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Using Tg(vtg1:mCherry) zebrafish embryos to test the estrogenic effects of endocrine disrupting compounds

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Dear Editor,

On behalf of the authors I would like to thank you for sending us the reviewers comments on the manuscript entitled “Using Tg(vtg1:mCherry) zebrafish embryos to test the estrogenic effects of endocrine disrupting compounds” (JoVE60462R2).

We were pleased to see the comments of the editors who considered our manuscript and video. We have carefully considered editors comments and revised the manuscript and the video according to each comment.

We hope that you will find the revised version sufficiently improved to be suitable for publication and we are looking forward to hearing from you.

Yours sincerely,
Zsolt Csenki

TITLE:

Using Tg(Vtg1:mcherry) Zebrafish Embryos to Test the Estrogenic Effects of Endocrine Disrupting Compounds

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KEYWORDS:

endocrine disrupting compounds, EDC, zearalenone, zearalenol, biomonitor, bioindicator, xenoestrogens, vitellogenin

SUMMARY:

Present here is a detailed protocol for the use of zebrafish embryos *Tg(vtg1: mCherry)* for the detection of estrogenic effects. The protocol covers the propagation of the fish and treatment of embryos, and emphasizes the detection, documentation, and the evaluation of fluorescent signals induced by endocrine disrupting compounds (EDC).

ABSTRACT:

There are many endocrine disrupting compounds (EDC) in the environment, especially estrogenic substances. The detection of these substances is difficult due to their chemical diversity; therefore, increasingly more effect-detecting methods are used, such as estrogenic effect-sensitive biomonitor/bioindicator organisms. These biomonitoring organisms include several fish models. This protocol covers the use of zebrafish *Tg(vtg1: mCherry)* transgenic line as a biomonitoring organism, including the propagation of fish and the treatment of embryos, with

an emphasis on the detection, documentation, and evaluation of fluorescent signals induced by EDC. The goal of the work is the demonstration of the use of the *Tg(vtg1: mCherry)* transgenic line embryos to detect estrogenic effects. This work documents the use of transgenic zebrafish embryos *Tg(vtg1: mCherry)* for the detection of estrogenic effects by testing two estrogenic substances, α- and β-zearalenol. The described protocol is only a basis for designing assays; the test method can be varied according to the test endpoints and the samples. Moreover, it can be combined with other assay methods, thereby facilitating the future use of the transgenic line.

INTRODUCTION:

There is a significant number of endocrine disrupting compounds (EDC) that are among the most hazardous substances in our environment. These are mainly estrogenic compounds that contaminate water from natural resources. The chemical diversity of the substances belonging to the group makes testing for their presence difficult, as different analytical methods are required for their detection. Based on their chemical structure it is very difficult to determine whether a substance is actually able to act as an estrogen. In addition, these substances are never present in a pure form in the environment, so their effects may be affected by other compounds, too[1]. This problem can be solved by effect-detecting methods, such as the use of biomonitor/bioindicator organisms that show estrogenic effects[2–5].

Recently, a variety of cell line[6] and yeast-based test systems[2,3] have been developed to detect estrogenic effects. However, these are generally only able to detect the binding of the substance to the estrogen receptor[2,3]. In addition, they are unable to model complex physiological processes in the organism, or to detect hormone-sensitive phases of life stages; thus, they often lead to false results.

It is known that certain genes react sensitively to estrogen in living organisms[7]. The detection of gene products by molecular biology methods is also possible at the protein or mRNA level[8,9], but usually involves animal sacrifice. Animal protection laws have become stricter, and there is a growing demand for alternative test systems that minimize the number and suffering of animals used in experiments or the replacement of the animal model with another model system[10]. With the discovery of fluorescent proteins and the creation of biomarker lines, transgenic technologies provide a good alternative[11]. With these lines, the activation of an estrogen-sensitive gene can be tested in vivo.

Among vertebrates, the potential of fish in environmental risk assessment is outstanding. They offer many advantages over mammalian models: being aquatic organisms, they are able to absorb pollutants through their entire body, produce a large number of offspring, and some of their species are characterized by short generation time. Their endocrine system and physiological processes show great similarities with other vertebrates and even with mammals, including humans[12].

Several genes for the detection of estrogenic effects in fish are also known. The most important are the estrogen receptors aromatase-b, choriogenin-H, and vitellogenin (vtg)[7,13]. Recently, several estrogen-producing biosensor lines have also been created from fish models used in the

laboratory, such as from zebrafish (*Danio rerio*)[4,5,14–17]. The main advantage of zebrafish in creating biosensor lines is the transparent body of the embryos and larvae, because the fluorescent reporter signal can then be easily studied in vivo without sacrificing the animal[10]. In addition to animal protection, it is also a valuable feature as it allows for studying the reaction of the same individual at different times of the treatment[18].

These experiments use a vitellogenin reporter transgenic zebrafish line[15]. The transgene construct used for the development of *Tg(vtg1:mCherry)* has a long (3.4 kbp) natural vitellogenin-1 promoter. The estrogen receptor (ER) is an enhancer protein activated by ligands that is a representative of the steroid/nuclear receptor superfamily. ER binds to specific DNA sequences called estrogen response elements (EREs) with high affinity and transactivates gene expression in response to estradiol and other estrogenic substances, so the more ERE in the promoter causes a stronger response[19]. There are 17 ERE sites in the promoter region of the Tg(vtg1:mCherry) transgene construct and they are expected to mimic the expression of the native vtg gene[15]. There is a continuous expression of the fluorescent signal in sexually matured females. However, in males and embryo the expression in the liver is only visible upon treatment with estrogenic substances (**Figure 1**).

[Insert **Figure 1** here]

Similar to the endogenous vitellogenin, the mCherry reporter is only expressed in the liver. Because vitellogenin is only produced in the presence of estrogen, there is no fluorescent signal in the controls. Because the expression is only in the liver, the evaluation of the results is much easier[15].

The sensitivity and usability of this line's embryos have been investigated on various estrogenic compound mixtures and also on environmental samples[15,20], and in most cases dose-response relationships were documented **(Figure 2**). However, in the case of highly toxic, mainly hepatotoxic, substances (e.g., zearalenone), only a very weak fluorescent signal may be visible in the liver of treated embryos and the maximum intensity fluorescent signal caused can be reached within a very small concentration range, which makes it difficult to establish dose-effect relationships[20].

[Insert **Figure 2** here]

There are several estrogenic substances present in the environment, such as 17-β-estradiol (environmental concentration: 0.1–5.1 ng/L)[21], 17-α-ethynylestradiol (environmental concentration: 0.16–0.2 µg/L)[22], zearalenone (environmental concentration: 0.095–0.22 µg/L)[23], bisphenol-A (environmental concentration: 0.45–17.2 mg/L)[24]. When testing these substances in a pure active form with the help of mCherry transgenic embryos, the lowest observed effect concentrations (LOEC) for fluorescent sign detection were 100 ng/L for 17-ß-estradiol, 1 ng/L for 17-α-ethynilestradiol, 100 ng/L for zearalenone, and 1 mg/L for bisphenol-A (96–120 hpf treatment), which is very close to or within the range of environmental concentrations of the substances[15]. The *Tg(vtg1:mCherry)* transgenic line can help detect estrogenicity in wastewater

samples after direct exposure. The line is as sensitive as the commonly used yeast estrogen test, the bioluminiscent yeast estrogen (BLYES) assay[15]. With the help of this line, the protective effects of beta-cyclodextrins against zearalenone-induced toxicity has been confirmed using chemical mixtures[20].

In a recent report, the in vivo use of the transgenic line was demonstrated with the help of two estrogenic zearalenone (ZEA) metabolites, α- and β-zearalenol (α-ZOL and β-ZOL)[25]. The protocol baseline is appropriate to study the estrogenic effects of several compounds or environmental samples on *Tg(vtg1:mCherry)* embryos.

PROTOCOL:

The Animal Protocol was approved under the Hungarian Animal Welfare Law and all studies were completed before the treated individuals would have reached the free feeding stage.

1. Embryo harvest and treatment

1.1. Maintain *Tg(vtg1:mCherry)* zebrafish at 25.5 ± 0.5 °C, pH = 7 ± 0.2, conductivity between 525 ± 50 µS/m, oxygen level ≥80% of saturation, and 14 h light and 10 h dark cycle.

1.2. Fill the mating tanks with system water and set up the fish for mating the afternoon before harvesting eggs.

1.3. Place the male and female fish into the tank and separate them with the help of a divider.

1.4. Remove the divider from the tanks as the light switches on next morning. Check the mating tanks for eggs every 15–20 min.

1.5. Harvest all embryos using a tea strainer or densely woven fine mesh and combine them into one large Petri dish with E3 buffer (5 mM sodium chloride, 0.17 mM potassium chloride, 0.33 mM calcium chloride, 0.33 mM magnesium sulfate) or clear system water.

1.6. Place the embryos in the incubator set to 25.5 ± 0.5 °C.

1.7. After 1–1.5 h, remove and discard unfertilized or inadequately dividing eggs with a plastic transfer pipet under a dissecting microscope.

NOTE: Unfertilized eggs are opaque; fertilized eggs are transparent. To start the treatment in a later stage of development, pay attention to the normal development of the individuals being treated.

1.8. Place the selected embryos in the treatment vessels (e.g., in Petri dishes or tissue culture plates) that have already been labeled and filled with different concentrations of the test substance.

1.9. Incubate the embryos at 25.5 ± 0.5 °C until the end of the experiment.

NOTE: Embryos can react with significant individual sensitivity to various estrogenic compounds, so it is recommended to use at least 15 embryos in at least three repeat treatments in order to evaluate the experiment properly. When selecting the container, keep in mind that the development of an embryo requires at least 200 µL of water[26]. In this experiment the embryos were incubated until 120 hpf at 25.5 ± 0.5 °C.

1.10. Refresh the test solution if it is neccesary to maintain the treatment concentration. Be careful when changing the test solution in order to avoid damaging the embryos.

NOTE: It is also possible to investigate mortality or sublethal symptoms during the experiment. The concentrations used should remain as stable as possible to obtain reliable results. This can be easily achieved by refreshing the test solutions. The frequency of refreshing may vary depending on the test substance. Therefore, it is advisable to check the concentration of the test substance by analytical measurements to determine the frequency of the solution updates.

2. Larvae preparation for photography

2.1. Prepare 4% methylcellulose with MS-222 (tricaine-methane-sulfonate) beforehand.

2.1.1. To do so, add 4 mg/mL MS-222 to 100 mL double distilled water to a final concentration of 0.168 mg/mL, and bring the solution to 4 ˚C. Then add 4 g of methylcellulose. Stir it with a magnetic stirrer, then leave it in 4 ˚C overnight.

2.1.2. Next day, stir it again and the solution should be ready to use. If the methylcellulose is not completely dissolved put it back at 4 ˚C and wait a few more hours.

2.2. Place 5 day old larvae into a 5 cm Petri dish per treatment group with a Pasteur pipette.

2.3. Remove the treatment solution from the larvae with a plastic pipette, then fill the Petri dish with 2 mL of 0.02% MS-222 anesthetic solution.

NOTE: Anesthesia is usually effective in less than a minute. The larvae are anesthetized if they do not swim away in response to touch. An overdose of MS-222 can kill the larvae.

2.4. Fill each square of a specially designed 10 cm Petri dish with 4% methylcellulose with MS-222 (**Figure 3**).

NOTE: Glue two 1.5 x 1.5 cm square areas at the bottom of the Petri dish using plastic sheets (1 mm height, 5 mm wide). Twenty larvae can be placed next to each other in a square. Instead of a specially designed Petri dish, another low edge container can be used to fix the position of the embryos.

[Insert **Figure 3** here].

2.5. Transfer the anesthetized larvae to methylcellulose with a little water in one of the two squares. From the first square transfer the larvae into the second square.

NOTE: With the help of this transfer, the 4% methylcellulose used for photography will not be diluted and the larvae will not rotate during imaging.

2.6. In the second square, rotate and orient larvae to their left side and gently press them down to the bottom of the cellulose with a microloader pipette tip cut up to 2 cm.

NOTE: Do not use other pipette tips because they cause injury to the larvae.

3. Microscopy

NOTE: Photography does not kill the animals. Animals can be awakened by removing them from the methylcellulose and placing them in fresh system water or treatment solution, so the same individual can be examined several times during the treatment.

3.1. In order to evaluate the signal of the expressed reporter, image the embryos with the same view and settings.

NOTE: See step 2.6 for optimal embryo positioning. The photos in the manuscript were taken under the conditions described. Bright field: 60x magnification, 6 ms exposition, Iris 100%, Gain:1.1, fluorescence photos: 60x magnification, 300 ms exposition, Iris: 100%, Gain:1, mCherry filter, fluorescent light source: mercury metal halide bulb. For photo taking a fluorescent stereomicroscope, dedicated camera, and software for microscope were used.

3.2. Place the Petri dish on the stage of the microscope.

3.3. Focus on the liver of the embryo and capture a bright field image using the associated software.

3.4. Switch the microscope to the mCherry filter and take a fluorescent image of the liver under fluorescent light using the associated software.

NOTE: Perform all the fluorescence steps in the dark. Do not change the magnification, the focus, or the position of the embryo between capturing the bright field and the fluorescent images, as this will assist in the analysis of the images.

3.5. Repeat steps 3.3 and 3.4 until all the embryos in the experiment have been imaged.

4. Determining integrated density

NOTE: One of the best indicators for comparing fluorescent signal strength is the integrated density value (i.e., the product of the area and mean gray value). One of the easiest ways to determine integrated density is to use the ImageJ program[27]. The program is available on the internet and can be installed on the computer.

4.1. Open ImageJ then upload the fluorescent image to be analyzed by either dragging and droping the image or clicking on **File|Open**.

4.2. Click on **Image|Color|Split** Channels to split the image made by the fluorescent filter according to the RGB color chart.

4.3. Work with the red channel image spectrum, close the other channels.

4.4. Designate a similarly sized elliptical area in the image so false signals do not interfere with the evaluation. Using the **Oval** tools, draw an ellipse over the highlighted liver area as accurately as possible.

4.5. If the signal is weak use light microscopy images (i.e., the bright field pair of the fluorescent image) to determine the location of the liver.

4.6. Click on **Analyze|Measure** to determine the signal strength and the size of the affected area. The integrated density value is automatically calculated by the software (IntDen column in the chart).

4.7. Continue the analysis by repeating steps 4.1–4.6 until all the fluorescent images of all embryos in the treatment group are analyzed.

4.8. Save the data and then analyze the integrated density values.

NOTE: Always select the same area size in the images during analysis. The size of the area to be analyzed depends on the magnification, image resolution, other settings for shooting, etc. The analysis can be accelerated or made more accurate by using personal macros for analysis. For example, macros can be used to ensure that the selected area is always the same in the examined images. Detailed descriptions for creating macros that allow the best image analysis according to particular photo settings are found on the ImageJ website.

REPRESENTATIVE RESULTS:

In the experiment presented in this manuscript, the effects of two estrogenic substances were tested at five concentrations starting at fertilization for 5 days on *Tg(vtg1:mCherry)* zebrafish embryos. We investigated whether fluorescent signals appeared in the liver of fish by the end of the exposure time because of the substances and whether there were differences in the estrogenicity of the two substances. Results were evaluated on the basis of the fluorescent images and integrated density values. In general, both substances induced expression of the

transgene by the end of the exposure time at those test concentrations at which individuals survived. In the cases of untreated control fish, no fluorescent signal was visible.

In the case of α-ZOL, at the highest test concentration (8 µM) all individuals died, so in this case the fluorescent signal could not be examined. At lower concentrations (0.5 µM–4 µM), a strong fluorescent signal was observed in the liver of the embryos (**Figure 4A**). No significant difference was observed in the fluorescence intensity and the size of the fluorescent areas ($p < 0.05$). The α-ZOL integrated density values (**Figure 4C**), show that the substance induced the appearance of a fluorescent signal. No significant difference was found between the integrated density values and between treatments ($p < 0.05$). The average integrated density varied between 31.26 ± 13.95 (0.5 µM) and 34.25 ± 15.36 (4 µM).

No mortality was documented during the treatment with β-ZOL, and the substance induced transgene activity at all treatment concentrations. The fluorescence intensity and the size of the fluorescent area increased as the concentration increased, as seen in the fluorescent images (**Figure 4B**). Comparing the fluorescent images of α- and β-ZOL visually, both the signal strength and the size of the fluorescent area were visibly weaker for β-ZOL at the same treatment concentrations of the two substances. Studying the integrated values of β-ZOL (**Figure 4D**), the average integrated density value almost doubled between the lowest and the highest treatment concentrations. However, in the case of β-ZOL there was no significant difference between the integrated density values of the individual concentrations ($p < 0.05$). The average integrated density varied between 15.86 ± 4.08 (0.5 µM) and 21.73 ± 5.94 (8 µM).

[Insert **Figure 4** here]

By examining the integrated density values obtained from the same treatment concentrations of the two substances (**Figure 5**), α-ZOL presented higher integrated density averages in each case relative to β-ZOL, which is consistent with the differences between signal strengths observed in the fluorescent images. In the cases of all treatment concentrations, significant differences (0.5 µM, $p = 0.0011$; 1 µM, $p = 0.0003$; 2 µM, $p = 0.0329$; and 4 µM, $p = 0.0325$) were found.

[Insert **Figure 5** here].

FIGURE AND TABLE LEGENDS:

Figure 1: Red fluorescent signal in the liver of vtg1:mCherry transgenic adult zebrafish and 5 dpf embryos, following 17-ß-estradiol (E2) induction. In female and in male treated with E2 (25 µg/L exposure time:48hrs) strong fluorescence of the liver is visible even through the pigmented skin. No fluorescent signal is visible in untreated male (**A**). Following E2 induction (50 µg/L exposure time: 0-120 hpf), a red fluorescent signal in the liver of 5 dpf embryos can also be observed, which is not visible in control embryos (**B**). While the fluorescent signal is continuously present in adult females, primarily males and embryos of the line are suitable for detecting estrogenic effects. (BF: bright field, mCherry: red fluorescent filter view, single plain images, Scale bar A: 5mm, scale bar B: 250 µm)

Figure 2: Dose-response diagram (A) and fluorescent images (mCherry) of the liver (B) exposed to 17-α-ethynilestradiol (EE2), in 5 dpf vtg1:mCherry larvae. Results are expressed as integrated density generated from the signal strength and the size of the affected area (±SEM, n = 60). 100% refers to the observed maximum. Fluorescent signal intensity increased gradually with concentration. Scale bar = 250 µm.

Figure 3: A 10 cm Petri dish with glued 1.5 x 1.5 cm wide, 1 mm thick plastic sheet squares for larvae preparation for photography.

Figure 4: Presentation of integrated density values derived from the intensity of fluorescent signals in the liver and from the size of the affected area caused by α- and β-zearalenol treatment on 5 day old *Tg(vtg1:mCherry)* transgenic zebrafish embryos. In the experiment, estrogen-sensitive embryos of the biomarker zebrafish line (20 larvae per groups in three replicates in every treatment concentration) were treated with 0.5 µM–8 µM concentrations of α- and β-ZOL from fertilization onwards for 5 days. Images of the fish livers for α-ZOL (**A**), and β-ZOL (**B**) clearly show that the substances induced the appearance of the fluorescent signal. Integrated density data are presented as mean ± standard deviation (SD = error bar). Data were analyzed with the iterative Grubbs' for identify outliers, which were excluded. Data were checked for normality with the Shapiro-Wilk normality test and compliance with the requirements of parametric methods was established. Statistical analyses were performed using a one-way ANOVA followed a Dunnett's test. Studying the integrated density values, no significant difference was found between the treatments in the cases of α-ZOL (**C**) and β-ZOL (**D**) ($p < 0.05$). Scale bar = 200 µm.

Figure 5: Comparison of α- and β-zearalenol integrated density values. Integrated density data are presented as mean ± standard deviation SD = error bar. Data were analyzed with iterative Grubbs' to identify outliers, which were excluded. Data were checked for normality with the Shapiro-Wilk normality test and compliance with the requirements of parametric methods was established. Significant differences were verified with unpaired t-test between α-ZOL and β-ZOL in the case of each concentration (0.5 µM, $p = 0.0011$; 1 µM, $p = 0.0003$; 2 µM, $p = 0.0329$; and 4 µM, $p = 0.0325$).

DISCUSSION:

The use of biomonitors/bioindicators for estrogenic effects has been spreading in toxicological studies. In vivo models play an outstanding role, because unlike in vitro tests, they not only provide information about the response of a cell or a receptor, but also allow the investigation of complex processes in the organism. Several transgenic lines for studying estrogenic effects have been produced from zebrafish, one of which *Tg(vtg1:mCherry)* was used for these studies. The method described here illustrates a protocol for the testing of embryos of this line in order to detect estrogen activity in vivo in pure, active ingredients.

Males and embryos of the line are also suitable for detecting estrogenic effects, but embryos have several advantages that promote their usability. In particular, the body is transparent, so the fluorescent signal in the liver can easily be observed. The zebrafish liver begins to develop 6

hours after fertilization (6 hpf) and starts working after 50 hours (50 hpf). First, the left lobe of the liver is formed, and at 96 hours (96 hpf) the right lobe of the liver also appears. The final shape of the liver is developed by around day 5 (120 hpf)[28,29]. The liver is able to produce endogenous vitellogenin from the age of 2–3 days of an embryo[14], which coincides with the appearance of the fluorescent signal in the *Tg(vtg1:mCherry)* line[15]. Therefore, when designing experiments, it should be taken into account that a fluorescent signal can only be expected in the embryo liver of from that time. The liver of the 5 day old embryos is already well-defined in a relatively large area, where the fluorescent signal can be easily detected under a stereomicroscope. This makes the development of test protocols that are not subject to animal protection laws possible. Vitellogenin, and similarly the fluorescent protein, are produced by the left lobe of the embryos' liver[15]. Therefore, the spatial orientation of the embryos is important for the detection of the strongest signal when examining the fluorescent signal or taking photographs. This is why embryos were laid on the left in the protocol. As can be seen from the representative results, the estrogenic effect of a test sample is clearly indicated by the fluorescent signal in the liver, so the results can be evaluated visually too. If the quantification of the results is needed, then the integrated density value defined by the ImageJ program is appropriate. However, for proper evaluation, it is indispensable that images be taken with the same settings during the experiment, and that the size of the highlighted fluorescent areas is the same in each image. Together with the precise positioning of the embryos, these are the most critical steps in the protocol. It is important to mention that in the case of embryos the expression of the transgene, similarly to the production of endogenous vitellogenin, shows a large dispersion and differences in individual sensitivity. In some cases, this can cause large variations in the results, which should be taken into account when designing the experiments.

An important aspect in determining treatment concentrations is that the cells of the embryos, and hence the liver cells, can be damaged by high concentrations of highly toxic substances, which can lead to a decline in vitellogenin induction. Therefore, tests should be performed at concentrations below LC_{10}[15].

Comparing the sensitivity of estrogen-sensitive fish lines to each other is a difficult task, because the lines described so far have been tested according to different protocols[5,14–16]. The line tested in this protocol is capable of detecting dose-effect relationships in cases of pure active ingredients, mixes, and environmental samples, and the obtained results correlated well with results with BLYES tests and HeLa cells[15,20].

The utility of the embryos of the line to test agents has been proven, including zearalenone [15]. In this work, two metabolites of the toxin, α- and β-zearalenol, were tested. According to literature data, α-ZOL is more toxic than β-ZOL[30] and its estrogenity is also higher[31]. These results are confirmed by our studies. Thus, studies on the embryos of the line are also suitable for comparing the estrogenic effects of other estrogenic substances.

Mycotoxin contamination in the food chain is a global problem, so several procedures have been improved to reduce mycotoxin levels in animal feed and human food[25,32]. One of the most promising solutions is mycotoxin biodegradation by microorganisms or by their enzymes. It may

be an essential postharvest method to decrease or eliminate mycotoxin decontamination. The ZEA degrading ability of numerous bacterial strains has been tested in the literature so far, however, recent research findings that prove high degradation of the toxin rarely specify the adverse effect of metabolites[33]. Because the embryos of this line are theoretically suitable to test the estrogenic effects of samples with organic matter content[15], a treatment protocol can be developed that can help test the biodegradation products of ZEA and the qualification of the degrading strains.

This protocol can be altered in many ways according to the planned test endpoints (e.g., exposure onset and length) and to the samples (e.g., mixtures or environmental samples) that are going to be tested and can be completed with other test methods (e.g., molecular methods). Thus, we hope that the use of the *Tg(vtg1:mCherry)* line will become a model of estrogenicity tests and for standard testing methods.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

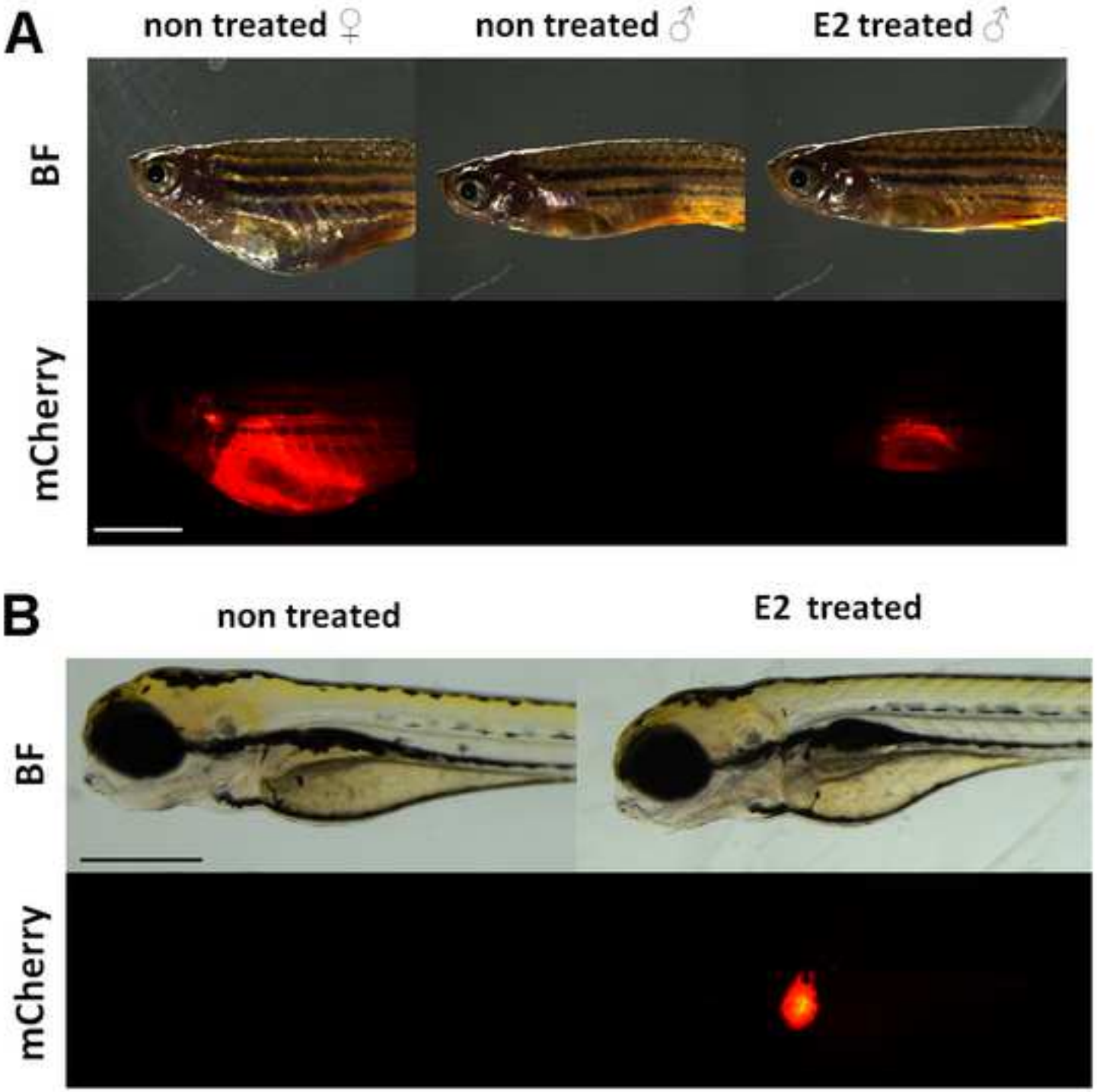

Figure 2

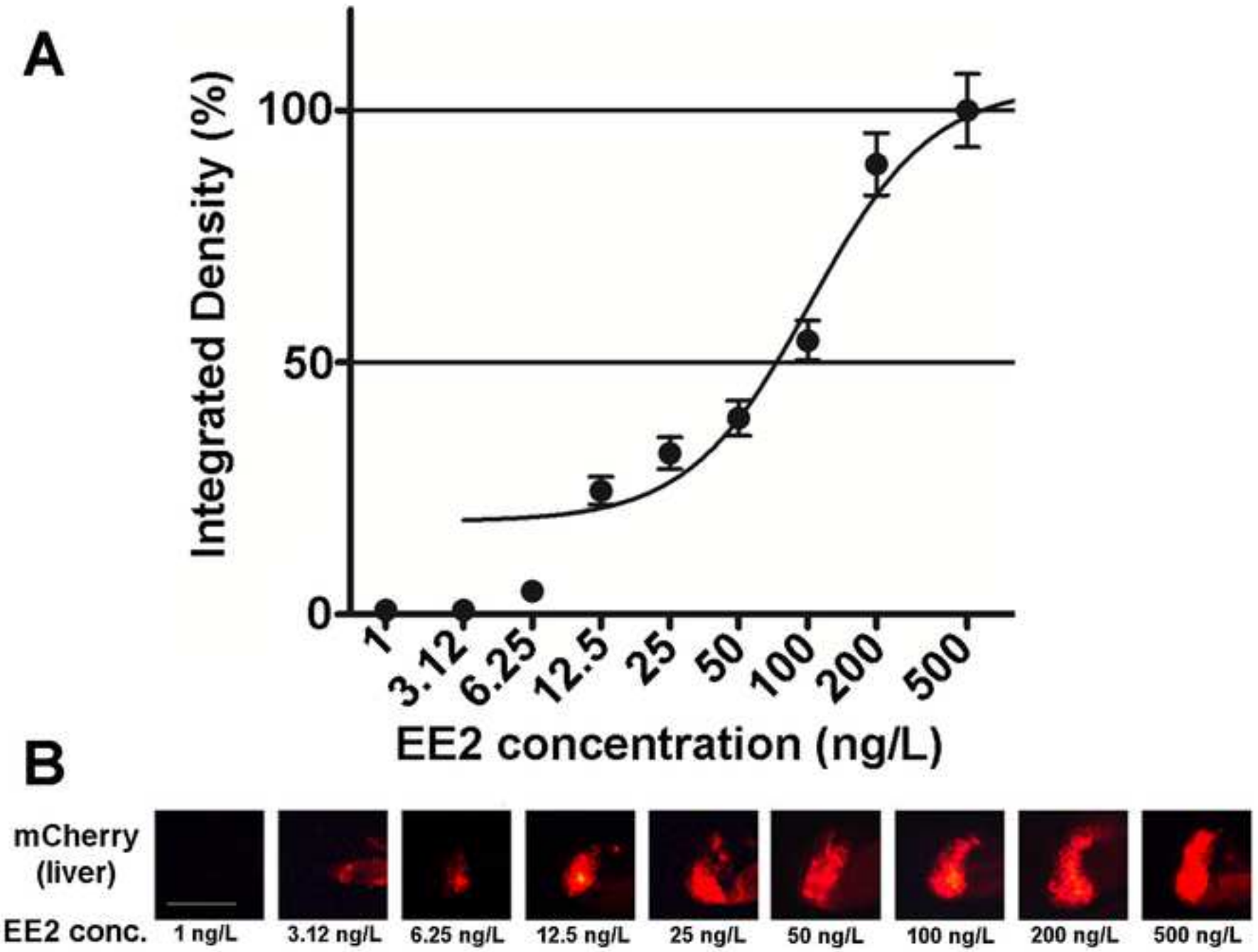

Figure 3

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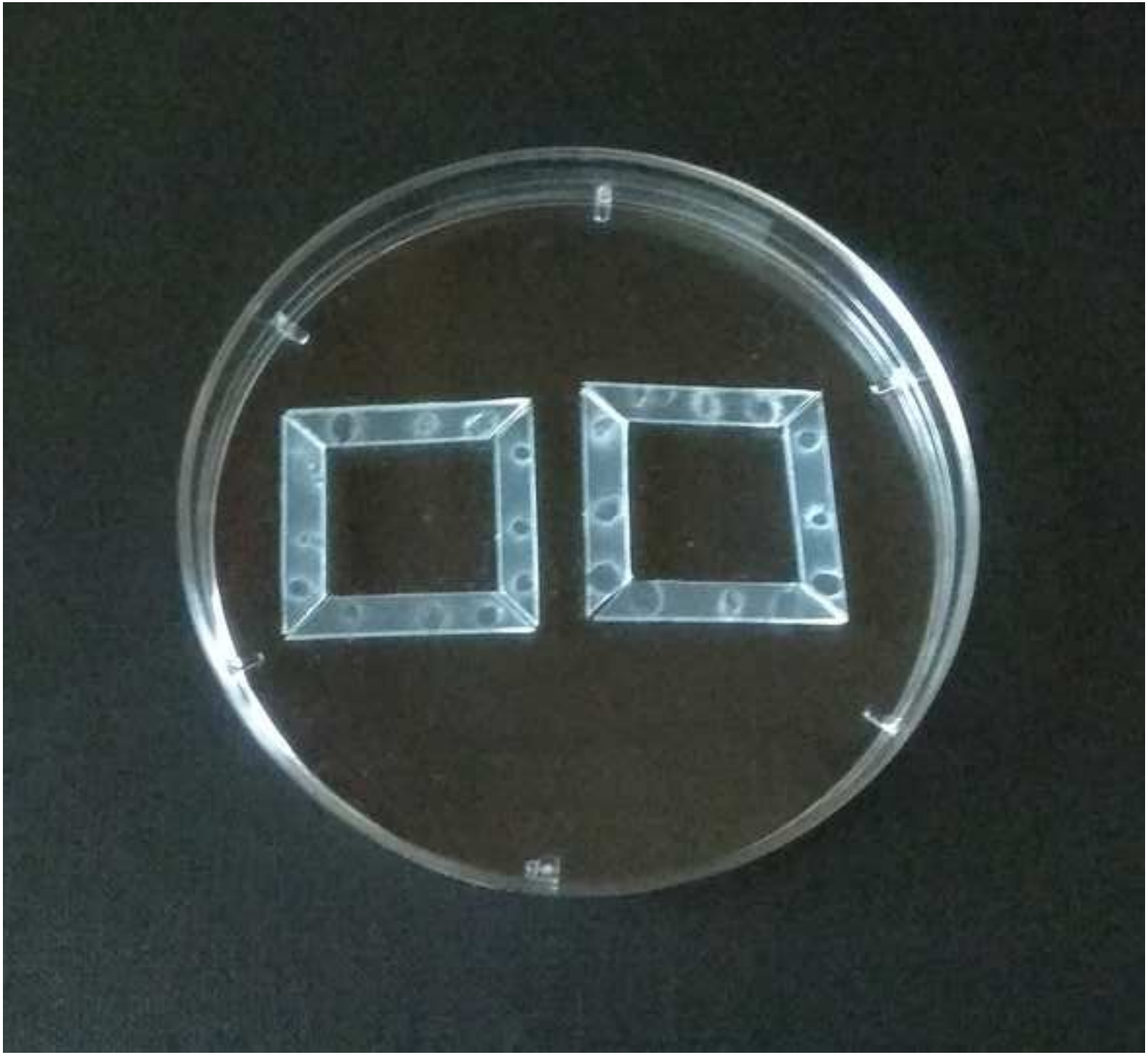

Figure 4

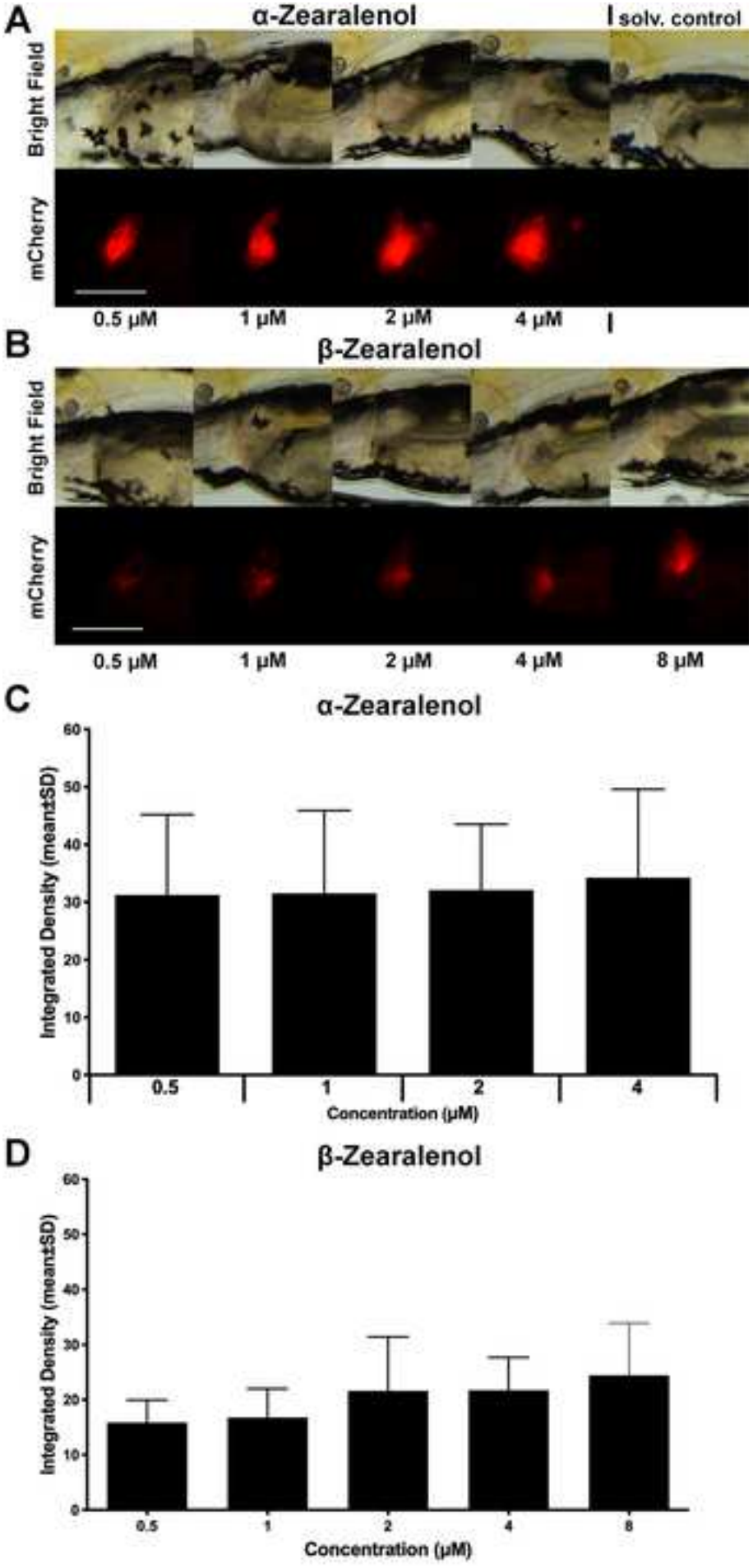

Figure 5

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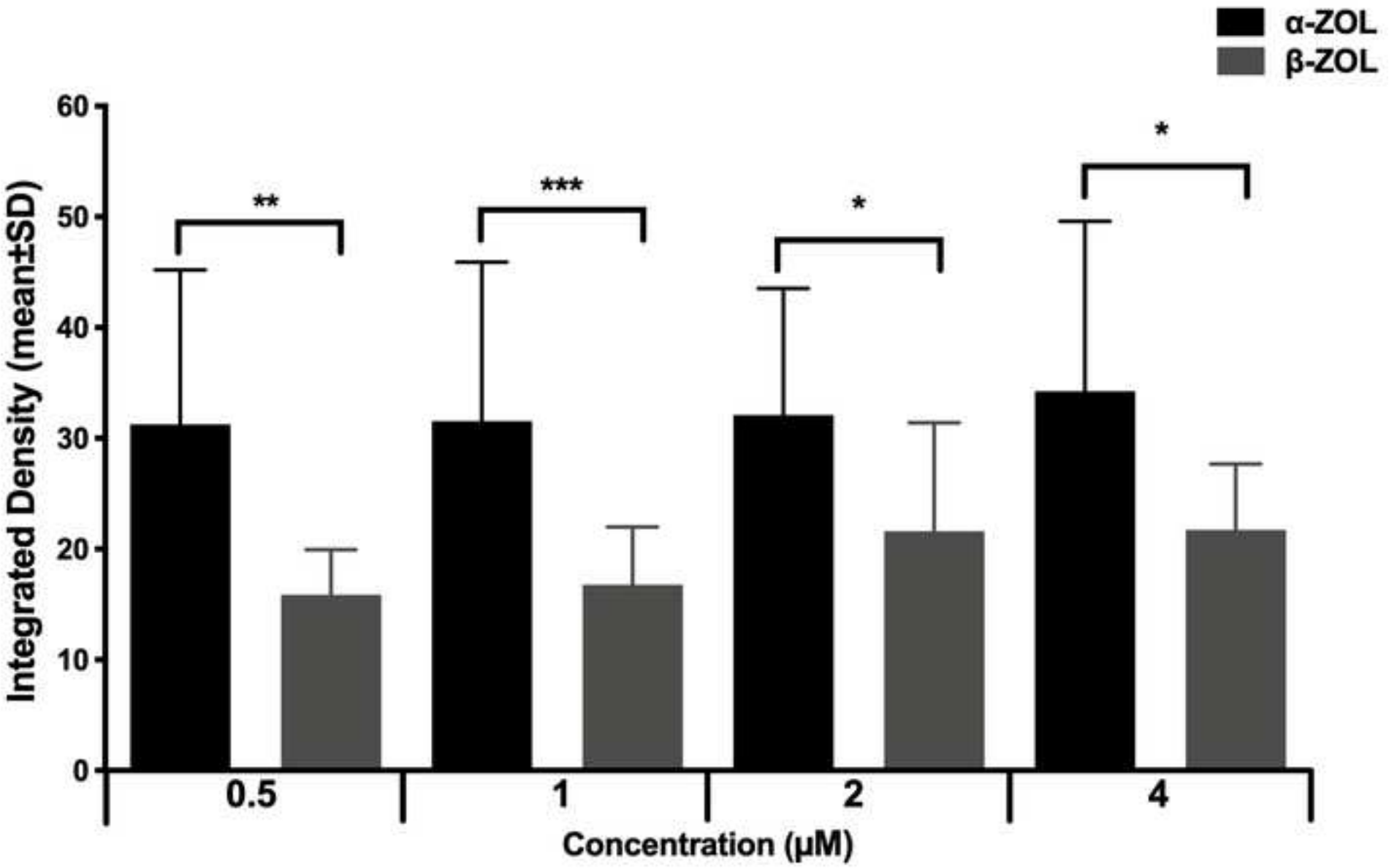

Name of Material/Equipment	Company	Catalog Number	Comments/Description
24 well tissue culture plate	Jet Biofil	TCP011024	
Calcium-chloride (CaCl2)	Reanal Laborvegyszer Ltd.	16383-0-27-39	
GraphPad Prism 6.01 software	GraphPad Software Inc.		
ImageJ software	National Institutes of Health, USA		Public access software, downloadable from: http://imagej.nih.gov/
Leica Application Suite X calibrated software	Leica Microsystems GmbH.		We used the softver described in the experiments, but any photographic software complies with the tests
Leica M205 FA stereomicroscope, Leica DFC 7000T camera	Leica Microsystems GmbH.		We used the equipments described in the experiments, but any fluorescent stereomicroscope is suitable for the tests
Magnesium-sulphate (MgSO4)	Reanal Laborvegyszer Ltd.	20342-0-27-38	
mCherry filter	Leica Microsystems GmbH.		
Mehyl-cellulose	Sigma Aldrich Ltd.	274429	
Microloader pipette tip	Eppendorf GmbH.	5242956003	
Pasteur pipette	VWR International LLC.	612-1684	
Petri-dish	Jet Biofil	TCD000060	
Potassium-chloride (KCl)	Reanal Laborvegyszer Ltd.	18050-0-01-33	
Sodium-chloride (NaCl)	Reanal Laborvegyszer Ltd.	24640-0-01-38	
Tricane-methanesulfonate (MS-222)	Sigma Aldrich Ltd.	E10521	

Editorial comments:

First of all we would like to thank you for reviewing our work and article. We greatly appreciate your work. We hope that our comments and corrections are acceptable, we were trying hard to complete the corrections according to our best knowledge. Our answers can be found below the comments.

1. **The editor has formatted the manuscript to match the journal's style. Please retain and use this version for revision.**

Thank you very much for your help. We kept the format.

2. **Please address specific comments marked in the manuscript.**

We responded to all specific comments and tried to comply with them.

3. **Please reword lines 94-99, 104-15, 124-125, 127, 129-130, 377-381 as it matches with previously published literature.**

The specified lines have been corrected.

4. **Once done please proofread the manuscript well for any grammar or spelling issues.**

Proofreading has been done to the best of our knowledge throughout the manuscript.

Video

1. Please ensure that the title is the same both in the video and in the text.
2. Please remove the numbering from the subheadings to avoid confusion.
3. 2:23 The two figures can be shown together for clarity.
4. 2:43 Please ensure that the subheadings are the same both in the video and in the text.
5. Please move the ethics statement before the start of the protocol. i.e., before showing the protocol subheading around 2:42.
6. Please ensure that the narration volume is the same throughout.

To the best of our knowledge, we have improved the video following the instructions provided.

Specific comments:

First of all we would like to thank you for reviewing our work and article. We greatly appreciate your work. We hope that our comments and corrections are acceptable, we were trying hard to complete the corrections according to our best knowledge. Our answers can be found below the comments.

Commented [A1]: The manuscript needs a thorough.

Proofreading has been done to the best of our knowledge throughout the manuscript.

Commented [A2]: The title needs rewording. Maybe change the marked worked with either endocrine disrupting compounds or nonsteroidal estrogens?

We have changed the title to “Using Tg(vtg1:mCherry) zebrafish embryos to test the estrogenic effects of endocrine disrupting compounds”

Commented [A3]: Reworded for clarity please check.
Thank you very much for the rewording, we have accepted it.

Commented [A4]: Citation? Commented [A5]: Citation? Commented [A6]: Citation for this zebrafish line.
Citations were inserted for the requested sections.

Commented [A7]: Please explain how these sites are helpful for your experiment, also please expand (estrogen response elements).
An explanation has been added. Lines:96-102

Commented [A8]: Please include this significance of using such a tragenic line with respect to your experiment being described below.
The requested information were added to the manusript. Lines: 104-107

Commented [A9]: What are the compounds used and how? Please expand this
An explanation has been added. Lines: 127-129

Commented [A10]: Before providing this explation, please first explain that these are EDCs present in the environment with citations. Then explain why you need to assess this individually. Please include citation along.
The requested changes and explanations have been made. Lines: 117-120

Commented [A11]: Please include citations, and please include the significance of using these compounds.
Citations were inserted for the requested sections. The rationale for choosing the materials is explained in the discussion section. Lines: 418-433

Commented [A12]: Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text. Commented [A13]: Please ensure that the subheadings are exactly same both in the video and in the text.
The video has been reworked as requested.

Commented [A14]: Any visual cues to look for before harvesting? Any time contrainsts?
No special visual cuest or time contrainsts are required for this step (1.5).

Commented [A15]: This part is not shown in the video. Please include if its important to incubate in the incubator at 25.5 degrees.
Step 1.6 was added to the video.

Commented [A16]: What is the time point in your case?
The Note following step 1.9 was completed with the requested information. Line: 178

Commented [A17]: After how long do you refresh the etest solution.
The Note following step 1.10 was completed with the requested information. Lines: 186-190

Commented [A18]: Moved here for clarity, please check this part.

The Larvae preparation for photography section has been redesigned as directed. Thank You for the help.

Commented [A19]: For how long, how do you check the anesthesia is enough?
The Note following step 2.3 was completed with the requested information. Lines: 210-211

Commented [A20]: What kind of squares?
We have reworded this section to avoid misunderstandings. Lines: 216-219

Commented [A21]: Included here to remove redundancy. Please check and change the narration accordingly.
We have changed the narration. Thank You for the help.

Commented [A22]: Please make this a separate section both in the video and in the text. Please include numbered action steps in imperative tense to show how the steps are performed. Some of the examples are given below. Please include all the button clicks.
We did a separate Microscopy section for the manuscript and the video. Lines: 232-259

Commented [A23]: How do you ensure this?
The Note following step 3.1 was completed with the requested information. Line: 241

Commented [A24]: These details can be moved to the microscopy section. Please do not include commercial names and use generic terms only. Please include all commercial terms in the Materials table.
The requested changes have been made and the table has been completed.

Commented [A25]: This needs clarity- how is this done in your experiment.
Step 4.6 was completed with the requested information. Lines: 284-285

Commented [A26]: Please move this this to the table of materials. This sentence can be removed from here.
The requested changes have been made and the table has been completed.

Commented [A27]: Presently the result shown doesnot match with the title and the hypothesis generated in the introduction. Please also include some results to dose specific response as asked by the reviewers. The results can be reprinted from the previous publication. If using previously published results, please obtain reprint permission and cite the publication accordingly. In the result section please explain yor observation and how did you use the results presented to conclude what you wanted to and how is it in line with the title
To support the usability of the transgenic line, we added a new figure (Fig. 1) to the introduction section to the manuscript (as well as in the video). The figure shows the dose-response diagram and fluorescent images (mCherry) of the liver exposed to 17-α-ethynylestradiol (EE2) in 5 dpf vtg1-mCherry larvae. The experiment for the image was based on the experiment described in Aquatic Toxicology and the structure of the figure is the same as found in the journal. However, these results and photos have not been published in any journal so far, they were prepared for a sensitivity test of a new generation of the transgenic line. The results are, in our view, a good indication of the utility of the line for assaying estrogenic substances and the appropriate sensitivity for displaying dose-response responses. The manuscript was supplemented in several places and explained the characteristics of the representative experiment. It is hoped

that the modifications made will be adequate to prove the utility of the transgenic line, since the steps of the test method described can be used independently of the properties of the test substance and of the sample being tested, can be adapted to the experiments and also can be quantified and evaluated using the method described.

Commented [A28]: Please check reworded for clarity. The narration in the video says 0.5 and 8 micro Molar… the results show different concentrations in between too. Please redo the narration.
The narration has been checked.

Commented [A29]: Instead of untreated, please show vehicle treatment to clearly show that there were no autofluorescence observed.
The images were modified for proper control.

Commented [A30]: Significance of this?
The requested information has been provided.

Commented [A31]: Figure number changed to match the figures being discussed. Please check.
Image numbering has been verified.

Commented [A32]: What is the significance of this? This line is not being used to differentiate between two estrogenic compounds (since both give red signal) but to test for the presence of estrogenic compounds in the environment. This can just be additional figure to show that the signal strength depends on the toxicity of the substances and can be measured using fish as the model system. Showing a dose dependent response to estrogenic compound / or showing how this line is used to test the presence of estrogens in environmental sample is helpful as asked by the reviewers.
We supplemented the manuscript to support the dose-response relationship of the line to estrogenic compounds (Fig.1). However, the line is also suitable for analyzing environmental samples and for monitoring changes in the estrogen effect due to mixtures of substances. We refer to these results in several places in the manuscript (Bakos et al., Faisal et al.) and we hope that our future readers will review these articles before working on the line. The line is also suitable for comparing the estrogen activity of different substances based on representative results, as it can be seen from the results in the chart. The differences between the fluorescent signals of the two degradation products are also apparent, and the difference between the numerical values can be statistically justified. The practical applicability of the results obtained with the two ZOLs was substantiated in the discussion.

Commented [A33]: Please include how error bars were derived. What does it represent? How many fishes were analyzed per concentration? How many experiments by substance was performed? Please include all details.
The figure caption was supplemented with the requested informations.

Commented [A34]: Please include.
The error bar has been supplemented.

Commented [A35]: Please reword.
The sentence has been reworded.

Commented [A36]: Not clear from the results presented in this manuscript.
We supplemented the manuscript to support the dose-response relationship of the line to estrogenic compounds (EE2) (Fig.1).

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Author(s): ZS. CSENKI, A. HORVATH, I. BOCK, E. GARAI, F. KEKESES, E. VASARHELYI, B. KOVACS, B. URBANYI, F. MUELLER, K. BAKOS

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