

# Drug Discovery Using Zebrafish

a report by

**May-Su You<sup>1</sup>** and **Yun-Jin Jiang<sup>2</sup>**

1. Manager, Zebrafish Facility; 2. Senior Scientist and Principal Investigator, Institute of Molecular and Cell Biology, Proteos

## Advantages of Using Zebrafish

Embryonic transparency, a large number of offspring per mating and a short generation time enable genetic screens to be carried out in zebrafish efficiently, and possibly on a large scale. The species has been a popular research model for genetic and developmental biology study for the past 30 years. During the past decade, following several large-scale genetic screens and the nearly completed zebrafish whole genome sequencing, a large number of mutants are available and the genome and gene functions of the fish are better understood. Therefore, the zebrafish has become an established model for genetic research of vertebrate development and human diseases. Recently, it has crossed the border and metamorphosed into a promising tool for drug discovery.

## The Value of Forward and Reverse Genetic Screenings

Forward and reverse genetic screenings have been successfully applied in zebrafish. Forward genetic screens look for the genes that are responsible for specific biological functions, while reverse screens aim to discover the corresponding mutants with mutations on the gene of interest. The targeting induced local lesion in genomes (TILLING) screen developed by Wienholds et al. in 2001 identified recombination activating proteins (rag-1) mutants and is the most well-known reverse genetic technology to select mutants with mutations in the target genes. Over the past five years, mutants with mutations in over 40 different target genes have been selected. More than 2,000 mutants from forward genetic screens have been identified with specific defects in various developmental processes, and some have relevance to human diseases.

Most of these precious lines are kept in the Zebrafish International Resource Center (ZIRC) and are accessible to the entire research community. The zebrafish website, Zfin (<http://www.zfin.org>), provides all the basic methods, tools and information needed for zebrafish research. To study the gene expression and function, for instance, whole mount *in situ* hybridisation and antisense morpholino injection (to knockdown gene function) can be rapidly applied to the embryos according to the Web-available protocol. These resources enable the beginner to easily initiate a zebrafish research programme. With the help of the well-organised international networking system, more researchers are attracted to devote themselves into this field. The number of published zebrafish papers per year doubled between 2000 and 2006. The zebrafish, a small vertebrate species with a unique combination of forward and reverse genetics, has become an important and powerful model system in addition to the existing systems such as mice and rats.

## The Uniqueness of the Zebrafish

Drug discovery is a complex process, and normally involves expensive, laborious and time-consuming tests. In order to tackle these limitations, people tend to look for a 'shortcut' by which to reach their desired result.

The zebrafish is suitable for small-molecule screening due to its unique embryonic nature. Taking full advantage of the fecundity, optical transparency and cheapness of zebrafish embryos, different chemical assays can be performed in a relatively short period of time. For drug-toxicity screening the end-point is simply represented by the survival rate. To study the drug efficacy in embryos, visual assessment of the phenotype is essential, and sometimes immunohistochemistry or whole mount *in situ* hybridisation is used to evaluate the phenotypes. The transparent embryos and well-established protocols for analysing phenotypes can reduce the experimental time. Some studies prove that the selected small molecules (e.g. inhibitors) can affect zebrafish development in a dosage-dependent manner. In these studies, the small zebrafish embryos were placed in the multiwells plates, into which different concentrations of small molecules were placed robotically. The number of embryos used and the high-throughput experimental design contribute to statistical power and facilitate easier decision-making.

## Promising Studies

A successful small-molecule screening using zebrafish was performed by Peterson et al.<sup>1</sup> They used wild-type zebrafish embryos to screen 1,100 randomly-selected small molecules that were added to the aqueous embryo medium. After one, two or three days of incubation, the morphological defects were observed and scored. They concluded that about 2% of these compounds lead to embryonic death and about 1% induced specific defects. Although the molecular targets of these compounds are unknown, they still managed to identify a small compound called '31S4' that affects general pigment production. Its chemical structure resembles phenylthiourea (PTU), suggesting that it is a potential tyrosinase inhibitor. Wild-type, disease-relevant mutants and



May-Su You is Manager of the Zebrafish Facility at the Institute of Molecular and Cell Biology (IMCB) in Proteos. She joined the IMCB in 2003, with the remit of setting up the Zebrafish Facility. She has published several papers on topics such as food science, nutrition, zebrafish and cell differentiation. Dr You obtained her PhD at the Hohenheim University in Stuttgart.



Yun-Jin Jiang is a Senior Scientist and Principal Investigator at the Institute of Molecular and Cell Biology (IMCB) in Proteos, and Adjunct Assistant Professor in the Department of Biochemistry at the National University of Singapore and at the School of Biological Sciences of Nanyang Technological University. He has written more than 50 papers on topics including Notch signalling, somitogenesis, neurogenesis and zebrafish development. Dr Jiang carried out his post-doctoral training at the Imperial Cancer Research Fund in London after receiving his PhD at the University of Tübingen, Baden-Württemberg.

transgenic lines are particularly useful for drug-target screening. For example, the gridlock mutants that have a hypomorphic mutation in the *hey2* gene have been used for suppressor screening. The mutant embryos develop a dysmorphogenesis of the dorsal aorta that prevents blood circulating to the trunk and tail, although perfusion of the head remains normal. Peterson and colleagues used gridlock mutant embryos to select two structurally related compounds called GS4012 and GS3999 from 5,000 small molecules. They concluded that these two molecules have to completely suppress the gridlock mutant phenotype, leading to the normal development of the vasculature network. GS4012 and GS3999 represent a novel class of compounds that were not previously known to influence vasculogenesis or angiogenesis. Therefore, this finding may point to a new direction for studying vasculogenesis/angiogenesis. These two studies proved that the zebrafish is an accurate and convincing model for small-molecule screening.

### Adult Zebrafish

In addition to embryonic and juvenile stages, the adult zebrafish have also been widely used for chemical screening. Adult zebrafish require more space and a special design to perform the experiments compared with embryonic zebrafish, which are less than 1mm in diameter and can be easily arrayed in multiwell plates. Although it may give rise to some logistical limitations, studies concerning regeneration and behaviour are still able to be carried out with great success. Berghmans et al.<sup>8</sup> developed an automated tracking system to measure the movement of zebrafish induced by exposure to a pro-convulsant, pentylene tetrazole (ptz), and then used it to screen and identify potential antiepileptic drugs (AED). Their results suggest zebrafish can be used for the primary screen to pick up potential AEDs. The toxicity test using adult zebrafish has also become a standard protocol for screening eco-toxicological substances for the Organisation for Economic Co-operation and Development (OECD).

Increasingly, more research is being carried out using zebrafish for human disease models, which is related to drug discovery. Graham et al. summarised studies in congenital and hereditary diseases, carcinogenesis, infection, inflammation and wound healing, immunological diseases, metabolic diseases, nutritional diseases, psychological and behavioural abnormalities and toxicity and poisoning. Cancer models in zebrafish will require creating fish with alterations of specific cancer genes, and forward and reverse genetic strategies and generations of transgenic lines are available to manipulate zebrafish genes. The studies have shown that there are excellent models of leukaemia and melanoma in the zebrafish.

### Whole-organism Models

Traditionally, drug screening relies on cell culture or protein-binding assays, which is an *in vitro* and cellular level test. The zebrafish provides an excellent whole-organism model for forward drug screening. The screening outcome involves not only the cellular but biochemical, physiological and even pathological analysis of an entire organism. The whole-organism approach allows biological questions to be dressed that simply cannot be addressed *in vitro*. Zon and colleagues concluded

**Table 1: The Zebrafish Model for Drug Screening**

Advantages	<ul style="list-style-type: none"> <li>• Small embryos are transparent, large number of offspring, short-generation time</li> <li>• Inexpensive, easy handling, large-scale screen amenable</li> <li>• A vertebrate, <i>in vivo</i> system with combination of forward and reverse genetics</li> <li>• Phenotype-based screening can be performed in wild-type, mutant and promoter-driven reporter transgenic embryos</li> <li>• The screening is robust and high throughput</li> <li>• Relevance to human diseases, high degree of similarity to humans in drug response</li> </ul>
Disadvantages	<ul style="list-style-type: none"> <li>• Knock-down target genes can only rely on expensive and labour-intensive morpholino injection</li> <li>• No knock-down technology available</li> </ul>

that some results obtained by whole-organism screen might be more relevant than those obtained using the *in vitro* and cell-culture-based screen. Many of the small molecules identified on their zebrafish cell-cycle screen were not identified in analogous *in vitro* cell-line screens. The reason is that the physiologically relevant organismal context is required for the action mechanisms of the compounds that they have discovered. This phenomenon is most likely applicable to all drug screens.

### Potential Drawbacks?

Although the zebrafish offers an inexpensive and uncomplicated way for testing the efficacy and safety of small compounds in a high-throughput manner, some significant questions remain unclear (see *Table 1*). How closely does the zebrafish model resemble human disease processes? How reliably can zebrafish results be translated to humans? How often will small molecules discovered in zebrafish screens have similar effects in humans? These unsolved concerns will compromise the idealistic of zebrafish model, even though a high degree of similarity in drug response has been proved between zebrafish and humans. Consequently, the sophisticated mammalian assay remains indispensable. However, the phenotype-based discovery of novel drug leads and preliminary assessments of compounds in zebrafish is especially efficient and powerful. The US Food and Drug Administration (FDA) reported that an estimated 10% in improvement in predicting failures before clinical trials would save US\$100 million per drug in development. Using zebrafish to perform a pre-screen on new compounds for disease treatment is definitely useful and money-saving. The zebrafish genes have more than 75% similarity to human genes, and its genome is 1.7x10<sup>9</sup> base pairs in size, which is just more than half of humans and mice. It indicates the function of individual genes might be less complex in fish than that of human, and may be easier to study.

### Conclusion

The zebrafish is no doubt a powerful model organism with a combination of forward and reverse genetics, low cost, amenable high-throughput and rapid *in vivo* analysis. With these unique features, it can be expected that the zebrafish will become more frequently used for drug discovery. ■

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