

# Analysis of gene function in the zebrafish retina

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## Abstract

Mutagenesis screens in zebrafish have uncovered several hundred mutant alleles affecting the development of the retina and established the zebrafish as one of the leading models of vertebrate eye development. In addition to forward genetic mutagenesis approaches, gene function in the zebrafish embryo is being studied using several reverse genetic techniques. Some of these rely on the overexpression of a gene product, others take advantage of antisense oligonucleotides to block function of selected loci. Here we describe these methods in the context of the developing eye.

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## 1. Introduction

The vertebrate retina is remarkably conserved in evolution. The major cell classes of the retina and their organization are very similar in phyla as divergent as primates and teleost fish. This striking anatomical conservation leads one to believe that genetic regulatory mechanisms underlying development of the retina have been essentially unchanged throughout vertebrate evolution. Owing to this exceptional conservation, the study of lower vertebrates is likely to reveal principles of retinal development relevant to all vertebrate species, including humans. The zebrafish, a relatively simple vertebrate, is one of the leading model systems for conducting research on eye development. The appeal of this organism relies on the combination of genetic and embryological qualities. Zebrafish are easy to propagate in large numbers, can be mutagenized using several methods, and numerous approaches have been developed to detect mutant phenotypes (reviewed in [1,2]). Owing to its transparency and rapid extrauterine development, defects resulting from genetic manipulations are particularly easy to analyze in the zebrafish embryo [3].

Genetic experiments in zebrafish initially relied on screens for mutant alleles. Numerous mutagenesis ex-

periments of varying scale yielded several hundred mutations affecting the zebrafish visual system [4–7]. While the random mutagenesis approach allows for a relatively unbiased identification of genes involved in a particular developmental process, it is an inefficient route for studying specific genes of known molecular structure. To analyze such genes, several reverse genetic approaches are available in zebrafish. One particularly effective method relies on the use of morpholino-modified antisense oligonucleotides to block gene function. These compounds are thought to specifically block the expression of target genes by interfering with translation and are effective throughout the first days of development. Complementary techniques exist to overexpress genes of choice by the injection of mRNA into early embryos or by the injection of DNA overexpression constructs. In more advanced scenarios, expression constructs can be activated at a desired stage of development using inducible promoters. Here we concentrate on well-established reverse genetic approaches to analyze gene function during eye development in zebrafish.

## 2. Knockdown analysis

Morpholino phosphorodiamidate oligonucleotides (MOs) have recently entered the growing pantheon of tools to manipulate gene function in several model

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systems, including the zebrafish [8,9]. MOs are synthetic DNA analogues that contain a neutral charge backbone in which the deoxyribose moiety is replaced with a morpholine (hydro-1,4-oxazine) ring. Morpholino antisense oligonucleotides overcome many limitations known to hinder the usefulness of other antisense approaches. One advantage of MOs over other RNA antisense reagents is that they are resistant to nucleases such as RNase A, RNase T1, nuclease P1, mung bean nuclease, and others [10]. MOs show low toxicity and low nonspecific binding to proteins. In contrast to DNA antisense compounds, such as phosphorothioate DNA analogues, MO activity is not RNase H-dependent, which is thought to contribute to their specificity [10]. Another important reason for the remarkable specificity of morpholino antisense compounds is that they appear to be effective only when targeted to the vicinity of the translation initiation codon or the splice sites [11,12]. It is currently thought that MOs act by creating a steric hindrance that prevents access of translation or splicing factors to the RNA template. The strongest experimental evidence for the specificity and efficiency of morpholino oligonucleotides comes from the comparison of phenotypic effects produced by chemically induced null mutant alleles and morpholino knockdown experiments. For numerous loci, the phenotypes of null alleles can be closely reproduced using antisense morpholino oligonucleotides [11,13–16].

### 3. Design of morpholinos

Several criteria must be met while designing an MO against a particular gene target. MOs that contain over 36% guanines or stretches of more than 3 guanines in a row display reduced water solubility and thus are less effective. Similar to PCR primers, the MO should also have little or no self-complementarity since the formation of secondary structure will render it incapable of binding to the target mRNA. MOs should be targeted to the transcript sequences located between the 5' cap and approximately 25 bases 3' of the translation initiation codon. Probes targeted to sequences more than about 20–30 bp downstream of the translation start site are significantly less effective (GeneTools, LLC). This lack of inhibition outside of specific sites is one of the key factors underlying the exquisite specificity of MOs; MOs that bind nonspecifically to other transcripts will not decrease translation unless they interact with very specific sites. This dramatically limits the number of sequences that can mediate nonspecific interactions [10]. The length of the MO also affects its activity. Efficacies increase substantially with increasing length—the longest commercially available MOs contain 25 bp (GeneTools, LLC).

MOs also effectively block gene function by interacting with splice sites [12]. The design parameters of splice site-

targeted morpholinos are generally the same as those discussed above except that their target sequence should encompass a splice donor site. As nuclear processing events occur soon after transcription, MOs exert their effect on splicing in a narrow window of time. As a result, the concentration of MOs injected needs to be considerably higher than would be required to suppress translation (GeneTools LLC; [17]). Because morpholino interference with splicing results in a change of transcript size, the efficacy of the knockdown can be quantitated by RT-PCR amplification of the splice product [12]. This is an important advantage that obviates the need for antibodies, which are not always available.

A useful strategy in MO knockdown experiments is the simultaneous use of two MOs targeted to nonoverlapping sequences. The use of two MOs against a single gene has been shown to result in a synergistic increase in inhibitory activity [12,18]. In the case of two MOs designed against *sonic hedgehog*, an eightfold higher incidence of somite defects is observed compared to injection of either MO alone [8]. A similar synergistic effect of two MOs designed against *frizzled-2* is observed. An amount of 2.5 ng of each MO administered simultaneously elicits a severe phenotype in 14% of injected embryos, while either MO alone fails to produce a severe phenotype [19].

A clear advantage of using MOs instead of other antisense oligonucleotide strategies is their low toxicity. This makes it possible to use high concentrations of MOs before nonspecific toxic effects are observed. One to two nanoliters of MOs at 3 mg/ml (3–6 ng) is usually sufficient to induce a phenotype. A dose dependence has been observed when up to 12 ng of MO has been injected per embryo. For example, injection of increasing amounts of an anti-*cyclops* MO induces increasing degrees of cyclopia [14]. For many MOs, high dosage can nonetheless produce nonspecific effects, including cell death [15,20], epiboly defects [13], neural degeneration [11], and anteroposterior axis truncations [8]. The appropriate amount of MO to be injected must be determined empirically by the user. The injection procedure is described below.

### 4. Potential problems in knockdown analysis

Morpholino-mediated interference may be ineffective for several reasons. The intracellular concentration of MOs may decrease during development due to dilution or degradation, consequently reducing their efficacy. Our observations suggest that MOs become less efficient past 50 h postfertilization (hpf). Consequently, an attempt to disrupt the activity of a gene that is known to become biologically relevant at or beyond 50 hpf may be difficult in the zebrafish. This limits the use of MOs to processes occurring relatively early during development.

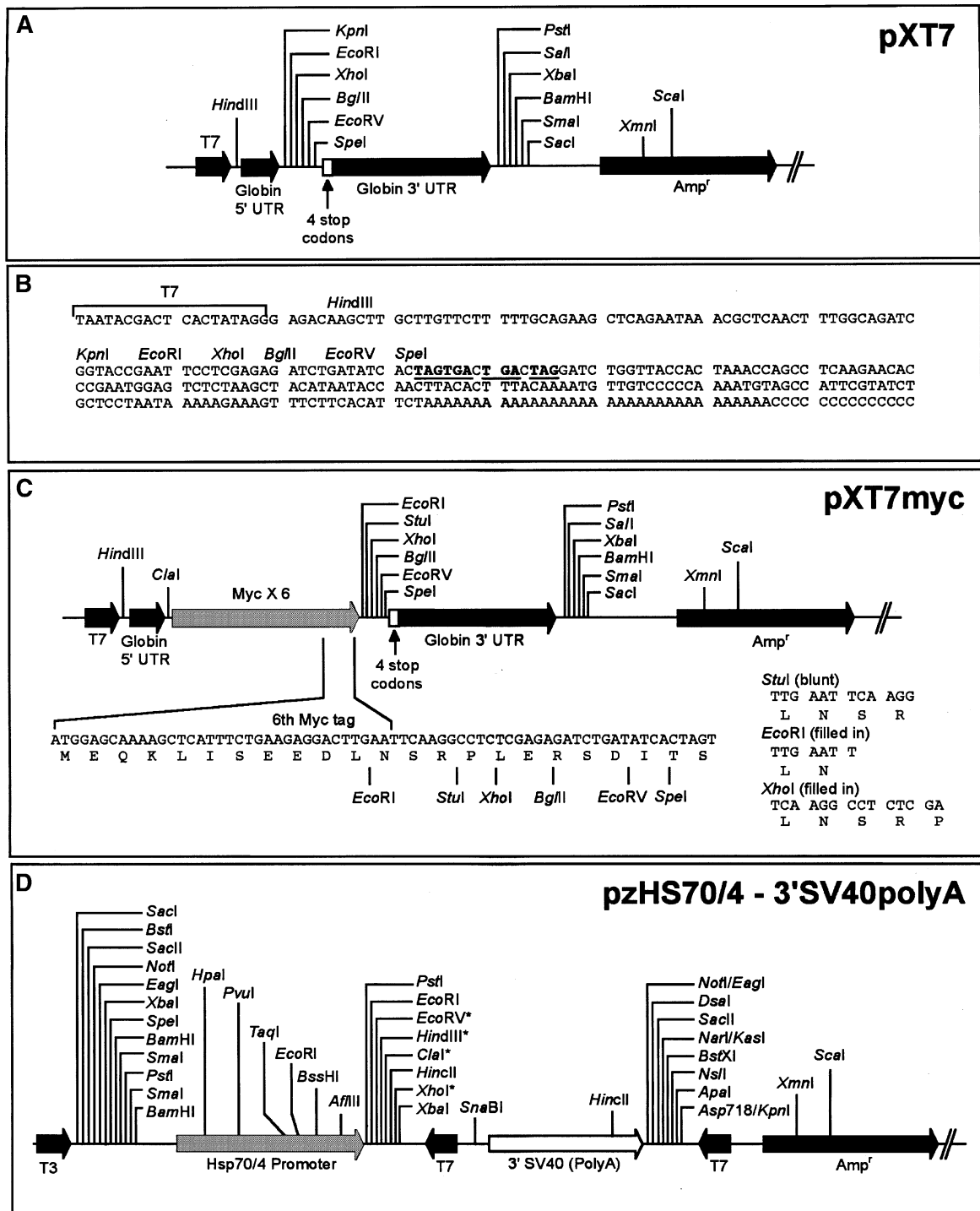
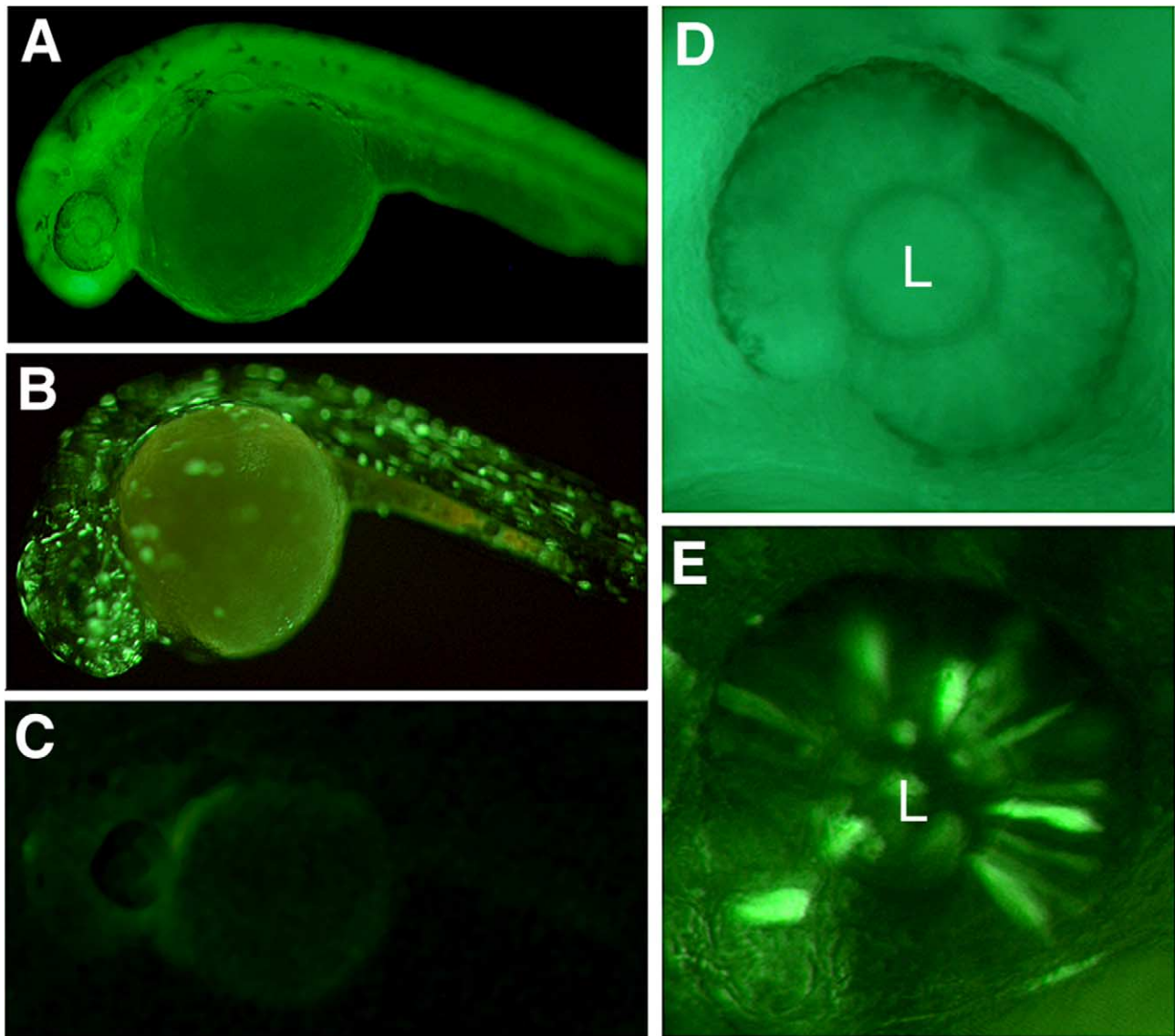


Fig. 1. The design of the expression vectors pXT7, its *myc*-tagged derivative pXT7myc (gifts from Dr. Sergei Sokol, Harvard Medical School), and the heat-shock-inducible pzHSP70/4-SV40polyA (a gift from Dr. John Kuwada, University of Michigan, and Drs. Bernard and Christine Thisse, Institut de Genetique et de Biologie Moleculaire et Cellulaire). (A, B) pXT7 is based on pGEM-4Z (Promega) and contains 5' and 3' globin UTR sequences. The ORF of a desired gene is inserted into the polylinker located immediately downstream of the 5' globin UTR region. (B) The 5' and 3' globin UTR sequences. The 5' UTR is flanked by *HindIII* and *KpnI* sites. Four stop codons are located immediately upstream of the 3' globin UTR (underlined). (C) A derivative of pXT7, pXT7myc, contains six contiguous *myc* tags located between the 5' globin UTR sequence and the polylinker. Restriction sites, located immediately downstream of the sixth *myc* tag, allow the insertion of a desired gene ORF into any of three reading frames by cloning into the *StuI* (blunt end), *EcoRI* (filled in), or *XhoI* (filled in) site. (D) The pzHSP70/4-SV40polyA vector was constructed by cloning a ca. 1.5-kb promoter fragment of the zebrafish *hsp70-4* gene into pBluescript II SK(-). An SV40 polyadenylation signal was added 3' to the polylinker [23]. The ORF of a gene under investigation can be inserted into any of the unique restriction sites (denoted by asterisks) in the polylinker located downstream of the *hsp70-4* promoter sequence.



**F**

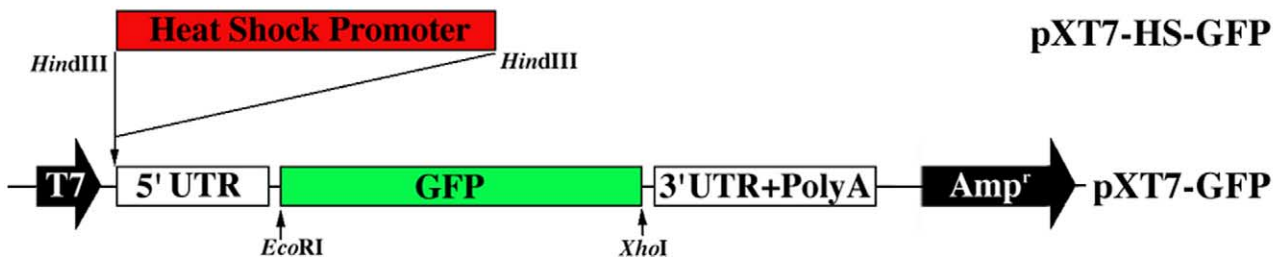


Fig. 2. GFP expression in zebrafish embryos produced using two alternative approaches. (A, D) Expression obtained by RNA injection. RNA was produced by in vitro transcription of GFP cDNA cloned into the pXT7 vector (see text for details). Embryos were injected at the 2- to 8-cell stage, reared at 28.5°C until 36 hpf, and photographed under UV illumination using a fluorescein filter. GFP fluorescence is present throughout the embryo (A) and in all cells of the eye (D). (B, E) Expression obtained by injecting the heat-shock-inducible vector, pXT7-HS-GFP, at the 2- to 8-cell stage. To activate expression, embryos were heat shocked (see text for details) and visualized under UV illumination. GFP fluorescence is visible 2 h following heat shock. In contrast to RNA injections that produce a uniform GFP fluorescence, DNA injections result in mosaic expression (B). A small fraction of retinal neuroepithelial and lens cells express GFP in the eye (E). (C) Uninjected control embryos show only weak endogenous fluorescence. (F) The design of the pXT7-HS-GFP vector used in experiments presented in (B) and (E). In all images dorsal is up and anterior is to the left. “L” indicates the lens.

Another factor that can limit the efficacy of an MO is the functional redundancy of many loci. Many zebrafish genes are present as multiple orthologues of one human or mouse gene [21]. The resulting functional redundancy can reduce the efficacy of an MO. This, of course, is a general limitation of any form of loss-of-function analysis. An example of functional redundancy is the lack of anterior midline defects in zebrafish embryos injected with MOs directed against either the *sonic hedgehog* or *tiggy winkle hedgehog* genes alone. It is possible to overcome the redundancy of zebrafish hedgehog loci by injecting simultaneously MOs directed against both genes. This approach produces severe cyclopia [11]. Morpholino oligonucleotides can be easily used to interfere with the function of two or even three potentially redundant genes as long as the molecular structure of these genes is known. In comparison to chemically induced alleles, this is clearly a strength of morpholino oligonucleotide analysis because generating homozygous carriers of multiple chemically induced mutations is very time consuming.

The genetic background of zebrafish strains is known to influence MO activity. A notable example is the *one-eyed pinhead* (*oep*) locus. In one wild-type strain, 43% of the embryos showed the mutant phenotype following treatment with an MO; in another strain, no embryos showed this defect [11]. We have found similar variation in efficacy when using MOs directed against the *nagie oko*, *glass onion*, and *CXCR4* genes when using fish of Tübingen or AB genetic backgrounds (personal observation, Z.P.). These strain differences may arise due to strain-specific DNA polymorphisms or the presence of currently unknown modifier loci that show different activity levels in genetically dissimilar strains. It is thus advisable to test each MO on at least two strains of wild-type animals. Despite these limitations, many research groups have used MOs with great success in the zebrafish.

## 5. Overexpression analysis

In conjunction with loss-of-function studies, such as the MO-based gene knockdown approach described above, gain-of-function analysis is an important element of reverse genetics studies. By examining the phenotypes of the animals overexpressing gene products of interest, one can further assess the biological functions of genes. This approach, unlike MO-based gene knockdown, also allows for the structure–function analysis of gene products. For example, by expressing a series of variant proteins lacking (or having mutations in) certain functional domain(s), one can identify functions of such domains in the context of the biological process being studied. In zebrafish, gain-of-function analysis can be achieved by the injection of DNA expression constructs or in vitro-synthesized mRNA into early stage embryos.

Generally, synthetic mRNA injections lead to a rapid and uniform expression, whereas DNA constructs result in a mosaic expression pattern. Due to these intrinsic differences in the pattern and the time course of expression, one of these methods may be advantageous depending on the nature of the phenotypic analysis. In some situations, both methods may be used together in a complementary way to better assess gene function. In this section, we focus on the strengths and weaknesses of two overexpression approaches by examining green fluorescent protein (GFP) expression in embryos injected with a synthetic mRNA or with a heat-shock promoter-driven DNA construct (Figs. 1D and 2F).

### 5.1. mRNA overexpression

The injection of mRNA is one of the most widely used methods of gene function analysis in zebrafish. It provides uniform expression, thus allowing one to upregulate gene activity fairly uniformly in all tissues. Below we describe the practical aspects of its application.

#### 5.1.1. (a) Expression vector

Since mRNA is produced in vitro using the T7, T3, or SP6 RNA polymerases, any vectors containing the corresponding promoters can be used. To increase the efficiency of translation and the stability of mRNA injected, the vectors should contain 5' and 3' UTR sequences, including the polyadenylation signal, from housekeeping genes such as  $\beta$ -globin. Fig. 1 shows a schematic diagram of one such vector, pXT7 (Figs. 1A and B), and its derivative that contains six tandem *myc* tags, pXT7myc (Fig. 1C). pXT7 was originally constructed based on pGEM-4Z (Promega, Madison, WI) [22].

#### 5.1.2. (b) mRNA preparation

Once a desired construct has been made, it needs to be completely linearized before in vitro transcription because undigested circular plasmid may yield high-molecular-weight (concatemered) transcripts and decrease the amount of the desired transcription product. To ensure complete digestion, ca. 5–10  $\mu$ g of vector DNA in a final volume of 100  $\mu$ l should be digested for a few hours and an aliquot (10  $\mu$ l) of the restriction digest should be run on a 1% agarose gel in parallel to the undigested control to ensure that only linearized plasmid is present. The digest can be subsequently extracted with phenol/chloroform and precipitated with alcohol. Alternatively, direct precipitation of the restriction digestion mixture in 500  $\mu$ l of 100% isopropanol may yield a sufficient quality of mRNAs. The resulting linearized DNA template (0.5–1  $\mu$ g) is then used for in vitro transcription using the mMessage mMachine kit (Ambion) according to the manufacturer's manual. Since the synthesized mRNA needs to be efficiently

translated in embryos, it is important that it contains a 5' cap (7-methylguanosine). Capped mRNA is produced by adding the cap analog m<sup>7</sup>G(5')ppp(5')G to the transcription reaction (included in the Ambion kit). Although the ratio of cap analog to GTP has been optimized by the manufacturer for each polymerase, for some transcripts it might be useful to adjust it further. The transcription reaction is typically carried out for 2.5 h at 37 °C. The concentration and quality of the mRNA are estimated by electrophoresis of an aliquot of the reaction mixture on a 1% agarose/TAE gel. Alternatively, mRNA concentration can be estimated by measuring its absorbance (*A*<sub>260</sub>). We find that mRNA synthesized in this way can be directly injected into embryos after appropriate dilution with H<sub>2</sub>O and without further cleanup such as a phenol/chloroform extraction. Before the injection into embryos, it may be useful to test whether the resulting mRNA yields the expected polypeptide size *in vitro* by incubating an aliquot of the reaction mixture with rabbit reticulocyte lysate (TNT Quick Coupled Transcription/Translation System; Promega) in the presence of [<sup>35</sup>S]methionine. An aliquot of the translation reaction is then electrophoresed through a polyacrylamide gel and the translation product is identified following <sup>35</sup>S autoradiography. This simple procedure will assess the integrity of the synthesized mRNA and will test whether it is efficiently translated. The diluted mRNA (10–100 µg/ml in water) is injected into one- to four-cell-stage embryos as described below. These experiments result in a fairly uniform expression in all tissues (Figs. 2A and D, compare to uninjected embryo in Fig. 2C).

## 6. Heat-shock promoter-driven overexpression

Temporal control of overexpression may become a necessity when a gene product has multiple functions at different developmental stages. DNA expression constructs carrying a gene of interest under the control of an inducible promoter allow one to manipulate gene activity in a temporally and spatially restricted manner. Recent isolation of a zebrafish heat-shock-inducible promoter from the *hsp70-4* gene provides a useful tool for this type of analysis [23]. An open reading frame (ORF) of choice may be inserted into the polylinker of a vector which contains the *hsp70-4* promoter to obtain temporal regulation of expression. Below we describe the use of such vectors to achieve temporal control of GFP expression in zebrafish embryos.

### 6.1. (a) Heat-shock-inducible expression vector

The pzHSP70/4-SV40polyA vector was constructed by cloning a 1.5-kb promoter fragment of the zebrafish *hsp70-4* gene into pBluescript II SK(-). An SV40 poly-

adenylation signal was added downstream of the *hsp70-4* sequence [23]. An ORF of choice can be introduced into the polylinker of pzHSP70/4-SV40polyA between the heat-shock promoter and the SV40 3' UTR (Fig. 1D). If compatible ends cannot be produced, the ORF may be inserted into the vector via blunt-end cloning into the polylinker *EcoRV* site. In such a case, the orientation of the insert can be determined by PCR using a reverse primer complementary to the gene ORF and a forward primer directed to the T3 promoter sequence in the vector. Only clones that contain an insert in the correct orientation will be PCR amplified. An alternative heat-shock-inducible vector was constructed based on pXT7 by inserting the *hsp70-4* heat-shock-responsive element into the *HindIII* site between the T7 promoter and the globin gene 5' UTR. To test the efficiency of this construct, we inserted a GFP reporter gene into its polylinker (Fig. 2F). The resulting construct, named pXT7-HS-GFP, was either injected into embryos after linearization (see above) or used as a template for PCR amplification of the HS-GFP fragment using the T7 (5'-TAATACGACTCACTATAGGG) and pXTHS-R (5'-GGTATTATGTAGCTTAGAGACTCC) primer pair. The resulting PCR product was inspected by electrophoresis on a 1% agarose/TAE gel, diluted in H<sub>2</sub>O to ca. 0.1 µg/µl, and injected into embryos as described below.

### 6.2. (b) Heat-shock protocol

Following injection of DNA expression constructs, appropriately staged embryos are placed in a 10-mm petri dish filled with 20 ml of egg water, prewarmed to 30 °C, and incubated in a water bath for 1 h at 38 °C [24]. GFP reporter gene expression is robust 2 h following heat shock (Figs. 2B and E).

## 7. Potential problems in overexpression strategies

Two major concerns should be kept in mind in overexpression experiments. First, the ubiquitous overexpression of a gene product at an early stage of embryonic development may cause developmental defects that preclude phenotypic analyses later on. Second, since the injected mRNA is gradually degraded, gene products with a short half-life may not last long enough to produce phenotypes. The development of caged RNA compounds may overcome these limitations and expand the applications of this method in the future [25].

In contrast to synthetic mRNA, the injection of DNA constructs results in a mosaic expression pattern (Figs. 2B and E). Consequently, simple injection of DNA is generally not useful for a whole-tissue level of gene function analysis. However, a mosaic expression pattern can present an advantage if one wishes to analyze phenotypes on a single-cell level. By being able to compare a

group of cells that overexpress the gene of interest with control construct-injected cells within the same tissue, one could determine the role of a gene product in cell morphology or cell migration. In the zebrafish embryo, the morphology of single cells overexpressing a gene of interest as a GFP fusion or co-injected with a GFP reporter construct can be monitored in living animals (Fig. 2E). If the mosaic expression presents an insurmountable problem, it can be circumvented by generating stable transgenic lines carrying a gene of interest under the control of an inducible promoter [23,26,27]. Such stable transgenic lines are generated by injecting linearized DNA into early embryos [28,29]. The  $G_0$  founder generation is usually mosaic and needs to be outcrossed to produce stable transgenic animals. As the level of transgene expression can vary depending on its integration site, it is important to maintain several independent transgenic lines to ensure that heat-shock-induced phenotypes are due to the expression of the gene of interest and not to position-specific effects.

In the overexpression experiments described above, it is important to monitor the efficiency. This sometimes poses a problem as suitable antibodies are not always available. In addition, even if an antibody has been generated, one may wish to distinguish the exogenous from the endogenous expression (e.g., mutant from wild-type protein). To accomplish this, several epitope tags and corresponding antibodies, such as anti-MYC, -HA, and -FLAG, are commercially available (anti-myc, Invitrogen, San Diego, CA, or Upstate Biotechnology, Lake Placid, NY; anti-HA, Roche Molecular Biochemicals, Indianapolis, IN; anti-FLAG, Sigma, St. Louis, MO) [30,31]. To facilitate the addition of an epitope tag, a concatemer of six *myc* tags has been incorporated into one of the variants of the pXT7 vector (shown in Fig. 1C). The same goal can be reached by expressing polypeptides as GFP fusions. An additional advantage of the latter method is that it allows one to monitor the expression level and the distribution of a protein product in living animals. In the cases of both epitope-tagged polypeptides and GFP fusions, one needs to ensure that modified proteins retain the biological functions of their wild-type counterparts. To avoid potential interference with protein function, one can use a bicistronic expression system, in which the gene of interest and a reporter gene are cloned in tandem in a single vector and are separated by an internal ribosome entry sequence [32]. Bicistronic vectors, however, are not yet in common use in the zebrafish.

## 8. Microinjection protocol

Several strategies may be used to deliver a reagent to a zebrafish embryo. Membrane-permeable reagents,

such as retinoic acid [33] or acridine orange [34], may be delivered by simply incubating the embryo in the proper medium. Large molecules such as mRNA, DNA, or MOs do not efficiently cross the cell membrane and so have to be injected. For injections into the yolk or individual blastomeres, pressure injections suffice. Delivering reagents to individual cells in the gastrula or later stage embryos requires the use of iontophoresis [35,36]. The following protocol is applicable to the microinjection of DNA, mRNA, or MOs into preblastula (1–16 cell)-stage zebrafish embryos.

To obtain two- to eight-cell embryos, mating pairs are setup in tanks (see below) with transparent dividers which separate male and female fish but allow water to circulate between the chambers. In the morning, the male is transferred to the female chamber and mating usually occurs within 30–60 min. Eggs are collected and immediately washed in egg water at 28.5°C. Embryos at the two- to eight-cell stage are arranged in agar troughs in 85-mm-diameter petri dishes filled with egg water. The troughs are made by placing a mold into molten 1% agar in egg water supplemented with 500 U/ml penicillin G and 500 µg/ml streptomycin sulfate, to inhibit microbial growth (Gibco BRL, Gaithersburg, MD). The design of the injection mold is important as the embryos, still in their chorions, must remain immobilized during injection. Our injection mold, modified from one described previously [24], is a rectangular plastic plate, 40 × 65 × 3 mm. On one side are six parallel rectangular ridges, each 1 mm wide, elevated by 1 mm, and separated by 5 mm from the others. Each ridge is 6 cm long and its imprint holds approximately 40 embryos. The imprints of the ridges are designed in such a way that embryos fit tightly into them and remain in place when the injection needle is retracted following delivery of a solution. As soon as possible after fertilization, the embryos are pressed into the troughs with a blunt metal or plastic rod. This approach obviates the need to dechorionate the embryos, a time-consuming and tedious procedure when done manually and frequently damaging to the embryo when performed through proteolytic breakdown of the chorion [24]. The dish containing the embryos is subsequently transferred to a stereomicroscope equipped with a heating/cooling stage set to 28.5°C (Heating/Cooling Thermal Stage System; Brook Industries, Lake Villa, IL). If a large number of injections need to be performed, the temperature can be lowered to 18–19°C. Embryos can tolerate such a temperature for 1 h during the early cleavage period; however, microtubule defects may occur if they are maintained at this temperature for longer periods of time.

The preparation of needles is crucial to the success of injection. Very thin needles will buckle when pressed against the chorion or clog during injection, while

thicker needles may seriously damage the embryo. Injection needles are made of thin-walled borosilicate glass capillaries (1 mm o.d., type MTW100-4; World Precision Instruments) and pulled to give a 10- $\mu$ m external diameter using a needle puller (Model P-30; Sutter Instruments). The tip is broken by pressing it against a petri dish wall and does not need to be beveled. Needles may be stored indefinitely in boxes by pressing them against a small wad of putty. The solution to be injected is backfilled into the needle using a Hamilton syringe (Hamilton, Adelaide, Australia; gas tight). The needle is then inserted into a needle holder (World Precision Instruments; 1.0 mm) attached via a thin polyethylene tube (Becton– Dickinson, Rutherford, NJ) to a hydrostatic pressure injection device (Stoelting; infusion/withdrawal pump). The needle is positioned for injections using a Narishige micromanipulator (M-152; Narishige) supported by a magnetic stand (GJ-1; Narishige). Approximately 1–4 nl of the working strength solution is delivered to each embryo. The setup described above delivers constant hydrostatic pressure. If a precise volume is to be delivered, pressure needs to be regulated using a pneumatic picopump (for example PV830; World Precision Instruments). A tracer dye, phenol red, for example (Sigma), may be included in the injection solution at 0.1–0.5% to visualize the volume of the injected material. The solution can be delivered into the yolk sac immediately adjacent to the blastomeres or it may be injected directly into the blastomeres themselves. A ca. 80- $\mu$ m-diameter bolus of injection fluid will contain approximately 2 nl of fluid. In the case of yolk injections, any reagent administered prior to the 16-cell stage will be delivered efficiently to all blastomeres as there is no physical boundary separating the blastomeres and the yolk [37]. If injection into the yolk is performed after the 16-blastomere stage, however, the injected reagent may not be delivered efficiently to all cells, resulting in a mosaic distribution [38]. Injection into only 1 or 2 blastomeres may be used to produce a phenotype only on one side of the embryo so that the other side can be used as a negative control [39,40]. This left–right asymmetry will not be present in all embryos as there is no strict correlation between the first cleavage furrow and the body axes [41]. A lineage tracer, such as dextran or GFP mRNA, should thus be included in the injection solution to monitor the distribution of the injected material. Following injection, embryos are reared as usual (see below) in embryo medium supplemented with penicillin G and streptomycin sulfate, to inhibit microbial growth (Gibco BRL). Inclusion of 0.003% 1-phenyl-2-thiourea (PTU; Sigma) in the egg water prior to 24 hpf prevents melanin synthesis, rendering the embryo transparent and making examination of retinal phenotypes easier [24]. One has to remember, however, that PTU itself can cause a developmental delay at 2.5 dpf and beyond.

## 9. Phenotype analysis

### 9.1. Techniques

Numerous and diverse approaches have been developed to study the phenotypic consequences of gene knockdown or overexpression in the zebrafish retina [3]. Here we list some of them and provide appropriate references as it is beyond the scope of this review to describe them all. Following an examination of gross morphology, the first and the simplest step in the characterization of a phenotype is histological assessment. Thin plastic sections (JB-4; Polysciences) stained with methylene blue/Azure II/basic fuchsin (Sigma) provide satisfactory morphology at the cellular level [42,43]. Electron microscopy delivers subcellular resolution but is technically more demanding [44–47]. Following microscopy, a common component of phenotypic analysis is the evaluation of expression patterns using single or double *in situ* hybridization [48,49]. Alternatively, immunolabeling of up to three antigens in whole embryos or sections is an elegant method for unraveling the colocalization of antigens [43,50]. Antibody staining combined with *in situ* hybridization can simultaneously localize proteins and mRNA in whole-embryo preparations [51].

Frequently, the manipulation of gene function will not affect the initial formation of cells but rather will produce defects in aspects of subsequent differentiation. In the case of ganglion cells, the development of axonal projections may be affected [52]; in photoreceptor cells the formation of outer segments or synaptic termini may display abnormalities [50,53]. Visualization of ganglion cells by anterograde or retrograde labeling using lipophilic tracers, DiI or DiO (Molecular Probes, Eugene, OR), has been used to reveal aberrations of retinotectal pathways in zebrafish mutants [54]. These tracers will dissolve in axonal membranes of ganglion cells and reveal their trajectories. Outer segments of photoreceptor cells are sometimes difficult to identify due to their contact with the retinal pigment epithelium. Once embryos are treated with PTU, however, the development of outer segments can be observed using simple light microscopy [55]. For more detailed analysis of photoreceptor differentiation, electron microscopic analysis is a necessity [50,53].

Apoptotic cell death is a frequent outcome of genetic manipulations and the assessment of its pattern is often informative. Apoptosis can be detected *in vivo* using a short incubation in acridine orange (Sigma), a membrane-diffusible aromatic molecule that preferentially stains dying cells by producing fluorescence inside acidic lysosomal vesicles [34]. An alternative approach, TUNEL (TdT-mediated dUTP nick-end labeling), is a more robust method for the detection of apoptosis. Most commercially available TUNEL kits rely either on

antibody detection of modified nucleotides (Intergen; ApopTag Peroxidase in Situ Apoptosis Detection Kit) or on fluorescent nucleotide derivatives which are incorporated into the fragmented DNA and allow the apoptotic nuclei to be visualized directly without the need for antibody staining (Roche Molecular Biochemicals; Apoptosis Detection Kit; Intergen; ApopTag Fluorescein Direct in Situ Apoptosis Detection Kit). It is possible to combine TUNEL with immunohistochemistry or in situ hybridization [56,57]; however, the use of antibodies requires that their target antigens be resistant to the proteinase K included in most TUNEL methods. When performing these double-labeling protocols, resistance of each antigen to proteinase K must first be empirically determined [57].

A number of more specialized tools are available for phenotype evaluation. Mosaic analysis is a powerful method that can determine whether a phenotype is cell autonomous or cell nonautonomous. Following gene knockdown or overexpression, it is possible to transplant donor blastomeres from embryos injected with morpholino oligonucleotides or RNA into wild-type animals and vice versa [58,59]. Birth-dating studies, which in zebrafish rely on the incorporation of bromodeoxyuridine into newly synthesized DNA, are applied to determine the timing of neurogenesis in the retina [60]. Iontophoretic cell labeling is used for the tracing of cell movements and cell lineage analysis [36,61]. Last, retinal function can be evaluated using electrophysiological [62] or behavioral techniques [63].

## 9.2. Markers

A large and expanding library of markers is available for evaluating the relative abundance of individual retinal cell classes and their architecture following knockdown or overexpression. In the ganglion cell layer, neurons and their axons are detectable with the 7A11 and zn-8 antibodies [64]. The latter recognizes a transmembrane antigen, neurolin [65]. Ganglion and amacrine cells can be labeled simultaneously using an anti-HuC/HuD antibody (Molecular Probes). As discussed above, anterograde or retrograde labeling with lipophilic tracers provides an efficient and specific method for visualizing ganglion cells [59,66].

The inner nuclear layer is very diverse in terms of cell content. In some vertebrate species, the amacrine cell class alone is estimated to contain over 20 different types of neurons [67]. Some subpopulations of amacrine cells can be specifically immunolabeled in the zebrafish retina with antibodies to somatostatin (DiaSorin Cat No. 20067), substance P (DiaSorin Cat No. 20064), serotonin (Chemicon Cat No. MAB352), GABA (Chemicon Cat No. MAB131), or neuropeptide Y (DiaSorin Cat No. 22940) [68–72]. Anti-calretinin (Chemicon Cat No. AB149) and anti-parvalbumin (Chemicon Cat No.

MB1572) antibodies are less specific. The first of these labels subsets of amacrine, ganglion, and bipolar cells; the second visualizes subset of cells in the ganglion and inner nuclear cell layers. The remaining classes of cells in the inner nuclear layer are more uniform in their appearance and histochemical properties. Interplexiform cells, a sparsely represented cell type, and their processes are recognized with anti-tyrosine hydroxylase antibodies (Chemicon MAB318; University of Iowa Developmental Studies Hybridoma Bank). Finally, the perikarya of Müller cells, the most abundant retinal glia, localize to the middle of the inner nuclear layer and can be visualized along with their processes with polyclonal antibodies to carbonic anhydrase II or glutamine synthetase [73]. Antibodies to glial fibrillary acidic protein (GFAP) also stain the processes of Müller glia. Among other sources, an anti-GFAP antibody, zrf-1, is available from the University of Oregon Monoclonal Antibody Facility (UOMAF).

The zebrafish photoreceptor cell layer contains rods and four types of cones [74]. Each of these cell types is uniquely characterized by the expression of a specific opsin(s). Nucleotide sequences of six opsin genes are available and can be used to design in situ probes [75]. Specific polyclonal antibodies to red, green, blue, UV, and rod opsins have also been generated [75]. A monoclonal antibody to zebrafish rod opsin, zpr-3 (formerly Fret-11), is also in use (available from UOMAF) [55,64]. In addition to anti-opsin antibodies, green/red double cones can be specifically visualized with the zpr-1 monoclonal antibody (formerly Fret-43), which recognizes an unknown antigen that is largely uniformly distributed throughout the entire cell membrane [74] (available from UOMAF).

In addition to evaluating the morphology or distribution of entire cells in the retina, it is frequently useful to examine subcellular structures such as cell junctions, cell nuclei, or centrosomes. Their integrity or localization is frequently informative. In retinal neuroepithelium, for example, the distribution of centrosomes, adherens junctions, or M-phase nuclei is an indicator of apicobasal polarity [16,43,59]. In the photoreceptor cell layer, on the other hand, the distribution of actin bundles allows one to evaluate the integrity of the outer limiting membrane. All these structures can be visualized in zebrafish using appropriate markers. Zebrafish centrosomes stain with anti- $\gamma$ -tubulin antibodies (clone TU-30, Pavel Draber; or Sigma, clone GTU-88 Cat No. T6557) [59,76,77]. Synaptic vesicles are good markers of synapses and are stained with an anti-SV2 antibody (University of Iowa Developmental Studies Hybridoma Bank Cat No. SV2). Mitotic nuclei are recognized with antibodies directed to phosphorylated H3 histone (Upstate Biotechnology Cat No. 06-570). Alternatively, the nuclear stains DAPI, Hoechst 33258, and YoPro also allow one to distinguish mitotic nuclei based on the

appearance of their chromatin (Molecular Probes Cat Nos. D-21490, H-21491, Y-3603) [16,43,59]. Adherens junctions can be localized with fluorophore-conjugated phalloidin, an F-actin-binding compound (Molecular Probes Cat No. A-12379). Alternatively, they are detectable with polyclonal antibodies to a synthetic  $\beta$ -catenin peptide [78]. Tight junctions provide a barrier to paracellular diffusion and mixing of apical and basal membrane proteins. They consist of several proteins, including the zonula adherens protein ZO-1, which in zebrafish is recognized by a mouse monoclonal antibody [79,80]. Finally, the orientation of mitotic spindles can be visualized by staining with an antibody to  $\alpha$ -tubulin (Sigma Cat No. T5168) [80].

The ease of phenotype evaluation is an obvious strength of the zebrafish model. Rapid external development is perhaps the main contributing factor to this state of affairs. Consistent with the fast overall rate of embryonic development, the zebrafish retina becomes functional by 72 hpf [81]. High fecundity of zebrafish females enables high-throughput approaches. Some experiments, in situ hybridization for example, can be performed in parallel on hundreds if not thousands of zebrafish embryos [82]. Transparency of early zebrafish embryos benefits the studies of gene expression, cell movements, or cell divisions, which can be performed in living animals. Gene expression in living zebrafish embryos is frequently monitored using GFP transgenes driven by appropriate promoters [83,84]. Finally, the zebrafish retina is large relative to other embryonic organs and its architecture and histochemical properties are well documented [2,85]. Morphological and histological data accumulated in many previous studies greatly aid the genetic analysis in this model organ.

## 10. Maintenance of animals

Conditions for the breeding of zebrafish, raising larvae to adulthood, and the maintenance of adult animals vary from laboratory to laboratory. A useful collection of protocols that can be used to propagate zebrafish strains is provided in *The Zebrafish Book* [24]. We consider here only those aspects of our fish facility maintenance that may be different from previously published protocols.

The water used to maintain zebrafish strains must be of a high quality. In some areas, water available through the municipal supply system is tolerated by adult animals but in general, embryos and larvae are much more sensitive to contaminants and require higher water quality. In our laboratory, water supplied to the fish room is cleaned using reverse osmosis (Osmonics; E4 Reverse Osmosis System) and ion-exchange mixed-bed columns (Resin-Tech). While this may not be necessary for adult animals, it is certainly required for the survival of larvae. On

purification, water is adjusted to pH 7.0–7.5 using sodium bicarbonate (baking soda for cooking use; Arm and Hammer) and the salinity of the water is adjusted to 600  $\mu$ S with Instant Ocean (Aquarium Systems). Embryos and larvae are stored in egg water (25 ml salt stock, 5 ml HCl, 10 g NaHCO<sub>3</sub>, 50 L deionized water; salt stock is 120 g Instant Ocean dissolved in 1 L of deionized water). Adult animals are fed with live brine shrimp (Brine Shrimp Direct; Grade A brine shrimp eggs; Argent Laboratories) or, if not used for frequent mating, their brine shrimp diet is partially replaced with flake food (Tetra).

To obtain embryos for experiments, fish are placed in mating cages (custom built) that consist of a clear external tank (8.4  $\times$  10  $\times$  20 cm) and an internal container (6  $\times$  8.8  $\times$  16.6 cm) that features a steel-mesh bottom and is supported by short plastic legs. Prior to breeding, the external tanks are filled with fish system water, the internal containers are inserted into them, and a tuft of artificial plastic “algae” (purchased at a local pet shop) is placed inside each of the internal containers. Subsequently, breeding pairs are transferred for mating. During mating, externally fertilized eggs fall through the steel mesh bottom of the internal container to the bottom of the external tank and remain protected from being eaten by their parents. To obtain large numbers of embryos, many (up to 150) mating pairs are setup. If embryos are required at a specific time of day, males and females are kept separated from each other in the same tank overnight by a clear plastic dividing wall and allowed to mate only at a specific time, after the fish facility lights turn on. Alternatively, fish can be maintained on a different light/dark cycle.

Raising embryos to adulthood is the most challenging aspect of zebrafish husbandry as high larval mortality is a common problem. Zebrafish embryos and early larvae are stored in petri dishes in egg water until 5 dpf. On the fifth day, healthy larvae will have developed swim bladders and are transferred to “baby tanks” that are autoclaved before each use because zebrafish larvae are sensitive to infections. Baby tanks are filled with a shallow volume of egg water (approximately 2 in. from the bottom) and air supply tubes that release ca. 1 air bubble per second are inserted into them. Larvae are fed a 1:1 blend of grade 1 (50–150  $\mu$ m) and grade 2 (150–250  $\mu$ m) hatchfry encapsalon (Argent Laboratories) for 2–3 weeks. Food particles remain on the surface of the water and healthy larvae forage for food in the upper extreme of the water column. The bellies of healthy animals are distended by food. Usually by the third week, larvae have grown large enough to eat live food as well and so their diet of hatchfry is supplemented with brine shrimp. In our experience, once larvae start eating brine shrimp, their survival rates are very high. We transfer them at this stage to the main fish system.

To provide a continuous supply of live food, brine shrimp cultures are set up daily in hatcheries (Florida

Aqua Farms; Hatch-Rite 1) filled with artificial seawater (250 g of Instant Ocean per 17 L of distilled water) and provided with a vigorous supply of air. After ca. 48 h, the air supply is removed, allowing live shrimp to settle to the bottom of the hatcheries while eggshells and unhatched eggs rise to the top. Shrimp are removed through a valve at the bottom of a hatchery, strained using a fine-mesh nylon net (Blue Ribbon Pet Products), and suspended in fish system water. To prevent bacterial growth, hatcheries are thoroughly washed and disinfected with 40% ethanol after each use.

Finally, infectious agents are potentially a very serious problem in fish facilities. Adult fish originating from other laboratories are maintained in a quarantine system that is physically isolated from the main fish facility. To prevent spreading of diseases, only bleached embryos are introduced into the main fish system from outside sources. Bleaching is performed for 5 min using a 0.005% solution of sodium hypochlorite (Sigma) in egg water, preferably during the first 36 hpf followed by several washes with egg water. A good discussion of fish diseases and methods of treating them is provided in *The Zebrafish Book* [24].

## 11. Future directions

The tools of gene function analysis in the zebrafish continue to improve. The ability to reduce or increase gene activity at a specific time point or in a specific tissue is of great importance in developmental genetic studies. Although we chose not to comment in depth on methods that are not well established, several recently developed approaches appear very promising for targeting gene expression to specific tissues. First, when using heat-shock constructs, the expression can be targeted to a desired group of cells by increasing the temperature in a specific area with a laser beam [23]. Alternatively, a similar effect can be achieved using the GAL4-UAS system [86]. This system, essentially copied from *Drosophila*, has been recently used to analyze the *notch* locus function in the retina [87]. Finally, overexpression in a chosen tissue and at a desired stage of development can be accomplished by using Bhc-caged RNA that is activated using UV light [25]. An attractive, although as yet unexplored, avenue of gene expression regulation in transgenic zebrafish could involve the use of tetracycline-inducible promoters [88]. Undoubtedly, soon even more advanced approaches will become available and further enrich the repertoire of tools for genetic analysis in the zebrafish model.

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