

# Using zebrafish in human disease research: some advantages, disadvantages and ethical considerations

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## Introduction

The zebrafish, *Danio rerio*, is a small teleost fish originating from the rivers of northern and eastern India (Engeszer et al. 2007). It possesses a number of advantageous physical characteristics that have resulted in its common use today as a laboratory model. These include its relatively small adult size of 2–3 cm, its rapid generation time of approximately 3 months, its ability to produce large clutches of externally fertilised eggs and the ease with which this fish can be kept and bred in aquaria.

The development of zebrafish as a genetic model began with the work of George Streisinger in the 1960s (reviewed in Grunwald & Eisen 2002). Streisinger developed a number of techniques to manipulate the ploidy of zebrafish eggs and zygotes and was ultimately able to use these techniques to produce homozygous, genetically identical individuals (clones) (Streisinger et al. 1981). Streisinger and his coworkers also established techniques for forward genetic screening in zebrafish (Walker & Streisinger 1983; Grunwald et al. 1988).

The transparent embryos of zebrafish develop synchronously and extremely rapidly. Published studies examining the development of zebrafish embryos can be found as early as 1952 (Battle & Hisaoka 1952). Zebrafish embryo development has even been examined in orbit aboard the Soyuz 19 (and other)

spacecraft (Dubinin et al. 1977) where weightlessness was seen to have no deleterious effects.

Zebrafish embryos can hatch after 48 hours of development. At 24 hours their overall body plan has been laid out and the primordia of many organ systems are in place (Thisse & Zon 2002). Thus, embryos of the vertebrate zebrafish develop at a rate similar to the rapidly developing embryos of the insect model organism, *Drosophila melanogaster*. Like *Drosophila* (and in contrast to the mouse) the embryos of zebrafish develop predominantly by cell division without extensive growth. This means that early embryos are large enough to manipulate easily (e.g., inject with substances to regulate gene expression) but they remain small enough during development to allow the use of particular analytical techniques. For example, the technique of “whole mount *in situ* transcript hybridisation”, in which cells in whole embryos are stained if they transcribe a particular gene (Jowett & Lettice 1994), can be used throughout zebrafish embryogenesis but only up until day 11 of the 20 days of mouse embryogenesis. The external fertilisation of zebrafish eggs means that it is possible to observe the normal or abnormal development of living zebrafish embryos. This is not generally possible with mice where observation of embryo development usually requires sacrifice of the mother and death of the embryo. Importantly, mouse embryos that die during development are rapidly broken down and reabsorbed whereas dead zebrafish embryos tend to persist for a while in their final state.

With Streisinger’s work as a foundation, Christiane Nüsslein-Volhard and Wolfgang Driever conducted independent, large-scale forward genetic screens for mutations affecting embryo development. The descriptions of the mutant phenotypes they

discovered were published in a single issue of the journal *Development* in 1986 (volume 123). While mutations affecting the development of vertebrates had previously been identified (e.g., in mice early in the 20<sup>th</sup> Century in inbred strains and in radiation mutagenesis screens in the 1950s, Lyon 2002) no large-scale screens had previously been attempted. The results of the large scale zebrafish mutation screens led to the establishment of a number of models of human genetic disease (reviewed by Lieschke & Currie 2007).

## Advantages of work with zebrafish

The current and growing popularity of using zebrafish for research into human disease can be attributed to its favourable physical characteristics, ready experimental manipulation and the extensive knowledge base on this organism that now exists. The low relative cost of maintaining a zebrafish facility compared to mice is also important.

## Zebrafish genetics

Zebrafish are vertebrates and so share a closer structural and physiological relationship to humans than invertebrate models. The zebrafish and human lineages diverged approximately 450 million years ago (Kumar & Hedges 1998) and their genomes are of similar size. Orthologues of most human genes can be found in zebrafish and these commonly show similar patterns of expression. Orthologous proteins are commonly approximately 70% identical in terms of their amino acid residue sequence. Interestingly, a widespread gene duplication event appears to have occurred in the zebrafish lineage such that duplicate orthologues of some zebrafish genes exist (reviewed by Postlethwait 2007). Frequently, the functions of a single human gene are found to be divided between the two zebrafish orthologues (i.e. “subfunctionalisation” has occurred, Lynch & Force 2000). This can be advantageous when loss of function of only one of the two zebrafish genes produces a simpler phenotype that is more amenable to analysis than the human disease phenotype. If needed, it is also possible to abolish simultaneously the function of both zebrafish genes, at least during embryo development, by injection of antisense morpholino oligonucleotides (see below).

The ability to perform large-scale random mutagenesis screens in zebrafish allows objective investigation of control biological processes that are not hamstrung by a researcher’s preconceptions of how a process might function. When zebrafish models of human genetic diseases are available the ability to screen for modifier mutations (that suppress or enhance the disease phenotype) using zebrafish allows dissection of the biological processes underlying the disease.

## Manipulation of gene activity

The technology for manipulation of zebrafish gene activity is less sophisticated than that available for the mouse but, nevertheless, quite extensive. While targeted mutagenesis (“knock-out”) or gene replacement (“knock-in”) by homologous recombination is not practical, very high rates of random mutagenesis can be achieved using chemical mutagens such as N-ethyl-N-nitrosourea (ENU) (Grunwald & Streisinger 1992). It is possible to screen for mutations in any particular gene when these are in a heterozygous state in the progeny of fish exposed to ENU. Isolated mutations can then be bred to homozygosity. This process, known as TILLING (Targeted Induced Local Lesions in Genomes) is now in common use in zebrafish (Wienholds et al. 2003). Recently, a method of inducing small deletions in targeted genes has been developed that relies on creation of proteins that can bind specifically to the desired gene and then cause cleavage of the DNA (Doyon et al. 2008; Meng et al. 2008).

## Transgenics

Considerable work has been done to develop transgenic technologies for the zebrafish (reviewed by Amsterdam & Becker 2005). In particular, the transposons Sleeping Beauty (reconstructed from genomic “fossil” evidence, Davidson et al. 2003) and Tol2 from the fish *Oryzias latipes* (common name medaka, Kawakami 2007) can be used both as vectors for transgenesis as well as for insertional mutagenesis. Transgenesis is also possible either through injection into fertilised oocytes of naked DNA fragments or DNA coinjected with I-SceI endonuclease (Grabher et al. 2004). Conditional expression of genes is possible either through use of heat shock promoters (Shoji & Sato-Maeda 2008) or the Gal4/UAS system (Asakawa & Kawakami 2008). In general, reliable expression of

transgenes is obtained in zebrafish only when fish, not mammalian, promoters are used.

Both transient and stable transgenesis of zebrafish can be used to create models of human disease states. In particular, this has allowed construction of zebrafish cancer models (e.g., Onnebo et al. 2005; Chen et al. 2007). Human cancer cells have been successfully grafted into zebrafish embryos where their ability to reorganise surrounding tissues and their sensitivity to anti-cancer treatments can be assessed (Geiger et al. 2008; Stoletov & Klemke 2008). The recent generation of mutant zebrafish that are completely transparent (i.e. do not develop pigment cells) will assist analysis of tumour formation and treatment (White et al. 2008).

### Ease of drug treatment

Much of the enthusiasm for modelling of human disease in zebrafish is due to the ease of exposure of embryos and fish to drugs to produce or alleviate particular phenotypes and the ability to do this on a large scale (also known as “chemical genetics”; for a review see Berger & Currie 2007). Zebrafish embryos develop externally to the mother and can be exposed to drugs by placing these in the embryo support medium. Unlike in placental mammals, the drugs are not subject to metabolism by the mother before reaching the developing embryo. Also, since zebrafish embryos are available in large numbers, it is possible to array them in microtitre dishes for exposure to different drugs, doses and drug combinations. A converse approach can also be used to understand the biology of drug response – genetic screens can be conducted to find mutants that are resistant or particularly sensitive to the effects of a particular drug (Baraban et al. 2007).

An interesting disease model, that combines transgenic manipulation with the ease of exposure of zebrafish embryos to drugs, is an attempt to model the effects on the pancreas of the loss of  $\beta$  cells that occurs in type I diabetes. Expression of the *nfsB* gene (encoding a nitroreductase) from *Escherichia coli* was driven in the beta-islet cells of the developing zebrafish pancreas using the promoter of the zebrafish insulin gene. Exposure of transgenic embryos to the pro-toxin metronidazole then caused formation of a cytotoxin in the beta-islet cells and the ablation of, specifically, these cells in the embryo. The *nfsB* gene

was fused to the fluorescent protein coding gene *mCherry* to create a fusion protein that marked  $\beta$  cells before ablation without affecting nitroreductase activity (Pisharath et al. 2007).

### Morpholino antisense oligonucleotides

Morpholino oligonucleotides are polymers of nucleic acid bases attached to morpholine rings (rather than deoxyribose rings) linked through phosphorodiamidate groups (Summerton & Weller 1997). They are highly resistant to degradation by cellular nucleases. Antisense morpholino oligonucleotides can be designed that can bind to complementary sequences in gene transcripts before splicing (to inhibit splicing) or in mRNA (to inhibit translation). When injected into newly fertilised zebrafish eggs they can effectively and specifically suppress mRNA translation for at least two days of development (i.e. until hatching). Therefore they are very useful for analysing the function of identified genes in embryo development.

Many genes involved in human disease have important functions during embryo development. Using morpholinos the effects of loss-of-function of these genes can be analysed in embryos and this can illuminate their normal functions. An example (mostly unpublished) from our own work is analysis of the function of the gene *PSEN2* that is a locus for mutations causing inherited early onset Alzheimer's disease in humans. A knockout of this gene in mice causes no obvious phenotype (Steiner et al. 1999). However, inhibition of translation of transcripts from the orthologous gene in zebrafish, *psen2*, causes phenotypes reminiscent of loss of Notch receptor signalling similar to those caused by loss of the related gene *psen1* (Nornes et al. 2008), namely reduced pigmentation and expansion of brain ventricles. Interestingly, we have also found that loss of *psen2* activity changes the number of a particular cell type in the developing embryonic spinal cord, the dorsal longitudinal ascending interneuron. The number of these neurons appears insensitive to changes in *psen1* activity. This gives us a unique bioassay for, specifically, *psen2* activity and this bioassay has been useful for demonstrating the dominant-negative effects of particular aberrant forms of Psen1 protein (Nornes et al. 2008).

There are distinct advantages of the ability to reduce gene expression through morpholino oligonucleotide injection. First, the cost relative to production of a

gene “knockout” in mice is very low. Second, it is easy to analyse the effects of loss of activity from a combination of genes by simultaneously injecting multiple morpholinos. Third, by injecting different amounts of morpholino, it is easy to assess the phenotypic effects of partial loss of gene activity. For these reasons, researchers sometimes examine whether the effects of loss of a particular gene function in zebrafish are as expected/interesting before proceeding with the time and expense of knocking out the gene’s function in mice.

### **Fertilised zebrafish eggs as test cells in molecular biological analysis**

Zebrafish embryos are outstanding in their utility for analysis of the genetic control of development. However, their macroscopic size (~0.5 mm diameter), and ease of injection also make them an exceptional system for manipulation and analysis of cellular molecular biology. Most analysis of this type is conducted using cultured cells, often transfected with various transgenes to alter gene activity. However, these cells often have highly abnormal patterns of gene expression due to their highly unusual environment and/or genetic changes that may be necessary to allow their continuous growth in culture (e.g., see Izadpanah et al. 2008). Introduction of a transgene often involves the addition of very large numbers of gene copies into the cell. All these factors can give experimental results that do not reflect the normal cellular action of genes and proteins. A fertilised zebrafish egg is a single cell that can be injected with varying concentrations and combinations of morpholino oligonucleotides and/or mRNAs to suppress or alter endogenous gene activity or drive ectopic gene expression. The subtlety of the manipulations and the physiological relevance of examining endogenous levels of gene expression mean that information on gene function obtained from manipulation of zebrafish embryo may more closely represent reality. Also, after morpholino or mRNA injection, the subsequent development of the embryo can provide a phenotypical readout of gene activity.

An example of the utility of using fertilised zebrafish eggs to analyse gene activity is our discovery that subtle changes in transcript splicing in both *psen1* and *psen2* can have potent dominant negative effects—something not previously observed for these genes despite many years of intensive research using, primarily, cultured cells (Nornes et al. 2008). The fact

that dominant negative effects can produce similar, but more extreme, phenotypes than simple blockage of *Psen1* translation led us to suspect that dominant negative forms of *Psen1* protein were also suppressing *Psen2* protein function. We found support for this hypothesis using our assay for *Psen2* activity (see above). This discovery was only possible due to the ability to subtly manipulate the splicing of endogenous *psen* gene transcripts and the subsequent developmental readout from zebrafish embryos. These observations would have been very difficult to make in cultured cells. Also, the dominant lethal nature of these changes in *psen* gene activity would preclude their discovery in mutation screens and would require complex inducible forms of transgene expression for observation in mice.

### **Disadvantages of the zebrafish model**

The advantages of zebrafish are many and increasing as the technology for manipulation of this model develops. However, for some biological research questions, there are particular characteristics of these embryos and fish that may be problematic. The most obvious shortcoming of zebrafish, particularly for questions of human relevance (e.g., disease modelling) is that it is not a mammal. Instead, it is poikilothermic and developing embryos lack a placenta. This means that some drugs may be metabolised in a different manner or, at least, at a different rate compared to mammals and this can alter their function. Zebrafish embryos exposed to drugs in their growth medium absorb these directly without modification by the mother’s or placenta’s metabolism. One example of this is the utility of the anaesthetic tricaine methanesulfonate for work with zebrafish compared to its lack of effectiveness in mammals where it is broken down too rapidly by their homeothermic metabolism (Wayson et al. 1976).

Gender in zebrafish also differs from mammals in that it does not appear to be genetically determined. This is not to say that zebrafish development cannot be altered by hormones, e.g., oestrogens, also active in mammalian sex determination. Indeed, zebrafish may be useful in environmental toxicological studies examining the effects of environmental levels of steroid hormone analogues on embryo development (Holbeck et al. 2006).

## Ethical considerations

Finally, we should consider the ethical aspects of work with zebrafish. As our appreciation of the complex behaviours and modes of consciousness of animals grows, so does our apprehension that our experimentation may inflict suffering. It is vital to consider the possible experience/consciousness of pain of animal models and to ensure that the value of the possible outcomes of the experimentation justifies the possible harm.

There is some debate over whether fish can in fact experience pain. Experiments conducted by Sneddon, Braithwaite and Gentle at the Roslin Institute indicated that fish possess neurons that respond appropriately to stimuli commonly known to cause pain. They also behave in unusual manners when subjected to what should be painful stimuli (Sneddon et al. 2003).

Sneddon's conclusions have been strongly criticised by Rose (Rose 2007) who distinguishes between pain and nociception as conscious and unconscious phenomena respectively. Rose asserts that fish lack the neural anatomy to experience pain and that quite complex responses to painful stimuli are possible in an unconscious state and he points to some inconsistencies in Sneddon et al.'s interpretation of their observations.

While the ability to respond to harmful stimuli is present in all animals, it is questionable whether many animals experience consciousness of suffering. Using the human brain as a standard for self-consciousness, we may obtain a very crude appreciation of the differing capacities for consciousness among animals by comparing brain size. The brain of an adult zebra fish probably has a mass of less than 0.1 g (Packard 1972) while a human brain typically has a mass 13,000-fold greater. The majority of work with zebrafish involves manipulation of embryos and, while there is no reliable published estimate of neuron number in a zebrafish embryo, one source ([http://www.exploratorium.edu/imaging\\_station/research/neurons/story\\_neurons.pdf](http://www.exploratorium.edu/imaging_station/research/neurons/story_neurons.pdf)) apparently indicates a neuron number of 10,000 at hatching. This is nearly one hundredth of that found in a honey bee (950,000 neurons, Menzel & Giurfa 2001). In developing zebrafish embryos, the first neurons form after 7.5 hours post fertilisation (Mendelson 1986) and one would expect the neuronal interconnections within a zebrafish embryo's brain to be less complex than in an adult.

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