

A novel bioluminescent NanoLuc yeast-estrogen screen biosensor (nanoYES) with a compact wireless camera for effect-based detection of endocrine-disrupting chemicals

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Received: 28 July 2017 / Revised: 8 September 2017 / Accepted: 19 September 2017
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Abstract The presence of chemicals with estrogenic activity in surface, groundwater, and drinking water poses serious concerns for potential threats to human health and aquatic life. At present, no sensitive portable devices are available for the rapid monitoring of such contamination. Here, we propose a cell-based mobile platform that exploits a newly developed bioluminescent yeast-estrogen screen (nanoYES) and a low-cost compact camera as light detector. *Saccharomyces cerevisiae* cells were genetically engineered with a yeast codon-optimized variant of NanoLuc luciferase (yNLucP) under the regulation of human estrogen receptor α activation. Ready-to-use 3D-printed cartridges with immobilized cells

were prepared by optimizing a new procedure that enables to produce alginate slices with good reproducibility. A portable device was obtained exploiting a compact camera and wireless connectivity enabling a rapid and quantitative evaluation (1-h incubation at room temperature) of total estrogenic activity in small sample volumes (50 μ L) with a LOD of 0.08 nM for 17 β -estradiol. The developed portable analytical platform was applied for the evaluation of water samples spiked with different chemicals known to have estrogen-like activity. Thanks to the high sensitivity of the newly developed yeast biosensor and the possibility to wireless connect the camera with any smartphone model, the developed configuration is more versatile than previously reported smartphone-based devices, and could find application for on-site analysis of endocrine disruptors.

Published in the topical collection *Microbial Biosensors for Analytical Applications* with guest editor Gérald Thouand

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00216-017-0661-7>) contains supplementary material, which is available to authorized users.

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Keywords Bioluminescence · NanoLuc luciferase · Endocrine disruptors · Effect-based analysis · Estrogenic activity · Yeast-based biosensor

Introduction

The monitoring of micropollutants in the aquatic environment represents both a key technical and regulatory challenge that has been addressed in the EU Directive 2008/105/EC (the Environmental Quality Standards Directive, EQSD), later amended with the Directive 2013/39/EU under the European WFD [1]. In particular, several environmental contaminants are known to affect endocrine functions resulting in adverse health effects in humans and wildlife. These compounds, falling under the umbrella of endocrine-disrupting chemicals (EDCs), interfere

at different levels with the endocrine system, e.g., by binding to the receptors of several hormones (e.g., estrogens, androgens and progestogens, corticosteroids, and thyroid hormones). Different unrelated molecules have been classified as EDCs, including synthetic hormones, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, dibenzofurans, and alkylphenols [2]. This high heterogeneity in chemical structure and physicochemical properties poses significant technical issues for the development of analytical methods and for the identification of a harmonized regulatory framework [3, 4]. Although there are no legal discharge limits in the environment, some micropollutants have been recently prioritized at EU level according to their suspected health risks and to the current unavailability of adequate monitoring methods [5]. In 2015, the Joint Research Center published a technical report with the first Watch List containing the following substances: diclofenac, 17 β -estradiol (E2), Estrone (E1), 17 α -ethinylestradiol (EE2), oxadiazon, methiocarb, 2,6-di-tert-butyl-4-methylphenol, triallate, imidacloprid/thiacloprid/thiamethoxam/clothianidin/acetamiprid, erythromycin/clarithromycin/azithromycin, and 2-ethylhexyl 4-methoxycinnamate. Of these ten substances, three compounds, i.e., E2, E1, and EE2, share the same mechanism of action, i.e., via activation of estrogen receptor α (ER α) [6]. The monitoring of these chemicals is challenging and only expensive and sophisticated laboratory equipment (e.g., mass spectrometry) can provide suitable detection limits for their detection.

An approach complementary to chemical analysis is represented by effect-based analysis, relying on the evaluation of actual biological activity of a sample, measured as the ability to activate receptors or other molecular targets [7–9]. Receptor-mediated effects are generally measured with bioassays or cell-based assays in which cells are re-programmed to express a reporter protein as a consequence of activation of a specific receptor. Cell-based assay have proven highly valuable tools to understand the level of estrogenic contamination in water bodies [3, 10] and for eco-toxicological studies [11, 12]. In particular, assays based on both human cell lines and yeasts have been developed by engineering living cells with the human estrogen α or β receptor, whose activation drives the expression of a reporter protein such as a luciferase or a green fluorescent protein [13–22].

As an alternative to reporter gene technology, [23] achieved detection at sub-ppb levels of estradiol and ppm levels of bisphenol A by engineering *Escherichia coli* cells to express on the surface native estrogen receptors and exploiting impedance. These assays are able to assess the effective biological activity of a sample taking into account mixture effects and even the presence of unidentified and unknown chemicals. Such information is crucial in the analysis of complex samples containing a high number of chemicals, for example to rapidly detect tap water contamination. Other biosensing approaches have been also explored to develop new tools able to measure estrogenic activity in environmental

samples; for example, [24] proposed receptor-based optical biosensors that can be reused for up to 300 sensing cycles.

Among cell-based assays for estrogen-like activity, the most applied are ER-CALUX based on human U2-OS osteosarcoma cell line [25], E-SCREEN based on MCF-7 human breast adenocarcinoma cell line [26], and the yeast estrogen screen (YES) developed by Routledge and Sumpter [27]. In particular, YES assay is based on a recombinant *Saccharomyces cerevisiae* yeast strain expressing the human estrogen receptor hER and a reporter plasmid carrying the reporter gene lac-Z encoding the enzyme β -galactosidase for colorimetric detection.

Despite their widespread use in laboratory settings, these assays have not yet been implemented in portable formats. The availability of new methods enabling the on-site analysis would be an extremely helpful tool for routine screening and for providing a rapid alert in case of accidental disasters in order to rapidly undertake proper countermeasures [6].

We previously reported proof-of-principle devices integrating yeast cells for endocrine disruptors, nevertheless the low bioluminescence (BL) emission of the cells required highly sensitive light detectors such as cooled charge-coupled device (CCD) cameras for astrophotography [28]. We recently reported the obtainment of general toxicity cell biosensors exploiting the smartphone-integrated camera as detector [29, 30]. Yet these biosensors integrated mammalian cell lines, with the well-known limitations related to cell handling and shelf-life. A 3D-printed cartridge was fabricated to integrate cells with a smartphone, simplifying the device itself but limiting its applicability to a single smartphone model. Considering the high number of commercial smartphones and their short lifespan, this represents a serious limitation for real-life applications. Therefore, taking advantage of our experience, we addressed main limitations of previous portable cell biosensors in terms of detectability, universality of the device, and shelf-life of the cells. To increase detectability, we developed a new yeast biosensor by exploiting NanoLuc luciferase [31] as reporter protein, to develop a device of general use we replaced the smartphone with a compact wireless camera that can be connected to any smartphone, and we optimized a novel strategy for obtaining reproducible ready-to-use alginate slices with embedded cells.

Materials and methods

Chemicals and reagents

All the reagents required for yeast cell culture maintenance were from Sigma-Aldrich (St. Louis, MO). Synthetic complete (SC) liquid medium was prepared by adding 6.7 g yeast nitrogen base w/o amino acids, 1.4 g yeast synthetic drop-out medium supplement, 10 mL adenine hemisulfate solution to 1 L of H₂O. The solution was autoclaved and then 40 mL of

glucose 50% w/v solution (0.22 μ m filter sterilized) was added. SC-ura-trp-leu medium was prepared by supplementing SC medium with L-histidine (2 g/L). SC (-Ura-Trp-Leu-His) medium was supplemented with L-histidine (0.02 g/L), L-leucine (0.1 g/L), L-tryptophan (0.02 g/L), and uracil (0.02 g/L). 17 β -estradiol (E2), 17 α -ethynylestradiol (EE2), diethylstilbestrol (DES), estrone (E1), and bisphenol A (BPA) were from Sigma-Aldrich. Dialysis tubing cellulose membrane and the kit for plasmid extraction and purification were from Sigma-Aldrich. FastDigest restriction enzymes, FastAP, and T4 DNA Ligase required for cloning and yeast protein extraction reagent (YPER) were from Thermo Fisher Scientific (Waltham, MA, USA). The bioluminescent substrate furimazine was from Promega (Madison, WI, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Obtainment of NanoLuc estrogen responsive *S. cerevisiae* strain

Yeast expression plasmid pRSII426 and pBEVY-L were from Addgene (Cambridge, Massachusetts, USA). The yeast codon optimized version of the NanoLuc-PEST luciferase (yNLucP) was a kind gift from Prof. C. Andréasson (Stockholm University, Sweden) [32]. The sequence encoding the human estrogen receptor was amplified with polymerase chain reaction (PCR) using pSP72hER α as template and cloned into pBEVY-L (Leu2 marker) vector under the control of constitutive ADH1 promoter, using *KpnI* and *EcoRI* sites. The generated vector was called pBEVY-L-ER α .

The reporter vector was created by cloning, into a pRSII426 plasmid (Ura3 marker), five copies of an ERE response element (-AGGTCAgagTGACCT-) [33] upstream of a minimal cytochrome C promoter (CYCmin) driving the expression of the yNLucP coding sequence, giving the plasmid pRSII426-ERE-yNLucP. The correctness of sequences and vectors was confirmed by restriction analysis and sequencing.

The yeast *S. cerevisiae* BMA64-1A (MATa, ura 3-52, trp1 Δ 2 leu2-3 112his3-11 ade2-1, can1-100) wild-type strain was used as recipient strain [34] and transformed with plasmids pBEVY-L-ER α and pRSII426-ERE-yNLucP using the LiAc/SS-DNA/PEG method [35]. Colonies harboring both vectors were selected in SC-ura-leu plates after incubation at 30 °C for 4 days. Fifteen percent glycerol stocks of the recombinant strain were prepared and stored at -80 °C.

Laboratory-based assay procedure and luminescence measurements

The novel NanoLuc yeast estrogen screen (nanoYES) assay was carried out in a type II laminar flow cabinet to reduce aerosol formation. Before running an assay, a single colony from an agar plate containing the selective medium was used to inoculate

3.0 mL of SC medium. This culture was grown overnight at 30 °C with orbital shaking at 200 rpm in selective SC medium.

Briefly, a 3 mL overnight yeast culture was diluted in fresh SC medium to optical density (OD₆₀₀) of 0.6 and grown for about 4 h until OD₆₀₀ = 1 was reached. Then, 150 μ L of culture was dispensed in 96-well microplates and incubated at 25 °C with different concentrations of E2 (from 0.001 to 100 nM) at 1% ethanol final concentration, for 1 h. Control wells (CTR) were incubated with 1% ethanol final concentration. BL emission kinetics were recorded using a Varioskan Flash multimode reader (5 min with 300 ms integration time) after addition of 50 μ L of an optimized BL substrate containing 10 μ M furimazine diluted in YPER reagent (YPERS-Nano). Light emissions were expressed as relative light units (RLU). The detection limit is defined as the E2 concentration that corresponds to the blank signal plus three times the standard deviation. All experiments were performed in triplicate and repeated at least three times.

Fabrication of the mobile platform 3D-printed cartridges and GoProHero5 adaptors

The compact GoPro HERO 5 video camera (GoPro, Inc., San Mateo, CA, USA) was chosen as light detector. A cartridge of 60 $\times$ 40 mm, 7 mm high, containing an array of 16 square wells (5 mm wide and 5 mm deep each) was created with a desktop 3D printer (Makerbot Replicator 2X) using black and white thermoplastic polymer acrylonitrile butadiene styrene (ABS) (FormFutura, Nijmegen, NL) using the dual extrusion option. The GoProHero5 adaptors and dark-box were printed using black ABS. All pieces were printed at 300 μ m layer resolution, 30% infill.

Preparation of cartridges with immobilized yeast biosensors.

An overnight culture of yeast cells was diluted in 30 mL of fresh medium to optical density (OD₆₀₀) of 0.6 and grown for about 4 h until an OD₆₀₀ = 1 was reached. The culture was centrifuged and resuspended in 3 mL SC-ura-leu medium containing 10% trehalose and 1.5% sodium alginate.

This mixture was then poured into a dialysis tubing cellulose membrane (avg. flat width 10 mm, molecular weight cut-off = 14 kDa) and immersed in a 0.2 M CaCl₂ solution for 1 h at room temperature (25 °C) to allow the formation of the gel inside the membrane. Using a 3D-printed “microtome-like” device, that has a slot for a surgical blade placed at 2 mm from the edge, the obtained gel (about 12 cm length, avg. diameter 5 mm) was repeatedly cut in several slices, then placed into the wells of the 3D-printed cartridges. A 50 μ L volume of SC medium was added to each well and the cartridges were covered with Parafilm M® and stored at 4 °C until use.

The stability of the yeast biosensor kept at 4 °C was daily tested by incubating the cells in duplicate with 10 nM E2 (50 µL) for 1-h incubation at room temperature; a 50 µL volume of YPER-Nano substrate was added to each well and image was acquired with the GoProHero5 in night mode (30 s, ISO 800) equipped with the 3D-printed black-box accessory. Images were analyzed with ImageJ software and data plotted using GraphPad Prism v.5 (GraphPad Software, Inc. La Jolla, CA). BL emission was normalized with respect to BL signal obtained at day 0 (freshly immobilized cells). All measurements were performed in duplicate and repeated at least three times with different cell cartridges.

Analytical performance of the GoPro-based yeast estrogen screen

The analytical performance of the developed platform was performed by incubating yeast estrogen biosensors with increasing concentrations (from 0.05 to 10 nM) of E2, selected as model estrogenic analyte. Briefly, a cell-cartridge containing immobilized yeast cells stored at 4 °C was equilibrated at room temperature for 15 min, then a 50 µL volume of E2 dilutions (1% ethanol final concentration) were added in duplicate wells. Control wells were incubated with 1% ethanol final concentration (50 µL). After 1-h incubation at room temperature, a 50 µL volume of YPER-Nano substrate was added to each well, then the cartridge was inserted into the 3D-printed black-box accessory, and BL emission was acquired with the GoProHero5 in night mode (30 s, ISO 800). Images were quantified with ImageJ software by selecting a square region of interest (ROI) around each well and measuring the BL emission of duplicate wells. E2 dose-response curves were obtained by calculating the fold response with respect to control and plotted using GraphPad Prism. Non-linear regression was performed by fitting the experimental data using a four-parameters sigmoidal curve, then the EC₅₀ value for E2 was calculated as the effective concentration which produces the midpoint *y* value (50%) of the dose-response curve. All measurements were performed in duplicate and repeated three times with different cell cartridges.

Yeast-estrogen cartridge configuration for effect-based analysis

To obtain yeast-estrogen biosensors for real sample analysis, easy-to-use cartridges with immobilized cells were prepared as follows: half of the cartridge was used to test in duplicate the yeast bioreporter response to 0.5, 1, and 5 nM E2 or 1% EtOH as control, while the remaining wells were incubated in duplicate with tap water samples (25 µL) spiked with different concentrations of diethylstilbestrol (0.1 and 10 nM) and bisphenol A (10 nM). Sample wells were also co-incubated with 0.5 nM E2 (25 µL). Dilution of estrogenic compounds

were prepared to provide a 1% ethanol final concentration in every well.

Cartridges were incubated for 1 h at room temperature, then a 50 µL volume of YPER-Nano substrate was added to each well and BL emission was acquired with the GoProHero5 as described above. Images were quantified with ImageJ software by selecting a square ROI around each well and measuring the mean BL emission of duplicate wells. BL signal were then normalized with respect to 0.5 nM E2 (selected as reference and set to 100%) and plotted using GraphPad Prism. All measurements were performed in duplicate and repeated three times with different cell cartridges.

Results and discussion

The possibility to use the smartphone camera to detect the BL emission from living whole-cell biosensors has been previously demonstrated by us exploiting genetically engineered mammalian cell lines [29, 30]. Despite adequate analytical performance, one of the main limitations of this approach is surely related to the short shelf life of mammalian cells when maintained outside an incubator with controlled temperature and humidity. In addition, considering the short life span of smartphones, mainly due to short battery life and software updates changes, the general applicability of such devices is questionable with the necessity of upgrading and fabricating new devices, with subsequent assay optimization and calibration, for each smartphone model.

In this scenario, a mobile platform based on the use of a compact camera, such as the GoProHero camera, and robust yeast cells as living biosensors could represent a suitable solution to overcome these issues. Indeed, yeast cells are particularly suitable for the development of whole-cell biosensors integrated into a portable mobile platform as they provide the analytical robustness typical of bacterial cells, with the possibility to express functional human receptors and regulatory elements to obtain predictive information about actual biological activity of samples [36].

The GoProHero5, optimized for sport and outdoor activities, represents a robust, waterproof CMOS camera (12 MP, UHD 4K) which can be directly controlled using the built-in touch-screen display or can be connected to any smartphone via dedicated GoPro-App (paired wireless network), making it a very versatile light sensor for the development of portable devices. In addition, the long exposure time (up to 30 s at ISO 800) makes this camera a powerful choice for low-light imaging applications, including bioluminescence measurements.

Development of a novel yeast-estrogen screen exploiting NanoLuc luciferase (nanoYES)

The novel yeast-estrogen strain (Fig. 1a) was obtained by genetically engineering *S. cerevisiae* cells with two vectors: (i) a plasmid for the expression of the human estrogen receptor α (hER α) under the control of the constitutive ADH1 promoter and (ii) a reporter plasmid containing five copies of an ERE response element (-AGGTCAgagTGACCT-) upstream of a minimal cytochrome C promoter (CYCmin) which drives the expression of the yeast codon-optimized NanoLuc luciferase coding sequence (yNLucP). Both vectors contain the 2 μ origin, thus ensuring consistent replication of the two plasmids during cell division and maintaining each plasmid at about 50 copies/cell. Due to the high copy number, the expression of the human estrogen receptor was placed under a weak promoter to avoid strong overexpression that may lead to artifacts in the biosensor response. Indeed, the high copy number of reporter vector allows consistent production of yNLucP reporter enzyme upon induction.

The small size (19 kDa) and the absence of post-translational modifications and disulfide bonds of NanoLuc luciferase enable its rapid synthesis and folding, thus reducing total assay time; moreover, the use of its destabilized version provides a half-life of 5 min in yeast, compared to 40 min of the yNluc [32], thus faithfully reflecting mRNA levels. Yeast cells were sequentially transformed with the two plasmids and single colonies screened for their responsiveness with 10 nM E2. A new BL substrate composition was formulated to optimize BL emission in yeast cells. The NanoGlo® substrate (containing a lysis buffer optimized for mammalian cells) showed suitable for yeast cells [32]. However, to increase light output, we formulated an alternative substrate by diluting furimazine (10 μ M) in YPER buffer, a specific yeast-cell lysis reagent used for the extraction of functionally active solubilized proteins from yeast. The use of this formulation (YPER-Nano) provides both an increased BL emission (20%) and more stable emission kinetics (signal half-life > 20 min) compared to NanoGlo® substrate or furimazine alone (signal half-life < 2 min). Dose-response curve for E2 (0.001–100 nM) were obtained using liquid cultures of three positive clones in 96-well microplate format and benchtop luminometer. The yeast-estrogen biosensor incubated for 1 h with different concentrations of E2 shows a limit of detection of 0.010 ± 0.002 nM and an EC₅₀ of 0.6 ± 0.1 nM (Fig. 1b). The nanoYES shows a LOQ of 0.020 ± 0.005 nM E2 and mean recovery rate of $93 \pm 11\%$. The nanoYES response to other estrogenic compounds was also evaluated (Fig. 1b) and corresponding limit of detection and EC₅₀s are shown in Table 1. The developed yeast estrogen bioassay shows comparable results in terms of ranking of estrogenic activity (E2 > EE2 > DES > E1) and EC₅₀ values, obtained by previous works based on recombinant yeast cells [37, 38].

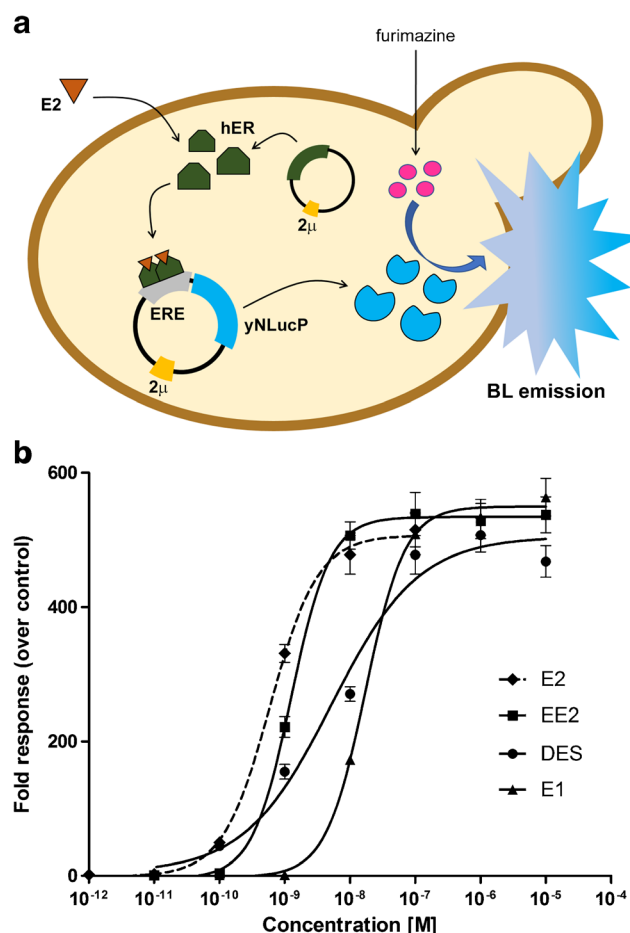


Fig. 1 **a** Schematic representation of the bioluminescent yeast-estrogen strain. *S. cerevisiae* cells were transformed with a plasmid for the expression of the human estrogen receptor α (hER α) under the control of the weak constitutive ADH1 promoter. Cells were also transformed with a vector containing five copies of estrogen response element (ERE) and the cDNA encoding for the yeast codon optimized NanoLuc luciferase destabilized variant (yNLucP). Both vectors contain a 2 μ replication origin for their propagation during yeast growth. The binding of estrogens such as 17 β -estradiol (E2) to hER α activates the intracellular signaling pathway (receptor dimerization) which leads to the expression of yNLucP luciferase. Light emission is obtained after addition of an optimized substrate solution containing furimazine (2-furanylmethyl-deoxy-coelenterazine). **b** Dose-response curves for different estrogenic compounds (E2: 17 β -estradiol; EE2: 17 α -ethinylestradiol; DES: diethylstilbestrol; E1: estrone) obtained using the nanoYES performed in 96-well microplate format and benchtop instrumentation. Data represent the mean values $\pm$ the standard deviation (SD) obtained with three replicates and repeating the experiments three times

Design and 3D-printing fabrication of the mobile platform based on GoPro Hero camera

The mobile platform was designed to create an all-in-one device based on the GoPro Hero5 camera (see Electronic Supplementary Material (ESM) Fig. S1). A series of modular adaptors were fabricated with black ABS using a desktop 3D printer, providing a dark box of compact size (65 $\times$ 65 mm,

Table 1 nanoYES response to estrogenic compounds, obtained using liquid cultures, in 96-well plate format and benchtop luminometer

Compound	LOD (nM)	EC50 (nM)
17 β -estradiol (E2)	0.010 $\pm$ 0.002	0.6 $\pm$ 0.1
17 α -ethynylestradiol (EE2)	0.05 $\pm$ 0.01	1.2 $\pm$ 0.3
Diethylstilbestrol (DES)	0.019 $\pm$ 0.005	5.2 $\pm$ 0.5
Estrone (E1)	0.5 $\pm$ 0.1	17 $\pm$ 2

60 mm height) for the acquisition of BL emission. This accessory also includes a slot to insert a 3D-printed cell-cartridge containing the immobilized nanoYES. The multi-well cartridges (60 $\times$ 40 mm), containing 16 wells of 5 $\times$ 5 mm (150 μ L volume each), were printed with white and black ABS using the dual extrusion option provided by the Makerbot Replicator 2X. In particular, the bottom of the wells was printed using white ABS, while black ABS was used for the remaining parts. This configuration allowed to increase the intensity of acquired BL signal by reflection while avoiding crosstalk between adjacent wells, outperforming cartridges printed with only white or black ABS that suffered of higher crosstalk and lower signals, respectively.

Immobilization procedure and stability of nanoYES

To obtain ready-to-use cartridges, the nanoYES was immobilized into alginate slices. Cells at OD = 1 (about 1.8×10^7 cells/mL) were tenfold concentrated in culture medium to achieve a sufficient BL emission for a sensitive detection with the GoPro camera. The immobilization medium also contains 10% *w/v* trehalose to increase the shelf life of yeast cells [39].

A straightforward procedure was developed to generate yeast-alginate slices of defined and reproducible dimension (2.1 ± 0.2 mm) (see ESM Fig. S2).

To evaluate the reproducibility of immobilized nanoYES, a whole cell-cartridge (Fig. 2a) was incubated with 10 nM E2 for 1 h, and BL image acquired with the GoPro camera (Fig. 2b). The quantification of BL emission of 16 slices provided a coefficient of variation (CV%) of 11%, which is comparable to previously reported whole-cell bioassays [40].

The efficacy of our procedure was compared to conventional method for obtaining alginate beads. A 50 μ L volume nanoYES/alginate mixture, containing the same cell number for each slice, was added dropwise into a CaCl₂ solution and let to harden for 1 h. Individual beads of about 4.5 ± 0.3 mm diameter were obtained, placed into the cell-cartridge wells, and treated as for the slices, with 10 nM E2 for 1 h. The mean BL signal using alginate beads is 8.5 times lower and less reproducible than those obtained with slices, most probably due to a slower and non-uniform distribution of analyte and BL substrate inside the beads (see ESM Fig. S3).

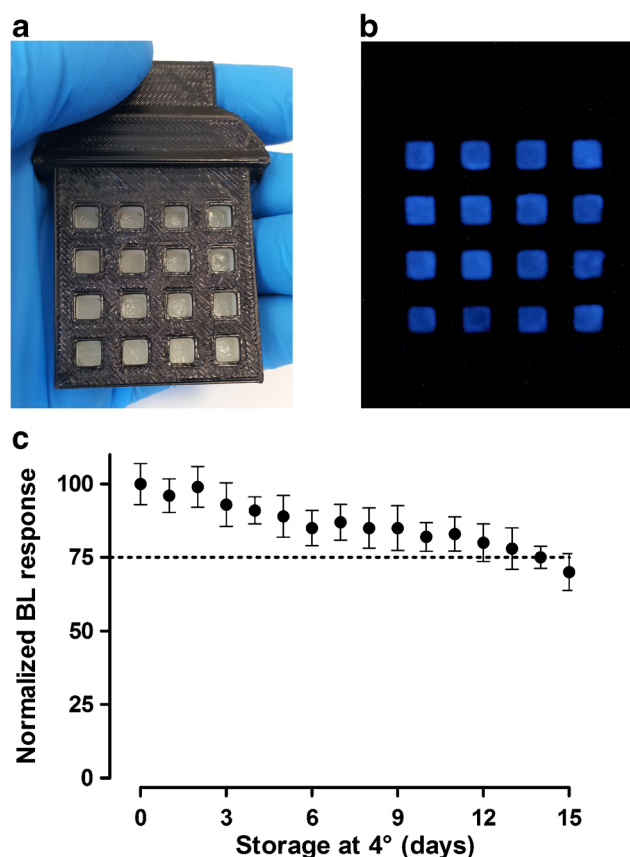


Fig. 2 **a** Picture of a 3D-printed cartridge containing 16 alginate slices of yeast-estrogen bioreporters. **b** BL image of 16 yeast-estrogen biosensors slices induced with 10 nM E2, acquired with the GoProHero5 camera in night mode (30 s at ISO 800), after addition of 50 μ L YPERNano solution. **c** Responsiveness of immobilized yeast-bioreporters stored at 4 °C. Each day, duplicate wells were treated with 1 nM E2 and BL emissions were acquired with the GoPro camera. BL signals are normalized with respect to day 0 (freshly immobilized cells)

The stability and responsiveness of yeast-estrogen bioreporters immobilized into alginate slices stored at 4 °C in the 3D-printed cartridges was evaluated. Each day, duplicate wells were incubated with 1 nM E2 for 1 h and BL images were acquired with the GoPro camera. As shown in Fig. 2c, the nanoYES response was consistently maintained within 7 days (85% of initial response at day 7) and even after 2 weeks, the nanoYES maintains about 70% of the responsiveness obtained at day 0 (freshly immobilized cells). To guarantee good analytical performance in terms of LOD and sensitivity, we decided to use cell-cartridges not older than 10 days, which still provide a BL emission over the 75% of initial response (arbitrarily selected threshold).

Analytical performance of GoPro-based nanoYES

The assay in optimized conditions consists of incubation of 50 μ L of E2 dilutions (concentration range from 0.05 to 10 nM) per each cartridge well containing (about 9.0×10^5

cells/slice) for 1 h at room temperature. Cell cartridge is then imaged with the GoProHero5 camera in night mode for 30 s at ISO 800, after addition of 50 μ L YPER-Nano substrate (Fig. 3a). Since cells are lysed during BL emission acquisition, the cartridges are single-use. Figure 3b shows a detailed BL image corresponding to the dose-response curve for E2 acquired 10 min after substrate addition. Dose-response curves obtained with three different cartridges showed a LOD of 0.08 ± 0.02 nM and EC₅₀ of 0.7 ± 0.1 nM E2 (Fig. 3c). In the GoPro-based platform, the limit of detection is about one order of magnitude higher compared to those obtained with the nanoYES using conventional benchtop luminometer while the EC₅₀ is comparable (0.6 ± 0.2 nM E2). Conversely, the LOD is comparable to other previously reported yeast estrogen bioassays, such as those reported by Leskinen et al. (0.03 nM for E2) [15]. Also, the EC₅₀ value is consistent with those obtained with similar yeast-based screening assays performed in laboratory settings, such as the conventional YES (EC₅₀: 0.32 nM) [7]. These results confirmed the suitability of this configuration for the straightforward and quantitative detection of estrogenic activity in water samples.

GoPro-based nanoYES: analysis of spiked samples

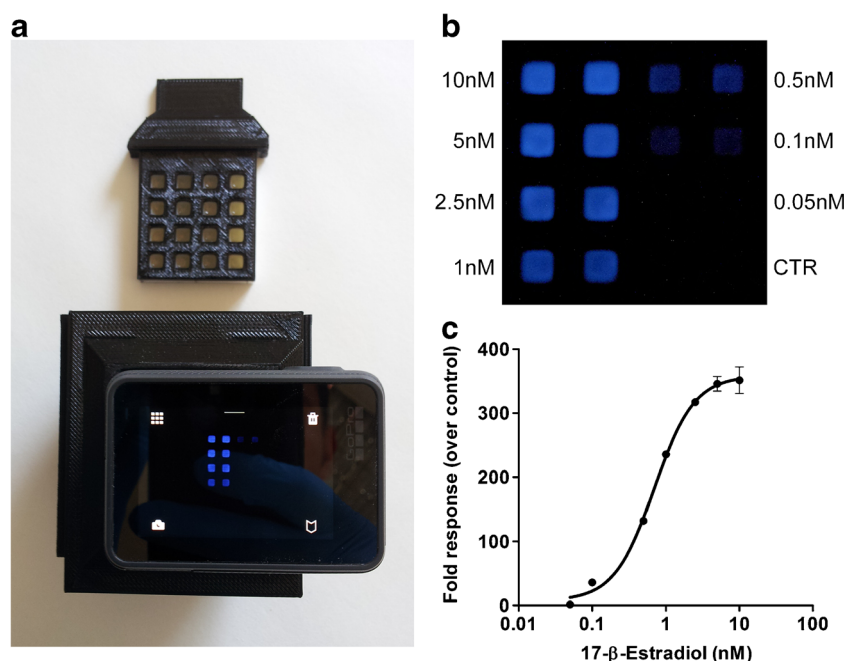
As a proof of concept, tap water samples spiked with different concentrations of diethylstilbestrol (DES) and bisphenol A (BPA) were analyzed with the GoPro-based nanoYES to explore its actual feasibility for on-site testing, especially for monitoring of sites affected by high pollution such as downstream of industrial manufacturing plants and agricultural areas.

BPA was selected because of its ability to act as weak ER binder and interfere with the endogenous E2, causing severe effects on the reproductive system [41, 42] while DES is a synthetic nonsteroidal estrogen widely encountered in influents and effluents from municipal water treatment plants with concentrations levels in the range of 4–12 ng/L for primary influents [43, 44]. BPA is present at relatively high concentrations in several site areas, for example according to a recent review BPA concentrations in groundwater vary between 1 ng/L and 20 μ g/L [45]. Each disposable cartridge also contains a calibration curve obtained by inducing the nanoYES with 0.5, 1, and 5 nM E2 allowing a rapid evaluation and subsequent interpolation of estrogenic activity of the samples (Fig. 4a). Control wells (CTR) were also included and incubated with 1% EtOH final concentration. Sample-wells were also induced with 0.5 nM E2, a concentration near the EC₅₀, to evaluate both estrogenic and anti-estrogenic activity of samples.

Mean BL emission of each sample was quantified and normalized with respect to 0.5 nM E2, set to 100% (Fig. 4b). Sample no. 1, which contains 10 nM BPA (simulating a high contaminated sample), shows a remarkable decrease (35%) of BL emission. At this concentration, BPA (a weak hER α binder) interferes with the transactivation induced by 0.5 nM E2, thus decreasing BL emission. Acting as partial agonist, BPA competes with E2 (full agonist) for receptor occupancy, thus producing a net decrease in the receptor activation compared to that observed with the E2 alone.

Sample no. 2, which contains a very high concentration of a potent estrogen (10 nM DES), shows a remarkable increase compared to 0.5 nM E2 and was selected to simulate a strong estrogenic effect as in samples containing mixtures of

Fig. 3 **a** Picture of a typical yeast estrogen assay performed by incubating the cell-cartridge containing immobilized yeast bioreporters with 50 μ L of sample and by acquiring BL emission with a GoProHero5 camera in night mode (30 s at ISO 800), after addition of 50 μ L YPERNano solution. **b** BL image obtained by incubating the yeast-bioreporters with 17 β -estradiol (concentration range from 0.05 to 10 nM) and **c** corresponding dose-response curve for 17 β -estradiol obtained after quantification of BL emission with ImageJ software. Data are plotted as fold response with respect to control (1% EtOH)



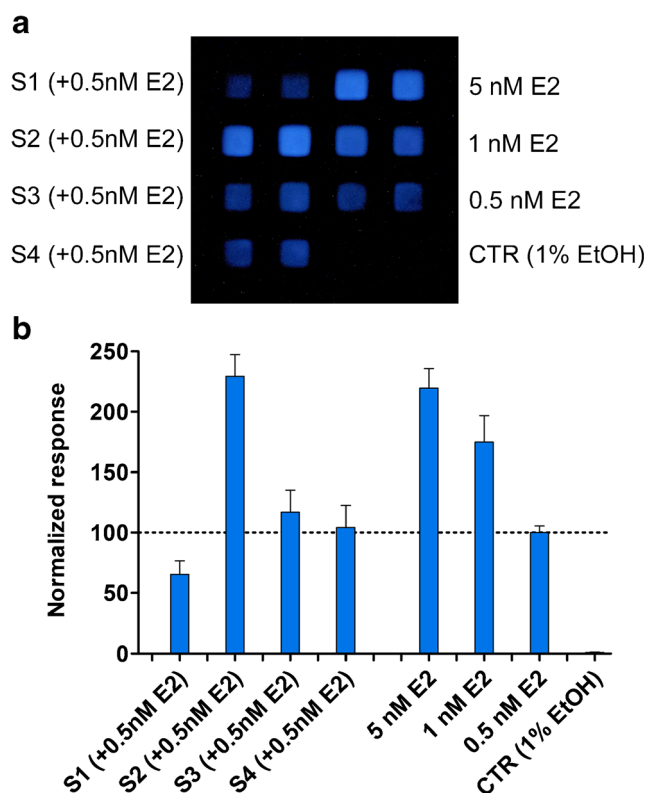


Fig. 4 **a** BL Image of a proof-of-concept analysis of spiked water samples and **b** quantitative elaboration where BL emissions were normalized with respect to 0.5 nM E2 set at 100. The configuration of the cell-cartridge allows to test the estrogenic activity of four samples in duplicate. The response of nanoYES to 0.5, 1, and 5 nM E2 was also included in each cartridge, allowing a quick evaluation and quantification of estrogenic activity. Sample wells are co-incubated with 0.5 nM E2 to detect both pro- and anti-estrogenic activity. As a proof-of-concept, three tap water samples were analyzed: sample no. 1 (S1) was spiked with 10 nM bisphenol A (BPA) shows anti-estrogenic activity. BPA (which does not produce a detectable BL signal at this concentration) is able to bind to the estrogen receptor thus interfering with the transactivation induced by 0.5 nM E2. Sample no. 2 (S2) which contains 10 nM diethylstilbestrol (DES) shows a remarkable increase compared to 0.5 nM E2 (comparable to BL signal obtained in the presence of 5 nM E2). Sample no. 3 (S3) was spiked with 0.1 nM diethylstilbestrol (DES); this concentration does not produce any estrogenic effect. Sample no. 4 (S4) contains only 50 μ L tap water and has no estrogenic effect. All measurements were performed in duplicate and repeated with three different cell cartridges

estrogenic compounds. Sample no. 3, spiked with 0.1 nM DES (whose concentration does not produce a significantly increased BL signal), and sample no. 4, containing only tap water, do not show any alteration with respect to 0.5 nM E2-induced transactivation.

It must be pointed out that the envisaged application of the platform is for rapid monitoring of effluents of wastewater and critical areas, such as agricultural and industrial sites, in which a high concentration of EDCs is expected. Moreover, future studies will be aimed at evaluating the analytical performance of the nanoYES in the presence of EDCs mixtures.

Conclusion

In this work, we proposed a mobile platform for effect-based analysis of endocrine disruptors, based on bioluminescent yeast estrogen biosensors and a compact wireless camera as light detector.

The newly developed yeast estrogen biosensors exploit a yeast codon optimized variant of Nanoluc luciferase to achieve rapid and sensitive detection of estrogen-like compounds within 1 h. A straightforward procedure to immobilize the yeast biosensors into 3D-printed cartridges was also developed, obtaining reproducible ready-to-use disposable cartridges that can be stored for 10 days at 4 °C without significant decrease in analytical performance.

The GoPro camera proved suitable for the sensitive detection of light emission from bioluminescent yeast biosensors, using the night mode setup with 30-s integration time. In addition, thanks to the possibility to connect this sensor with any smartphone model via dedicated GoProApp, the developed configuration results in a more standardized and versatile platform, compared to smartphone-based devices. A custom application (APP) running on different operative systems could be developed to either provide instructions to the user and for the quantification of BL images to obtain immediate results about estrogenic activity of samples.

In the proposed configuration, the mobile platform, containing robust microbial bioluminescent whole-cell bioreporters, allows a rapid evaluation of estrogenic activity in water samples and could find application for on-site analysis of EDCs.

Acknowledgements This research was sponsored in part by PRIN 2015 “Securing and ensuring sustainable use of agriculture waste, co- and by-products: an integrated analytical approach combining mass spectrometry with health effect-based biosensing” (Prot. 2015FFY97L), PRIN 2015 “Multifunctional nanotools for advanced cancer diagnostics” (Prot. 2015TWP83Z), and the NATO Science for Peace and Security Programme under Grant No. 985042.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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