Application Note

Developmental Biology

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Automated Image Screening of Zebrafish Embryos Exposed to Developmental Toxins

Using Agilent BioTek Gen5 data analysis software to quantify morphological changes in zebrafish embryos after toxin treatment

Author

Sarah Beckman, PhD
Agilent Technologies, Inc.

Abstract

High-throughput screening is a valuable method used to discover new drugs, as well as to uncover novel uses for existing compounds. Numerous biological systems are used for toxicity screening, however zebrafish are an attractive model for medium to high-throughput screening due to their small size, low cost, and transparent vertebrate anatomy. They are especially amenable to general toxicology screens which are able to capture a broad range of phenotypes and which have historically delivered more first-in-class drugs than targeted screens. In this application note we demonstrate high-throughput screening and analysis of zebrafish embryos treated with several developmental toxins. We expose embryos to ethanol, retinoid acid, and cyclopamine, then capture brightfield images of the embryos in multi-well plates with an Agilent BioTek Lionheart automated microscope. The images are analyzed for size and shape using Agilent BioTek Gen5 data analysis software.

Introduction

Humans are exposed to a myriad of environmental toxins in their everyday life though fine particulates in the air, chemicals found in food packaging, household items, personal care products, and naturally occurring compounds such as metals. This exposure is associated with both acute toxicity and long term consequences, which include congenital abnormalities, chronic diseases, cognitive disabilities, cancer and death.[1] These toxic effects are well conserved between humans and zebrafish. Zebrafish are complex vertebrates and maintain elaborate mechanisms for activating or mitigating the effects of exogenous chemical substances. Although differences in pharmacological effects between zebrafish and humans certainly do exist, there are hundreds of examples of small molecules that have conserved biological activities in fish and humans.[2] This conservation of biological activity makes zebrafish a useful model for toxicity screening.

Another attractive feature of zebrafish for studying toxicity is their amenability to medium to high-throughput experiments. Embryos can be placed in multi-well plates to assess the effect of their exposure to drugs. In this manner, zebrafish have been used to identify teratogens, to uncover mechanisms of action of common toxicants, and to elucidate tissue specific effects.[1] The zebrafish model provides an opportunity to combine the power of rapid toxicology screens with the ability to study the effect of exposures on a vertebrate, making zebrafish an invaluable system for research in toxicology.

One of the benefits of performing toxicology assays with a whole organism is that phenotypic screens can easily be carried out. Phenotype driven approaches to drug discovery have out-performed target driven approaches.[3] This is partially due to the fact that phenotype driven screens can identify chemical modifiers of virtually any biological process, while target-based approaches are dependent upon *a priori* selection of a protein target, and as such are biased toward discovery of small molecule inhibitors of well-studied proteins. As a result, this approach is less likely to reveal the functions of novel targets. Furthermore, the most effective drugs may hit multiple targets. Target-based screens will by their very nature overlook drugs that have more than one target. Another advantage of using whole organisms for drug discovery is the physiological context they provide. The vast majority of small molecules discovered by *in vitro* target-based screening exhibit undesirable characteristics (such as lack of specificity or toxicity) when tested in the context of a whole organism. In contrast, a small molecule discovered by virtue of its ability to cause a desirable phenotype in a whole organism is more likely to be cell permeable, devoid of obvious toxicities, and effective.[4]

To improve throughput and remove subjectivity, advances in high content imaging (HCI) techniques have provided ways to automate both data collection and image analysis.[5] Zebrafish are amenable to high content imaging owing to their permeability to small molecules and their transparency. Furthermore, large amounts of embryos can quickly be obtained and used to assess the effects of multiple drugs in a screening approach. Automation of the imaging of zebrafish embryos has the potential to decrease time and increase reproducibility and objectivity of the assay. However, one of the rate-limiting procedures in a developmental zebrafish screen is the morphological assessment of each larva. What is needed are objective, accurate, and rapid methods for screening zebrafish for dysmorphology.

This study uses zebrafish, in combination with time-lapse imaging and Agilent BioTek Gen5 microplate reader and imager software to analyze zebrafish embryo shape following toxin exposure. Embryos are treated at early developmental time points with several toxins and then grown to 2 to 6 days post fertilization (dpf). Embryos are imaged in 96-well plates and size and shape measurements are determined using Gen5. Within these parameters the embryos are classified into basic shapes such as round, straight, short, and curved.

Materials and methods

Zebrafish maintenance

Zebrafish were maintained essentially as described in Westerfield.[6] Adult zebrafish, both male and female, were mixed and maintained at 28 °C with a 14/10 hour light/dark cycle. To collect embryos, male and female zebrafish were put into a breeder basket the night before and embryos were collected in the morning. Zebrafish embryos were kept in a 28 °C incubator in E3 media (5 mM NaCl, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$, 0.17 mM KCl, and 0.1% methylene blue).

Drug treatment

Twelve embryos were placed in each of 8 wells of a 12-well plate. Cyclopamine (Tocris, Minneapolis, MN), retinoic acid (Tocris), and ethanol (Sigma, St. Louis, MO), were all diluted in E3 media. Two milliliters were added to each well of the plate. Toxin treatment was started at various stages of development. Cyclopamine treatment was started at the 8-cell stage. Ethanol treatment was started at tail bud. Retinoic acid treatment was started at shield stage. Except for ethanol treatment, embryos were grown to 2 dpf before imaging. Ethanol treated embryos were grown to 6 dpf.

Imaging

Following treatment, embryos were dechorionated with pronase (Sigma), treated with tricaine (Sigma) until they were unresponsive to touch, and placed into 96-well plates (Corning 3904, Canton NY). In the case of 96-well plates, embryos were positioned in the center of the well and a 3x4 montage was taken at 4x. For further visual analysis, images were taken in manual mode at 10x using a combined z-stack montage. In all cases, images were taken with the Agilent BioTek Lionheart FX automated microscope using the brightfield channel.

Image analysis

Image montages were stitched together using Gen5 default settings. Next, images were preprocessed to remove background and facilitate identifying the shape of the embryo. The background was set to "light" and the rolling ball was left at the Gen5 default settings. Finally, image analysis was performed, which created a primary mask to outline the shape of each embryo. The parameters for the primary mask and the preprocessing steps are detailed in Table 1 and Figure 1.

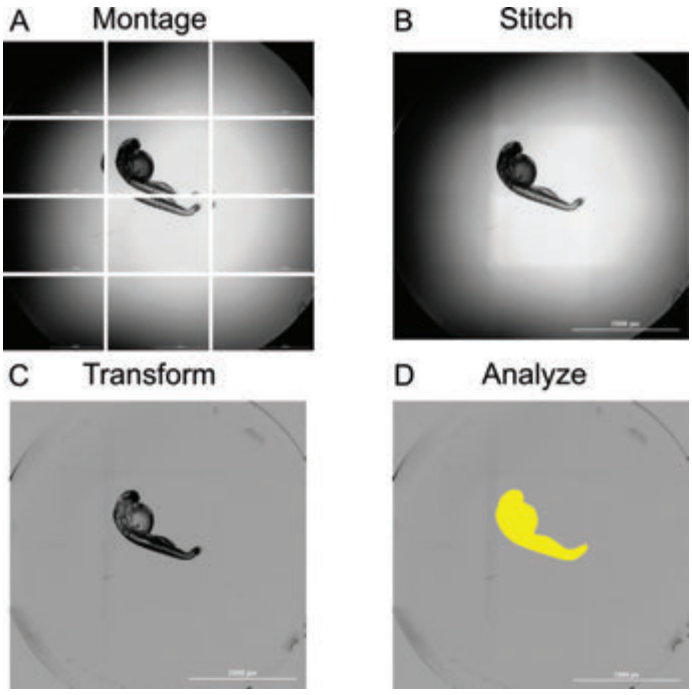

Figure 1. Imaging and analysis parameters. 4×3 montage was taken of the well (A) and the images were then stitched together (B). Image preprocessing was applied to subtract background (C) and the embryo shape was analyzed with a primary mask (D).

Table 1. Agilent BioTek Gen5 software settings for stitching, image preprocessing, and cellular analysis. After a montage of images is taken, individual image frames are stitched together. Image preprocessing removes background from the image to facilitate masking around the embryo. Cellular analysis creates a mask around the embryo in order to calculate image shape parameters.

Stitching	
Registration Channel	Brightfield
Fusion Method	Linear blend
Crop stitched image to remove black rectangles on the borders	Checked
Downsize Stitched Image	Checked
Image Preprocessing	
Image Set	Brightfield
Background	Light
Rolling Ball Diameter	Auto
Priority	Fast speed
Image Smoothing Strength	0
Cellular Analysis	
Detection Channel: Primary Mask and Count	Brightfield
Threshold	500
Background	Light
Split Touching Objects	Unchecked
Fill Holes in Masks	Checked
Minimum Object Size 800 µm	
Maximum Object Size	100,000 µm
Include Primary Edge Objects	Unchecked
Analyze Entire Image	Checked
Advanced Detection Options	
Background Flattening: Rolling Ball Diameter	100
Image Smoothing Strength	5

Results and discussion

Ethanol is the most commonly consumed and abused drug. Embryo shape after exposure to a specific toxin is often directly related to the pathway that the drug targets. In this case, zebrafish larvae exposed to ethanol develop developmental malformations including skeletal dysmorphogenesis, axis shortening, and developmental delay.[7] Zebrafish embryos were treated with 0, 1, or 2 percent ethanol at the tail bud stage (10 hours post fertilization (hpf)) and grown until 6 dpf. Embryo morphology parameters examined include length, elongation, length-width ratio, circularity, form factor, and width. These parameters are

detailed in Table 2. Little difference is seen between control embryos and 1% ethanol treatment. However, embryos treated with 2% ethanol show a shortened axis, circular morphology, and increased width. This is quantified in Figure 2.

Table 2. Custom and default Agilent BioTek Gen5 image analysis parameters are used to describe the size and shape of the primary mask surrounding zebrafish embryos.

Parameter	Description
Length	Longest dimension of object
Elongation	(Length-width)/length
Length-Width Ratio	Length/width
Circularity	Measure of roundness (aspect ratio)
Form Factor	(4*π*Area)/Perimeter²
Width	Measurement from side to side of object

The steroidal alkaloid cyclopamine has both teratogenic and antitumor activities arising from its ability to specifically block cellular responses to vertebrate hedgehog signaling.[8] Sonic Hedgehog (shh) is required to specify ventral structures in the spinal cord. If this signal is missing, the spinal cord becomes dorsalized and midline structures fail to form normally which results in a curved body structure.[9] In this assay, zebrafish embryos were treated with 8 two-fold dilutions of cyclopamine starting at 100 µM. Treatment began at the 8-cell stage (1.25 hpf) until 2 dpf. Embryo morphology was quantified with Agilent BioTek Gen5 and one can observe a roughly dose-dependent effect on embryos size and shape, including embryo curvature. Embryos appear to be most affected at treatments >12.5 µM: embryos using treatments of ≤12.5 µM show phenotypes similar to untreated embryos; while embryos with treatments >12.5 µM present with a curled phenotype and twisted tails. This is quantified in Figure 3.

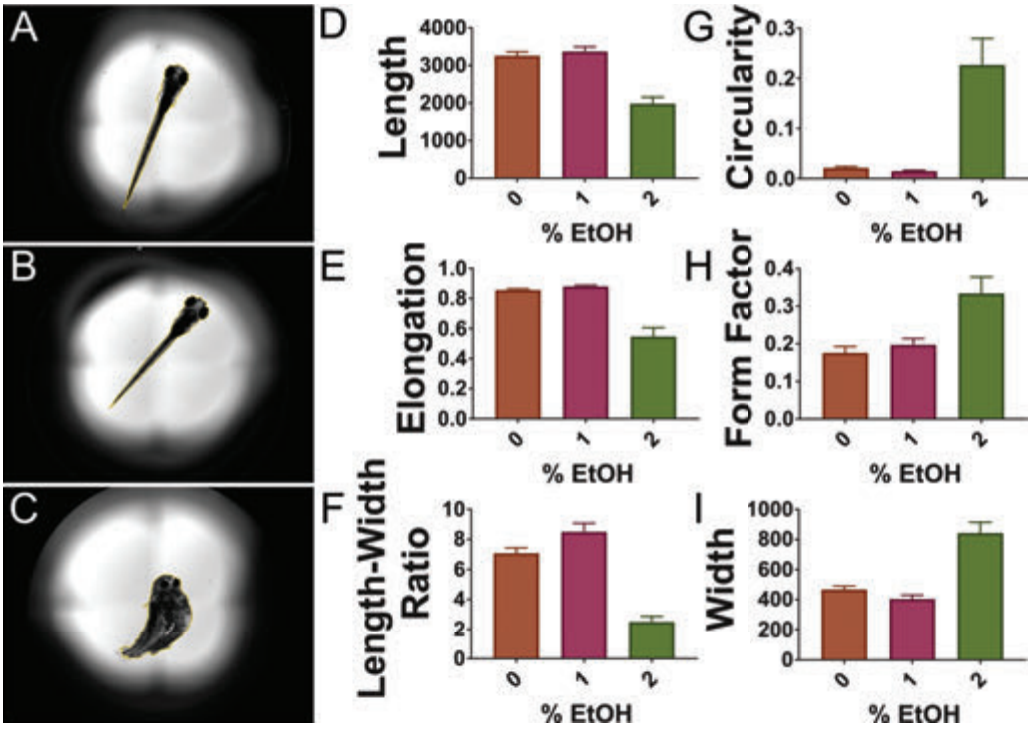

Figure 2. Embryos exposed to ethanol present with a short, round phenotype. (A) Control embryos (B) embryos exposed to 1% ethanol (C) embryos exposed to 2% ethanol. (D-G) Comparison of the shape parameters calculated for each treatment.

Retinoic acid (RA) is a natural morphogen derived from vitamin A. Treatment with RA perturbs normal anterior-posterior axis development. Excess RA acts as a posteriorizing agent. As such, there is a lack of anterior head structures and a shortened body length at the highest doses of treated embryos.[10] Tail growth is also affected, which is a later effect of RA and results in shortened tails.[11] Zebrafish embryos were treated with 8 two-fold dilutions of retinoic acid starting at 64 µM. Treatment went from the shield cell stage (6 hpf) until 2 dpf. Embryo morphology was again quantified with Gen5. Embryos appear to be affected at all treatments and present with short tails and a curved phenotype which is dose-dependent. This is quantified in Figure 4. At the highest doses, embryos present as more circular, which gives way

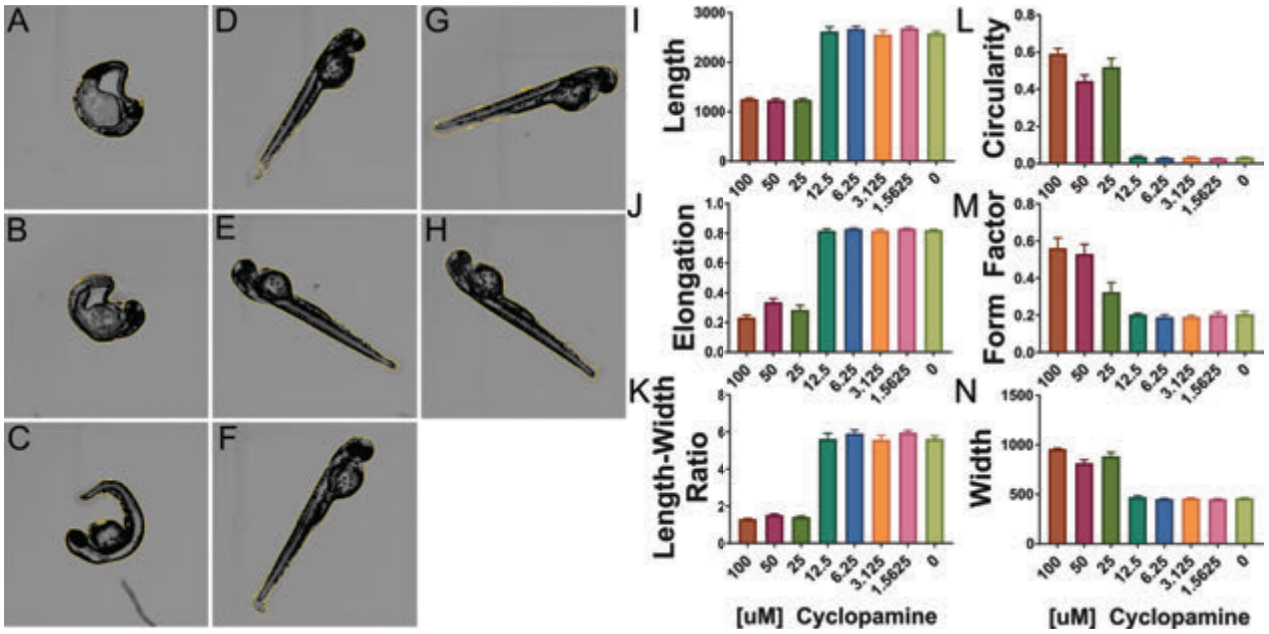

Figure 3. Embryos exposed to cyclopamine present with a curved phenotype and twisted tails. (A) 100 µM cyclopamine (B) 50 µM (C) 25 µM (D) 12.5 µM (E) 6.25 µM (F) 3.125 µM (G) 1.5625 µM (H) 0 µM (I-L) comparison of the shape parameters calculated for each treatment.

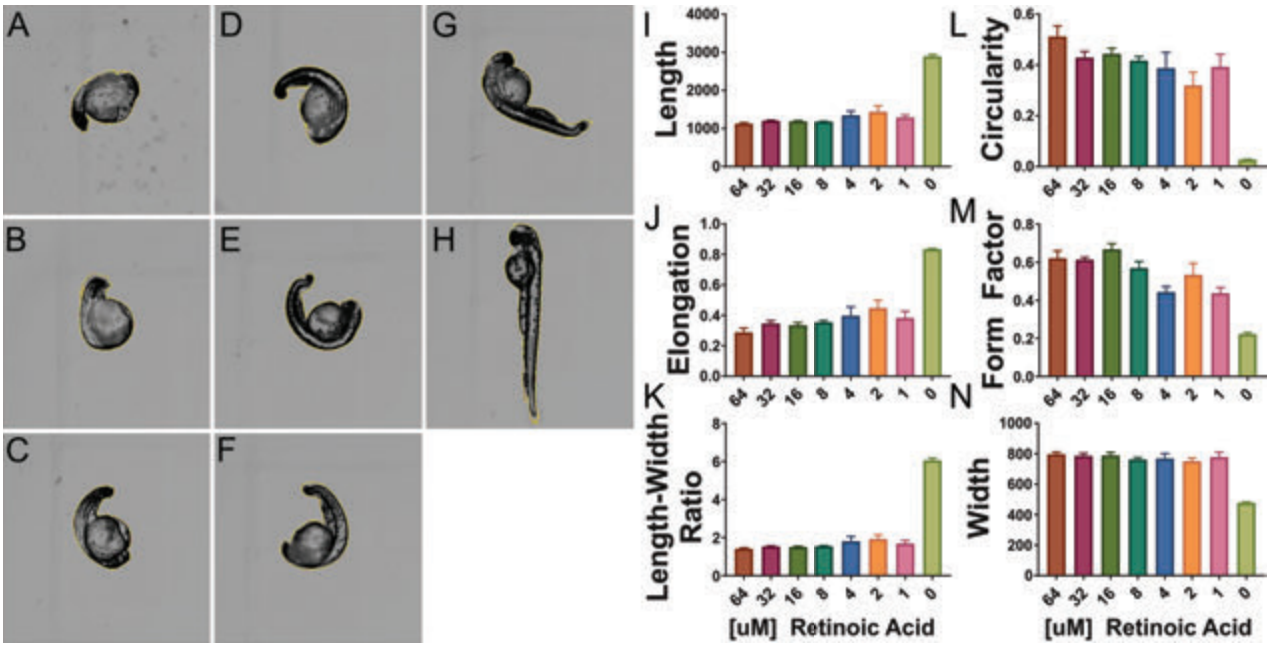

Figure 4. Embryos exposed to retinoic acid posteriorized and have shortened tails. (A) 64 µM (B) 32 µM (C) 16 µM (D) 8 µM (E) 4 µM (F) 2 µM (G) 1 µM (H) 0 µM (I-L) comparison of the shape parameters calculted for each treatment.

to a more curved, and then straight phenotype. This can be quantified with Gen5 subpopulation analysis.

Use the parameters outlined in Table 3 to identifiy general morphological features of the embryos such as straight, circular, curved, and short. A quick view of the plate map, shown in Figure 5, gives a general idea of the morphology of embryos in each well of the 96-well plate. Three of the wells are excluded due to debris on the bottom of the well which affected image analysis.

After basic shape assessments are made, researchers will want to dive down further into more detailed morphological assessment. For this, select embryos can be further imaged to acquire more detail. This is shown in Figure 6 where a 10x montage z-stack is taken of the embryos which gives details to further determine the extent of features such as axis shortening, posteriorization, amount of pigmentation, and edema.

Table 3. Definition of the parameters used to determine which embryos are straight, circular, curved, or short.

Object Shape Parameters		
Straight	Length/width ratio >5	
Circular	Form factor >0.55	or circularity >0.5
Curved	Form factor >0.3	and form factor <0.55
Short	Elongation <0.4	

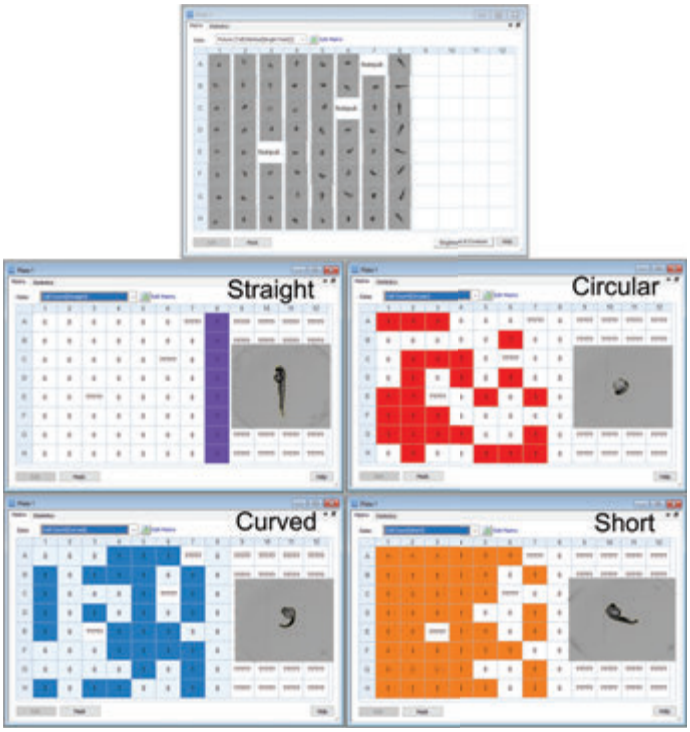

Figure 5. Object shape parameters of RA treated embryos. Images are divided into 4 classes: straight, circular, curved, or short based upon the parameters outlined in Table 3.

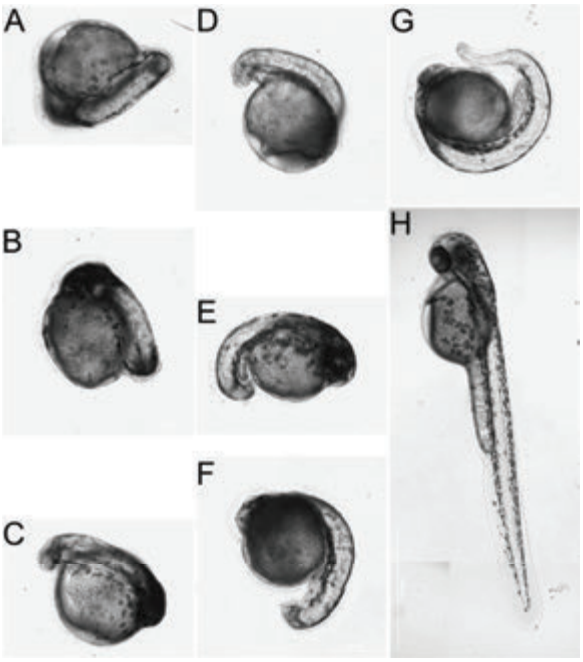

Figure 6. 10x images of retinoic acid treated embryos allows for further assessment of toxicity. (A) 64 µM (B) 32 µM (C) 16 µM (D) 8 µM (E) 4 µM (F) 2 µM (G) 1 µM (H) 0 µM.

Conclusion

The ability to perform high-throughput imaging and analysis is essential for toxicology screens. This application note demonstrates that using the Agilent BioTek Lionheart FX automated microscope and Agilent BioTek Gen5 data analysis software can image and analyze zebrafish in 96-well plates treated with different doses of developmental toxins. This note demonstrates dose-dependent effects on image shape parameters and a strategy to categorize embryos by basic morphological features such as round, curved, straight, or circular. As form is directly related to function, the shape of an embryo may give researchers an idea of what pathway to begin exploring when there is an interesting hit from an unknown compound. The combination of high-throughput imaging and analysis using a vertebrate model makes this an attractive method for medium to high-throughput toxicology screening.

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