Application Note

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

Using Agilent BioTek Gen5 microplate reader and imager software to analyze cell death in zebrafish embryos

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Abstract

Ethanol exposure during development can have devastating effects on the fetus. This study used zebrafish, an excellent model to study both normal development and toxicity, 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 were assessed using acridine orange staining. It was 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 approximately 1 in 100 children born in the United States each year.[2] The range of outcomes following fetal alcohol exposure is broad, and includes 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 the 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]

This study used acridine orange (AO) staining combined with the Agilent BioTek Gen5 microplate reader and imager software to determine the amount of apoptotic cell death in zebrafish embryos after 24 hours of ethanol exposure. The study also performed image processing and analysis to generate a focused image from an image stack, then used 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. 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 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, part number 4520, Corning, NY).

Imaging

Images were acquired using a 2x objective on an Agilent BioTek Lionheart FX automated microscope 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. Twelve stacks were taken with a height of 12 µm per stack for a total height of 144 µm.

Image processing

Two preprocessing steps were used 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 Stack option under Z-projection (Figure 1A, Table 1).

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

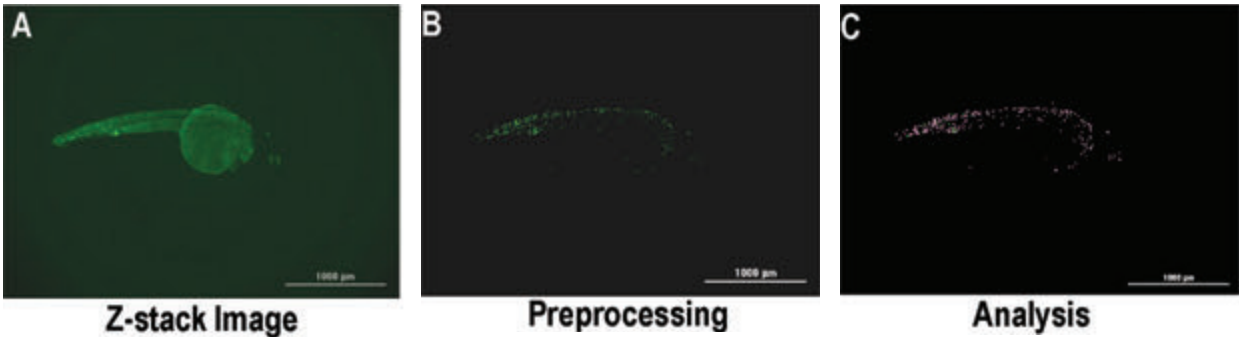

Figure 1. Acridine orange spot counting workflow. (A) 2x image after using focus stacking to create a z-projection (B) Image after applying preprocessing to the image stack. (C) Object masks highlighting cells between 1-10 µm are highlighted in pink. The highlighted cells represent acridine orange positive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

Table 1. Agilent BioTek Gen5 image analysis software settings for Z-projection. The z-project function creates a focused image projected from an image stack.

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

Table 2. Agilent BioTek Gen5 microplate reader and imager software settings for cell counts. Image preprocessing removed background from the resulting image to facilitate image analysis. The image analysis parameters generate cellular masks in the GFP channel to count AO-stained cells in each embryo.

Image PreProcessing	
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	5,000
Background	Dark
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Minimum Object Size	1 µm
Maximum Object Size	10 µm

Table 3. Agilent BioTek Gen5 image analysis software settings for determining the AO integral. Image preprocessing 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 the integral of AO fluorescence to be determined within the embryo.

Image PreProcessing	
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
Minimum Object Size	300 µm
Maximum Object Size	10,000 µm

In the case of images that 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.

Results and discussion

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 shown in Figure 2. Image stacks were taken in both the brightfield and GFP channels.

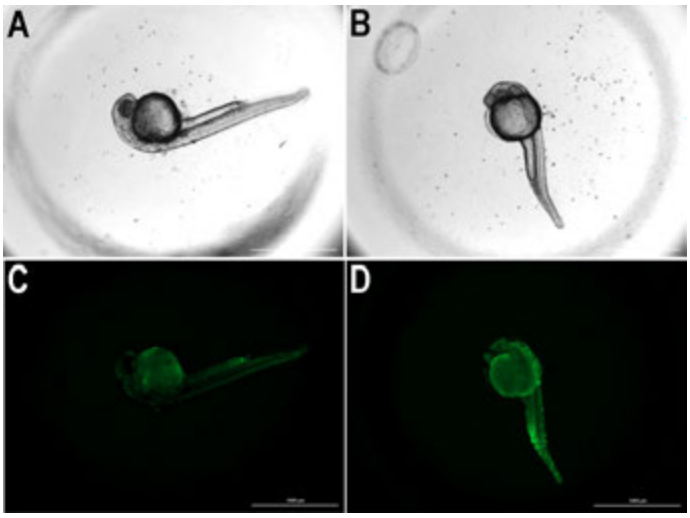

Figure 2. Zebrafish in round bottom 96-well plates. Zebrafish treated with 0% ethanol (A,C) or 0.3% ethanol (B,D) were placed into round bottom 96-well plates, then image stacks were taken in both the brightfield (A,B) and GFP (C,D) channels with a 2x objective. All embryos are positioned roughly in 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 preprocessing 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 shows a histogram of the size of the AO positive cells analyzed after preprocessing. Zebrafish cells are smaller than mammalian cells and cell size can range from 3 to 12 µm.[12] In this case, an average of 4.82 µm was seen, which is reasonable since the rolling ball used to process the image was 5 µm.

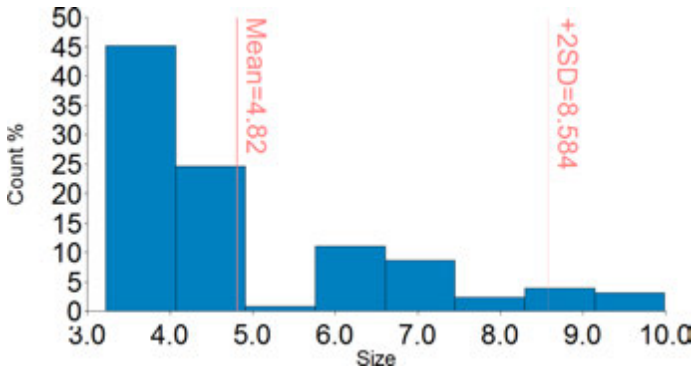

Figure 3. Size of cells analyzed after preprocessing.

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, masks were created around each embryo (shown in red in Figure 5) and Gen5 software was used to calculate the integral of AO fluorescence in each embryo. It was found, as with the cell-counting data, that with higher amounts of ethanol treatment there was increased AO fluorescence, which corresponds to a dose-dependent increase in cell death in the embryos (Figure 5).

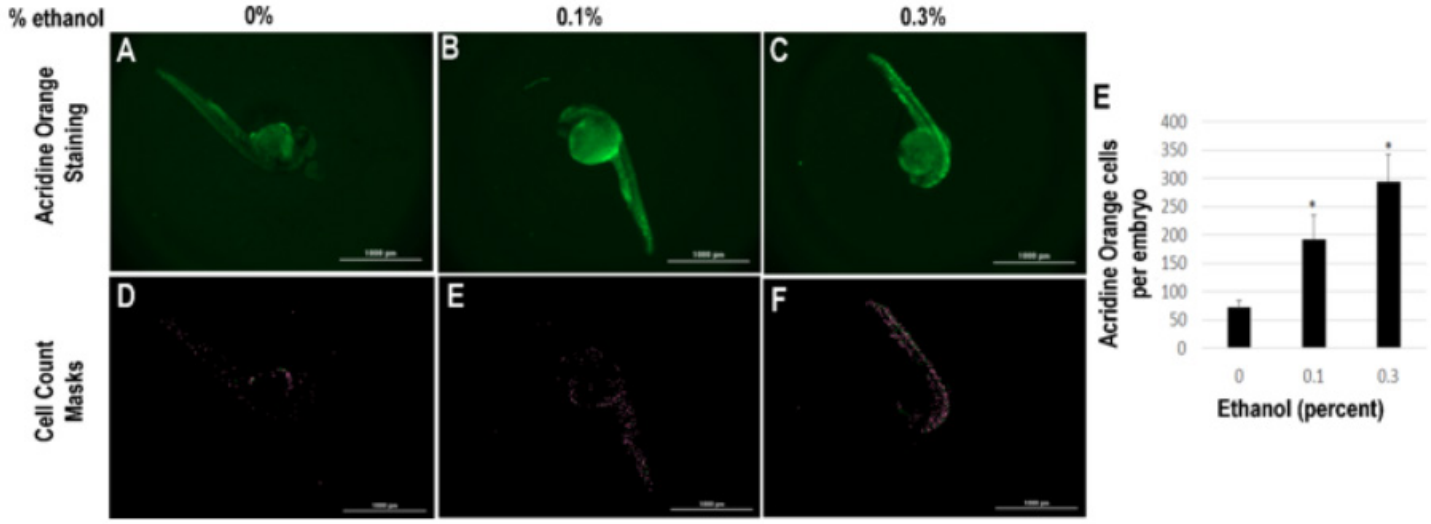

Figure 4. Cell death in zebrafish embryos increases in a dose-dependent manner after treatment with ethanol as shown by a number of AO-positive cells. Image stacks of acridine orange-stained embryos treated with 0 (A,D), 0.1% (B,E), and 0.3% (C,F) ethanol are shown in the GFP channel. The preprocessed 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).

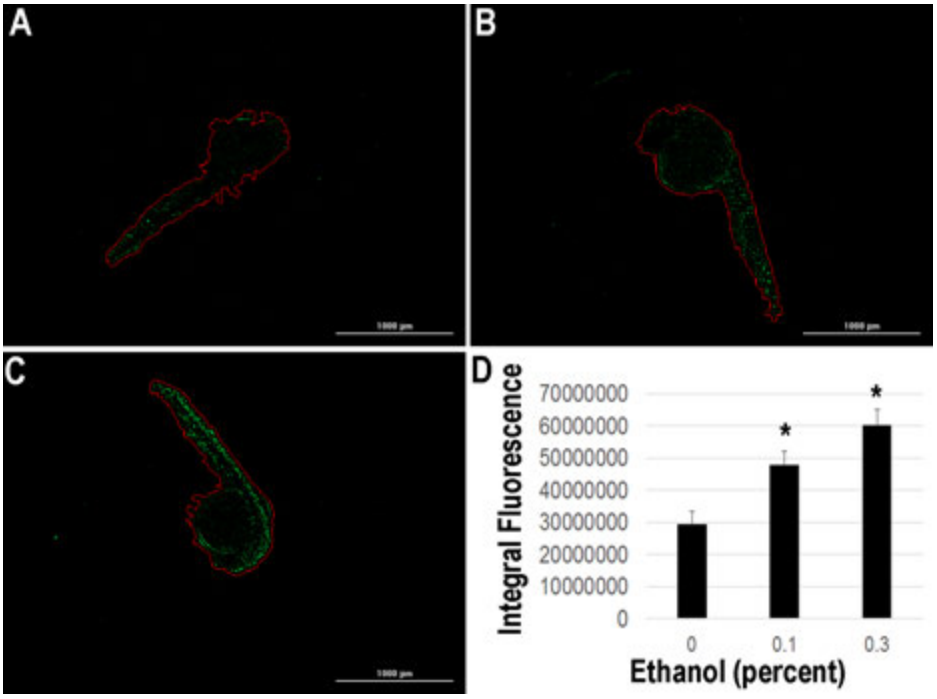

Figure 5. Cell death in zebrafish embryos increases in a dose-dependent manner after treatment with ethanol, as shown by AO fluorescence. Embryos treated with 0 (A), 0.1% (B), or 0.3% (C) ethanol are shown. Image masks were created around each embryo and the integral of AO fluorescence for each embryo was calculated using Agilent BioTek 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).

Conclusion

The ability to analyze cell death in zebrafish embryos in a high-throughput manner is critical for toxicology screens. This application note demonstrates that zebrafish treated with ethanol have a dose-dependent increase in apoptotic cell death. Use of acridine orange dye in combination with the Agilent BioTek Gen5 microplate reader and imager software allows rapid and consistent analysis of the number of AO-positive apoptotic cells in live zebrafish embryos in a 96-well plate. Analysis of the level of AO staining per embryo was demonstrated, with an increase in AO fluorescence correlating with increased cell death.

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