Using Acridine Orange to Measure Cell Death in Ethanol Treated Zebrafish Embryos

Using Gen5 to Analyze Cell Death in Zebrafish Embryos

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Abstract

Ethanol exposure during development can have devastating effects on the fetus. In this study we use zebrafish, an excellent model to study both normal development and toxicity, in order to determine the effects of ethanol exposure during development. Zebrafish embryos were treated with ethanol during the first 24 hours of development and the effects of ethanol treatment on cell death was assessed using acridine orange staining. We found that exposure of embryos to ethanol results in a dose-dependent increase in cell death overall in the embryo.

Introduction

Ethanol exposure from maternal consumption of alcoholic beverages has been linked to developmental abnormalities in both humans and animal models.[1] Alcohol is a teratogen that can have devastating effects on the developing embryo and fetus. In humans, exposure to ethanol during development may lead to fetal alcohol syndrome (FAS). FAS affects about 1 in 100 children born in the United States each year.[2] The range of outcomes following fetal alcohol exposure are broad, and include growth retardation, craniofacial malformations, and central nervous system malformation.[3] Furthermore, the consequences of gestational alcohol exposure are a major public health issue, which have a wide range of effects at a great cost.[3]

Zebrafish are an excellent vertebrate model to study development toxicity as they share molecular, biochemical, cellular and physiological characteristics with higher vertebrates. Furthermore, the transparent embryos rapidly develop externally, which allows changes in development to be continuously observed. This greatly facilitates developmental time course studies. The zebrafish is a powerful model for studying the genes that regulate sensitivity and resistance to developmental toxins, such as ethanol.[1] Previous studies have shown that zebrafish embryos have a range of effects after exposure to ethanol including abnormal eye development, reduction in body length, and higher mortality. Since this cluster of defects overlaps with human FAS, zebrafish are an excellent model to study the effects of ethanol on development.[4]

Ethanol is a well-established developmental toxicant, however the mechanisms of cellular and molecular toxicity remain unclear. Cell death has been implicated as a potential explanation for ethanol-dependent toxicity. Animal models have played a crucial role in the study of FAS, including confirming that alcohol is a teratogen and providing insights into the mechanisms by which alcohol exerts its effects.[5] Excessive cell death has been reported to underlie ethanol-induced nervous system pathogenesis during various stages of embryonic or fetal development. During the embryonic stages, mouse neural crest cells were vulnerable to ethanol induced cytotoxicity.[6] In developing rat forebrain, ethanol triggers widespread apoptotic neurodegeneration.[7] In the zebrafish model ethanol induces cell death throughout the embryo, including the retina[8] and central nervous system.[1]

Ethanol exposure in the developing animal has been shown to induce cell death through apoptosis. Apoptosis is a critical component of the normal development of multiple tissues and organ systems. The process of apoptotic cell death is highly conserved and follows a morphologically distinctive pattern. Apoptosis describes the specific cellular shrinkage, membrane blebbing, nuclear condensation and nuclear fragmentation commonly recognized as hallmarks of this type of cell death. This process is strictly regulated and can be induced by developmental toxins including ethanol.[9]

In this study we use acridine orange (AO) staining combined with Gen5™ Microplate Reader and Imager oftware to determine the amount of apoptotic cell death in zebrafish embryos after 24 hours of ethanol exposure. We perform image processing and analysis to generate a focused image from an image stack and then use the object masking capabilities of Gen5 to count the number of green AO positive cells per embryo as well as determine the total amount of AO fluorescence.

Materials and Methods

Zebrafish Maintenace

Zebrafish were maintained essentially as described in Westerfield.[10] Adult zebrafish, both male and female, were mixed and maintained at 28 °C with a 14/10 hour light/dark cycle. In order 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).

Ethanol Treatment

Zebrafish eggs were collected, sorted to remove dead and unfertilized embryos, and placed in 24-well culture plates at a density of <10 embryos per well. Embryos were treated at the 64 cell stage with ethanol for 24 hours.

Acridine Orange Staining

Live zebrafish embryos were stained with the vital dye acridine orange (AO) in order to determine the amount of apoptotic cells per embryo.[11] To perform the assay, embryos were placed in 10 µg/mL of AO (Sigma, St. Louis, MO) in E3 media. After 60 minutes of staining embryos were washed three times in E3 media. After staining, embryos were transferred to round bottom 96-well plates for imaging (Corning, catalog #4520, Corning, NY).

Imaging

Images were acquired using a 2x objective on the Lionheart FX Automated Microscope (BioTek Instruments, Winooski, VT) configured with a brightfield as well as a GFP light cube. The GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter. Stacks of images were taken in both the brightfield and GFP channels. 12 stacks were taken with a height of 12 µm per stack for a total height of 144 µm.

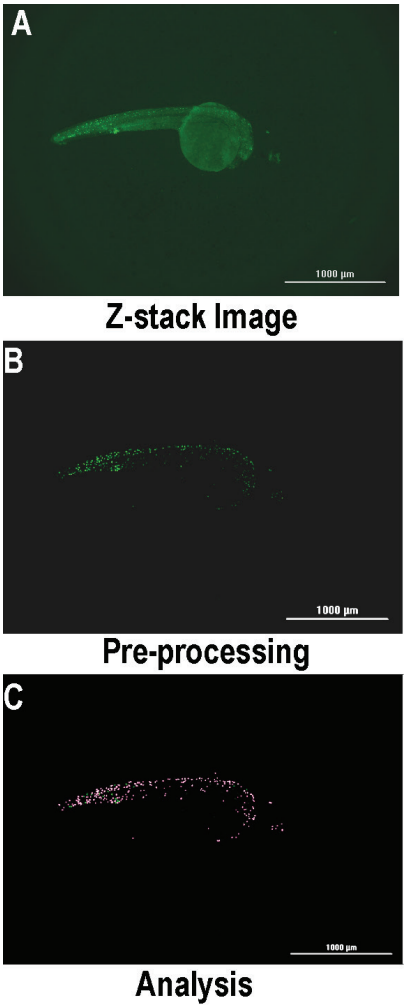

Figure 1. **Acridine orange spot counting workflow.** (**A**) 2x image after using focus stacking to create a z-projection (**B**) Image after applying pre-processing to the image stack. (**C**) Object Masks highlighting cells between 1-10 µm are highlighted in pink. The highlighted cells represent acridine orange positive apoptotic cells in the zebrafish embryo.

Image Processing

Two pre-processing steps were employed to analyze the stacks of GFP images obtained from the Lionheart FX. Prior to image analysis, the GFP image stacks were flattened into one image using the Focus Stacking option under Z-Projection (Figure 1A, Table 1).

Z Projection	
Channel	GFP 469,525
Method	Focus Stacking
Size of Max Filter	11 px
Top Slice	12
Bottom Slice	1

Table 1. **Gen5 Image Analysis software settings for Z-projection.** The z-project function creates a focused image projected from an image stack.

The image projection was then pre-processed with either a 5 µm rolling ball in order to obtain the best distinction between individual cells (Figure 1B, Table 2), or a 15 um rolling ball to minimize background fluorescence (Table 3). Imaging pre-processing and analysis parameters are described in detail in Tables 1-3.

Image Analysis

For cellular analysis, object counting analysis was performed on the GFP channel to highlight each individual cell according to the parameters outlined below in Table 2 (Figure 1C).

Image Pre-Processing	
Image Set	ZProj[GFP 469, 525]
Background	Dark
Rolling Bar Diameter	5 µm
Priority	Fine Results
Image Smoothing Strength	0
Cellular Analysis	
Detection Channel Primary Mask and Count	Tsf[ZProj[GFP 469,525]]
Threshold	5000
Background	Dark
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Min. Object Size	1 µm
Max. Object Size	10 µm

Table 2. **Gen5 Image Analysis software settings for cell counts.** Image pre-processing removed background from the resulting image to facilitate image analysis. The image analysis parameters generate cellular masks in the GFP channel in order to count AO stained cells in each embryo.

In the case of the images which were analyzed to determine the total AO fluorescence, a primary mask was created in the GFP channel to encompass the whole embryo (Table 3). Then, the AO fluorescence integral of the whole embryo was determined using Gen5.

Image Pre-Processing	
Image Set	ZProj[GFP 469, 525]
Background	Dark
Rolling Bar Diameter	15 µm
Priority	Fine Results
Image Smoothing Strength	0
Cellular Analysis	
Detection Channel Primary Mask and Count	Tsf[ZProj[GFP 469,525]]
Threshold	150
Background	Dark
Split Touching Objects	Not Checked
Fill Holes in Masks	Checked
Min. Object Size	300 µm
Max. Object Size	10000 µm

Table 3. **Gen5 Image Analysis software settings for determining the AO integral.** Image pre-processing removed background from the resulting image to facilitate image analysis. The image analysis parameters generate a cellular mask in the GFP channel to outline the entire zebrafish, which allows for the integral of AO fluorescence to be determined within the embryo.

Results

Zebrafish embryos were treated with 0, 0.1 or 0.3% ethanol for 24 hours starting at the 64 cell stage. Directly following treatment embryos were stained with AO to mark apoptotic cells. Following staining, zebrafish embryos were washed three times and treated with tricaine until immobile and placed into 96-well round bottom plates to image as is shown in Figure 2. Image stacks were taken in both the bright field and GFP channels.

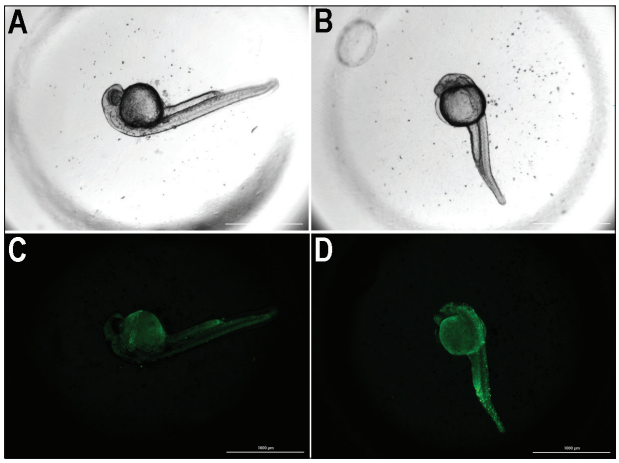

Figure 2. **Zebrafish in round bottom 96 well plates.** Zebrafish treated with 0% ethanol (**A**,**C**) or 0.3% Ethanol (**B**,**D**) were placed into round bottom 96 well plates and then image stacks were taken in both the bright field (**A**,**B**) and GFP (**C**,**D**) channels with a 2x objective. All embryos are positioned roughly in a lateral orientation and are in the center of the well.

Image stacks were then flattened with the focus stack function of Gen5 and the resultant image was subjected to pre-processing to remove background AO staining. Then, the number of AO positive cells per embryo was determined with the cell counting function of Gen5 software.

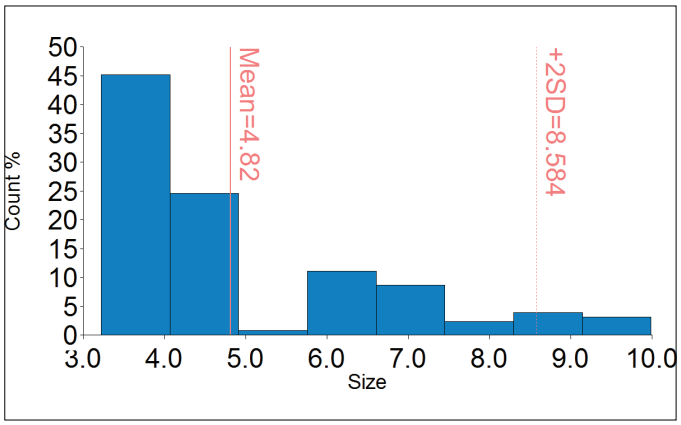

Figure 3. Size of cells analyzed after pre-processing.

Figure 3 shows a histogram of the size of the AO positive cells analyzed after pre-processing. Zebrafish cells are smaller than mammalian cells and cell size can range from 3-12 µm.[12] In this case we see an average of 4.82 µm, which is reasonable since the rolling ball used to process the image was 5 µm.

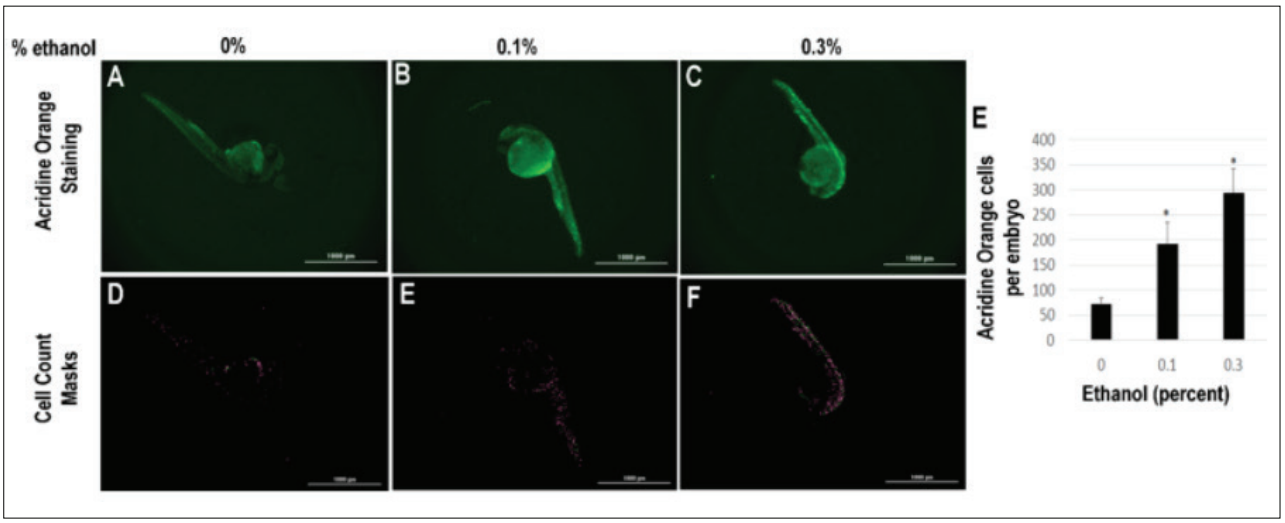

Figure 4. **Cell death in zebrafish embryos increases in a dose dependent manner after treatment with ethanol as shown by number of AO positive cells.** Image stacks of acridine orange stained embryos treated with 0 (**A**,**D**), 0.1% (**B**,**E**), 0.3% (**C**,**F**) ethanol are shown in the GFP channel. The pre-processed images with pink masks around the AO positive cells are shown for each treatment. As ethanol concentrations increase, the number of apoptotic cells also goes up. Quantification is shown in panel E. As percent ethanol increases the number of apoptotic cells per embryo increases in a dose dependent manner. Both 0.1% ethanol and 0.3% ethanol treatment result in significantly more cell death than the control (*$p<0.05$).

Ethanol has been shown to induce cell death in the zebrafish embryo[1] and indeed with increasing concentrations of ethanol there are more AO positive cells per embryo. The 0.1% and 0.3% ethanol treatment groups both contained significantly more AO positive cells per embryo than the control embryos (Figure 4).

An increase in fluorescence in the GFP channel correlates with increased AO staining and increased cell death in the embryo. Therefore, an alternative way to analyze the data is by determining the integral fluorescence in the GFP channel for each zebrafish. In this case we created masks around each embryo (shown in red in Figure 5) and used Gen5 software to calculate the integral of AO fluorescence in each embryo. We found, as with the cell counting data, that with higher amounts of ethanol treatment there is increased AO fluorescence, which corresponds to a dose dependent increase in cell death in the embryos (Figure 5).

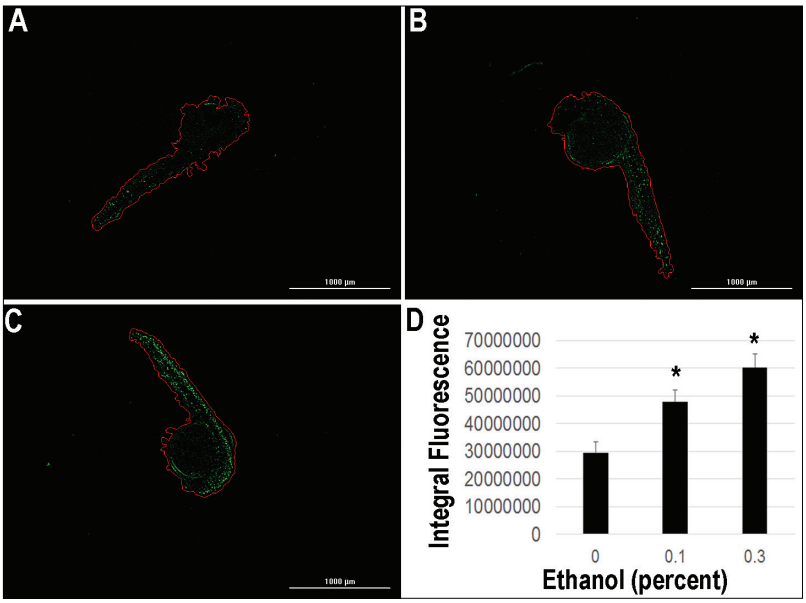

Figure 5. **Cell death in zebrafish embryos increases in a dose dependent manner after treatment with ethanol as shown by AO fluorescence.** Embryos treated with 0 (**A**), 0.1 (**B**) or 0.3 (**C**) percent ethanol are shown. Image masks were created around each embryo and the integral of AO fluorescence for each embryo was calculated using Gen5 software. (**D**) The integral of AO fluorescence goes up in a dose dependent manner, with 0.1 and 0.3 percent ethanol treatment both significantly higher than the control (*p<0.05).

Conclusions

The ability to analyze cell death in zebrafish embryos in a high-throughput manner is critical for toxicology screens. Here, we demonstrate that zebrafish treated with ethanol have a dose dependent increase in apoptotic cell death. Use of acridine orange dye in combination with Gen5™ image processing and analysis allows for rapid and consistent analysis of the number of AO positive apoptotic cells in live zebrafish embryos in a 96-well plate. We also demonstrate analysis of the level of AO staining per embryo, with an increase in AO fluorescence correlating with increased cell death.

References

1. Carvan, M. J., Loucks, E., Weber, D. N. & Williams, F. E. *Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis.* Neurotoxicol Teratol 26, 757–68 (**2004**).

2. Lovely, C. B., Fernandes, Y. & Eberhart, J. K. *Fishing for Fetal Alcohol Spectrum Disorders: Zebrafish as a Model for Ethanol Teratogenesis.* Zebrafish 13, 391–8 (**2016**).

3. Riley, E. P., Infante, M. A. & Warren, K. R. *Fetal alcohol spectrum disorders: an overview.* Neuropsychol Rev 21, 73–80 (**2011**).

4. Ali, S., Champagne, D. L., Alia, A. & Richardson, M. K. *Large-scale analysis of acute ethanol exposure in zebrafish development: a critical time window and resilience.* PLoS ONE 6, e20037 (**2011**).

5. Murawski, N. J., Moore, E. M., Thomas, J. D. & Riley, E. P. *Advances in Diagnosis and Treatment of Fetal Alcohol Spectrum Disorders: From Animal Models to Human Studies.* Alcohol Res 37, 97–108 (**2015**).

6. Chen, S & of and Experimental , S.-K. *Iron-mediated free radical injury in ethanol-exposed mouse neural crest cells.* Journal of Pharmacology and Experimental (**2000**)

7. Ikonomidou, C. et al. *Ethanol-Induced Apoptotic Neurodegeneration and Fetal Alcohol Syndrome.* Science 287, 1056–1060 (**2000**).

8. Muralidharan, P., Sarmah, S. & Marrs, J. A. *Zebrafish retinal defects induced by ethanol exposure are rescued by retinoic acid and folic acid supplement.* Alcohol 49, 149–63 (**2015**).

9. Cole, LK & Ross, LS. *Apoptosis in the developing zebrafish embryo.* Developmental biology 240, 123–142 (**2001**).

10. *The zebrafish book: a guide for the laboratory use of zebrafish.* http://zfin. org/zf_info/zfbook/zfbk. html (**2000**).

11. Abrams, JM, White, K, Fessler, LI & Development, S.-H. *Programmed cell death during Drosophila embryogenesis.* Development (**1993**).

12. Samsa, L. A., Fleming, N., Magness, S., Qian, L. & Liu, J. *Isolation and Characterization of Single Cells from Zebrafish Embryos.* J Vis Exp (**2016**). doi:10.3791/53877