Introduction

There is growing interest in imaging and analysis of zebrafish and C. elegans as model organisms for drug discovery and toxicity studies. Adult zebrafish can grow to about 4 cm in length, and nearly transparent larval zebrafish has been developed using the nearly transparent larva at its 5 days post-fertilization stage (5-dpf) where its size is about 9 mm. For this purpose microplates with 96-well format are most suitable test plates, as the wells have sufficient capacity for aquatic water to keep the fish viable. Additionally, it is desirable to have no more than 1 fish per well so that the health of one does not affect the visibility of its neighbors.

These requirements slow down the throughput of most microscopes and high-content imaging systems since, even under the 2X magnification, multiple fields of view (FOV) need to be imaged to ensure the whole well is covered. Here we show how the features of the new IN Cell Analyzer 2000 system overcome these difficulties: (1) A large-chip camera enables rapid single-image whole-well imaging under the 2X objective with minimal image shading; (2) for higher magnification imaging, user-controlled commands can manually change the objective over the area of interest and sometimes the specimen image stitching enables high-resolution whole-well images; (3) two independent modalities of autofocus enable precise z-stacking to that vertical distances along any two organs can be determined.

In an accompanying paper [1], the system's whole-well imaging capability is demonstrated for applications involving stem cell colonies.

Methods

Sample preparation

Zebrafish larvae samples were kindly provided by Phylino Inc. The 2dpf wild larvae which were first anesthetized in Tricaine before being fixed by 4% paraformaldehyde. Larvae were placed in 1:159 Corning Costar 96-well plates in 150 µl of the fixing media.

Image acquisition

Images were acquired on IN Cell Analyzer 2000. Objectives were 2X0.1 NA, 40X1.3 NA, 100X.4X NA using transmitted light and fluorescent modalities with appropriate filter sets and exposure times. The large CCD cameras (Photometrics CoolSnap Ha) in conjunction with the 2X objective, enables a FOV of 7.6X7.6 mm, covering the whole well diameter (6.4 mm) of the 96-well plate with a single image capture.

To capture the whole zebrafish in the 2-dimension, 2-stacks were acquired (Fig. 4), 100 images per stack, spaced 10 µm (Table 2), over a total distance of 1000 µm.

Image stitching and analysis

For higher resolution whole-well imaging, the automated image overlap feature of the instrument was used at 5% overlap. In this way the entire well, or an area of interest in the image, was extended, image files were transferred to IN Cell Investigator 1.5 where automated image stitching generated a single image for each well.

Larvae Placement, Alignment and Pose in Well Bottoms

In the samples studied, 5-dpf zebrafish larvae, anesthetized with the agent used for well bottoms, Alignment of body cells relative to the plate sides is rather random but there is a tendency for attachment of the zebrafish head to well walls. The pose is mostly lateral, such that only one eye is imaged (Fig. 3). About 10% of larvae lie with their heads started partially toward a ventral pose, where 1½ to 2 eyes are visible in the images (Fig. 4).

Field of View (FOV) Size with IN Cell Analyzer 2000

The system's large CCD chip camera allows 96-well plates for FOV with 2048 pixels on each side. FOV dimensions depend on the choice of objective as shown in Table 1.

Need for Single-Shot Whole-Well Imaging of Zebrafish

In automated imaging, throughout requirements demand that the sample of interest be placed and imaged within a single field of view. In our experience, the best plate format for zebrafish larvae is the 96-well format because it can contain sufficient volume of aerated water (>150µl) to keep the fish viable. A single fish should be held in each well to avoid cross contamination effects.

Since placement of larvae in wells is rather random, one needs to image the whole so that the specimen is not missed. Figure 1 shows that under the 2X objective with FOV >7.6x7.6 mm, the IN Cell Analyzer 2000 large CCD camera readily covers the whole area of each well with a single shot (well diameter 6.4 mm). In this way, thousands of zebrafish can be automatically imaged per day.

Automated Magnification High Imaging of Zebrafish

In IN Cell Analyzer 2000 system allows two approaches for acquisition of higher magnification images to observe the cellular level or fixed details and examples. In the first approach, one might be interested in detection of neurotoxicity effects by measurements on fluorescently stained cells. In the first approach, one can run rapid automated whole-well imaging of the larvae in each well under a 2X objective, followed by computer controlled change of objective to 10X (Fig. 3, and IV stage movement and positioning away the over organ area of interest, e.g. as in Fig. 3). In the second approach, particularly useful if high-resolution coverage of the whole well area is needed (e.g. multiple organisms per well), one may choose the field-overlap feature of the system (at 5% overlap) and acquire the appropriate number of images. For example, under the 10X objective, one requires acquisition of 25 FOVs to cover the whole well (Table 1). Next, the set of images are sent to the IN Cell Investigator 1.5 application for seamless image stitching and analysis.

Automated Z-Stack Imaging of Zebrafish

Depending on the body area under imaging, the axial thickness of the specimen can vary between 0.2 to 1 mm. The z-stack imaging feature of IN Cell Analyzer 2000 can be used to extract 3-dimensional information from the larvae.

For normal imaging without Z-sectioning, one needs to consider the effect of the image blurring that occurs for thick specimens. If the sample is thinner and more transparent so that its high resolution images appear relatively sharp, as in Fig. 3. Head and mid body areas in the larvae and the brain are thinner and can be blurred by the choice of the objective in use (Fig. 3). This is because images appear in focus only for sections of the brain, but not that lie within the depth of field (DOF) of the objective.

In Table 2A/B show calculated values of DOF for the list of objectives (Fig. 2). The system's field overlap functionality + automated image stitching with IN Cell Investigator 1.5. It is possible to acquire (Fig. 4), 100 images per stack, spaced 10 µm (Table 2), over a total distance of 1000 µm for seamless image stitching and analysis.

Image stitching and analysis

For higher resolution whole-well imaging, the automated image overlap feature of the instrument was used at 5% overlap. In this way the entire well, or an area of interest in the image, was extended, image files were transferred to IN Cell Investigator 1.5 where automated image stitching generated a single image for each well.

Summary

IN Cell Analyzer 2000 was used for automated imaging of zebrafish in well plates, with fluorescence and transmitted light modalities.

- The system's large CCD camera under the 2X objective captures a field of view 7.6x7.6 mm. This enables rapid single-image whole-well imaging from 96-well plates.
- The 2X objective has a lateral resolution of 3.4 µm, for higher resolution whole-well imaging, the automated system overcome these difficulties: (1) A large-chip camera readily covers the whole area of each well with a single image capture.
- Images were acquired on IN Cell Analyzer 2000. Objectives were 2X NA, 40X1.3 NA, 100X.4X NA using transmitted light and fluorescent modalities with appropriate filter sets and exposure times. The large CCD cameras (Photometrics CoolSnap Ha) in conjunction with the 2X objective, enables a FOV of 7.6x7.6 mm, covering the whole well diameter (6.4 mm) of the 96-well plate with a single image capture.
- To capture the whole zebrafish in the 2-dimension, 2-stacks were acquired (Fig. 4), 100 images per stack, spaced 10 µm (Table 2), over a total distance of 1000 µm.
- For higher resolution whole-well imaging, the automated image overlap feature of the instrument was used at 5% overlap. In this way the entire well, or an area of interest in the image, was extended, image files were transferred to IN Cell Investigator 1.5 where automated image stitching generated a single image for each well.

Conclusions

IN Cell Analyzer 2000 enables fully automated and continuous high-content imaging of the zebrafish model organism.

References