Evaluating Ethanol Induced Cell Death in Zebrafish

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Abstract

Zebrafish are a powerful model organism used for studying numerous biological processes. Some of the benefits of the zebrafish model include that they mature within a short period of time, are optically transparent, develop outside of the mother's body, and are vertebrates. Because of these, and other reasons, zebrafish are used in a wide variety of applications including drug discovery, developmental biology, and molecular genetics. One specific application that zebrafish are well-suited for is toxicity testing. The zebrafish is an excellent model organism for identifying and understanding the genes that regulate sensitivity and resistance to developmental toxins.

Most toxicology screens use cultured cells, which limits the assays to cell-autonomous phenotypes or those end points that can be observed in relatively simple culture systems. A cell monolayer in a dish fails to capture the complexity of a complete organism, and cell based reporters give little indication of disease phenotype modulation. Furthermore, small molecules may act differently in whole organisms compared to cell culture. In contrast, zebrafish screens are carried out in living zebrafish embryos, which possess fully integrated vertebrate organ systems. As such, a much broader range of phenotypes can be assayed in zebrafish than cultured cells. Whole organism screening has the advantage of being less targeted than cell-based screens, allowing the drug to interact with any biological pathway. The readout is an alteration of a whole organism phenotype which relates well to disease.

In this study, we take advantage of the utility of the zebrafish organism to assess the effect of the known toxicant ethanol on cell death. After 24 hours of ethanol treatment, we evaluate the effect of ethanol on cell death using acridine orange staining in combination with automated microscopy. Spot count analysis was performed which allows the determination of the number of acridine orange positive cells per zebrafish.

BioTek Instrumentation

Figure 1. Lionheart™ FX Automated Microscope with Augmented Microscopy™

All inclusive microscopy system: Optimized for live cell imaging with brightfield, color brightfield, phase contrast and fluorescence channels. Up to 100x air and oil immersion magnification.

Up to 20 frames per second (fps) image capture: Enables characterization of rapid cellular events and addition of reagents with uninterrupted monitoring of cellular response.

Integrated environmental control: Incubation up to 40 °C with CO_2 /O_2 and available humidity control provides optimal conditions for long-term imaging of live cells.

Powerful Gen5™ Microplate Reader and Imager Software: Automated image capture, processing, and analysis tools, including dual masking for cell counting and subpopulation analysis, object based spot counting, and annotation and movie maker functions.

Materials and Methods

Zebrafish Maintenance

Zebrafish were maintained according to standard conditions. 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 Mg SO_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 0-3% 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. To perform the assay, embryos were places in 10 µg/mL of AO (Catalog No. A6014, Sigma, St. Louis, MO) in E3 media. After 30 minutes of staining, embryos were washed three times in E3 media. After staining, embryos were transferred to round bottom 96 well plates for imaging (Catalog No. 4520, Corning, Inc., Corning, NY).

Materials and Methods *(Cont.)*

Imaging

Images were acquired using a 2x objective on the 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 with a height of 12 µm per stack for a total height of 144 µm.

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 (Figures 2 and 3A). The image projection was then pre-processed with a 5 µm rolling ball in order to obtain the best distinction between individual cells (Figure 3B). Image analysis was similar for the images which were analyzed by the GFP integral. In this case a 20 µm rolling ball was used for pre-processing (Figure 3C).

Image Analysis

In the case of the cellular analysis, object counting analysis was performed on the GFP channel to highlight each individual cell (Figure 3D). In order to determine total GFP expression per embryo, a mask was created in the GFP channel to encompass the entire embryo (Figure 3E). Then, the GFP integral of the whole embryo was determined using Gen5 software.

Results

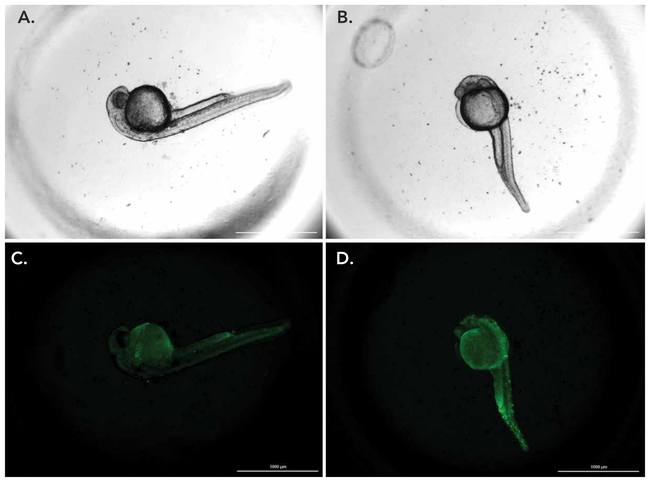

Figure 2. Zebrafish in round bottom 96 well plates. Zebrafish embryos were treated with 0 **(A,C)**, 0.1, or 0.3% **(B,D)** ethanol for 24 hours starting at the 64 cell stage. Directly following treatment, embryos were stained with acridine orange to mark apoptotic cells. Following staining, zebrafish embryos were washed three times and treated with tricaine until immobile and placed into 96 well round bottom plates to image. Image stacks were taken in both the brightfield **(A, B)** and GFP channels **(C,D)** with a 2x objective. All embryos are positioned roughly in a lateral orientation and are in the center of the well.

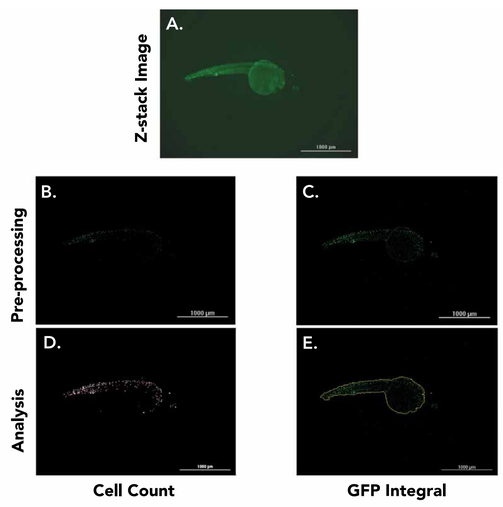

Figure 3. Acridine orange spot counting workflow. Image stacks were flattened with the focus stack function of Gen5 software. **(A)** 2x image after using focus stacking to create a Z-projection. The resultant image was subjected to pre-processing to remove background GFP staining. **(B)** Image after applying a 5 µm rolling ball background subtraction to the image stack. **(C)** Image after applying a 20 µm rolling ball background subtraction to the image stack. **(D)** The number of GFP positive cells per embryo was determined with the cell counting function of Gen5 software. 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. **(E)** Object mask surrounding the whole embryo. Threshold is set at 150 in the GFP channel. The GFP integral is then taken over the object mask. This amount of GFP expression is consistent with the amount of cell death in the embryo.

Results *(Cont.)*

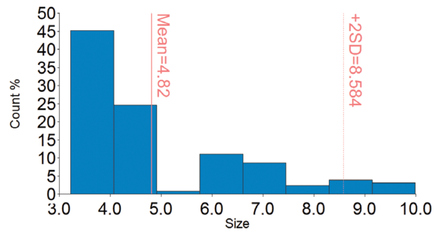

Figure 4. Size of cells analyzed after pre-processing. This figure shows a histogram of the size of the GFP positive cells analyzed after pre-processing. Zebrafish cells are smaller than mammalian cells and cell size can range from 3-12 µm. In this case there is an average of 4.82 µm, which is reasonable since the rolling ball used to process the image was 5 µm.

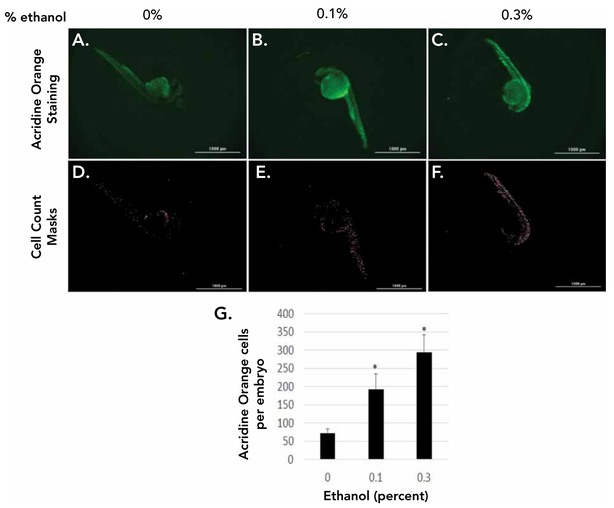

Figure 5. Cell death in zebrafish embryos increases in a dose dependent manner after treatment with ethanol as shown by number of GFP positive cells. Image stacks of acridine orange stained embryos treated with 0 **(A,D),** 0.1 **(B,E),** or 0.3% **(C,F)** ethanol are shown in the GFP channel. The pre-processed images with pink masks around the GFP positive acridine orange cells are shown for each treatment. As ethanol concentrations increase, the number of apoptotic cells also goes up. Quantification is shown in panel **G.** 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).

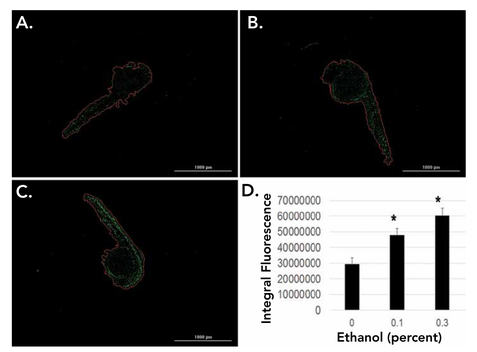

Figure 6. Cell death in zebrafish embryos increases in a dose dependent manner after treatment with ethanol as shown by GFP expression. Embryos treated with 0 **(A)**, 0.1 **(B)** or 0.3% **(C)** ethanol are shown. Image masks were created around each embryo, and the integral of GFP expression for each embryo was calculated using Gen5 software. **(D)** The integral of GFP expression goes up in a dose dependent manner, with 0.1 and 0.3% ethanol treatments both significantly higher than the control (*p<0.05).

Conclusions

1. The ability to analyze cell death in zebrafish embryos in a high-throughput manner is critical for toxicology screens.
2. Zebrafish treated with ethanol have a dose dependent increase in apoptotic cell death.
3. Cell death can be measured by number of GFP positive cells per embryo or total amount of GFP expression per embryo.
4. Use of acridine orange dye in combination with Gen5 image processing and analysis allows for rapid and consistent analysis of the number of GFP positive apoptotic cells in live zebrafish embryos in a 96-well plate.