# Researchers Find Their Nemo

**With its genome almost fully sequenced, the zebrafish has gone from bit player to rising star among model organisms. Its lower maintenance costs and the ease of tissue imaging in live zebrafish are among the benefits that have cast this organism as an emerging player in disease research and drug screening.**

The zebrafish is a small, active fish native to India. The hardiness of this striped fish makes it popular in home aquaria; aficionados seeking a genetically modified pet can even buy transgenic GloFish. But the zebrafish, *Danio rerio*, is also the bright star among model organisms for biologists. Within the next year, the genome sequence of the zebrafish is expected to leap to a level of completeness for a vertebrate surpassed only by the human and the mouse genomes (http://www.ensembl.org/Danio_rerio). This will hoist it toward the A-list of model organisms. Already, the fish shows potential in studies of the basic biology of human diseases, including cancer, and in high-throughput screens for drug discovery.

The zebrafish emerged as a potential model three decades ago and was put to work in the study of early development. Advances in cloning, mutagenesis, and transgenesis in zebrafish allowed invertebrate-style experiments on a vertebrate. Large genetic screens for randomly generated mutants began in Europe and the US in the early 1990s, on a scale usually associated with cultured cells or invertebrates. Basic numbers tell the story: one hundred fish fit in a 20 l tank and each female produces over two hundred embryos a week. Most organs are recognizable 24 hr after fertilization. Within 5 days of development, larvae are mobile and foraging.

Funders are keen on zebrafish as a model but could become keener when the complete genome sequence becomes available. "Within the NIH, there has been a steady trans-institute support for expanding the utility of zebrafish as a model organism," says Shawn Burgess of the NIH's Developmental Genomics Section in Bethesda, Maryland. "I think outside the NIH, because fish physiology is somewhat distant from its mammalian counterparts, there is still a lot of skepticism about whether zebrafish will have as central a role in human biomedical research as say mouse research does. Some skepticism is justified, but perhaps not enough emphasis is placed on how much cheaper research in zebrafish can be compared to mice, and how much can be learned from the differences as well as the similarities," says Burgess.

"The zebrafish is underutilized in modeling in almost all areas of human disease," according to Tim Chico of the UK's MRC Centre for Developmental and Biomedical Genetics, who blames our mammal supremacist mindset. But fundamental cellular processes like development of the vascular system, which he studies, are conserved across vertebrate species, and zebrafish bring a good haul of benefits to researchers. Chico points out that a fish costs 7 pence per week to maintain, whereas a mouse costs £4. Also, the zebrafish genome sequencing project begun in 2001 by the UK's Wellcome Trust Sanger Institute has put reverse genetics on the menu. Key techniques in use include TILLING (targeting local lesions in genomes), gene overexpression, knockdown models, and use of the yeast Gal4-UAS marker system.

Derek Stemple of the Sanger Institute says the eighth and final version of the zebrafish genome sequence at 6.5–7× coverage will be available next year. "It is 85 to 90% complete. The trouble is that there is so much repetitive DNA, just like in humans, and sometimes it is difficult to know physically where that belongs in a genome." Burgess adds: "The eighth version will be a 'finished' version like human was in 2003, meaning that the error rate is about 1 in 50,000 bases and all non-repetitive DNA is sequenced. There should be no unincorporated sequence, which has not been true to date." Researchers believe the complexity and polymorphic nature of the starting material—from multiple fish—caused initial delays and some express frustration at the speed of the sequencing effort. "The availability of the genome is transformative and there has been some consternation at the pace it has become available. But that just speaks to how hungry everyone is to have access," according to Randall Peterson of Harvard Medical School in Boston.

"Sanger is spending more resources to help the zebrafish genome catch up with human and mouse," says neuroscientist Monte Westerfield of the University of Oregon. "There is an ongoing effort by the Sanger VEGA team to annotate individual genes and transcripts in collaboration with us in Oregon. And then ZFIN incorporates data from the literature. That is significantly improving the genome sequence and assembly. And that aids in all sorts of experiments." ZFIN is a centralized repository for all things "zebbie" and a first port of call for anyone interested in starting research on zebrafish. It has almost 5000 members.

Embryologist Marnie Halpern of the Carnegie Institute of Washington stresses the time the Sanger sequence saves, pointing out that her lab can confirm the identity of a new mutation affecting the brain in a matter of months instead of years. However, there are still some regions of the genome her lab is working on where the sequence is poor, causing trouble in cloning regulatory regions of a gene of interest, for example.

"Recently there has been a second wave of researchers looking for creative new ways to exploit zebrafish," says Burgess. Tissue regeneration is one area, with studies revealing that fish can regenerate heart, nervous tissue, retina, and fins. "We are specifically looking at how adult hearing regenerates in zebrafish after exposure to intense sound or ototoxic drugs. My lab uses a mixture of

genetics and genomics approaches to define the gene network that activates during hearing regeneration," says Burgess. Hearing loss is permanent in mammals, but all other vertebrates, including birds, are able to repair hearing damage, he notes; "Therefore I believe that the loss of regenerative ability in mammals must be a relatively recent occurrence and perhaps activation of a very small set of genes could actually cause a 'reactivation' of the regeneration process. Support for this idea comes from the recent discovery that it takes as few as four genes to convert somatic cells to embryonic stem cells."

Validating new drug targets remains a bottleneck in drug discovery, and the fish can help. Most small molecule discovery efforts in industry and academia rely on high-throughput screening and simplified in vitro reactions or cell-based assays, but Peterson is testing those same approaches on living zebrafish. He has shown that drugs known for their effects in humans have similar effects in zebrafish larvae. In 2004, Peterson's group screened 5000 small molecules and discovered two that overcame a blood circulation problem in a mutant called *gridlock*. Large numbers of fish can be tested simultaneously because early zebrafish embryos measure less than a millimeter and several fit in each well of a 96-well plate. Peterson notes: "In some assays, we screen as many as 5000 animals per day." There are also benefits from an ethical standpoint, as work can be done on embryos rather than adults and does not require dissection, destruction, or invasive imaging of the fish.

Peterson uses a robotic microscope to record responses of zebrafish embryos or larvae to various stimuli. Adding compounds to each well, he can assess the way they affect the animal's baseline behavior. His team is using this approach to identify new stimulants, sedatives, and other behavior-modifying compounds that could be used for treating nervous system disorders such as Alzheimer's disease. In another screen, a molecule was discovered that blocks the progression of bone overgrowth disease and is advancing toward the clinic. "We designed a zebrafish screen, screened through thousands of molecules and identified an intriguing molecule. We then used chemistry to optimize it, moved it into mouse models and showed it was effective," says Peterson.

Uwe Strähle of the University of Heidelberg recalls how he was warned off the zebrafish with: "Don't do it. The mouse is much, much better." He feels his decision has been amply justified, pointing to the almost 5000 mutants and transgenic lines for fish, including those with altered cholesterol, metabolism, heart physiology, or cancer. And he can study systems biology in a live and intact organism. "There is an initiative in early planning stages where people in the community are exploring whether or not a large scale project to knock out every gene in the genome is feasible and valuable," says Peterson. "It is clear to me that this isn't science fiction and we could, in a short period of time, be able to deconstruct every gene in the zebrafish genome." Stemple sees the fish racing ahead of the mouse: we will be able to identify phenotypes for all protein-encoding genes well before it is done in the mouse, he says (although currently the mouse is ahead of the game with about 5000 genes deleted, versus 700 deleted in the zebrafish).

The NIH was an early starter in supporting zebrafish research. "There was a lot of excitement over the past years at NIH; it seemed like every institute wanted to expand their portfolio to include zebrafish research," notes Westerfield. "To a great extent, I think that is still true." Moreover, the community has expanded dramatically in just 5 years, with capacity numbers at last year's international zebrafish conference in Madison, Wisconsin (http://www.union.wisc.edu/zebrafish/). The big growth area has been clinicians wanting to understand the basic biology of disease genes. The NIH has supported the development of tools that would "help promote zebrafish as an animal model for studying development and diseases," says Lorette Javois at the NIH's National Institute for Child Health & Human Development and a member of the Trans-NIH Zebrafish Coordinating Committee. "A lot of times those types of applications that aren't strongly hypothesis driven don't fare well in the standard NIH peer review systems," she adds. "We have two different solicitations for applications that are currently active. One is soliciting research tools and techniques generally that would enhance zebrafish research; the other is soliciting more specifically genetic screens that would enhance zebrafish research." For fiscal year 2009, NIH funding for research tools and genetic screens totaled almost $11 million spread over 33 grants.

Meanwhile, the European Commission (EC) launched a substantial zebrafish project in 2004 (ZF-MODELS), with over 20 research teams receiving €12 million (http://www.zf-models.org/). "This was mainly a large scale mutagenesis program in zebrafish to identify genes linked with development, health and disease," says Jacques Remacle, a scientific officer with the EC. Another €11 million is now being negotiated for a followup project, ZF-HEALTH. "We want to build on the mutagenesis and understand the roles of genes in health and disease," explains Remacle. Other large funders include the Dutch government, providing €14 million spread over several years through its Smart Mix program, and the Wellcome Trust, which is funding large-scale TILLING at Sanger as well as most of the zebrafish genome sequencing project.

Physician scientist Leonard Zon, a Howard Hughes investigator at Harvard, is renowned for his zebrafish models of human disease. He began by creating zebrafish mutants that had problems making blood cells and worked on the first zebrafish mutant that predicted a new human disease gene. A recent chemical genetics screen using zebrafish revealed that prostaglandins stimulate blood stem cell production; prostaglandin E2 in particular boosted the number of blood stem cells in zebrafish and mice. Zon's lab is now treating human cord blood with prostaglandin E2 to try to increase stem cell numbers prior to transplant. "Recently we studied a clinical trial for patients with leukemia who are getting a cord blood transplant," he explains. "And we are treating the cord blood with the chemical that we found in zebrafish. So it is an interesting story on how zebrafish could facilitate us in finding new therapies for disease."

Zon also has developed a melanoma model in zebrafish and recently discovered a gene that when mutated accel-

erates cancer formation. The gene encodes an enzyme, he says, "so in theory, if we could make a drug to that particular gene product, then you would have a new therapy." The advantage of zebrafish cancer models is that they absorb small molecules directly from the water, thereby enabling rapid screening for anticancer agents in vivo (Figure 1). And it is in cancer that the zebrafish and its newly sequenced genome may gain its stripes. "Early tumors are very difficult to detect and in most organisms you can't follow them live," explains Ralf Dahm, now at the Spanish National Cancer Research Centre in Madrid, who managed the ZF-MODELS project in Europe. "If you transplant a human tumor into the fish, however, you can visualize the very early steps in cancer at high resolution." You can see how the young tumor recruits blood vessels and interacts with its microenvironment, and how the immune system reacts, he notes. A high-quality sequence will be critical, and Dahm says even the first version released by Sanger in 2002 helped his research.

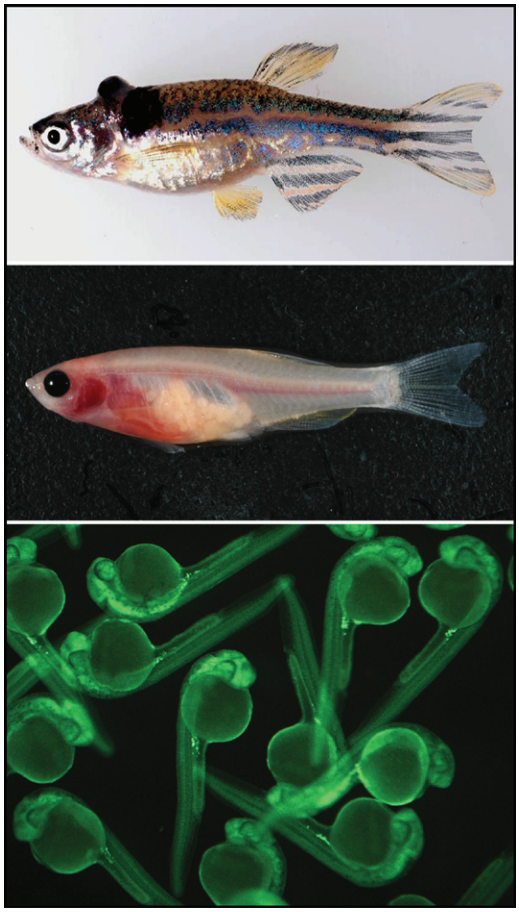

**Figure 1. The Zebrafish in Close Up**

(Top) *BRAF*$^{V600E}$ is the most common oncogene in human melanomas. Zebrafish carrying mutant *p53* and expressing a *BRAF*$^{V600E}$ transgene in melanophore pigment cells develop melanomas. (Middle) In the zebrafish *casper* mutant, development of pigment cells is disrupted. These optically clear fish are valuable for imaging studies as their internal organs are visible. (Bottom) Zebrafish embryos expressing a fluorescent protein in their primordial germ cells. Photos courtesy of C. Ceol (top), R.M. White (middle), and P. Schlueter and R. Peterson (bottom).

Clearly, the fish is not just a poor man's mouse. Cancer biologist Steven Leach at Johns Hopkins in Baltimore, Maryland, relies on zebrafish models in addition to mice. His institute has sequenced the exomes of 24 different human pancreatic cancers, but the challenge now is to annotate this vast catch and determine which mutations contribute to cancer pathogenesis. "Traditionally that would have been done in the mouse, but with this torrent of new data we really have a dramatic bottleneck in terms of evaluating the significance of these mutations," says Leach. "Based on the ease and relative low cost of establishing transgenic zebrafish, fish cancer models provide significantly higher throughput in the functional evaluation of candidate oncogenes and tumor suppressor genes."

Over the last decade it has become apparent that all vertebrates have the same sets of genes, and that biochemical and developmental pathways are highly conserved. Javois at the NIH is clear about the zebrafish sequence's potential here: "It allows people to go between animal model systems and look for homologous genes, so if you are working on some other animal model on a particular gene or pathway, you can look for the equivalent or vice versa." Almost all zebrafish genes have been found to have equivalent human genes (orthologs). "When I started with this model, a large number of people thought I was crazy," notes Zon. A few said we don't even know whether fish form blood the same way as humans, he recalls. Zon cloned the genes for all of his blood mutants, with the help of the Sanger sequence, and found human orthologs for all of them. With 300 million years of evolution between fish and humans, he discovered that the same carrier protein still brings iron from mother or yolk to embryo, for example. "With a full set of genes from the Sanger it is possible to create streams of fish for quantitative trait analysis. Because of the large number of fish you could get, it could really lead to an unprecedented jump in science," says Zon.

Stemple, who studies muscular dystrophies, stresses the advantages of the speed and scale of experiments possible with the fish: "In zebrafish you can already see the effects of a mutation two days after fertilization, so that you can usually score a muscular dystrophy in zebrafish very early in development. That is really useful, because you can get rapid development, and you can look at literally hundreds of fish and hundreds of genes very quickly. That is why people are turning to zebrafish as a model organism."

Researchers increasingly work on adult fish, and Dahm is enthusiastic about the possibilities of monitoring cells in the almost transparent *casper* mutant (Figure 1). Moreover, techniques such as "brainbow," which allows simultaneous labeling of many cells with individual "colors," permit analyses of complex neural networks in vivo, he notes. "Using this approach to study cell lineages and cell diversity in cancer, including testing the importance of the recently identified cancer stem cells to tumor development, clearly is a very exciting potential application of brainbow." Another recent development for targeting specific genes is zinc finger nuclease technology. "This is a really potentially transformative technology that is going to rapidly accelerate reverse genetic approaches in zebrafish, such as are commonly applied in mice and other model organisms. It obviously relies on very precise sequencing of the zebrafish genome," notes Leach. But despite all of this progress, as Leach emphasizes, "the field still carries the burden of demonstrating that zebrafish models of cancer truly

have utility beyond more traditional mouse models when it comes to impacting on patients with the disease."

Like all models, the zebrafish is not perfect. The tools and techniques to manipulate zebrafish genetically are not yet as sophisticated as they are for mice, notes Dahm. "There are, for instance, fewer promoters for tissue-specific expression, and targeted mutagenesis is only now becoming easier with the new zinc finger nucleases," he says. Anything to do with mother-embryo interactions is difficult to study, he adds, because fertilization and embryonic development are external. Moreover, zebrafish are not mammals. "So there are some human organs, processes, and physiology that you can't study in zebrafish, such as lung, mammary gland, and uterus," notes Westerfield. The size of the fish is a big advantage for microscopists but can also be a drawback when large amounts of tissue are required. Even adults are only about 2 inches long. "Taking blood from one of the tiny zebrafish larvae or having to dissect their livers for proteomics analyses, although possible, is no trivial task," says Dahm. And though many researchers think antisense oligonucleotides called morpholinos are the "silver bullet" for knocking down genes and studying their function, says Westerfield: "Nothing beats a heritable mutation, however, and results from morpholino studies should be confirmed by examining protein expression and, ultimately, mutants. Also, for translational work, therapeutics developed in zebrafish will typically need to be tested in mammals before moving to humans."

Despite the drawbacks, charitable trusts and foundations are recognizing the zebrafish's potential as a model organism. For example, Chico has received funding from the British Heart Foundation, and his institute has just received a £1.5 million Medical Research Council grant to set up a screening facility for small molecule drugs. He is also using the fish to study arterial occlusions, which are difficult to create in other model organisms. He can now observe developments in live animals and can watch the effects of temporarily knocking out genes using morpholinos.

And the fish has returned home, in a manner of speaking. The Institute of Genomics and Integrative Biology in Delhi, India, has collected wild specimens of *Danio rerio* from rivers and has begun sequencing their genomes using next generation sequencing machines. The Indian group are using the fish as a model to understand within-species genetic variation and to complement the Sanger sequence. Some researchers see this as providing extra impetus for Sanger to complete its efforts.

The fish model has migrated beyond Europe and the US, with significant zebrafish research groups now in Australia, New Zealand, China, and Singapore. According to Zon: "There's lots of opportunities for new investigators coming into the field and most institutions want a zebrafish researcher in their midst." Though there is no such thing as a perfect model system, notes Burgess, "the zebrafish is valuable because it strikes a compromise balance between the large number of animals that can be examined and its relatedness to humans." The zebrafish is underutilized in terms of its possibilities, comments Westerfield, who is upbeat about its future: "There's plenty of room for folks who have got crazy new ideas."

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