

Analysis of *Danio rerio* Embryos Utilizing High Content Analysis Methodology

Rebecca J. Henderson¹, Gang Lin¹, Debby Nickischer¹, Tim Baranowski², and Jeffrey Haskins¹

¹Thermo Scientific, Laboratory Automation and Cellular Imaging • 100 Technology Dr., Pittsburgh, PA U.S.A. 15219

²Zygon, LLC • 520 Kell Hall, 24 Peachtree Center Avenue, Atlanta, GA U.S.A. 30303

<http://www.thermo.com/cellomics>

Abstract

Live, 1-day old, Z-TagSM Fluorescent Blood Vessel zebrafish embryos (Zygon LLC, Atlanta, GA) were placed in a 384-well Falcon plate, one embryo per well in Holtfeter's solution supplied by Zygon. Z-Tag embryos are genetically engineered with *Aequorea coeruleolucens* GRFP under the control of the vascular endothelial growth factor receptor 2 (VEGFR2) promoter to label the developing vasculature. The plate was treated with two anti-angiogenic compounds, Sugen 4312 and PTK 787, both dissolved in dimethyl sulfoxide (DMSO). Sugen 4312 was tested between 0 and 30µM, and PTK 787 was tested between 0 and 10µM. Negative controls were 1% final concentration of DMSO. The embryos were handled at 27°C for 18 hours and were imaged at 2.5x magnification. An algorithm specific to imaging zebrafish was applied, demonstrating dose-dependent differences in angiogenic development between treated and non-treated embryos. This experiment shows the application of a high content analysis platform in zebrafish analysis.

Introduction

Zebrafish (*Danio rerio*) embryos are often utilized as a model biological system due to their well characterized stages of embryogenesis, the transparency of the externally fertilized embryos, and the organism's close similarity to vertebrate systems. Furthermore, zebrafish can be genetically manipulated, creating mutants and fluorescently tagged proteins of interest. In this study, Zygon's Z-TagSM Fluorescent Blood Vessel zebrafish embryos were used in conjunction with known anti-angiogenic compounds to demonstrate their applicability in an automated image acquisition and analysis platform.

The Z-Tag embryos are engineered with *A. coeruleolucens* GRFP tagged vascular endothelial growth factor receptor 2 (VEGF2), producing green fluorescent vessels throughout the developing embryos. Imaging these vessels illustrates the normal development of zebrafish angiogenesis which can be inhibited by anti-angiogenic compounds such as Sugen 4312 and PTK787. Here, high content analysis methodology was implemented to automate the image capture and image analysis of these fluorescent embryos exposed to various concentrations of Sugen 4312 or PTK787 for 18 hours. The Thermo Scientific Cellomics[®] ArrayScan[®] V1 HCS reader (Thermo Fisher Scientific, Pittsburgh, PA) and a specialized application for embryonic vessel identification were used together to create a fully automated method by which to replicate previous anti-angiogenesis studies carried out with these embryos. Once the images were captured, the application was automatically applied to the images, and the data were immediately available for viewing.

The data collected demonstrate that the zebrafish application identified embryos in the proper orientation, distinguished the embryos' trunk for vessel analysis, and disregarded the embryos' heads from the analysis. The application produced an overlay on intersegmental (IS) and dorsal longitudinal anastomotic (DLA) vessels on each embryo, and the data revealed dose-dependent decreases in angiogenesis as the concentration of drug increased, as expected. This study clearly demonstrates how automated imaging and analysis can be applied to whole-organism studies.

Materials and Methods

Embryo Handling

One-day-old, live, Z-TagSM zebrafish embryos arrived directly from Zygon and were handled at room temperature with wide bore pipet tips (provided by Zygon). Each embryo was aspirated with 30µL Holtfeter's solution (also provided by Zygon) and individually placed into wells of a clear Falcon 384-well microplate. The embryos were always manipulated going head first or tail first into the pipet to avoid damage to the embryo. Dead embryos and debris were carefully avoided to ensure that only one developing embryo was included in each well.

Compound Treatments, Incubation, and Anesthesia

Working stocks (2x) in Holtfeter's solution without antibiotics were made from thawed stock vials of Sugen 4312 (Su) and PTK787 (PTK). Working stocks were made serially by 1:3 dilutions to create final concentrations of 0.30µM - 30µM for Su and final concentrations of 0.03µM - 10µM for PTK. Dimethyl sulfoxide (DMSO) at 2% working concentration served as a negative control. The 2x stocks were added to all wells with embryos, and the plate was centrifuged at 32 x g for 1 min to ensure proper mixing. The plate was incubated overnight in a 27°C waterless incubator.

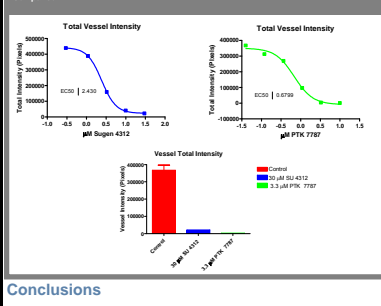
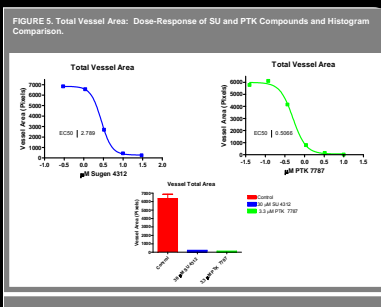
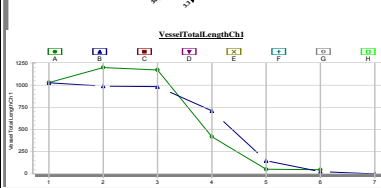
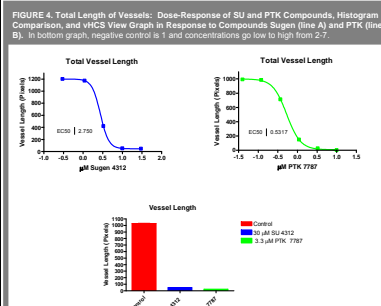
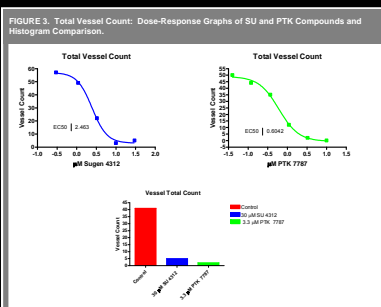
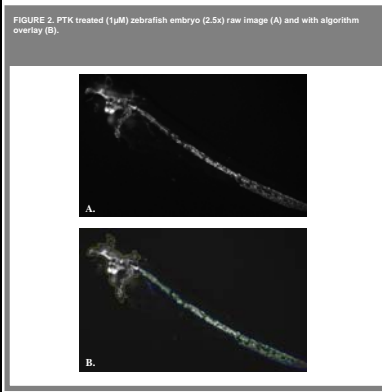
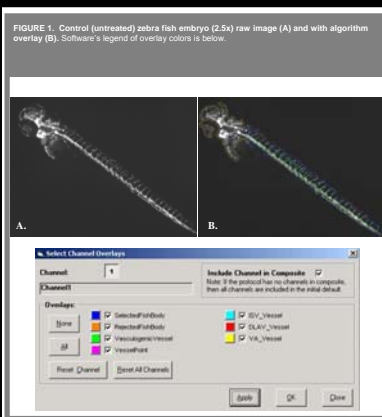
The next morning, the embryos were incubated with a final concentration of 0.016% tricaine (provided by Zygon) to anesthetize the embryos. Each well received 2.4µL of the tricaine solution, and the plate was slightly vortexed to mix. After a 45 min incubation, the plate was again centrifuged at 32 x g for 1 min to align embryos on their lateral sides for imaging.

High Content Analysis of Embryos

Images of each embryo were taken at 2.5x magnification, one field per well. Embryos were imaged for GRFP fluorescence, and the embryos were analyzed with a specially designed application to detect variations in angiogenic development along the embryos' vertebral column. The application can quantify and report a comprehensive set of features such as vessel count, length, area, intensity, and width, as well as general features about the embryo's torso. The application automatically discards from analysis any embryo that is not in an optimal condition (wrong orientation, unhealthy, out-of-focus). Also, the embryo heads are automatically identified and excluded from consideration, and only the trunks are included for vessel analysis.

Results

- Zebrafish embryos were successfully imaged and analyzed with the specialized angiogenesis application (Figures 1 and 2)
- Application identified the following vessels within each embryo: intersegmental vessels (ISV) and dorsal longitudinal anastomotic vessels (DLAV)
- Dose-response curves of vessel total count, total length, total area, and total intensity were generated with Sugen 4312 and PTK787 (Figures 3-6)



Conclusions

- Zebrafish embryos are amenable to automated imaging and analysis.
- Sugen 4312 and PTK787 produced dose-dependent inhibition of angiogenesis in the zebrafish transgenic embryos as demonstrated by vessel total count, total length, total area, and total intensity.

References

TC Tran, et al. (Dec 2007) "Automated, quantitative screening assay for anti-angiogenic compounds using transgenic zebrafish." *Cancer Research*. 67(23): 11386-11392.