IDEA Bio-Medical Ltd.
Seeing Better

Benefits

- A.I. driven fish and organ identification
- Orientation-based fish inclusion or exclusion
- Coupled brightfield and fluorescence imaging
- Specialized plates obtain 80% side-oriented fish

Application Note

Automated, deep learning-based Zebrafish image analysis for stress-dependent stem cell response in the tail region.

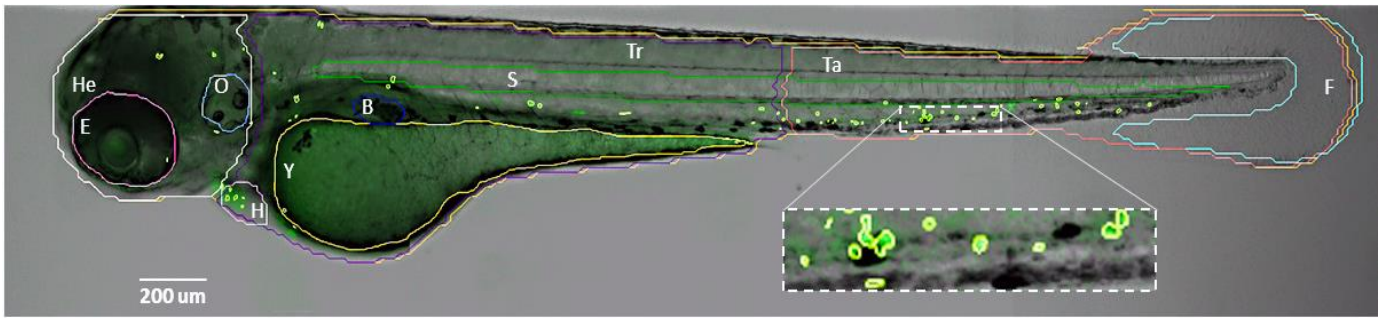

Figure 1 – Overlay image of a transgenic zebrafish having GFP-tagged stem cells (4 dpf). Z-stack images were acquired in brightfield and green fluorescence channel on the *WiScan® Hermes* HCS microscope at 4x magnification, then processed as described below. The novel A.I.-powered Zebrafish analysis algorithm automatically identifies the fish outer contour, internal organs and three body regions [Head, Trunk & Tail], as shown here. Structure outlines depict the two-channel co-analysis of fish anatomy together with fluorescently-tagged cells of interest (GFP-tagged stem cells, light-green clusters in zoom-in).
Organ labeling: E = eye [dark-pink], O = Otic vesicle [light blue], Y = Yolk sac [yellow], B = Bladder [dark blue], H = Heart [light pink], S = Spine [dark green], F = Fin [cyan]; body compartments: He = Head [white], Tr = Trunk [violet], Ta = Tail [orange].

INTRODUCTION

Zebrafish (*Danio rerio*) are an attractive model organism for the study of human disease genotype, phenotype and treatment, whose potential has yet to fully develop. Hematopoiesis, in particular, has already benefited from the use of whole animal studies to investigate the creation, maintenance, migration and differentiation of stem cells [1]. Despite their advantages as a disease model, drug screening studies do not commonly utilize zebrafish due to difficulties related to automating data acquisition and analysis to quantify morphological and other changes with high throughput. A unique challenge is proper side-orientation of the fish to permit proper structure visualization. Here, we describe a new artificial intelligence (A.I.) powered image analysis tool developed by IDEA Bio-Medical that enables screening and quantitation of zebrafish and internal organs visualized via microscopy in microwell plates in a parameter-free fashion (Figure 1). Combined with rapid screening technologies built into the *WiScan® Hermes* HCS system, our new product is the fastest, easiest image-based zebrafish screening tool available.

[1] Bolli, N., *et al.* "*cpsf1* is required for definitive HSC survival in zebrafish" *Blood* (2011) **117**, pp 3996-4007, DOI: 10.1182/blood-2010-08-304030

METHODS

Plate Preparation

The Hermes imaging system is compatible with virtually any sample format, such as 35mm dishes, or plates having 6, 12, 24, 48, 96, 384 or 1536 wells. In addition, the Hermes allows for custom definition of plates conforming to SBS standards, including round-bottom wells. Some plates available on the market are better suited for smaller fish, while others facilitate orienting fish on their side. Contact IDEA Bio-Medical for more details.

Users should first place the desired number of anesthetized fish into each well; one fish per well is recommended, but multiple fish per well can also be supported. Fish can be oriented on their side at the bottom of the well by gently centrifuging plates at ~200 *g* for 5-10 seconds, with maximum acceleration and deceleration. This step can be supplemented with manual orientation using a long, plastic gel loading tip.

Users can choose to automatically select side-oriented fish during post-processing analysis, with or without the steps listed above. In case of post-processing selection, fish having defined organs (see Table 1) are extracted from the whole plate population.

Note: it is important to change the culture water before imaging to remove large debris. It may be advisable to work under a fume hood to avoid dust particles.

Image Acquisition

Image acquisition of Zebrafish is intended to be done in brightfield with fluorescence using Z-stack acquisition. Single-plane imaging is also supported and increases throughput rate. In this fashion, the *Hermes* patented autofocus system may enable scans of a whole 96-well plate in less than two minutes. Any of the supported magnifications (2X – 60X) can be used to image the fish. Optimal imaging utilizes the 2X or 4X objectives and the accurate motion capabilities of the Hermes to create a stitched montage of the entire fish.

Higher magnification is recommended for higher resolution imaging of specific structures, rather than the entire fish. Such imaging is most efficient when coupled with the *Hermes* object-mapping application (Rare Event). In this way it is possible to detect a fish in a well using a lower magnification (2X – 10X), then automatically scan it with the same or higher magnification (4x – 60X) to obtain a Z-stack exclusively at the desired locations.

Image Pre-processing

Post-acquisition batch image processing software accompanying the *Hermes* system permits users to select among several acquisition options:

- Best slice selection and intensity projections of Z-stack acquisitions
- Stitching/montaging for fish spanning several fields of view
- Fluorescence deconvolution for enhanced spot-detection

Table 1 – Object Identification

Objects Identified Automatically			Features Extracted
– Fish Outline	– Brain	– Granules & Spots	– Area
– Yolk Sac	– Bladder	– Fish Regions	– Count
– Eye	– Heart	➢ Head	– Fluorescence Intensity
– Tail Fin	– User-definable Organ (Drawn Manually)	➢ Trunk	– Shape
– Spine		➢ Tail	

Image analysis in *WiSoft® Athena* Zebrafish application

Brightfield images are analyzed by a unique artificial intelligence (AI) powered algorithm developed by IDEA Bio-Medical. It automatically detects zebrafish and their internal organs in brightfield with no required user input.

Fluorescence images can be used in conjunction with brightfield to measure fluorescence within organs, and to identify granules, spots and other labeled structures in any number of colors.

User-defined, manually drawn objects can also be defined in the *Athena* Zebrafish application. This capability enables small-scale, exploratory projects where organs or structures of interest are not in a pre-defined category, but can be readily identified.

Sample Application

Counting clusters of stem cells specifically in the tail region

Data courtesy of Alexandra Lubin PhD, (Elspeth Payne lab, UCL Cancer Institute).

Transgenic zebrafish having GFP-tagged progenitor stem cells were cultivated to 3-days post fertilization. Fish were subjected to hemolytic stress, which causes an increase in the number of visible stem cell clusters. The effects of stress had been established using traditional, manual analysis methods. Here, the utilization of the AI-powered Athena image analysis application validates the expected results in a fully automated fashion. Following image acquisition, fish were subjected to DNA sequencing to confirm wild type (WT) and mutant genotypes.

To ensure that all stem cell clusters are visualized, the fish were acquired with Z-stack followed by maximum intensity projection and image stitching, as described above in *Image Pre-processing*. The fish were detected, then automatically segmented into the three regions – head, trunk and tail (Figure 1). Stem cell clusters were identified in the green fluorescence channel (Figure 2C).

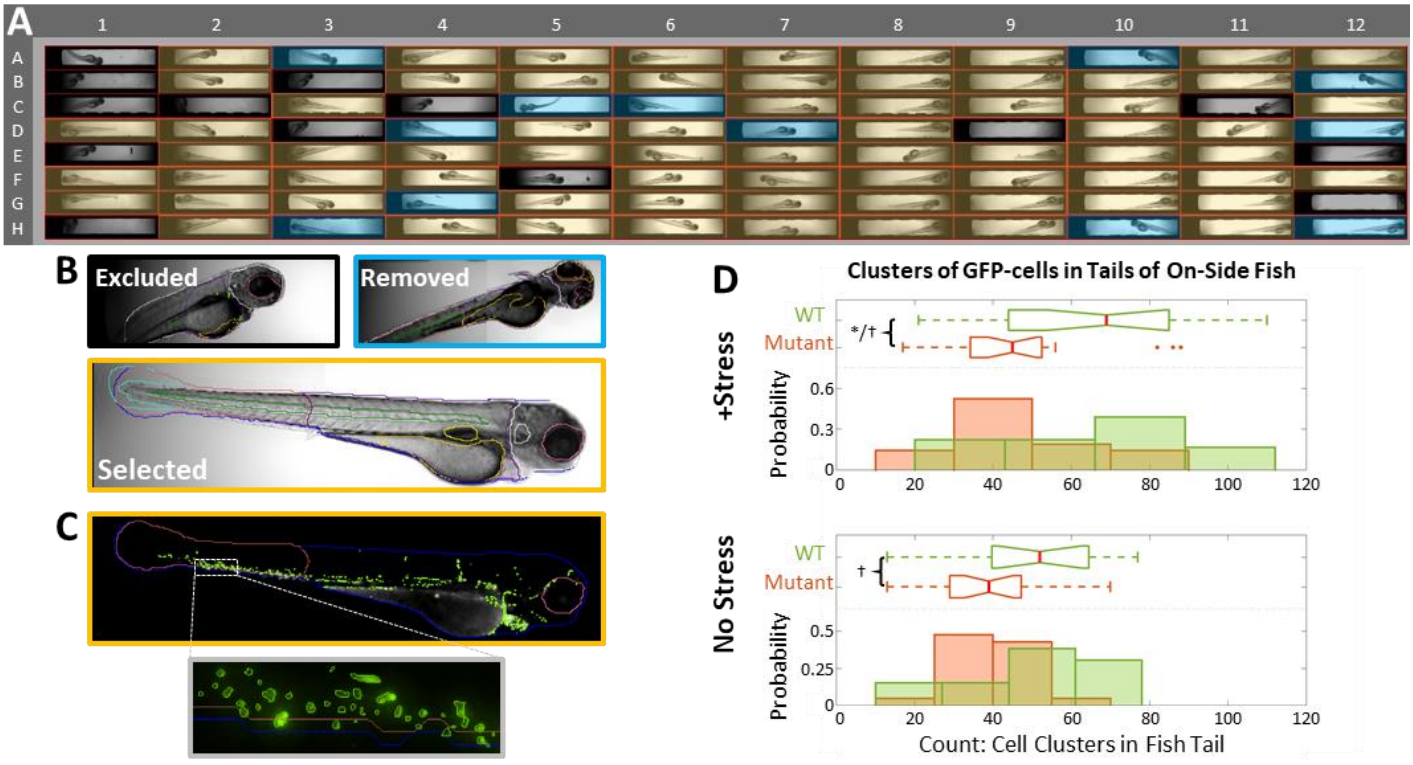

Figure 2 – Plate-wide screening with orientation selection. A) Plate-wide visualization of all wells, color-coded regarding inclusion of the fish: Side-oriented (Yellow), improperly oriented (Cyan), fragmented or not within the well imaging area (Black). **B)** Example images showing three fish automatically-categorized via identification of internal structures: the eye and tail region [Fig 1]. **C)** Fluorescence image of *Selected* fish from (B) showing the fish outline (blue), tail region (Red) and segmented cell clusters (green); zoom-in highlights the cell clusters in the tail. **D)** Quantitation of the number of cell clusters found in the tail region (C) for fish subjected to hemolytic stress vs. control following post-acquisition genotype classification (WT-Wild Type); probability density function (bottom) and boxplot (top) contain the same data and highlight the differing phenotypic response. Statistics: (*) $p < 0.05$ by two-sample *t*-test; (†) $p < 0.05$ by two-sample Kolmogorov-Smirnov test.

Cluster counting was restricted to the tail region of fish lying in a side orientation (Figure 2). Fish on their side were selected as those fish having only one eye visible and a tail region of at least 0.175 mm^2. Such size requirement ensured that the entire tail region fell within the field of view. Though the cell clusters are visible outside of the tail region, above the yolk sac for instance, they are not considered.

The number of cell clusters in each fish tail was extracted and exported for plotting. Figure 2D confirms, as expected, that WT fish exhibit a larger phenotypic change in response to hemolytic stress compared to the mutant variant. WT fish increase the median cell cluster count by 33% (from 52 to 69) whereas mutants increase by only 15% (from 39 to 45).

Region-specific quantification, as shown here, can be directly adapted for tumorigenic and immunological study of fluorescence intensity, structural colocalization or morphology.

Time Savings

Sample processing steps

Zebrafish microscopy entails three steps: fish mounting to define orientation, imaging and analysis. The Hermes Zebrafish application eliminates the need for manual intervention.

Table 2 – Sample Processing Comparison

Step	Hermes	Conventional Microscopy
Fish Orienting	Automated orientation detection	Manual manipulation
Image Acquisition	Automated Multi-well plate compatible	Manual
Image Analysis	Automated No parameter definition	Manual segmentation

Time Investment Comparison

Here, we compare the throughput obtainable via the Hermes imaging system with estimated time requirements for the three experimental steps from Table 2 using two conventional microscopy modalities. Sample mounting is the time to place fish into wells of a multi-well plate with desired orientation; the *Athena* software permits post-analysis orientation selection, and hence has less stringency for mounting requirements. For manual imaging, more fish may be mounted than imaged in the time described. Imaging estimates are for experienced users acquiring a multi-channel Z-stack data set. For conventional analysis methods, it is presumed that users will manually segment each image individually. Post-processing for image projections, best-slice selection, and stitching is not included.

Table 3 – Per-Fish Time Investment

Method	Hermes	Confocal (Manual)	Widefield (Manual)
Number of fish	96	3	15
Mounting (min)	15	15	15
Imaging (min)	13 *	90	60
Analysis (min)	5 ** 15 ***	15	30
Total Time (min)	48	120	105
Minutes per fish	**0.5**	40	7

* 4 Fields of view, 6 slices, 2 colors in specialized orientation-assistive 96 well plates.

** Estimated time needed for a user to set desired parameters for automated analysis.

*** Upper computation time estimate to run the AI-powered image analysis algorithm.

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