Automated Morphological Feature Assessment for Zebrafish Embryo Developmental Toxicity Screens

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ABSTRACT

Detection of developmental phenotypes in zebrafish embryos typically involves a visual assessment and scoring of morphological features by an individual researcher. Subjective scoring could impact results and be of particular concern when phenotypic effect patterns are also used as a diagnostic tool to classify compounds. Here we introduce a quantitative morphometric approach based on image analysis of zebrafish embryos. A software called FishInspector was developed to detect morphological features from images collected using an automated system to position zebrafish embryos. The analysis was verified and compared with visual assessments of 3 participating laboratories using 3 known developmental toxicants (methotrexate, dexamethasone, and topiramate) and 2 negative compounds (loratadine and glibenclamide). The quantitative approach exhibited higher sensitivity and made it possible to compare patterns of effects with the potential to establish a grouping and classification of developmental toxicants. Our approach improves the robustness of phenotype scoring and reliability of assay performance and, hence, is anticipated to improve the predictivity of developmental toxicity screening using the zebrafish embryo.

Key words: developmental toxicity; zebrafish embryo; alternatives to animal testing; image analysis.
an individual technician or researcher and limited standardization. Hence, the approach currently used for developmental toxicity screening in zebrafish embryos might be biased by the experience and accuracy of the observer. Furthermore, observations are often not documented by storing the relevant images, thus making verification and reanalysis of data difficult.

Previous phenotypic image analyses have focused on fluorescent imaging for measuring, that is, cardiovascular development (Leet et al., 2014), cardiovascular function (Burns et al., 2005; Leet et al., 2014; Letamendia et al., 2012; Yozzo et al., 2013), and angiogenesis (Letamendia et al., 2012; Vogt et al., 2009). There are few published studies using automatic phenotypic image analysis for bright-field microscope images without fluorescent markers or staining (Arslanova et al., 2010; Deal et al., 2011; Jeanray et al., 2015; Liu et al., 2012; Schutera et al., 2016). Some of these studies were limited to the identification of specific phenotypes such as lethality (Aishut et al., 2010; Liu et al., 2012), hatching status (Liu et al., 2012), changes in pigmentation (Arslanova et al., 2010; Schutera et al., 2016), or lack of eyes (Schutera et al., 2016). One study aimed at developing a computational malformation index through the use of morphometric parameters (eg, total body area, convexity) in combination with a very brief human visual assessment (Deal et al., 2016). That method was more objective as user scoring was based on microscopic observations and the cumulative degree of abnormality could be described, but the different phenotypes (eg, edema, small eyes) were not resolved. A different approach was developed by Jeanray et al. (2015) using supervised machine learning to identify developmental phenotypes. This approach is based on an initial expert classification of phenotypes and requires several rounds of classification and learning but can be used to establish concentration-response curves for cumulative phenotypic assessment. However, the same or similar instrumentation and settings would be required to apply their established models directly.

Crucial for a quantitative, unbiased approach to phenotype assessment using 2D images is a proper orientation of the fish embryos. Slight differences in the orientation and the subsequent 2D projection could lead to changes in feature detection. Therefore, in this study, an image-based detection and quantification of morphological features in zebrafish embryos was developed based on an automated system for positioning of the embryos in a capillary. Multiple morphological features were automatically extracted from zebrafish images using a custom MATLAB-based software called FishInspector. Although our workflow was developed for automated positioning in a capillary, it can also be applied to manually positioned embryos as conducted in other studies (eg, Pervali et al., 2011). However, this may be more time consuming and may introduce additional variability. In a second step, we used the analytical platform KNIME and R scripts for morphometric analysis and quantification using the coordinates of each feature detected by FishInspector.

Morphological features were complemented by video-based measurements of heart rate and behavioral effects (locomotor response at 96 h post-fertilization [hpf]). These two functional parameters provide further endpoints relevant for safety areas assessment and potentially linked to developmental toxicity. For instance, a comparative endpoint analysis (Ducharme et al., 2013) has revealed a high correlation of behavioral endpoints with (gross) malformations of fish embryos and hence may support quantitation of overall assessment of teratogenic effects.

To demonstrate the capacity of the software for the multi-endpoint analysis, it was applied to a set of 5 model compounds representing diverse drug classes. Three compounds (methotrexate, topiramate, and dexamethasone) known to cause developmental toxicity in mammals and two compounds (glibenclamide and loratadine) as non-developmental toxicants. The performance of this method was also analyzed in the context of sensitivity differences between 3 laboratories experienced with conventional visual assessment and scoring of developmental anomalies in the zebrafish embryo. The intention was, for example, to understand whether the automatic assessment provides increased sensitivity compared with conventional assessments in other laboratories.

MATERIALS AND METHODS

Chemicals. The following chemicals were used: loratadine (CAS-RN 79794-75-5, purity ≥ 98%, Sigma-Aldrich), methotrexate (CAS-RN 59-05-2, purity ≥ 98.5%, AppliChem), glibenclamide (CAS-RN 10238-21-8, purity ≥ 99%, Sigma-Aldrich), dexamethasone (CAS-RN 50-62-2, purity ≥ 97%, Fluka), topiramate (CAS-RN 97240-79-4, purity ≥ 98%, Sigma-Aldrich), all-trans retinoic acid (CAS-RN 302-79-4, purity: ≥ 98%, AppliChem Panreac), and N-phenylthiourea (PTU, CAS-RN 103-85-5, purity ≥ 98%, Sigma-Aldrich). Loratadine, glibenclamide, dexamethasone, and all-trans retinoic acid were dissolved in dimethyl sulfoxide (DMSO). Test solutions were obtained by dilution of the stock solutions in embryo test medium according to the OECD testing guideline 236 (OECD, 2013, pH 7.4–7.5) resulting in final DMSO concentrations of 0.01% (all-trans retinoic acid), 0.5% (loratadine and glibenclamide), and 1% (dexamethasone). The different DMSO concentrations reflect the different solubility in DMSO, that is, the concentration of DMSO was kept as low as possible to obtain full concentration-response curves for mortality and sublethal phenotypes.

Zebrafish Developmental Toxicity Assay Overview. Adult, healthy, and unsexed zebrafish were used for the production of fertilized eggs. We used the UFZ-DBI strain (generation F14–15), obtained originally from a local breeder and kept for several generations at the UFZ. Fish were cultured and used according to German and European animal protection standards and approved by the Government of Saxony, Landesdirektion Leipzig, Germany (Aktzenzeichen 75-9185.64). Just after fertilization eggs were treated against fungal infection with a diluted Chloramine-T bleaching solution (0.5% w/v) for 60 s with gentle periodic agitation, washed twice with embryo medium and transferred into a petri dish for egg selection. Bleaching did not affect the hatching of embryos at later stages. All control embryos were hatched at 96 hpf. The bleaching was conducted to avoid carry over of fungi or microbes from the tanks. Embryos were exposed to the test compound, a solvent control and a positive control (all-trans retinoic acid at 12.5 nM) from 2 to 48 hpf and from 2 to 96 hpf, at a temperature of 28 (±1)°C (14:10 light: dark cycle). Forty-eight-hour exposures were conducted in crystallization dishes covered with watchmaker glasses with a test volume of 16 ml and 16 embryos per dish. Ninety-six-hour exposures were conducted in rectangular 96-well microplates (Clear Polystyrene, flat bottom, Uniplate, Whatman, GE Healthcare, Little Chalfont, UK) covered by a lid with a test volume of 400 μl (one embryo per well, 16 wells per concentration tested). No evaporation was observed during the exposure period. The different protocols were used since manual decoloration is difficult to conduct in 96-well plates. For hydrophobic compounds (log P < 4) low exposure volumes in 96-well microplates (400 μl exposure volume per embryo) may result in
a (pronounced) decline in exposure concentration when compared with exposure in crystallization dishes (1000 μl volume per embryo). Therefore, for hydrophobic compounds (loratadine and glibenclamide) exposure was conducted in crystallization dishes for both the 48 and 96 h exposure in order to compensate for a potential loss of exposure concentration due to absorption in embryos and to the wells of the microplate. Tests were performed with at least 2 replicates. Renewal of the exposure solutions were performed every 24 h, except for methotrexate, for which, due to confirmation of stable exposure concentration, a 48 h renewal interval was selected (see Supplementary Table 2), and for topiramate, for which stability was assumed (Michael et al., 1998) and no renewal was done. Phenotypic assessment by automated imaging (“Image-Based Quantification of Morphological Features” section) was conducted after assessment of lethality, behavioral effects (at 96 hpf), and visual assessment using a stereomicroscope (Olympus SZX10, Massachusetts). Visual and automatic image-based assessment of phenotypes at the UFZ was conducted for the same experiment and same fish. Supplementary Table 1 shows the endpoints evaluated by visual observation. More details on the test protocol can be found in Supplementary Table 2.

Developmental Staging Analysis

Comparison of developmental stages of zebrafish incubated at 28 (±1)°C was done using untreated embryos from 5 different stages from 32 to 96 hpf (32, 48, 72, 82, and 96 hpf). Linear regression analysis was performed to determine which of the features quantified using the FishInspector exhibit a significant correlation during normal development.

Image-Based Quantification of Morphological Features

Automated imaging of zebrafish embryos. Images of zebrafish embryos were obtained using the VAST Bioimager (Union Biometrica, Gees, Belgium) (Pardo-Martin et al., 2010; Pulak, 2016) with the on-board camera of 10 μm resolution. Beforehand imaging embryos were dechorionated (required for 48 hpf stages only) and anesthetized with a tricaine solution (150 mg/l, TRIS 26 mM, pH 7.5). Embryos exposed in crystallization dishes were transferred to a 96-well microplate with rectangular wells. Loading of each fish from rectangular 96-well plates was done using the LP sampler (Union Biometrica, Gees, Belgium) and 4 pictures were automatically collected (two laterals, one dorsal, and one ventral image). Additionally, a video of 15 s at 30 frames per second was recorded of each embryo in lateral position for later video-based determination of the heart frequency. For the analysis, fish embryos were removed from the microtiter plates such that individuals from different concentrations were analyzed alternately. This was done to avoid time bias. The concentration of tricaine used here has been shown not to affect the heart rate frequency within the time frame (2 h) that was used for analysis (Yozzo et al., 2013).

Feature detection using the FishInspector software. Lateral control images of embryos at 48 and 96 hpf were used initially for software development. FishInspector was developed within MATLAB environment and the source code and an executable application do not require knowledge of computer programming languages. The complete workflow only requires the use of the standard open source tools (KNIME, R, and ImageJ). The workflow is provided in Dryad, (Teixido et al., 2018). Pigmentation was quantified by measuring the sum area of pigment cells along the lateral line, using the area covered by the notochord as the enclosure region. In order to validate the pigmentation analysis, embryos were exposed to increasing concentrations (0–150 μM) of N-phenylthiourea (PTU) (Supplementary Figure 5), a model compound that is known to inhibit melanization (Karlsson et al., 2001).

Subsequently, other features are identified in a stepwise manner (Supplementary Figure 1). Hence, the detection of specific morphological features is dependent on the detection of other features and is facilitated by excluding regions that may interfere. The identification of the regions of interest was driven by visual observation and measurement of generic object properties. For example, once the contour of the fish was localized, the eye was detected by searching for a dark object either in the right or left half of the zebrafish. The detection algorithms were successively improved by using images of embryos treated with all-trans retinoic acid (used as the positive control for gross changes in body morphology). Given that establishment of a 100% correct automated feature detection would be very challenging and to allow improvement by the user, the software permits modification of the parameters used for the automated feature detection, and also manual correction if the feature is not sufficiently detected. At present, jaw morphology analysis cannot be detected automatically with the FishInspector and requires a manual annotation step that is, label of the tip of the lower part of the mouth. The resulting output of the FishInspector is a set of xy coordinates of the morphological feature detected. For each image analyzed, data are exported to a single JSON file, which is a language independent open-standard file format typically used for transmitting data between applications. The boundary coordinates of multiple detected features can then be stored in a structured text file. This allows the seamless integration of the FischInspector output into custom post-processing algorithms, which can be implemented in any programming language.
Heart Rate Quantification

An automated image workflow was developed using the KNIME Analytics Platform (workflow available in Dryad, Teixido et al., 2018). The zebrafish heart as the region of interest (ROI) is detected by comparing the absolute difference in pixel intensity between two consecutive frames. By using a threshold method and morphological operations, irrelevant areas were removed from the analysis. Then the pixel variance of the ROI in each frame was used to determine the heart frequency using a Fast Fourier transform with the spectrum function included in the base package of R.

Locomotor Response (LMR)

The locomotor response was assessed at 96 hpf prior to the analysis with the VAST Bioimager system. Embryonic movement was tracked using the ZebraBox video tracking system (Viewpoint, Lyon, France) for 40 min in a series of light and dark periods to stimulate movement (10 min equilibration in light, followed by 20 min in dark, and a final 10 min light phase) as described in Irons et al. (2010). The movement in the light periods was recorded using maximum intensity (1200 lux). Movement in light and dark periods was recorded using an infrared camera and the video tracking mode with a detection threshold set to 20. The temperature was continuously maintained at 28(±1) °C. Live embryos, including malformed embryos and embryos showing no inflation of the swim bladder, were considered for the analysis of the locomotor response. The percentage of effects (EC0) was calculated on the basis of the mean travelled distance as described in Klüver et al. (2015) using the dark phase interval (10–20 min).

Inter-Laboratory Study Design

Three laboratories participated in this study. They were: Department of Bioanalytical Ecotoxicology, Helmholtz Center for Environmental Research (UFZ), R&D Preclinical Safety, Sanofi-Aventis Deutschland GmbH, and BBD BioPhenix-Biobide. The laboratories used an agreed test protocol (described in “Zebrafish Developmental Toxicity Assay Overview” section) with minor differences between laboratories as shown in Supplementary Table 2. The UFZ was the only laboratory to include an image-based quantification of morphological features using the FishInspector (as described in “Image-Based Quantification of Morphological Features” section), heart rate quantification (“Heart rate quantification” section), and behavior analysis (“Locomotor Response (LMR)” section). Testing of the compounds was done in a blind manner at 2 of the 3 laboratories (Biobide and UFZ), that is, identity of the compounds was only released after completion of the effect assessment. The test concentrations were not harmonized between the different laboratories and were individually adjusted based on range findings or to improve the description of the concentration response curves in replicates.

Data Evaluation

Two approaches were used for the concentration-response analysis: (a) effect quantification with continuous data normalized to the mean control value and, (b) threshold-based quantal effect data. The first approach was used for endpoints with high variability between controls of replicates, observed for heart rate, behavior, and pigmentation. For these endpoints, data were normalized to the mean control of each replicate and concentration-response curves were derived from these data. For all other endpoints (eye size, body length, yolk sac size, head size, swim bladder, jaw-eye distance, and otolith-eye distance), similar to the method proposed for obtaining benchmark responses with dichotomized continuous data (U.S. EPA 2012), a threshold value was established by analysis of the variability of about 130 control embryos of different replicates (Supplementary Table 3). Values deviating by ±2 SD were considered as indicating a deviation from the control and were used to calculate the fraction of embryos for which the appropriate endpoint was affected. For the overall cumulative effect assessment, a threshold of 2.5 SD was used given the higher likelihood that one of the features was affected randomly. Concentration-response curves were derived for all the morphological features and also for lethality and abnormalities (visual assessment) only when a clear concentration-response was observed and more than 30% of embryos were affected. To characterize responses for each chemical we derived an EC50 as the concentration at which 50% of the embryos were deviating from the feature as it was observed in controls. Lethal concentrations (LC50) and effect concentrations (EC50) for each endpoint were obtained with the sigmoidal dose-response (Hill-slope) equation (equation 1) calculated in SigmaPlot (version 13.0).

### Table 1. Morphological Features Measured in the Zebrafish Using the FishInspector Software

| Phenotypic Feature | Data Exported as Json Format | Parameter or Metric | Corresponding Endpoint in Visual Assessment |
|--------------------|------------------------------|---------------------|------------------------------------------|
| Eye size           | Eye xy coordinates           | Surface area (mm²)  | Reduced eye size                         |
| Body length        | Fish contour xy coordinates  | Length (mm)         | Not assessed                             |
| Yolk sac size      | Yolk sac contour xy coordinates | Surface area (mm²) | Increased yolk sac size or abnormal morphology |
| Otolith-eye distance | Otolith xy centroid (saccule, the largest otolith) | Length (mm)         | Not assessed                             |
| Pericard size      | Pericard contour xy coordinates | Surface area (mm²) | Increased pericard size                   |
| Tail malformations | Notochord xy coordinates     | Curvature           | Tail curvature                           |
| Swim bladder inflation | Swim bladder contour xy coordinates | Surface area (mm²) | Failure to inflate the swim bladder       |
| Head size          | Fish contour xy coordinates, otolith, and eye centroid | Surface area (mm²) | Reduced or abnormal head size             |
| Pigmentation       | Area (in pixels) of pigment cells from lateral line between eye centroid and lower jaw tip | Sum surface area (mm²) | Not assessed                             |
| Lower jaw position | Distance in the x coordinate | Distance (mm)       | Underdeveloped or abnormal jaw           |

Notes: The data are exported in Json file format and used to quantify different metrics by the use of a customized KNIME workflow. The corresponding assessment using the conventional visual assessment is also shown in the table.
Constraints for max and min were set to 100 and 0. In order to rank the capability of an agent to produce developmental toxicity in relation to lethal effects we calculated the teratogenic index (TI), which is defined as the ratio between the \( \text{LC}_{50}/\text{EC}_{50} \) and was successfully established in the Xenopus frog embryo’s developmental toxicity screening assay (Mouche et al., 2017). A chemical was classified as developmentally toxic if morphological alterations were concentration-dependent reaching more than the 30% effect level. For the automatic image-based assessment, effect concentrations (EC\(_{50}\)) for all endpoints were calculated based on a log-logistic model in R (LL.4 model from package drc [Ritz et al., 2015]). To reduce uncertainty, treatment groups with less than 4 surviving individuals were excluded from the analysis. Effect signatures of visual and image-based assessment were obtained by normalizing each effect concentration to the most sensitive feature (EC\(_{50}\) most sensitive feature/EC\(_{50}\) specific feature) for each time point (48 and 96 hpf). This allows for comparison of all features at the same scale. Hierarchical clustering was performed based on the “Manhattan” distance using the hclust function in R and “Ward.D2” method.

RESULTS

The FishInspector Software and Phenotype Characterization

A user-friendly platform for feature detection based on two-dimensional projection of fish embryos called FishInspector was developed. The graphical user interface of the software is illustrated in Figure 1. FishInspector is written in MATLAB and an executable version for Windows is freely available (latest software update available at Zenodo [Kießling et al., 2018]). The software has a modular structure and the MATLAB code can, in principle, be extended to include more features by programming appropriate plugins. In order to compensate for potential errors of the automated image analysis, particularly during the development of the software or in cases where it is difficult to establish error-free automated detection, the software allows user interaction and correction. Variability of image qualities depending on the source (camera and microscope settings, resolution, contrast, intensity) may impact on feature detection. Therefore, adjustable parameters were included in the software, making it possible to compensate for camera or microscope-dependent differences. In its current version the FishInspector is able to locate up to 10 different morphological features (Table 1), and export their coordinates to an open format (JSON—JavaScript Object Notation—file). The average processing time was 3 h per plate (2 h unsupervised for the image acquisition and 1 h for the FishInspector analysis). It should be noted that FishInspector is not intended to detect deviations from normal phenotypes. This is done by subsequent analysis using the identified feature coordinates and existing analysis routines. The identified feature coordinates are processed subsequently in a KNIME workflow to derive their metrics (Table 1, see Material and Methods, supplementary KNIME workflow in Dryad, Teixido et al., 2018). The features were chosen because of their relevance in zebrafish embryo development and the observed phenotypes of the model compound exposures.

Some features can be expected to change during the course of development. So, developmental retardation would lead to changes in those parameters in particular. If several features that correlate during the course of normal development change in a consistent manner, this could serve as an indicator for developmental retardation. Therefore, cross-correlation of the different features was analyzed in untreated embryos from 32 to 96 hpf (Figure 2b). Body length and eye size were the most highly correlated features (\( r = 0.94 \) and 0.87, respectively) following by yolk sac size (\( r = -0.84 \)). The eye-ear distance, a common morphological marker used to stage zebrafish embryos (Beasley et al., 2012; Kimmel et al., 1995), showed a slight correlation (\( r = 0.7 \)). However, if restricted to stages between 48 and 96 hpf, the correlation increased (\( r = 0.92 \), Supplementary Figure 6) and was therefore used to assess growth retardation at 96 hpf. The lower jaw position was analyzed between 72 and 96 hpf and also showed a positive correlation (Supplementary Figure 7).

In fish embryo toxicity assays, DMSO is often used as carrier solvent to accelerate solubilization of hydrophobic chemicals, up to concentrations of around 1%. Therefore, effects of DMSO were also evaluated using the FishInspector software and KNIME workflows. Most of the affected endpoints exhibited EC\(_{50}\) ≥ 2% (v/v) DMSO, except for noninflation of the swim bladder and locomotor response. Both showed an EC\(_{50}\) value of around 1% DMSO (Supplementary Table 4) representing the maximum solvent concentration that was used for analyzing the effects of dexamethasone (for loratadine, glibenclamide, and all-trans retinoic maximum DMSO concentrations of 0.5%, 0.5%, and 0.01%, respectively, were used).

Comparison of the Automated Quantitative versus Visual Analysis

To illustrate the performance of the software, we analyzed the phenotypic effects of six model compounds previously characterized in the zebrafish and mammalian models for developmental toxicity (Supplementary Table 5). First, the visual assessment and the automated quantitative assessment with the FishInspector were compared by calculating a cumulative EC\(_{50}\) representing the concentration where 50% of the embryos were affected by any of the quantified individual endpoints (swim bladder effects were excluded for this analysis). The two assessments revealed very similar effect levels (Figure 3a). However, the visual assessment did not reach an EC\(_{50}\) for dexamethasone, whereas the automated assessment—based mainly on morphological changes of pericard size, yolk sac size, and lower jaw position—was able to reveal an EC\(_{50}\) of 5\( \mu \)M after 96 h of exposure.

EC\(_{50}\) values were also derived for each individual endpoint analyzed with the visual and automatic image-based method (see Figure 3b for an example of concentration-response curve).

Figure 3c shows the comparison between visual and image-based specific altered endpoints using a color scale that represents the EC\(_{50}\) normalized to the most sensitive endpoint for each of the time points (48 and 96 hpf).

In addition to the morphological endpoints analyzed with the FishInspector, two functional endpoints, heart rate and locomotor response for behavior analysis, were added to our analysis to increase the diagnostic power of the phenotype assessment. Loratadine showed a strong reduction in heart rate at both measurement time points. Topiramate exposure was found to alter heart rate at 96 hpf. Methotrexate and all-trans retinoic acid showed reduced locomotion in the dark phase, in contrast to topiramate and loratadine, which showed increased locomotion during light phase.
Chemical Signatures
The measurement of each individual endpoint enabled the construction of a phenotypic signature for each compound according to the most affected endpoint. Figure 4 shows these signatures with a color code scaled from no effect (yellow, 0) to specific effect (red, 1).

Inter-Laboratory Assessment of the Zebrafish Developmental Toxicity Assay
The five selected compounds were also evaluated in two other laboratories that are currently using visual assessment to score for developmental toxic effect in zebrafish (Sanofi and Biobide). The overall results (LC50, EC50 values) are shown in Table 2. Only in 1 laboratory (Sanofi), dexamethasone showed a concentration-dependent increase in effects and an EC50 could be extrapolated. Based on the teratogenic index with individually set laboratory thresholds (Sanofi threshold for developmental toxicity liability of TI > 1.2), there were 4 compounds classified as developmentally toxic compounds (loratadine, methotrexate, topiramate, and dexamethasone) and 1 (glibenclamide) classified as nondevelopmentally toxic. Glibenclamide is not reported to cause developmental toxicity in mammals.
DISCUSSION

The FishInspector as a Flexible Platform for Detecting Morphological Features

Although large-scale toxicity screens have been carried out with zebrafish (Gustafson et al., 2012; Padilla et al., 2012; Truong et al., 2014), the phenotypic assessments are typically nonquantitative or semiquantitative at best. Morphological phenotyping remains a subjective process that may vary greatly between laboratories and could be affected by the fatigue, training, and expertise of those who perform the analysis and scoring. The use of a more unbiased, quantitative phenotypic assessment using image analysis, such as the one presented in this manuscript, can mitigate the subjectivity inherent in tests that rely on phenotype observations. Aiming to reduce this potential subjective bias from zebrafish embryo morphological analysis and to potentially link phenotype patterns to mode of action in subsequent analyses, we developed the software FishInspector. It provides an integrated and user-friendly platform for feature detection based on a two-dimensional projection of fish embryos. A crucial prerequisite is that embryos are analyzed out of their chorion (requiring manual dechorionation for stages <72 hpf) and that images are obtained after precise orientation of embryos. For instance, more than 75% eye overlap of the left and right eye in lateral two-dimensional projections was reported to be required for ear-eye distance analyses with less than 5% error (Beasley et al., 2012).

Correction of feature detection with the FishInspector is frequently required, but not for all features. For example, eye size, body length, notochord, and yolk are robust parameters that rarely need interaction or require only little correction. Other features like the jaw or pericard mostly require user correction. However, user interaction in the FishInspector is required only for the detection of the features and can also be conducted blind. Assessment of whether the chemical is provoking a certain phenotype or deviation from controls is made via concentrations-response modeling. This greatly reduces the bias if compared with visual microscopic observation and scoring. Furthermore, with the FishInspector one has an improved documentation of the analysis given that assessments can always be traced back to the original images.

Existing image analysis platforms (Molecular Devices ImageXpress, Definiens Developer software, Noldus Danioscope, Thermo Scientific Cellomics Zebabox, or GE Healthcare Lifesciences Cell Investigator Zebrafish Analysis) do not at present allow feature annotation to the same extent or with the same flexibility or future development potential as our approach. Moreover they are not freely available as open source software, and some of them require co-purchase of certain equipment and/or have been discontinued. The FishInspector software in our study has been used in conjunction with the VAST bioimager system which automatically positions embryos in a glass capillary prior to imaging (Pardo-Martin et al., 2010).

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However, in principle, it is possible to use conventional pictures obtained with a bright-field microscope (Supplementary Figure...
Therefore, we provide a simple workflow that automatically rotates the images and draws a virtual capillary. The user-friendly workflow processes multiple images simultaneously based on an imageJ macro embedded in a KNIME workflow (Teixido et al., 2018). Hence, it uses established and open source software. The workflow can easily be adapted to accommodate different image properties depending on the source of the image (eg, intensity, contrast). As for any type of image analysis, the quality of the images is critical even if manually positioned embryo images are used.

A limited number of features can be detected at present (Table 1). Due to the modular architecture of the FishInspector, the plan is to increase the number of detected morphological features, including support for dorsal and ventral images. Future versions may also implement self-learning algorithms to make automatic feature detection more robust. Manually approved feature contours could be used, for example, to train Active Shape/Appearance models (Cootes et al., 1998; Cootes and Taylor, 1992) and to minimize the need for manual correction.

Cross-correlation analysis of all the features with progressing development indicated that a subset of the morphological endpoints exhibit a high correlation and enable improved identification of growth retardation (Figure 2b), a common parameter evaluated in mammalian developmental toxicity studies. The potential confounding effects of DMSO on phenotypes and behavior was also revealed in this study. DMSO was used up to a concentration of 1%, representing the EC50 for nonswim bladder inflation and reduced locomotor activity. Effects of DMSO on locomotion have been previously reported in other studies at a concentration as low as 0.01% (Chen et al., 2011). The effect on these parameters should be carefully interpreted (eg, reduce locomotion in dexamethasone-treated embryos in combination with 1% DMSO in our study). Hence, we suggest, in general, minimizing the amount of DMSO especially for specific examinations or considering potential interference by solvents in the interpretation of results. However, for screening purposes, maximization of the compound solubility and uptake through standardized DMSO concentrations (eg, 1%) have been used effectively with good predictivity (Krupp, 2016).

**Software Performance and Differences between Visual and Automated Assessment**

The ability of our approach to detect developmental toxicity was demonstrated by using six compounds previously assessed by other laboratories for the optimization and performance evaluation of the zebrafish developmental toxicity assay (Gustafson et al., 2012). Our image-based quantitative approach eliminates possible observation bias whereas demonstrating consistency with the overall effect assessment by visual analysis of an experienced researcher. Furthermore, automated assessment included the evaluation of two additional endpoints,
body length, and pigmentation, which could not be properly evaluated by visual analysis due to its inherent subjectivity. Our approach slightly increases throughput given that the imaging is conducted unsupervised. However, the amount of data generated also increases the subsequent analysis workload. Indeed, we did not primarily aim or expect to increase throughput, rather to increase content and accuracy in the morphological assessment.

Comparison between visual and automatic specific altered endpoints reveals in general good agreement, with 3 major exceptions (Figure 3c): (1) Methotrexate exposure resulted in increased incidence of embryos showing bending of the tail after 48 h of exposure. However the visual analysis was not sensitive enough to capture this effect. (2) Using visual assessment we were only able to observe a concentration-dependent effect on swim bladder inflation for dexamethasone after 96 h of exposure. However the visual analysis was not sensitive enough to capture this effect. (3) For loratadine exposure after 96 h, the visual assessment indicated swim bladder malformations and heart rate decrease. Therefore, as swim bladder inflation seems to be affected by many chemicals, it may have a limited diagnostic value at the 96 hpf stage. Two functional endpoints, heart rate and locomotor response, allowed us to discover potential off-target effects of drugs, like reduced heart rate after loratadine exposure. Heart and jaw abnormalities are frequently analyzed as teratogenic indicators, using transgenic or stained fish embryos. Heart morphology has not yet been included in the automatic assessment with the FishInspector, but heart rate quantification may partially capture heart malformations.

**Comparative Effect Analysis**

Using the different morphological and functional endpoints quantified in our study, phenotypic signatures were derived for each chemical and scaled by normalization to the effect concentration of the most sensitive endpoint. Our data suggests that observed differences in phenotype patterns could reflect the differences in the underlying mechanism of action (Figure 4). Using the FishInspector software, a larger amount of chemicals with similar mechanisms could now be analyzed to reveal whether commonalities between compound effect patterns could be derived and linked to modes of action or common key events. In the present analysis, embryos exposed to all-trans retinoic acid and methotrexate both showed tail or body axis curvature as the most sensitive morphological feature. Both compounds are associated with neural tube defects in mammals. All-trans retinoic acid interferes with the retinoic pathway, which is especially important for anterior-posterior
patterning of the spinal cord and hindbrain, neuronal differentiation, and axis elongation (Tonk et al., 2015). Methotrexate is a folate analog that acts by competitively inhibiting dihydrofolate reductase, an enzyme involved in DNA biosynthesis. This impairment in nucleotide biosynthesis can decrease mitotic rates during critical morphogenetic windows (Lee et al., 2012). Hence, similarities in effect patterns may reflect the conversion of both pathways at neural tube organogenesis.

Our study also supports evidence for the known side effects of the antihistaminic loratadine. The most affected endpoint for loratadine exposure was reduced heart rate and body length of the embryos. Some antihistaminic compounds have been shown to reduce the heart rate by competitive inhibition of the muscarinic receptors in mammals (Liu et al., 2006). In zebrafish, knock-down of muscarinic receptors has been demonstrated to alter cardiac β-adrenergic receptor activity (Steele et al., 2009).

The phenotypic effect observed after exposure to the antiepileptic drug topiramate revealed growth retardation as the most affected endpoint after 48 and 96h exposure. The use of antiepileptic drugs during pregnancy has been associated with congenital defects and developmental delay in humans (Campbell et al., 2013), however the underlying mechanism is still unknown. Our approach allowed us to identify growth retardation as the main endpoint of topiramate exposure, rather than teratogenic effects. Antiepileptic drugs are also capable of inducing neurodevelopmental effects (Ornoy, 2006) and interfere with the GABA and AMPA/kainate glutamate receptor and block voltage-dependent sodium channels (Schneiderman, 1998). In our study we observed increased locomotion during the light phase of the locomotor response analysis, which may potentially relate to the MoA of topiramate.

Dexamethasone exposure caused reduced yolk sac size in zebrafish embryos, potentially related to the role of glucocorticoid in energy metabolism by mobilizing and relocating energy substrate stores (Nesan and Vijayan, 2013). Mammalian studies have demonstrated that glucocorticoids cause cleft palate and craniofacial development in zebrafish as well (Hillegass et al., 2008). Our study also revealed an alteration in jaw development by a reduced jaw-eye distance (Figure 3c).

Interlaboratory Assessment

The performance of our method was verified by comparing it with the visual assessments of 3 different laboratories experienced with conventional visual assessment of the zebrafish embryos. A previous interlaboratory assessment study showed that technical differences were the primary contributor to interlaboratory differences in classification of a compound as developmentally toxic using zebrafish embryos (Ball et al., 2014). Our approach avoids score assignment based on qualitative measures of effect. The interlaboratory study showed good agreement; however dexamethasone was classified as developmentally toxic by only one laboratory (Sanofi) using the visual inspection method. The quantitative approach showed a higher sensitivity for the detection of chemical effects and the sensitivity of effect assessment for dexamethasone was increased (Table 2). The overall weak effects caused by dexamethasone, however, could also be due to reduced bioavailability of the compound. It has been reported that embryonic concentrations reached only 20% of the exposure concentrations indicating a potential slow uptake and internal concentration not in equilibrium (Steenbergen et al., 2017). Uptake of the chemicals by zebrafish embryos was not analyzed in our study, as the focus was on feature detection and quantification of developmental toxicity. However, we consider it important that this be included in routine screens, either via appropriate TK models or by internal concentration analysis (Brox et al., 2014) since a slow and/or limited uptake of a substance by an embryo could represent a confounding factor in the assessment of effects. Loratadine was classified as a false positive in all laboratories including the automatic image-based assessment. This compound demonstrated a high uptake in previous studies, which may have contributed to the false positive results in the assay (Gustafson et al., 2012). Whether analysis with the FishInspector would lead to a higher number of false positives, however, requires a more thorough analysis of a greater number of chemicals.

CONCLUSIONS

This study has demonstrated the value of the FishInspector software and quantitative analysis has been demonstrated. The FishInspector software allows an unbiased and automated quantitative assessment of morphological changes in zebrafish embryos after chemical treatment, particularly for embryos positioned to a precise orientation. Its modular architecture allows users to implement the detection of additional features. Furthermore, to facilitate automatic recognition of features and reduce user interaction, self-learning algorithms for feature detection could be considered.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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