# Novel Imaging and Analysis of Zebrafish for High Throughput Screening

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## Abstract

**Large organisms, such as zebrafish embryos, have become attractive model systems for developing disease-related assays for drug discovery and toxicology studies. A limitation in performing these assays has been the availability of fast image acquisition systems with sufficient resolution and depth of field to accurately characterize such organisms. A second limitation has been in the analysis of the complicated images to provide fast and accurate quantification of the biology of interest in the assay. Here we report on the use of the IsoCyte® Laser Scanning Imager for high-throughput image acquisition of the large 3-dimensional objects coupled with MetaXpress® and AcuityXpress® software applications, for visualization and data analysis. The proprietary optical system has a large depth of field (400 µm) that allows whole well images to be acquired in less than 5 minutes per microplate. Images are obtained simultaneously in both laser scatter, which provides a label-free image of the object, and up to three other fluorescent channels (e.g., green fluorescence protein-labeled vessels). Two applications in a 384-well plate format will be presented. In one example, live Z-Tag zebrafish embryos (Zygogen) used for the identification of therapeutic agents affecting angiogenic blood vessels were analyzed. In another example, a panel of substances was tested for acute toxicity effects of varying doses on developing zebrafish embryos. The results show that a fast and accurate count of blood vessels is possible and toxic effects on morphology can be quickly measured with the combined data acquisition and analysis software.**

## Introduction

**Zebrafish make an attractive model for testing the effect of compounds because they combine the throughput of cell-based assays with the biological relevance of animal models. Since zebrafish embryos develop most major organ systems within the first 5 days and have a permeable, transparent skin it is easy to visualize specific tissues of interest, especially with the aid of fluorescent reporters. This study investigated 1) the dose-dependent effect of an angiogenesis-inhibiting compound (Vatalanib/PTK787) on vessel formation in the tail of developing embryos expressing Green reef coral Fluorescent Protein in their vasculature and 2) the effect of varying doses of known-toxic compounds on length and morphology of zebrafish embryos treated for 24 hours. To effectively utilize the embryos in a screening scenario they were dispensed alive into wells of a 384 well microplate, dosed with drugs, and scanned on an IsoCyte® DL Laser Scanning Imager. Images were simultaneously acquired showing both fluorescence signal and laser scatter (similar to brightfield) signal. The resulting images were analyzed either "on-the-fly" during acquisition or imported into MetaXpress® software for analysis.**

## Materials and Methods

- **Z-Tag℠ Angiogenesis Assay Starter Kit (Zygogen)**
- **Z-Tag Fluorescent Blood Vessel Embryos (Zygogen)**
- **Dimethyl sulfoxide (Sigma, PN 276855)**
- **Ethanol (Sigma, PN 459836)**
- **Clozapine (Sigma, PN C6305)**
- **Malathion (Spectracide 50%)**
- **Hexachlorophene (Sigma PN 4-0323)**
- **Melamine (Beacon Analytical, PN 20-0158)**
- **384 well glass-bottom microplates (Matrical, MGB1011-HG)**

## Angiogenesis Assay Procedure

**Embryos were enzymatically dechorionated at 1 day post-fertilization (dpf) by Zygogen and treated with varying doses of Vatalanib/PTK787 or 1% DMSO prior to shipping. Upon receipt, 1 embryo was transferred in 60 uL of solution to each well of a 384 well clear-bottom plate. Embryos were arrayed in columns of 5-8 replicates per dose. After 2 hours equilibrating in the plate, embryos were anesthetized by the addition of 2.5 uL of 0.4% tricaine. After 30 minutes, fish were centrifuged at 32xg for 1 minute to orient them flat on the bottom of the well. The 2 dpf fish were then scanned using the IsoCyte Imager with the settings shown in Table 1.**

| IsoCyte® Settings | Angiogenesis Assay | Acute Toxicity Assay |
|---|---|---|
| Excitation Source | 488 nm laser | 488 nm laser |
| GFP Emission | 510-540 nm | 510-540 nm |
| Laser Scatter Emission | 475-485 nm | 475-485 nm |
| Scan Area | 3200x3200 um (entire well+10%) | 3200x3200 um (entire well+10%) |
| Resolution | 5x5 um | 5x5 um |
| Data Averaging | 2 averages/pixel | 1 average/pixel |

Table 1. IsoCyte™ instrument settings for scanning zebrafish.

**IsoCyte data was automatically saved as a plate of raw images in addition to saving as MDCStore™ (database) compatible individual TIF files. TIF images were imported from the database into either MetaMorph® or MetaXpress® software for analysis using a simple journal containing the Neurite Outgrowth Module which was adapted to count the fluorescent blood vessels sprouting from the main vessel of the tail.**

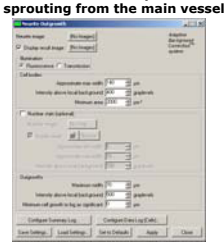

Figure 1. Neurite Outgrowth module of MetaXpress ® software

## Acute Toxicity Assay Procedure

**Embryos were dechorionated at 1 dpf by Zygogen. Upon receipt at 2 dpf, 1 embryo per well was transferred in 40 uL of solution to a 384 well plate. Embryos were arrayed in columns of 7 replicates per dose. Forty microliters of a 2X concentration of each of 6 compounds was added to the appropriate treatment well and embryos were incubated at 28°C for 24 hours. Thirty minutes before scanning, embryos were anesthetized by the addition of 5 uL of 0.4% tricaine. Fish were centrifuged at 32xg for 1 minute to orient them flat on the bottom of the well and the 3 dpf fish were then scanned using the IsoCyte Imager with the settings shown in Table 1.**

**IsoCyte® data was automatically saved as a plate of raw images in addition to saving as MDCStore™ compatible individual TIF files. TIF images were imported from the database into MetaXpress® software for analysis using a journal that created a mask using the combined fluorescent and scatter images then reported morphometry parameters (e.g. length) in addition to intensity measurements.**

## Results

### Angiogenesis Assay

**An entire 384 well plate was scanned and saved in 8 minutes on the IsoCyte® imager. The fluorescent and scatter images can be viewed as they are acquired. See Figure 2 for an example of the zebrafish GFP images with a Vatalanib dose response.**


Figure 2. Inhibition of tail vessel branch development is apparent in the fluorescent images of increasing Vatalanib dose.

**The user chooses which of 6 different file types to automatically save after a scan on IsoCyte imager. This scan was saved as a single 680 MB raw data file (for 2 wavelengths in 384 wells) and as a folder of TIF images for easy import into MetaMorph® software. A MetaMorph® analysis journal was created which cropped the image and utilized the Neurite Outgrowth application module for identifying and quantitating the fluorescent blood vessels in the tail. Figure 3 shows the steps of the analysis. The entire plate of images is then automatically analyzed using the journal and the data output either recorded to a database file or into an Excel spreadsheet.**

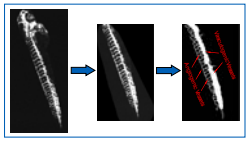

Figure 3. Illustration of MetaMorph® image analysis for angiogenesis.

**The number of counted vessels was plotted. Figure 4 shows averaged results normalized to the DMSO control. Error bars represent standard error on the replicates. The inhibition of vessel formation was consistent with results seen at Zygogen.**

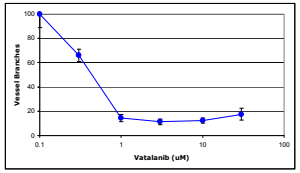


Figure 4. Decrease in tail vessel formation with increasing drug dose.

### Acute Toxicity Assay

**Since the Acute Toxicity Assay relies on gross morphological changes in the embryo's body, no pixel averaging was performed during the scan and an entire 384 well plate was imaged and saved in 4 minutes. Figure 5 is an overlay of the GFP and scatter images acquired on the IsoCyte imager.**

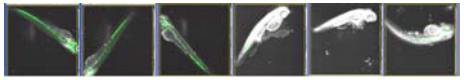


Figure 5. Toxic effects are visible with increasing dose. Laser scatter signal increases while GFP intensity decreases.

**Often the acutely toxic effects are measured as a difference in overall embryo length due to retarded development or curling of sick embryos. This analysis can be done by importing IsoCyte images into MetaXpress® software and using Integrated Morphometry Analysis to measure length. Length averages were compared to intensity of scatter from images analyzed during acquisition. Figure 6 illustrates that the scatter intensity is the best indicator of acute toxicity in this case. Figure 7 shows the toxicity effects of 6 compounds using total laser scatter signal determined with IsoCyte software.**

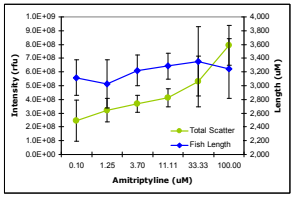


Figure 6. Toxic effects are visible with increasing dose. Laser scatter signal increases more obviously than fish length decreases.

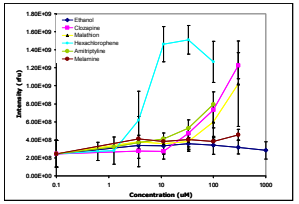


Figure 7. Laser scatter signal shows a comparison of the toxic effects of 6 different compounds.

## Summary

**The IsoCyte® Laser Scanning Imager is ideal for rapidly acquiring zebrafish images simultaneously in laser scatter and fluorescence without time-consuming focusing steps. On-the-fly analysis using IsoCyte software enabled automated zebrafish toxicity screens. Complex image processing for angiogenesis was accomplished in semi-automated fashion using MetaXpress® software and existing application modules.**

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