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Semi-automated detection of goitrogenic compounds using transgenic zebrafish embryos and the VAST BioImager platform

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Introduction and objectives

The knowledge on environmentally relevant pollutants that may interfere with thyroid signaling is scarce. Here, we present a method for the screening of thyroid disrupting chemicals (goitrogens) based on the transgenic zebrafish line tg(tg:mCherry) using the VAST (Vertebrate Automated Screening Technology) BioImager platform. While the strain tg(tg:mCherry) reflects the expression of thyroglobulin (the thyroid hormone precursor) [1], the VAST system allows an easier and relatively fast handling of zebrafish embryos compared to manual procedures [2]. This method may be also applied to environmental samples.

Materials and methods

Chemicals

For the exposure experiments, the following chemicals were used: 3,4-dichloroaniline (DCA, CAS–RN 95-76-1, purity ≥98%; Aldrich), dimethylsulfoxide (DMSO, CAS–RN 67–68–5, purity ≥99.5%; ≤200 ppm H2O, Merck), N, N'-ethylenethiourea (CAS–RN 96-45-7, purity ≥98%, Fluka), phloroglucinol (CAS–RN 108-73-6, purity ≥99%, Aldrich), potassium perchlorate (CAS–RN 7778-74-7, purity ≥99%, Sigma-Aldrich), 6-propyl-2-thiouracil (PTU, CAS–RN 51-52-5, analytical standard, Fluka), pyrazole (CAS–RN 288-13-1, purity ≥98%, Aldrich), resorcinol (CAS–RN 108-46-3, purity ≥99%, Sigma- Aldrich), sulfamethoxazole (SMX, CAS–RN 723-46-6, analytical standard, Fluka), thyroxine (T4, CAS–RN 51–48–9, purity ≥98%, Sigma).

Green algae samples

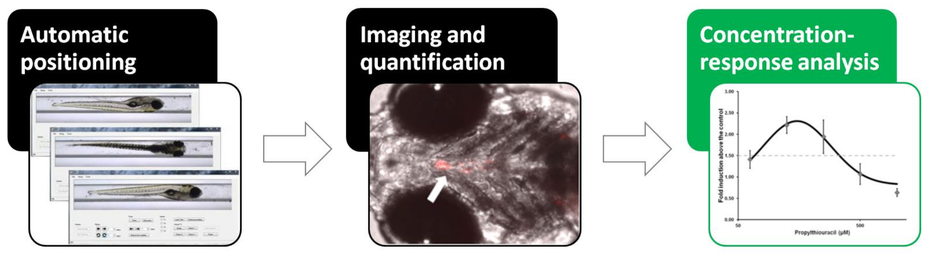
Chlorella kessleri (cultivation number 102) were cultivated 21 days in a mixture of Zehnder and Bristol medium with distilled water in the ratio of 1:1:2 (v/v/v). Organisms were grown for 21 days at 22◦C ± 2◦C under continuous light (cool white fluorescent tubes, 3000 lx) and aeration. The cultivations were started with a 10% (v/v) inoculum of a previous culture. Cells were separated by centrifugation at 3000 g. Concentrated biomass were stored frozen -18°C. The concentration of dry matter was determined gravimetrically after lyophilization (117 g DW /l).

Zebrafish maintenance and exposure

We used the UFZ-OBI strain (generation F12, established from a stock of a local breeder) crossed with the tg(tg:mCherry)1 zebrafish strain (generation F9) provided by the University of Brussels [1]. Fish were cultured at 26±1°C at a 14:10 h light: dark cycle in a recirculating tank system, and used according to German and European animal protection standards and were approved by the Government of Saxony, Landesdirektion Leipzig, Germany (Aktenzeichen 75–9185.64). Zebrafish embryos were exposed from 48–120 hours post fertilisation (hpf). Dilutions of the stock were prepared freshly before starting the exposure with embryonic medium according to the OECD testing guideline 236.3 The DMSO concentration never exceeded the 0.01% of the exposure solutions. Embryos were kept in the incubator at 26°C and a 14:10 hours light:dark cycle.

Screening of tg(tg:mCherry) embryos and image analysis

Embryos at 120 hpf were anesthetized with MS222 (600 mg/L) and were sampled using the VAST (vertebrate automated screening technology) BioImager platform (Union Biometrica, Gees, Belgium) [2]. The VAST platform was used in tandem with a Leica Microscope (Leica DM5000B) equipped with a Leica digital camera (DFC 365FX), an external excitation light source (Leica EL6000), and a 20X objective (HXC APO L-U-V-I 20x/0.50). Using the VAST BioImager, embryos were positioned ventrally and at least 10 embryos per sample were photographed using the same illumination conditions: 400 ms of fluorescent light exposure at maximal light intensity, a gain of 3, gamma value of 1 and grey scale from 0–255. The micrographs were analysed using the Image J software (http://rsbweb.nih.gov/ij/). For each micrograph a fold induction of fluorescence was calculated by comparing the integrated pixel density with that of the mean of the controls.



Real time PCR

Total RNA was extracted from control and exposed embryos at 120 hpf. 2 µg of RNA was reversely transcribed with RevertAid™ H Minus Reverse Transcriptase (Fermentas, Leon-Roth, Germany) in 20 µl reaction mix according to the manufacturer instructions . qPCR was carreid out using a Step-One-Plus PCR system (Applied Bioosystems, Darmstadt, Germany) and SensiMix ™ SYBR with ROX as passive reference dye (Bioline, Luckenwalde, Germany) in 12.5 µl reaction volume . The PCR reaction was conducted with an initial denaturalization of 10 min at 95 °C, followed by 35 cycles of 15 s denaturation at 95 °C, 20 s annealing at 57 °C and 20 s elongation at 72 °C. Efficiencies were in the range of 90 – 100 %. Sequences used (5'-3'): ef1a; Fw - CGTCTGCCACTTCAGGATGTG, Rv – ACTTGCAGGCGATGTGAGCAG / tg; Fw - CTGGTCACCTGTGGTTGATG, Rv – TCCCTGAAGCTGCTCAAAAT. Results are expressed as mRNA copies relative to the reference ef1a gene.

Statistical analysis

To obtain the concentration-response curves, the fold induction values were fitted to models describing exponential, hyperbolic, logistic, linear and peak-shaped gene expression profile. The applied models were ranked by calculating the Akaike's Information Criterion (AIC) for each fit. The curve fitting and the estimation of AIC were done using an R script6 based on the 'drc', 'DoseFinding', 'epicalc' and 'multicomp' R packages [3]. According to the comparison of different models, the Gaussian-log model (4 parameters) was selected as suitable model for all compounds tested:

$$y = c + (d - c)\exp\left(-0.5\left(\frac{\log(x) - \log(e)}{b}\right)^2\right)$$

Results

Chemical compounds

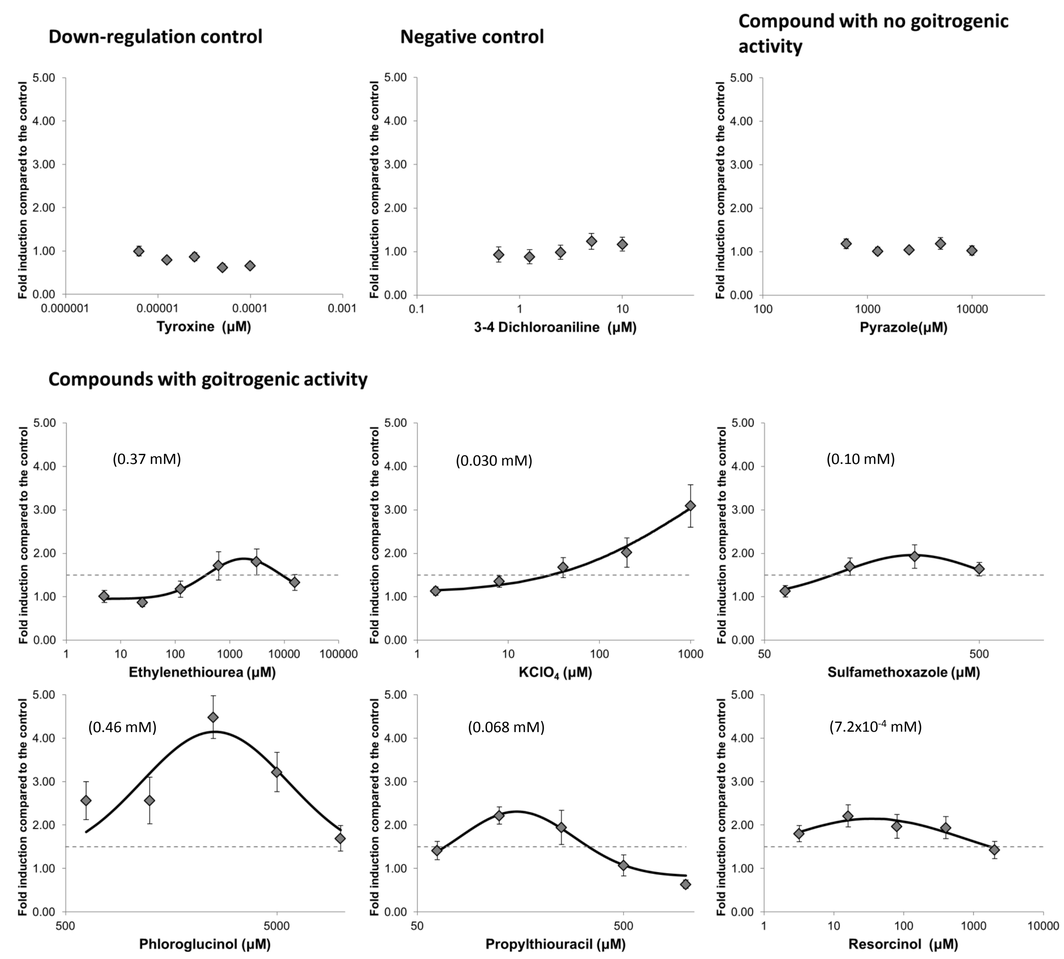


Figure 1. Concentration-dependent induction of tg:mCherry fluorescence in zebrafish embryos. The concentration-dependency of compounds with induction > 1.5-fold was fitted to a logarithmic Gaussian (4 parameters) model. The dashed line highlights the 1.5-fold inductions levels, which was used to calculated effect concentrations (numbers in brackets). Grey diamonds indicate the mean of fluorescence signal for at least 10 embryos. Error bars represent the standard error.

Green algae samples

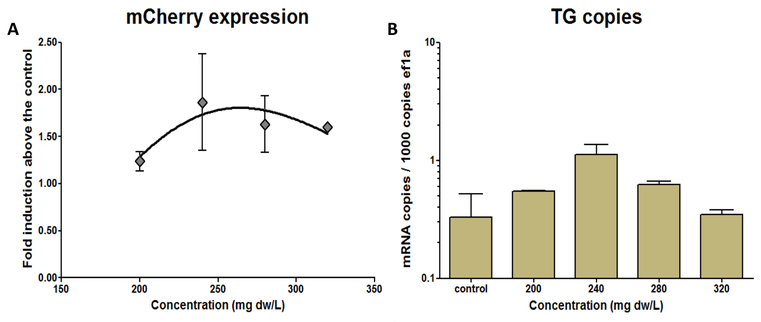


Figure 2. (A) Induction of tg:mCherry fluorescence in zebrafish embryos exposed to *Chorella Kessleri* samples **(B)** qPCR expression levels of thyroglobluin (TG)

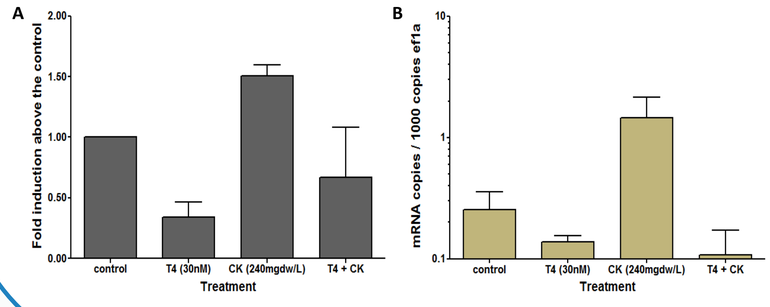


Figure 3. (A) Induction of tg:mCherry fluorescence after exposure and coexposure to T4 and *Chorella Kessleri* samples **(B)** TG expression levels corresponding to the same exposures represented in A

Conclusions

i) The combination of the VAST system, fluorescence imaging and concentration-response analysis represents an efficient method for the screening of compounds with goitrogenic activity.
ii) The resulting semi-automated method facilitates and speeds up the screening process and can easily be conducted by a user with little technical expertise.
iii) This detection system may not only be applied to identify individual potentially goitrogenic compounds but also be applied for the assessment of potential goitrogenic activities associated to environmental samples or cell/tissue extracts.

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References:
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