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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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2  
3 **A novel bioluminescent NanoLuc yeast-estrogen screen biosensor (nanoYES) with**  
4  
5 **a compact wireless camera for effect-based detection of endocrine disrupting**  
6  
7 **chemicals**  
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10  
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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. *S. cerevisiae* cells were genetically engineered with a yeast codon-optimized variant of NanoLuc luciferase (yNLucP) under the regulation of human estrogen receptor  $\alpha$  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  $\mu$ L) with a LOD of 0.08 nM for 17  $\beta$  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.

**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 an 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 $\beta$ -estradiol (E2), Estrone (E1), 17 $\alpha$ -ethinylestradiol (EE2), oxadiazon, methiocarb, 2,6-ditert-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  $\alpha$  (ER $\alpha$ ) [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.

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3 An approach complementary to chemical analysis is represented by effect-based analysis, relying  
4 on the evaluation of actual biological activity of a sample, measured as the ability to activate  
5 receptors or other molecular targets [7-9]. Receptor-mediated effects are generally measured with  
6  
7 bioassays or cell-based assays in which cells are re-programmed to express a reporter protein as a  
8  
9 consequence of activation of a specific receptor. Cell-based assays have proven highly valuable tools  
10  
11 to understand the level of estrogenic contamination in water bodies [3,10] and for eco-toxicological  
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13 studies [11-12]. In particular, assays based on both human cell lines and yeasts have been developed  
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15 by engineering living cells with the human estrogen  $\alpha$  or  $\beta$  receptor, whose activation drives the  
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21 expression of a reporter protein such as a luciferase or a green fluorescent protein [13-22]-15-..

22  
23 As an alternative to reporter gene technology [23] achieved detection at sub-ppb levels of estradiol  
24 and ppm levels of bisphenol A by engineering *E. coli* cells to express on the surface native estrogen  
25 receptors and exploiting impedance. These assays are able to assess the effective biological activity  
26  
27 of a sample taking into account mixture effects and even the presence of unidentified and unknown  
28  
29 chemicals. Such information is crucial in the analysis of complex samples containing a high number  
30  
31 of chemicals, for example to rapidly detect tap water contamination. Other biosensing approaches  
32 have been also explored to develop new tools able to measure estrogenic activity in environmental  
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34 samples; for example [24] proposed receptor-based optical biosensors that can be reused for up to  
35  
36 300 sensing cycles.

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39 Amongst cell-based assays for estrogen-like activity, the most applied are ER-CALUX based on  
40  
41 human U2-OS osteosarcoma cell line [25-46], E-SCREEN based on MCF-7 human breast  
42  
43 adenocarcinoma cell line [26-47], and the yeast estrogen screen (YES) developed by Routledge and  
44  
45 Sumpter [27-48]. In particular YES assay is based on a recombinant *Saccharomyces cerevisiae*  
46  
47 yeast strain of the which contains a gene for the expression expressing of the human estrogen  
48  
49 receptor hER and a reporter plasmid carrying the reporter gene lac-Z encoding the enzyme  $\beta$ -  
50  
51 galactosidase for ....colorimetric detection.  
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3 Despite their widespread use in laboratory settings, these assays have been not yet implemented in  
4 portable formats. The availability of new methods enabling the on-site analysis would be an  
5 extremely helpful tool for routine screening and for providing a rapid alert in case of accidental  
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Despite their widespread use in laboratory settings, these assays have been not yet 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 [2819]. We recently reported the obtainment of general toxicity cell biosensors exploiting the smartphone-integrated camera as detector [29-3020-21]. 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 [3122] 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 H<sub>2</sub>O. The solution was autoclaved and then 40 ml of glucose 50% w/v solution

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3 (0.22  $\mu\text{m}$  filter sterilized) was added. SC-ura-trp-leu medium was prepared by supplementing SC  
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5 medium with L-histidine (2 g/L). SC (-Ura -Trp -Leu -His) medium was supplemented with L-  
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7 histidine (0.02 g/L), L-leucine (0.1 g/L), L-tryptophan (0.02 g/L) and uracil (0.02 g/L). 17 $\beta$ -  
8  
9 estradiol (E2), 17 $\alpha$ -ethynylestradiol (EE2), diethylstilbestrol (DES), estrone (E1) and bisphenol A  
10  
11 (BPA) were from Sigma-Aldrich. Dialysis tubing cellulose membrane and the kit for plasmid  
12  
13 extraction and purification were from Sigma-Aldrich. FastDigest restriction enzymes, FastAP and  
14  
15 T4 DNA Ligase required for cloning and YPER (Yeast Protein Extraction Reagent) were from  
16  
17 Thermo Fisher Scientific (Waltham, MA, USA). The bioluminescent substrate furimazine was from  
18  
19 Promega (Madison, WI, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis,  
20  
21 MO, USA).  
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#### 24 25 26 27 *Obtainment of NanoLuc estrogen responsive S. cerevisiae strain*

28  
29 Yeast expression plasmid pRSII426 and pBEVY-L were from Addgene (Cambridge,  
30  
31 Massachusetts, USA). The yeast codon optimized version of the NanoLuc-PEST luciferase  
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33 (yNLucP) was a kind gift from Prof. C. Andréasson (Stockholm University, Sweden) [3223]. The  
34  
35 sequence encoding the human estrogen receptor was amplified with polymerase chain reaction  
36  
37 (PCR) using pSP72hER $\alpha$  as template and cloned into pBEVY-L (Leu2 marker) vector under the  
38  
39 control of constitutive ADH1 promoter, using *KpnI* and *EcoRI* sites. The generated vector was  
40  
41 called pBEVY-L-ER $\alpha$ .  
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44  
45 The reporter vector was created by cloning, into a pRSII426 plasmid (Ura3 marker), five copies of  
46  
47 an ERE response element (-AGGTCAGagTGACCT-) [3324] upstream of a minimal cytochrome C  
48  
49 promoter (CYCmin) driving the expression of the yNLucP coding sequence, giving the plasmid  
50  
51 pRSII426-ERE-yNLucP. The correctness of sequences and vectors was confirmed by restriction  
52  
53 analysis and sequencing.  
54

55  
56 The yeast *S. cerevisiae* BMA64-1A (MAT $\alpha$ , ura 3-52, trp1 $\Delta$ 2 leu2-3 112his3-11 ade2-1, can1-100,  
57  
58 wild type strain was used as recipient strain [3425] and transformed with plasmids pBEVY-L-ER $\alpha$   
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3 and pRSII426-ERE-yNLucP using the LiAc/SS-DNA/PEG method [3526]. Colonies harbouring  
4  
5 both vectors were selected in SC-ura-leu plates after incubation at 30 °C for 4 days. 15% glycerol  
6  
7 stocks of the recombinant strain were prepared and stored at –80 °C.

#### 8 9 10 11 *Laboratory-based assay procedure and luminescence measurements*

12  
13 The novel NanoLuc yeast estrogen screen (nanoYES) assay was carried out in a type II laminar  
14  
15 flow cabinet to reduce aerosol formation. Before running an assay, a single colony from an agar  
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17 plate containing the selective medium was used to inoculate 3.0 mL of SC medium. This culture  
18  
19 was grown overnight at 30 °C with orbital shaking at 200 rpm in selective SC medium.  
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22  
23 Briefly, a 3 mL overnight yeast culture was diluted in fresh SC medium to optical density (OD<sub>600</sub>)  
24  
25 of 0.6 and grown for about 4 h until OD<sub>600</sub> = 1 was reached. Then, 150 µL of culture was dispensed  
26  
27 in 96-well microplates and incubated at 25 °C with different concentrations of E2 (from 0.001 nM  
28  
29 to 100 nM) at 1% ethanol final concentration, for 1 h. Control wells (CTR) were incubated with 1%  
30  
31 ethanol final concentration. BL emission kinetics were recorded using a Varioskan Flash multimode  
32  
33 reader (5 minutes with 300 ms integration time) after addition of 50 µL of an optimized BL  
34  
35 substrate containing 10 µM furimazine diluted in YPER reagent (YPER-Nano). Light emissions  
36  
37 were expressed as relative light units (RLU). The detection limit is defined as the E2 concentration  
38  
39 that corresponds to the blank signal plus three times the standard deviation. All experiments were  
40  
41 performed in triplicate and repeated at least 3 times.  
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#### 46 47 *Fabrication of the mobile platform 3D-printed cartridges and GoProHero5 adaptors*

48  
49 The compact GoPro HERO 5 videocamera (GoPro, Inc, San Mateo, CA, USA) was chosen as light  
50  
51 detector. A cartridge of 60 x 40mm, 7 mm high, containing an array of 16 square wells (5 mm wide  
52  
53 and 5 mm deep each) was created with a desktop 3D printer (Makerbot Replicator 2X) using black  
54  
55 and white thermoplastic polymer acrylonitrile butadiene styrene (ABS) (FormFutura, Nijmegen,  
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3 NL) using the dual extrusion option. The GoProHero5 adaptors and dark-box were printed using  
4  
5 black ABS. All pieces were printed at 300  $\mu\text{m}$  layer resolution, 30% infill.  
6

#### 7 8 *Preparation of cartridges with immobilized yeast biosensors.*

9  
10 An overnight culture of yeast cells was diluted in 30 mL of fresh medium to optical density ( $\text{OD}_{600}$ )  
11  
12 of 0.6 and grown for about 4 h until an  $\text{OD}_{600} = 1$  was reached. The culture was centrifuged and  
13  
14 resuspended in 3 mL SC-ura-leu medium containing 10% trehalose and 1.5% sodium alginate.  
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16  
17 This mixture was then poured into a dialysis tubing cellulose membrane (avg. flat width 10 mm,  
18  
19 molecular weight cut-off = 14 KDa) and immersed in a 0.2 M  $\text{CaCl}_2$  solution for 1 h at room  
20  
21 temperature (25  $^\circ\text{C}$ ) to allow the formation of the gel inside the membrane. Using a 3D printed  
22  
23 “microtome-like” device, that has a slot for a surgical blade placed at 2 mm from the edge, the  
24  
25 obtained gel (about 12 cm length, avg. diameter 5 mm) was repeatedly cut in several slices, then  
26  
27 placed into the wells of the 3D-printed cartridges. A 50  $\mu\text{L}$ -volume of SC medium was added to  
28  
29 each well and the cartridges were covered with Parafilm M<sup>®</sup> and stored at 4  $^\circ\text{C}$  until use.  
30  
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32  
33 The stability of the yeast biosensor kept at 4  $^\circ\text{C}$  was daily tested by incubating the cells in duplicate  
34  
35 with 10 nM E2 (50  $\mu\text{L}$ ) for 1 h incubation at room temperature; a 50  $\mu\text{L}$ -volume of YPER-Nano  
36  
37 substrate was added to each well and image was acquired with the GoProHero5 in night mode (30 s,  
38  
39 ISO 800) equipped with the 3D printed black-box accessory. Images were analysed with ImageJ  
40  
41 software and data plotted using GraphPad Prism v.5 (GraphPad Software, Inc. La Jolla, CA). BL  
42  
43 emission was normalized with respect to BL signal obtained at day 0 (freshly immobilized cells).  
44  
45 All measurements were performed in duplicate and repeated at least three times with different cell  
46  
47 cartridges.  
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#### 50 51 52 53 *Analytical performance of the GoPro-based yeast estrogen screen*

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55 The analytical performance of the developed platform was performed by incubating yeast estrogen  
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57 biosensors with increasing concentrations (from 0.05 nM to 10 nM) of E2, selected as model  
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3 estrogenic analyte. Briefly, a cell-cartridge containing immobilized yeast cells stored at 4°C was  
4  
5 equilibrated at room temperature for 15 min, then a 50 µL-volume of E2 dilutions (1% ethanol final  
6  
7 concentration) were added in duplicate wells. Control wells were ~~treated~~ incubated with 1% ethanol  
8  
9 final concentration (50 µL) ~~of 1% EtOH~~. After 1h incubation at room temperature a 50 µL-volume  
10  
11 of YPER-Nano substrate was added to each well, then the cartridge was inserted into the 3D printed  
12  
13 black-box accessory and BL emission was acquired with the GoProHero5 in night mode (30 s, ISO  
14  
15 800). Images were quantified with ImageJ software by selecting a square region of interest (ROI)  
16  
17 around each well and measuring the BL emission of duplicate wells. E2 dose-response curves were  
18  
19 obtained by calculating the fold response with respect to control and plotted using GraphPad Prism.  
20  
21 Non-linear regression was performed by fitting the experimental data using a four-parameters  
22  
23 sigmoidal curve, then the EC50 value for E2 was calculated as the effective concentration which  
24  
25 produces the midpoint y value (50%) of the dose-response curve. All measurements were performed  
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27 in duplicate and repeated three times with different cell cartridges.  
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#### 34 *Yeast-estrogen cartridge configuration for effect-based analysis of real samples*

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36 To obtain yeast-estrogen biosensors for real sample analysis Ready-to-use cartridges with  
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38 immobilized ~~yeast-estrogen biosensors~~ cells were prepared as follows: half of the cartridge was  
39  
40 used to test in duplicate the yeast bioreporter response to 0.5, 1 and 5 nM E2 or ~~vehicle only~~ (1%  
41  
42 EtOH) as control, while the remaining wells were incubated in duplicate with tap water samples (25  
43  
44 µL) spiked with different concentrations of diethylstilbestrol (0.1 and 10 nM) and bisphenol A (10  
45  
46 nM). Sample wells were also co-incubated with 0.5 nM E2 (25 µL). Dilution of estrogenic  
47  
48 compounds were prepared to provide a 1% ethanol final concentration in every well.  
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52 Cartridges were incubated for 1 h at room temperature, then a 50 µL-volume of YPER-Nano  
53  
54 substrate was added to each well and BL emission was acquired with the GoProHero5 as described  
55  
56 above. Images were quantified with ImageJ software by selecting a square ROI around each well  
57  
58 and measuring the mean BL emission of duplicate wells. BL signal were then normalized with  
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3 respect to 0.5 nM E2 (selected as reference and set to 100%) and plotted using GraphPad Prism. All  
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5 measurements were performed in duplicate and repeated three times with different cell cartridges.  
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## 9 **Results and discussion**

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11 The possibility to use the smartphone camera to detect the BL emission from living whole-cell  
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13 biosensors has been previously demonstrated by us exploiting genetically engineered mammalian  
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15 cell lines [29-3020-21]. Despite adequate analytical performance, one of the main limitations of this  
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17 approach is surely related to the short shelf life of mammalian cells when maintained outside an  
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19 incubator with controlled temperature and humidity. In addition, considering the short life span of  
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21 smartphones, mainly due to short battery life and software updates changes, the general  
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23 applicability of such devices is questionable with the necessity of upgrading and fabricating new  
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25 devices, with subsequent assay optimization and calibration, for each smartphone model.  
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29 In this scenario, a mobile platform based on the use of a compact camera, such as the GoProHero  
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31 camera, and robust yeast cells as living biosensors could represent a suitable solution to overcome  
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33 these issues. Indeed, yeast cells are particularly suitable for the development of whole-cell  
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35 biosensors integrated into a portable mobile platform as they provide the analytical robustness  
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37 typical of bacterial cells, with the possibility to express functional human receptors and regulatory  
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39 elements to obtain predictive information about actual biological activity of samples [3627].  
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43 The GoProHero5, optimized for sport and outdoor activities, represents a robust, waterproof CMOS  
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45 camera (12 MP, UHD 4K) which can be directly controlled using the built-in touch-screen display  
46  
47 or can be connected to any smartphone via dedicated GoPro-App (paired wireless network), making  
48  
49 it a very versatile light sensor for the development of portable devices. In addition, the long  
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51 exposure time (up to 30 s at ISO 800) makes this camera a powerful choice for low-light imaging  
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53 applications, including bioluminescence measurements.  
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58 *Development of a novel yeast-estrogen screen exploiting NanoLuc luciferase (nanoYES)*  
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3 The novel yeast-estrogen strain (Fig.1a) was obtained by genetically engineering *S. cerevisiae* cells  
4 with two vectors: i) a plasmid for the expression of the human estrogen receptor  $\alpha$  (hER $\alpha$ ) under the  
5 control of the constitutive ADH1 promoter and ii) a reporter plasmid containing five copies of an  
6 ERE response element (-AGGTCAgagTGACCT-) upstream of a minimal cytochrome C promoter  
7 (CYCmin) which drives the expression of the yeast codon-optimized NanoLuc luciferase coding  
8 sequence (yNLucP). Both vectors contain the 2 $\mu$  origin, thus ensuring consistent replication of the  
9 two plasmids during cell division and maintaining each plasmid at about 50 copies/cell. Due to the  
10 high copy number, the expression of the human estrogen receptor was placed under a weak  
11 promoter to avoid strong overexpression that may lead to artefacts in the biosensor response.  
12 Indeed, the high copy number of reporter vector allows consistent production of yNLucP reporter  
13 enzyme upon induction.  
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34 The small size (19 kDa) and the absence of post-translational modifications and disulphide bonds of  
35 NanoLuc luciferase enable its rapid synthesis and folding, thus reducing total assay time, moreover  
36 the use of its destabilized version provides a half-life of 5 min in yeast, compared to 40 min of the  
37 yNluc [3223], thus faithfully reflecting mRNA levels. Yeast cells were sequentially transformed  
38 with the two plasmids and single colonies screened for their responsiveness with 10 nM E2. A new  
39 BL substrate composition was formulated to optimize BL emission in yeast cells. The NanoGlo®  
40 substrate (containing a lysis buffer optimized for mammalian cells) showed suitable for yeast cells  
41 [3223]. However, to increase light output, we formulated an alternative substrate by diluting  
42 furimazine (10  $\mu$ M) in YPER buffer, a specific yeast-cell lysis reagent used for the extraction of  
43 functionally active solubilized proteins from yeast. The use of this formulation (YPER-Nano)  
44 provides both an increased BL emission (20%) and more stable emission kinetics (signal half-life >  
45 20 min) compared to NanoGlo® substrate or furimazine alone (signal half-life < 2 min). (~~data not~~  
46 ~~shown~~).

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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

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3 incubated for 1 h with different concentrations of E2, shows a limit of detection of  $0.010 \pm 0.002$   
4 nM and an EC50 of  $0.6 \pm 0.1$  nM (Fig.1b). The nanoYES shows a LOQ of  $0.020 \pm 0.005$  nM E2  
5 and mean recovery rate of  $93 \pm 11\%$ . The nanoYES response to other estrogenic compounds was  
6  
7 also evaluated (Fig.1b) and corresponding limit of detection and EC50s are shown in table 1. The  
8  
9 developed yeast estrogen bioreporter assay shows comparable results in terms of ranking of  
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11 estrogenic activity (E2>EE2>DES>E1) and EC50 values, obtained by previous works based on  
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13 recombinant yeast cells [37-38]. \_  
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#### 23 *Design and 3D-printing fabrication of the mobile platform based on GoPro Hero camera*

24 The mobile platform was designed to create an all-in-one device based on the GoPro Hero5 camera  
25 (Fig.S12a). A series of modular adaptors were fabricated with black ABS using a desktop 3D  
26  
27 printer, providing a dark box of compact size ( $65 \times 65$  mm, 60 mm height) for the acquisition of BL  
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29 emission. This accessory also includes a slot to insert a 3D-printed cell-cartridge containing the  
30  
31 immobilized nanoYES. The multi-well cartridges ( $60 \times 40$  mm), containing 16 wells of  $5 \times 5$  mm  
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33 ( $150 \mu\text{L}$  volume each), were printed with white and black ABS (Fig.2b) using the dual extrusion  
34  
35 option provided by the Makerbot Replicator 2X. In particular, the bottom of the wells wasere  
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37 printed using white ABS, while black ABS was used for the remaining parts. This configuration  
38  
39 allowed to increase the intensity of acquired BL signal by reflection while avoiding crosstalk  
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41 between adjacent wells, outperforming cartridges printed with only white or black ABS, that  
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43 suffered of higher crosstalk and lower signals, respectively.  
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#### 52 *Immobilization procedure and stability of nanoYES*

53 To obtain ready-to-use cartridges the nanoYES was immobilized into alginate slices. Cells at OD =  
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55 1 (about  $1.8 \times 10^7$  cells/mL) were 10-fold concentrated in culture medium to achieve a sufficient BL  
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3 emission for a sensitive detection with the GoPro camera. The immobilization medium also  
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5 contains 10% w/v trehalose to increase the shelf life of yeast cells [39,28].

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7 A straightforward procedure was developed to generate yeast-alginate slices of defined and  
8  
9 reproducible dimension ( $2.1 \pm 0.2$  mm) (Fig.S12).

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11 To evaluate the reproducibility of immobilized nanoYES, a whole cell-cartridge (Fig.32a) was  
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13 incubated with 10 nM E2 for 1 h, and BL image acquired with the GoPro camera (Fig.23b). The  
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15 quantification of BL emission of 16 slices provided a coefficient of variation (CV%) of 11%, which  
16  
17 is comparable to previously reported whole-cell bioassays [40]~~which is acceptable for a whole-cell~~  
18  
19 ~~bioassay.~~  
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23 The efficacy of our procedure was compared to conventional method for obtaining alginate beads.  
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25 A 50  $\mu$ L-volume nanoYES/alginate mixture, containing the same cell number for each slice, was  
26  
27 added dropwise into a CaCl<sub>2</sub> solution and let to harden for 1 h. Individual beads of about  $4.5 \pm 0.3$   
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29 mm diameter were obtained, placed into the cell-cartridge wells and treated as for the slices, with 10  
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31 nM E2 for 1 h. The mean BL signal using alginate beads is 8.5 times lower and less reproducible  
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33 than those obtained with slices, most probably due to a slower and non-uniform distribution of  
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35 analyte and BL substrate inside the beads (Fig.S23).

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38 The stability and responsiveness of yeast-estrogen bioreporters immobilized into alginate slices  
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40 stored at 4 °C in the 3D printed cartridges was evaluated. Each day duplicate wells were incubated  
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42 with 1 nM E2 for 1 h and BL images were acquired with the GoPro camera. As shown in Fig.32c,  
43  
44 the nanoYES response was consistently maintained within 7 days (85% of initial response at day 7)  
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46 and even after two weeks the nanoYES maintains about 70% of the responsiveness obtained at day 0  
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48 (freshly immobilized cells). To guarantee good analytical performance in terms of LOD and  
49  
50 sensitivity we decided to use cell-cartridges not older than 10 days, which still provide a BL  
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52 emission over the 75% of initial response (arbitrarily selected threshold).  
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58 *Analytical performance of GoPro-based nanoYES*  
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3 The assay in optimized conditions consists of incubation of 50  $\mu$ L of E2 dilutions (concentration  
4 range from 0.05-10 nM) per each cartridge well containing (about  $9.0 \times 10^5$  cells/slice) for 1 h at  
5 room temperature. Cell cartridge is then imaged with the GoProHero5 camera in night mode for 30  
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10 s at ISO 800, after addition of 50  $\mu$ L YPER-Nano substrate (Fig.43a). Since cells are lysed during  
11 BL emission acquisition, the cartridges are single-use. Fig.43b shows a detailed BL image  
12 corresponding to the dose-response curve for E2 acquired 10 min after substrate addition. Dose-  
13 response curves obtained with three different cartridges showed a LOD of  $0.08 \pm 0.02$  nM and  
14 EC50 of  $0.7 \pm 0.1$  nM E2 (Fig.43c). In the GoPro-based platform the limit of detection is about one  
15 order of magnitude higher compared to those obtained with the nanoYES using conventional  
16 benchtop luminometer while the EC50 is comparable ( $0.6 \pm 0.2$  nM E2). Conversely, the LOD is  
17 comparable to other previously reported yeast estrogen bioassays, such as those reported by  
18 Leskinen et al (0.03 nM for E2) [15]. Also, the EC50 value is consistent with those obtained with  
19 similar yeast based screening assays performed in laboratory settings, such as the conventional YES  
20 (EC50: 0.32 nM) [7]. These results confirmed the suitability of this configuration for the  
21 straightforward and quantitative detection of estrogenic activity in water samples.  
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#### 39 *GoPro-based nanoYES: analysis of ~~real~~ spiked samples*

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41 As a proof of concept, tap water samples spiked with different concentrations of diethylstilbestrol  
42 (DES) and bisphenol A (BPA) were analysed with the GoPro-based nanoYES to explore its actual  
43 feasibility for on-site testing, especially for monitoring of sites affected by high pollution such as  
44 downstream of industrial manufacturing plants and agricultural areas.  
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49 BPA was selected because of its ability to act as weak ER binder and interfere with the  
50 endogenous E2, causing severe effects on the reproductive system [41-4229-30] while DES is a  
51 synthetic nonsteroidal estrogen widely encountered in influents and effluents from municipal water  
52 treatment plants with concentrations levels in the range of 4-12 ng/L for primary influents [43-  
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3 4434]. BPA is present at relatively high concentrations in several site areas, for example according  
4 to a recent review BPA concentrations in groundwater vary between 1 ng/L and 20 µg/L [45].

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7 Each disposable cartridge also contains a calibration curve obtained by inducing the nanoYES with  
8  
9 0.5, 1 and 5 nM E2 allowing a rapid evaluation and subsequent interpolation of estrogenic activity  
10 of the samples (Fig.54a). Control wells (CTR) were also included and incubated with 1% EtOH  
11 final concentration. Sample-wells were also induced with 0.5 nM E2, a concentration near the  
12 EC50, to evaluate both estrogenic and anti- estrogenic activity of samples.

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Mean BL emission of each sample was quantified and normalized with respect to 0.5 nM E2, set to  
100% (Fig.54b). Sample no. 1, which contains 10 nM BPA, (simulating a high contaminated  
sample) shows a remarkable decrease (35%) of BL emission. indicating the presence of a weak  
hER $\alpha$  binder. This sample contains 10 nM BPA, simulating a high contaminated sample. At this  
concentration, BPA (a weak hER $\alpha$  binder) which binds to hER $\alpha$  interfering interferes with the  
transactivation induced by 0.5 nM E2, thus decreasing BL emission. Acting as a 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) and  
shows a remarkable increase compared to 0.5 nM E2 and, was selected to simulate the effect-based  
response a strong estrogenic effect as in samples containing mixtures of 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, did do not show any alteration  
with respect to 0.5 nM E2, induced transactivation and were spiked with 0.1 nM DES (whose  
concentration is too low does not to produce a significantly increased estrogenic effect) or contains  
tap water only, respectively.

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

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3 concentration of EDCs is expected. Moreover, future studies will be aimed at evaluating the  
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5 analytical performance of the nanoYES in the presence of EDCs mixtures.  
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## 8 9 **Conclusion**

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

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15 The newly developed yeast estrogen biosensors exploit a yeast codon optimized variant of Nanoluc  
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17 luciferase to achieve rapid and sensitive detection of estrogen-like compounds within 1 h. A  
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20 straightforward procedure to immobilize the yeast biosensors into 3D printed cartridges was also  
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22 developed, obtaining reproducible ready-to-use disposable cartridges that can be stored for 10 days at  
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24 4 °C without significant decrease in analytical performance.

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26 The GoPro camera proved suitable for the sensitive detection of light emission from bioluminescent  
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28 yeast biosensors, using the night mode setup with 30 s integration time. In addition, thanks to the  
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30 possibility to connect this sensor with any smartphone model via dedicated GoProApp, the  
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32 developed configuration results in a more standardized and versatile platform, compared to  
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34 smartphone-based devices. A custom application (APP) running on different operative systems  
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36 could be developed to either provide instructions to the user and for the quantification of BL images  
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38 to obtain immediate results about estrogenic activity of samples.  
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42 In the proposed configuration, the mobile platform, containing robust microbial bioluminescent  
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44 whole-cell bioreporters, allows a rapid evaluation of estrogenic activity in water samples and could  
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46 find application for on-site analysis of EDCs.  
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## 48 49 **Notes**

50  
51 The authors declare no competing financial interest.  
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37 | Environ Sci Pollut Res Int. 2015; 22(8): 5711-5741. Bisphenol A, nonylphenols,  
38 | benzophenones, and benzotriazoles in soils, groundwater, surface water, sediments, and  
39 | food: a review).  
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## Figure legends

**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  $\alpha$  (hER $\alpha$ ) 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 $\mu$  replication origin for their propagation during yeast growth. The binding of estrogens such as 17 $\beta$ -estradiol (E2) to hER $\alpha$  activates the intracellular signalling 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 $\beta$ -estradiol; EE2: 17 $\alpha$ -ethynylestradiol; 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.

~~**Fig. 2S1 a)** 3D printed accessory designed to hold a GoPro Hero5 creating a self supporting device. The adaptor (65x65 mm, 60 mm height) provides a dark box for acquisition of bioluminescent emission and integrates a slot to house the cell cartridge. **b)** Multi-well cartridge (60x40 mm) printed with white and black ABS containing 16 wells of 5x5 mm (150  $\mu$ L volume each) to house the cell alginate slices.~~

**Fig. 32 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  $\mu$ L YPERNano solution. **c)** Responsiveness of immobilized yeast-bioreporters stored at 4  $^{\circ}$ C. Each day duplicate

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3 wells were treated with 1 nM E2 and BL emissions were acquired with the GoPro camera. BL  
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5 signals are normalized with respect to day 0 (freshly immobilized cells).  
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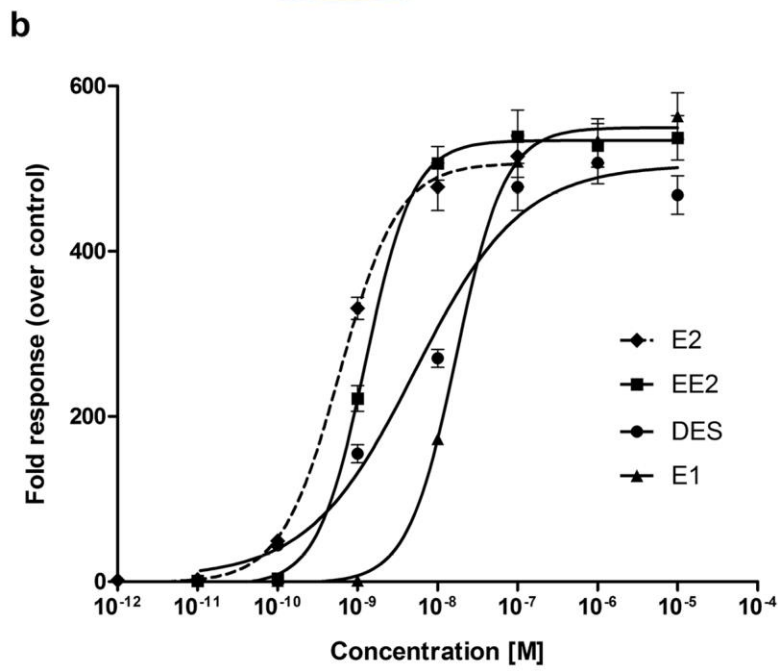
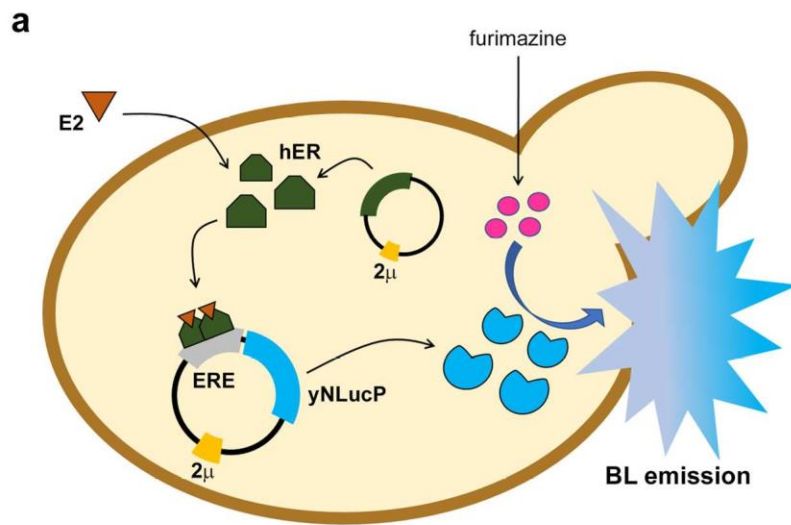
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10 **Fig. 34 a)** Picture of a typical yeast estrogen assay performed by incubating the cell-cartridge  
11 containing immobilized yeast bioreporters with 50  $\mu$ L of sample and by acquiring BL emission with  
12 a GoProHero5 camera in night mode (30 s at ISO 800), after addition of 50  $\mu$ L YPERNano  
13 solution. **b)** BL image obtained by incubating the yeast-bioreporters with 17 $\beta$ -estradiol  
14 (concentration range from 0.05 nM to 10 nM) and **c)** corresponding dose-response curve for 17 $\beta$ -  
15 estradiol obtained after quantification of BL emission with ImageJ software. Data are plotted as fold  
16 response with respect to control (1% EtOH).  
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27 **Fig. 54 a)** BL Image of a proof-of-concept analysis of spiked water samples and **b)** Quantitative  
28 elaboration where BL emissions were normalized with respect to 0.5 nM E2 set at 100. ~~graphical~~  
29 ~~elaboration of a proof-of-concept analysis of real samples.~~ The configuration of the cell-cartridge  
30 allows to test the estrogenic activity of four samples in duplicate. The response of nanoYES to 0.5,  
31 1, and 5 nM E2 was also included in each cartridge, allowing a quick evaluation and quantification  
32 of estrogenic activity. Sample wells are co-incubated with 0.5 nM E2 to detect both pro- and anti-  
33 estrogenic activity. As a proof-of-concept, three tap water samples were analyzed: sample no. 1  
34 (S1) was spiked with 10 nM bisphenol A (BPA) shows anti-estrogenic activity. BPA (which does  
35 not produce a detectable BL signal at this concentration) is able to bind to the estrogen receptor thus  
36 interfering with the transactivation induced by 0.5 nM E2. Sample no. 2 (S2) which contains 10 nM  
37 diethylstilbestrol (DES) shows a remarkable increase compared to 0.5 nM E2 (comparable to BL  
38 signal obtained in the presence of 5 nM E2). Sample no. 3 (S3) was spiked with 0.1nM  
39 diethylstilbestrol (DES), this concentration does not produce any estrogenic effect. Sample no. 4  
40 (S4) contains only 50  $\mu$ L tap water and has no estrogenic effect. All measurements were performed  
41 in duplicate and repeated with three different cell cartridges.  
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**Table 1.** nanoYES response to estrogenic compounds, obtained using liquid cultures, in 96 well plate format and benchtop luminometer.

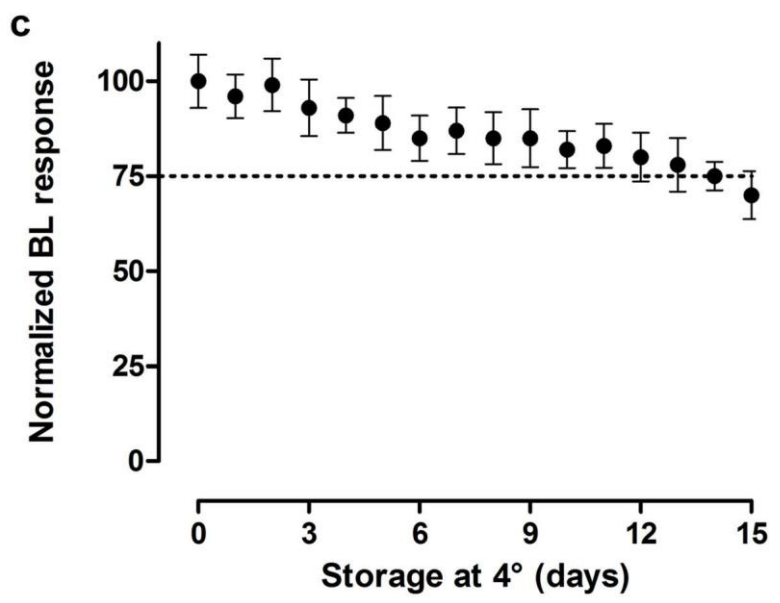
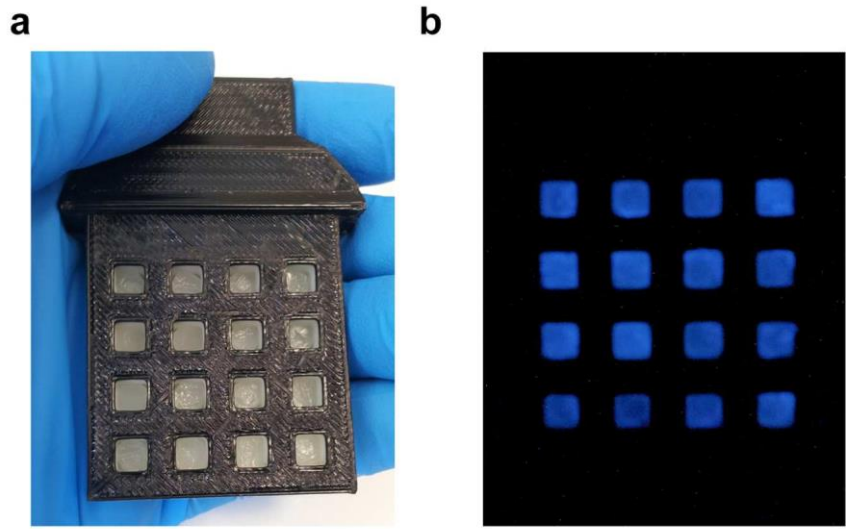
<b>Compound</b>	<b>LOD (nM)</b>	<b>EC50 (nM)</b>
<b>17<math>\beta</math>-estradiol (E2)</b>	0.010 $\pm$ 0.002	0.6 $\pm$ 0.1
<b>17<math>\alpha</math>-ethynylestradiol (EE2)</b>	0.05 $\pm$ 0.01	1.2 $\pm$ 0.3
<b>Diethylstilbestrol (DES)</b>	0.019 $\pm$ 0.005	5.2 $\pm$ 0.5
<b>Estrone (E1)</b>	0.5 $\pm$ 0.1	17 $\pm$ 2

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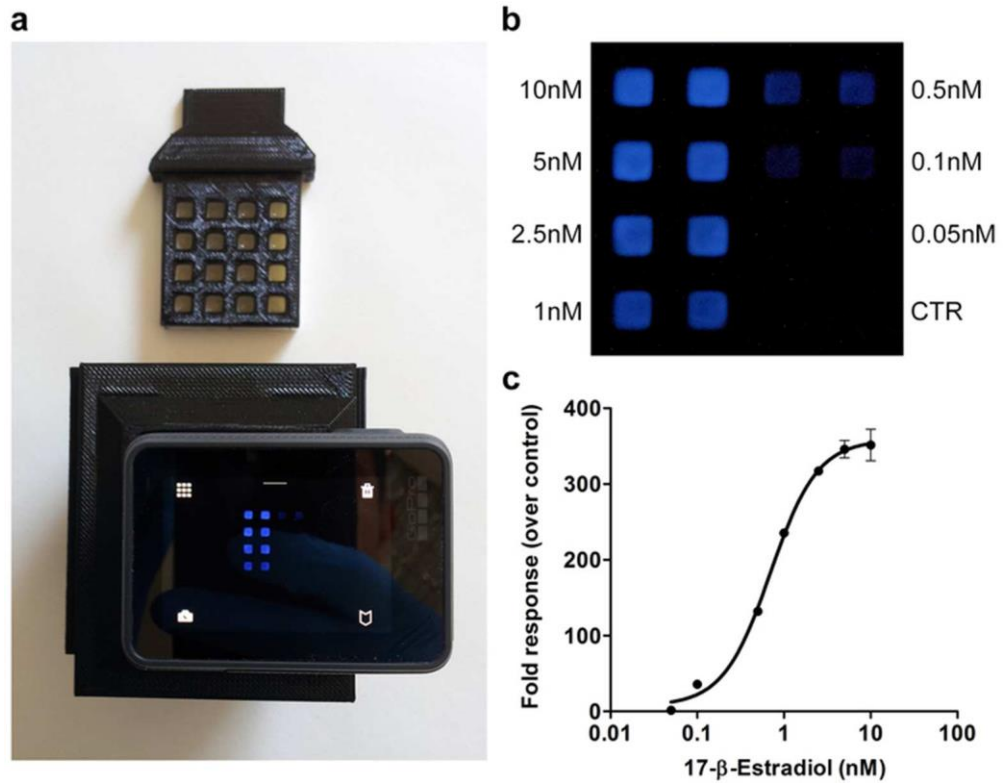
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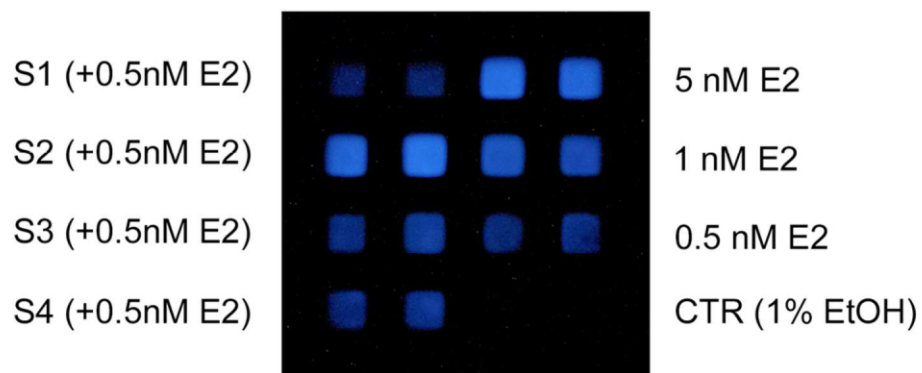
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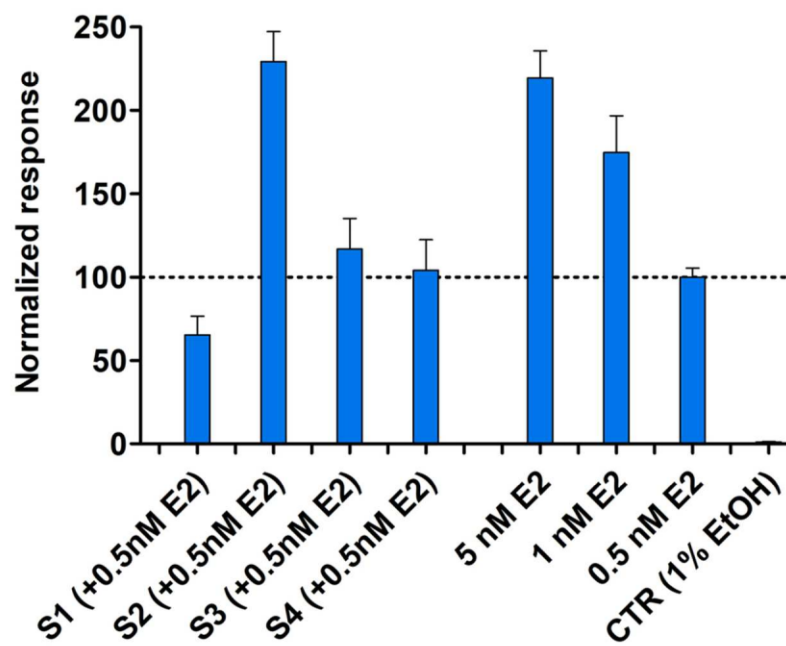
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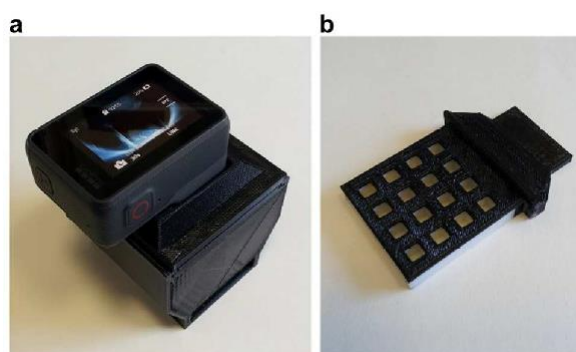
533x312mm (96 x 96 DPI)

## Supplementary material

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

*Cevenini Luca<sup>1</sup>, Lopreside Antonia<sup>1</sup>, Calabretta Maria Maddalena<sup>1</sup>, D'Elia Marcello<sup>2</sup>, Simoni*

*Patrizia<sup>3</sup>, Michelini Elisa<sup>\*1,4,5</sup>, Roda Aldo<sup>\*1,4</sup>*



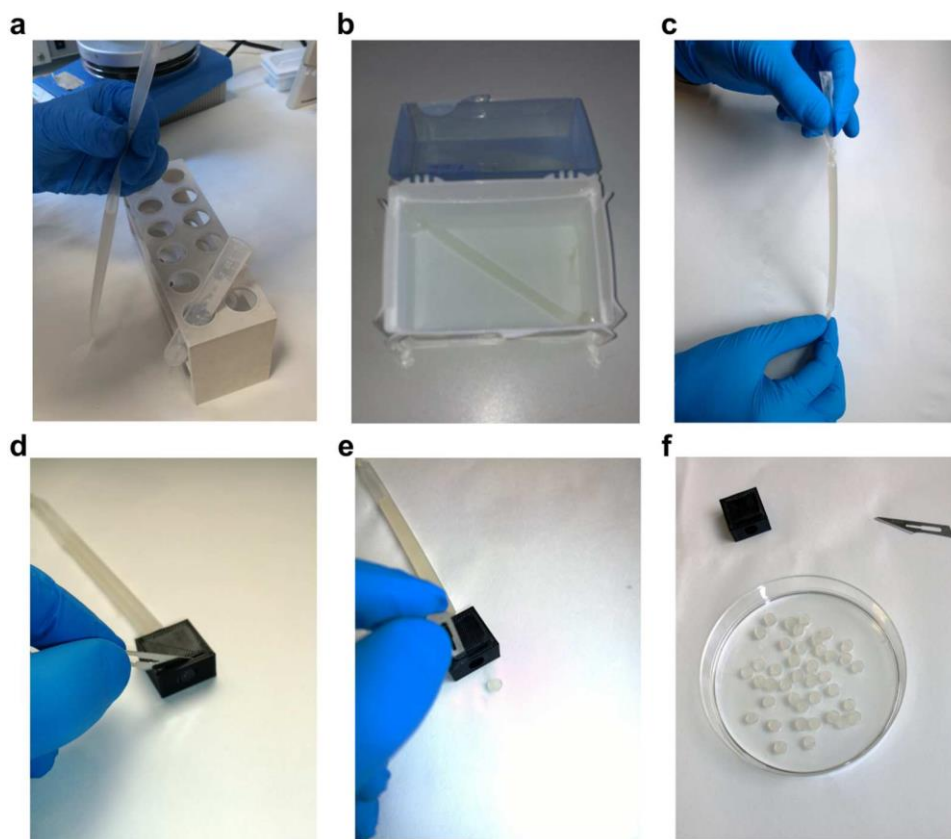
**Fig. S1 a)** 3D printed accessory designed to hold a GoPro Hero5 creating a self-supporting device.

The adaptor (65 x 65 mm, 60 mm height) provides a dark box for acquisition of bioluminescent

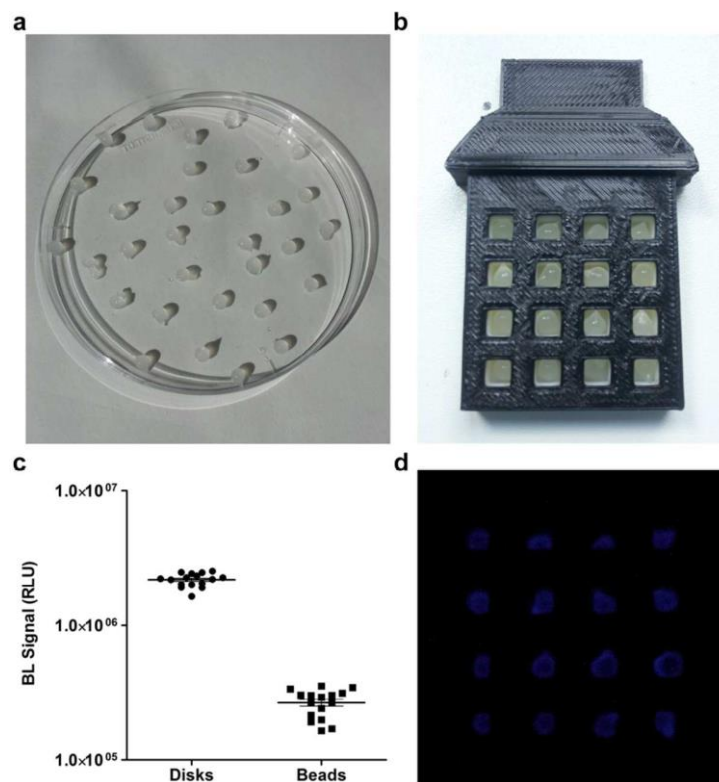
emission and integrates a slot to house the cell-cartridge. b) Multi-well cartridge (60 x 40 mm)

printed with white and black ABS containing 16 wells of 5 x 5 mm (150  $\mu$ L volume each) to house

the cell-alginate slices.



**Fig. S12** Immobilization of nanoYES in alginate slices. **a)** Yeast cultures at  $OD_{600}=1$  (about  $1.8 \times 10^7$  cells/mL) were concentrated 10X in culture medium containing trehalose (10% w/v) and poured (3 mL of yeast culture) into a dialysis tubing cellulose membrane (avg. flat width 10 mm, molecular weight cut-off = 14 KDa). **b)** The membrane was then immersed into a  $CaCl_2$  solution to allow the formation of the gel inside the membrane. **c)** After 1 h incubation at room temperature ( $25^\circ C$ ) the yeast-bioreporter are immobilized into calcium alginate matrix and ready for the next steps. **d)** The obtained gel (about 12 cm length, avg. diameter 5mm) is inserted in a 3D printed “microtome-like” device fabricated for the straightforward production of slices with defined dimension. **e)** The microtome has a slot for a surgical blade placed at 2 mm from the edge, allowing to repeatedly cut the gel by simply realigning it to the edge after each slice. **f)** Using this technique about 48 slices of  $2.1 \pm 0.2$  mm were obtained, which are sufficient for the production of three cartridges.



**Fig. S23** Comparison with yeast immobilized in alginate beads. **a)** Picture of alginate beads obtained with the conventional procedure by dripping 50  $\mu$ L of nanoYES-alginate mixture into a 0.1 M  $\text{CaCl}_2$  agitated solution. **b)** Picture of the alginate beads (avg. diameter 4.5 mm) into a 3D printed cartridge. **c)** BL emission intensities and distribution of nanoYES immobilized in alginate slices or beads and induced with 10 nM E2. **d)** BL image of nanoYES immobilized in alginate beads and induced with 10 nM E2.