambers. Synthetic wastewater spiked with different diclofenac concentrations was pumped through the supply chambers for 12 h. Change of fluorescence intensity is analyzed as a function of time and concentration. (c, d) To verify the data, respective cells were cultured in the presence of synthetic wastewater spiked with different diclofenac concentrations and the percentage of fluorescing cells was analyzed by flow cytometry as a function of time and diclofenac concentration.

environmentally relevant diclofenac concentrations do already exist, none provides information regarding the biologically active portion of the analyte. Moreover, current analyses are time consuming and expensive, as they require specially trained personnel and laboratory equipment. Further research is ongoing in order to develop a small dedicated detection device which will replace the fluorescence microscope and can be easily handled outside the laboratory. Thus, our microfluidic flow-through device forms the basis for a platform for fluorescence detection in living cells which allows an easy-to-handle as well as cost-efficient analysis of diclofenac directly on-site. Moreover, by replacing the diclofenac reporter cells with other genetically modified yeast strains, the scope of applications can be easily enlarged. Thus, our system may delineate a sensor for advanced water monitoring in future.

Declarations of conflicting interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.08.003.

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