Zebrafish: A Multifaceted Tool for Chemical Biologists

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Zebrafish: A Multifaceted Tool for Chemical Biologists

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1. INTRODUCTION AND SCOPE OF THE REVIEW

In 1868, Friedrich Miescher completed his medical training and joined the physiological chemistry laboratory of Felix Hoppe-Seyler to study the chemical composition of biological tissues.[1] From leucocytes isolated from pus-soaked bandages, he identified an acid-insoluble compound he called "nuclein", which he determined was 14% nitrogen, 3% phosphorus, and 2% sulfur in composition.[2] Nuclein or nucleic acids, the molecules that code for life, was thus the product of a marriage between chemistry and biology. Since then we have made much progress in deciphering the DNA code and reading the entire genome of a number of organisms. With the current sophistication of molecular techniques and the ability to handle large amounts of information, we are today able to address the molecular complexity of life and design therapeutics to diseases at a scale and detail previously not attempted. Today more than ever chemistry and biology can complement each other toward improved knowledge, healthcare, and environmental management.

Multicellular life forms rely heavily on intercellular and interorgan communication to survive, maintain homeostasis, and reproduce. It is not possible to capture these complex interactions in the in vitro systems such as purified preparations or even cultured cells. Model organisms allow the study of biological processes in their natural context. Zebrafish, a small pet-shop fish, has been serving as an informative model for understanding human biology. In this Review, we shall discuss the various facets of zebrafish (*Danio rerio*) that has made it a versatile model organism for biologists as well as chemists. We will showcase examples and instances of how this model organism can serve as a tool for enhancing interdisciplinary studies in biology and chemistry and developing new tools and techniques and novel therapeutic options.

2. ZEBRAFISH: THE MODEL ORGANISM

Zebrafish or *Danio rerio* is a small tropical fresh-water fish endemic to south-Asian rivers, canals, streams, and slow-moving, shallow water bodies (Figure 1A). It belongs to the subphylum *Vertebrata* and the subclass *Teleostei*. It is commonly found in pet stores and can be easily maintained at home and the laboratory. The adults are about 2 cm long and can be maintained in fresh-water tanks at 28 °C in the laboratory. These fish breed easily in the lab conditions and have a

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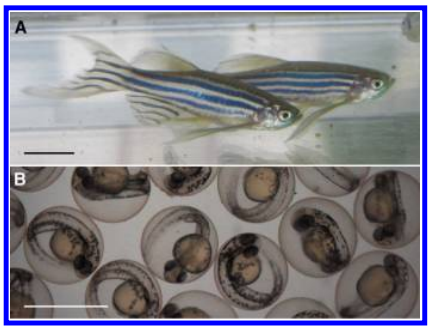

Figure 1. *Danio rerio*, the model organism. (A) Zebrafish adults are small fresh-water fish that have blue iridescent stripes along their body, which give them their name. Scale bar is 1 cm. (B) The 48 hour old embryos, still in their chorion. Scale bar is 1 mm.

generation time of ~3 months. Females are highly prolific egg layers with one individual capable of laying 100−200 eggs in a day, on a weekly basis. The females spawn in the dawn hours and only in the presence of the males, laying unfertilized eggs into the water, which are externally fertilized by the males. In the laboratory, the timing of the egg-laying can be controlled using a regulated light−dark cycle. For optimum breeding and collection of eggs, males and females are maintained in a tank with shallow water overnight, and the newly laid eggs are separated from the adults by a mesh to prevent them from eating the eggs.[3]

A number of "wild type" strains are routinely used in the laboratory, and some of the very commonly used lines such as the Tubingen (TU) and the AB lines have been incrossed and lethal mutations segregated out through many generations of breeding in the laboratory. Although the eggs and embryos cannot be preserved alive, zebrafish lines may be preserved long-term by freezing the sperm of the males in liquid nitrogen. The frozen sperm are revived by thawing, and freshly collected eggs from a female fish are fertilized in vitro to recover frozen lines.[3] The external fertilization and the transparent nature of the zebrafish embryo allow study of the embryonic development from the single-cell stage until 10 days or more using simple stereomicroscopes (Figure 1B). The zygote undergoes very rapid cell division and development, and the general body plan is laid out in 48 hours.[4] The organ systems have matured by 5 days, and the animals start feeding at this stage.

Thus, the small size, high fecundity, external fertilization, transparent embryos, and rapid development of zebrafish allow it to be easily adapted for high-throughput molecular as well as genetic studies efficiently.

George Streisinger of the University of Oregon is credited with pioneering the use of this organism for developmental biology studies in his laboratory in the early 1980s.[5,6] The accessibility of the embryo made it easy to follow the embryonic development in live animals, and the study was systematized by Charles Kimmel and colleagues in 1995, when they first described in detail the developmental stages of the zebrafish embryo.[7] This gave impetus to the first large-scale mutagenesis screen to identify genes involved in vertebrate development by the Nusslein−Volhard lab at the Max Planck Institute-Tubingen and the Driever lab and Fishman lab at Massachusetts General Hospital, Boston, MA.[8,9] This developmental screen spawned numerous new zebrafish laboratories around the world that focused on identifying the mutated loci in the zebrafish and studying the role of the mutated genes in development. The initial discoveries laid the foundation for using zebrafish as a model for dissecting vertebrate development and human biology, as many of the genes identified proved to be orthologues of human genes with developmental functions conserved from drosophila to humans.[10−12]

The sequencing of the zebrafish genome and the development of a number of powerful resources (described in coming sections) have now expanded the use of zebrafish into studies on adult diseases,[13] behavior,[14] drug discovery,[15] and many others.

3. GENOME

Wellcome Trust Sanger Institute began sequencing the genome of the lab strain of zebrafish (TuAB) in 2001. Although the data have been available in the public domain (ensembl.org) since 2002, this sequence has recently been formally published.[16] At the same time, Patowary and colleagues also published the sequence and the variation map of the Indian wild strain of zebrafish.[17]The zebrafish genome contains around 1.4 billion base pairs distributed along 25 chromosomes or linkage groups (LG)[16] coding for approximately 26,000 proteins. A number of resources such as the Zebrafish Genome Browser at Ensembl, Zebrafish Genome Browser at UCSC, Zebrafish Genome Resources at NCBI, and FishMap Zebrafish Genomics Knowledgebase[18,19] provide information on comparative genomics, annotation of new genes, identification and functional annotation of known and novel functional elements in the genome, and help organize and extract information about gene expression and function (Table 1).

The sequencing projects revealed that more than 71% of human protein-coding genes have at least one zebrafish orthologue.[16] Systematic comparison of mapped genes and expressed sequence tags (ESTs) showed a surprising degree of synteny in the arrangement of genes between the zebrafish and human genomes.[20,21] Analysis of this gene synteny led to the discovery that the zebrafish genome has undergone a duplication that can be traced back to the teleost lineage, long before the emergence of zebrafish.[22] The duplication was followed by several events of chromosome rearrangements. Thus, it is common to find two orthologues for many human genes in the zebrafish genome. Many of them appear to have acquired complementary expression patterns and functions demonstrating significant evolution following the duplication event.[23]

In the past few years, the discovery of a number of novel noncoding RNAs such as microRNAs and piRNAs with important cellular functions hints at many more functional transcripts in the genome than previously appreciated. A number of groups have identified and functionally characterized miRNAs in zebrafish[24−26]highlighting the pervasive nature of these noncoding transcripts in evolution. Recent whole genome analysis studies of the zebrafish genome have further led to the identification and annotation of a large number of long noncoding RNAs.[27,28] These studies will hasten our understanding of functional elements in the zebrafish genome and in turn the human genome.

4. GENETICS AND FUNCTIONAL GENOMICS

4.1. Forward Genetics

Historically, zebrafish were adopted as a model for developmental genetics. Forward genetics approach is a powerful and unbiased means to identify hitherto unknown players in a

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Table 1. Resources for Zebrafish Research

Resources	Website	Description
		General
ZFIN	zfin.org	A publicly funded website that hosts extensive information on *Danio rerio* including genomic databases, developmental stages, publication and molecular tools[29].
EuFishBioMed	http://www.eufishbiomed.kit.edu/	Resource website for fostering exchange of information, techniques, materials and expertise within and beyond the fish community
ZIRC	zebrafish.org/zirc	A publicly funded facility, housed in the University of Oregon, Eugene, USA, that distributes mutant lines, antibodies and probes for use by the zebrafish community.
European Zebrafish Resource Center (EZRC)	http://www.itg.kit.edu/ezrc	Housed in Karlsruhe Institute of Technology, Germany EZRC has recently started collecting mutant lines for distribution. The center will also provide facilities for high throughput screening.
ZHA: Zebrafish Husbandry Association	www.zhaonline.org/main/	A professional organization involved in improvement of husbandry standards for usage of zebrafish in research.
Zfishbook	zfin.org/zf_info/zfbook	Zebrafish protocol book, online version[3].
The Zebrafish: Disease Models and Chemical Screens		Methods in Cell Biology Volume 105, Pages 1-568 (2011) Edited by H. William Detrich, Monte Westerfield and Leonard I. Zon ISBN: 978-0-12-381320-6
Zebrafish: A Practical Approach (The Practical Approach Series)		Edited by Christiane Nusslein-Volhard, Ralf Dahm (2002) ISBN 978-0-19-963808-6
		Genomics
ZF-HEALTH	http://zf-health.org/	A publicly funded effort to understand the functional importance of 1000 genes implicated in human diseases using a combination of phenotyping, genomics and chemical genetics.
The Sanger Institute's Zebrafish Mutation Resources	www.sanger.ac.uk/Projects/D_rerio/zmp/	An online resource of zebrafish mutant lines generated by the Wellcome Sanger Institute, UK.
Welcome Trust Sanger Institute, Zebrafish Resources	www.sanger.ac.uk/resources/zebrafish/	Lists the Sanger Institute's zebrafish resources and reagents.
FishMap	http://miracle.igib.res.in/fishmap/	A community resource for zebrafish genomics, gene annotation[19].
Charles River's Laboratories	info.criver.com/research_models_and_services/zebrafish_testing	Provides genotyping services for zebrafish mutants and transgenics.
ZFN designing tools	pgfe.umassmededu/ZFPsearch.html	Zinc finger nuclease designing algorithm for creating mutants in zebrafish genes.
TALEN toolkit	http://www.addgene.org/Talen/	Designing and cloning tools required to create TALENs for injection into zebrafish (and other organisms)
		Anatomy and imaging
ZETRAP	plover.imcb.a-star.edu.sg/webpages/home.html	Provides information on enhancer trap transgenic zebrafish lines generated by the Korzh lab[30]
ZTrap	kawakami.lab.nig.ac.jp/	Provides information on enhancer trap transgenic zebrafish lines generated by the Kawakami lab (Kawakami et al., 2010).
Atlas of Zebrafish Anatomy	www.zebrafish.uni-freiburg.de/anatomy.html	A collection of sections through zebrafish embryos at various different developmental stages.
FishNet	http://www.fishnet.org.au/	An online database of 3-D models of zebrafish from embryonic to adult stage created using optical projection tomography (OPT).[31]
Fishscope	depts.washington.edu/fishscop/	A resource of time-lapse movies of various stages of zebrafish development.
FishFace	Fishbase.org	An anatomical and reporter expression atlas of the craniofacial structures of zebrafish
		Commercial screening services
Evotec	www.evotec.com	Provides zebrafish screening platforms for diseases, *in vivo* toxicity and safety pharmacology testing.
Phylonix	phylonix.com	Provides zebrafish screening services to assess compound safety, toxicity and efficacy.
ZF BioLabs	www.zfbiolabs.com	Zebrafish screening for biomedical and toxicological research.
In Danio Biosciences	www.indanio.com	Offers a ligand trap system, which includes 48 transgenic zebrafish lines, each of them expressing one of the 48 human Nuclear Receptors, allows simultaneous assessment of drug delivery, stability, specificity, tissue-selectivity and toxicity.

process (Figure 2). In the 1990s, three research groups initiated a systematic forward genetic screen to identify novel mutations that affect embryonic development in zebrafish. Single nucleotide mutations were introduced at random sites in the genome of zebrafish adult male germ cells using the chemical mutagen N-ethyl-nitrosourea (ENU, an alkylating agent that preferentially causes A to T transversions).[32] The progeny of the ENU-treated males were then screened for phenotypes of interest identifying over a 1000 mutant lines with diverse effects on development. In December 1996, the journal *Development* dedicated an entire issue to publish the genetic screen and the various zebrafish mutations generated in this screen.[8,9] Many of these mutations have later been mapped to genes and have been further studied to reveal the molecular pathways important for development. The same approach has been used many times since then to identify mutations affecting behavior, cancer, and other such adult-associated phenotypes. For example, in 2005 Zon and colleagues performed a cell proliferation assay-based screen on mutant zebrafish embryos, uncovering mutations that increase susceptibility to cancer.

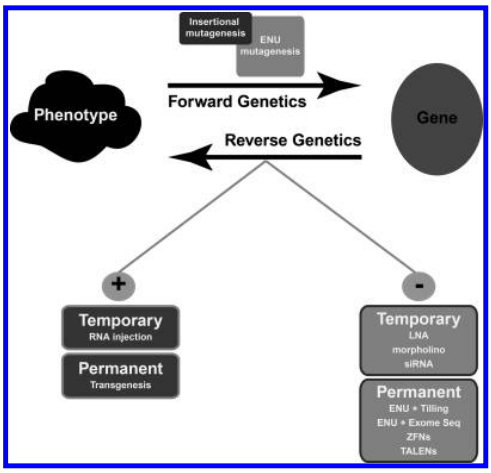

Figure 2. Schematic depiction of forward and reverse genetics approaches to analyze gene function.

They identified a novel gene *bmyb* from zebrafish that was found to be mutated in human tumor samples.[33] In another innovative screen using fluorescent reporters for lipid processing, zebrafish mutants were identified, which had defective lipid absorption and processing.[34]

Zebrafish are capable of complex behavior, many of them common to humans and fish. Upon repeated exposure to psycho-stimulant addictive drugs, adult zebrafish exhibit a reward-seeking behavior. Using this simple reward-associated behavior assay, adult zebrafish were screened to identify mutants with reduced sensitivity to cocaine[35] and resistance to addiction.[36] ENU mutagenesis screens thus have the power to yield very interesting insights into biology and need to be more thoroughly explored with new and innovative screening strategies.[37] One of the limitations of chemical mutagenesis is the difficulty in identification of the single base mutation in the whole genome, which involves mapping of the causative mutation to a single locus.[38] In compensation, however, one single effort can identify a number of different genes and multiple alleles of each, resulting in a particular phenotypic readout.

Alternative strategies of mutagenesis have been evolved that involve insertion of DNA cassettes to disrupt the host genes. These DNA cassettes can be designed to reveal important information on expression and location of the disrupted gene. Also, in these cases, identification of the mutant locus of interest is as simple as cloning the flanking DNA regions using either inverse PCR- or RACE-based techniques and sequencing them.[39] To generate these mutants, the gene-disrupting DNA segments are microinjected into zebrafish zygotes, which integrate into the genome randomly causing disruptions. The progeny of the mutagenized adults are then screened for phenotypes of interest. Retroviruses and transposons, both natural DNA disruptors, have been used to drive precise integration of DNA segments in the genome.[40,41] The first retroviral insertional mutagenesis screens performed in zebrafish identified over 300 insertion events that caused detectable developmental phenotypes.[42]A thorough analysis of all of the insertion sites revealed that only 16% of the total insertions fell in the coding exons of the genes, revealing a large number of unproductive insertions.[43] In 2011, Clark and colleagues published a new gene-breaking transposon mutagenesis strategy, which enables the disruption of protein coding regions (also noncoding regions) at the same time reporting on the expression of the trapped gene[44,45] (Figure 3). This

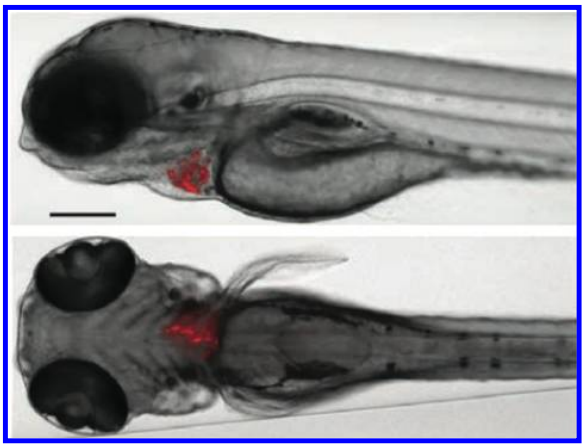

Figure 3. Protein trap into troponin T2A, a heart specific gene. The red fluorescence protein expression marks the expression of the endogenous gene trapped and disrupted by the protein trap construct. Reprinted with permission from ref 44. Copyright 2011 Macmillan Publishers Ltd.

protein-trapping construct, for the first time, enables the excision of the trap construct from the zebrafish genome to revert the genotype and phenotype back to wild type.

Thus, there are a number of strategies for efficiently generating mutants in random regions of the genome. Harnessing the power of high-throughput sequencing and genotyping technology, these methods are also being adapted to identify mutations in genes of interest. Mutations created by either ENU, transposon, or retroviral-based mutagenesis may be screened rapidly by sequencing to identify the mutations. The Zebrafish Mutation Project at Wellcome Sanger Institute aims to identify knockouts in every protein coding gene in the zebrafish genome by intensive sequencing. The Zebrafish Tilling Project has created a cryopreserved sperm library of more than 8000 mutagenized fish, which are being reiteratively screened for mutations in genes of interest.[46,47] Thus, a large compendium of mutant zebrafish lines is becoming available to the zebrafish community for interrogating different aspects of biology (Table 1).

4.2. Reverse Genetics

The sequencing of the human genome has accelerated the discovery of disease-associated genes by genome wide association studies (GWAS) and other genome-scale mapping techniques. It has also given birth to a whole field of noncoding biology focusing on DNA elements in the intergenic regions and noncoding transcription units. To be able to harness the full potential of the human genome project, it is essential to be able to assay these putative functional elements for function. Zebrafish is well placed to be a good model for functional genomics with its highly conserved genome sequence now available in public domain. Apart from such large-scale functional annotation, the identification of the genetic basis of many human diseases has spurred studies focused on individual genes and gene families in zebrafish. The reverse genetics approach attempts to understand the function of genes

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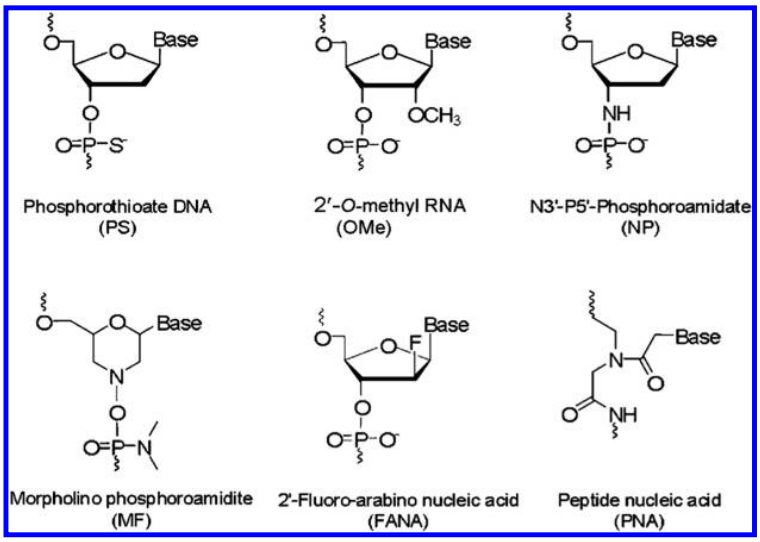

Figure 4. Examples of synthetic nucleic acid analogues containing backbone modifications. Reprinted with permission from ref 56. Copyright 2007 American Chemical Society.

of interest by targeted modulation of their activity and levels (Figure 2).

Loss or disruption of gene activity may be achieved by transient knock-down (downregulation of functional copies) of gene products or by generating zebrafish lines with inactivating mutations in the gene.

Morpholino antisense oligonucleotides (or phosphoramidate morpholino oligo) are the most popular method for transient knock-down of gene expression in zebrafish. Morpholino oligonucleotides were developed in the 1980s as an alternative to antisense DNA oligonucleotides that were being used in research as well as for developing therapeutics.[48]Modified sugar-containing ribonucleosides were found to be cheaper and simpler to assemble (Figure 4). These oligos display specific complementary nucleic acid binding as well as higher stability as compared to DNA/RNA oligos.[48,49]Morpholinos are now synthesized and marketed by Gene Tools Inc. and are extensively used for transient knock-down of gene expression in zebrafish embryos. Genome scale functional analysis of genes may be carried out using high-throughput microinjections of antisense morpholinos and phenotype mapping.[50]

Other chemically modified oligonucleotides that have been used successfully to knock-down gene expression in zebrafish embryos are peptide nucleic acids (PNAs) and locked nucleic acids (LNAs).[51] Peptide nucleic acids (PNAs) contain a backbone of *N*-(2-aminoethyl)-glycine linked by peptide bonds and string together purines and pyrimidines to create a chimera of peptide and nucleic acids[52](Figure 4). PNAs have higher specificity and binding affinities in DNA−PNA or RNA−PNA interactions than normal DNA−DNA or RNA−DNA hybrids. Because of their unnatural backbone, they are also quite resistant to enzymatic degradation and have increased stability.[53−55]LNA or locked nucleic acids have a modified "locked" ribose ring (Figure 4) that stabilizes the Watson−Crick base pairing and thus reduces the length of the oligo needed to achieve specificity.[56] Their modified structure also affords resistance to endonuclease cleavage. However, the antisense LNA strategy has not been very successful in zebrafish due to toxicity issues.[51]

Although extremely versatile and simple to perform, oligomer-based gene expression knock-down is transient, ubiquitous, and constitutive. For precise spatiotemporal control of gene expression, the oligonucleotides have to be available or active in an inducible manner. Tang and colleagues used a photocleavable linker to link a short 2-*O*′-methyl RNA to an antisense PNA such that incident light would cleave and liberate the PNA for knock-down of the target gene.[57]A number of kinds of synthetic caged morpholinos have been designed that can be uncaged and activated by using light and have been demonstrated to be effective in knocking-down genes in a temporally regulated manner.[58−62] All of these strategies are able to inactivate the gene of interest at will using light irradiation. However, it is important to be able to both activate and inactivate genes in a conditional manner. This was achieved by Tallafuss and colleagues through the introduction of a photocleavable bond between the monomers in the antisense oligo such that upon irradiation with light, the cleavage of the antisense oligomer relieves repression and activates the target gene.[63]

Most oligomer-based antisense technologies interfere with mRNA expression by blocking translation or splicing and are thus required consistently and in a stoichiometry similar to the endogenous RNA. RNA enzymes or ribozymes are constantly regenerating agents that can cleave, and thus render inactive, their sequence-specific target. Jadhav and colleagues demonstrated that ribozymes designed against miRNA, which they called Antagomirzymes, can be very effective in cleaving and inactivating endogenous miRNA.[64]Further using modified nucleotides to create LNAzymes, they were able to achieve better efficiency in cultured cells[65]and could efficiently knock-down miRNA in zebrafish embryos with minimal toxicity.[66] The LNAzyme-based knock-down can be very useful in case of noncoding RNA where antisense-based oligos might be ineffectual.

RNA interference (RNAi) commonly used in mammalian cells and other model organisms such as *Caenorhabditis elegans* and *Drosophila* has recently been successfully applied in zebrafish. Microinjection of double-stranded RNA to induce

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siRNA has been used to silence gene expression of mRNAs[67−71] transiently.

Most of the antisense-based strategies described above provide quick results, but the utility is limited by the transient nature of the gene "knock-downs". The effect of the antisense lasts only until the injected RNA is degraded and removed from the cell. Very recently, two groups have generated DNA-based constructs that express short hairpin RNA (shRNA), which can then be processed to generate siRNA.[72,73]Using promoters of microRNA (miRNA) and intron-based expression, they were able to achieve significant tissue-specific knock-down of the target genes in zebrafish embryos. An advantage of this system is that transgenic animals can be generated containing the shRNA sequence, which can be used for long-term experiments including adult knock-down in a spatiotemporally controlled fashion.

With the many different manipulation techniques available, the most consistent and reliable way to assess the function of a gene is still the mutagenesis strategy. Unlike the mouse, gene knockouts using homologous recombination have not been very efficient in the zebrafish.[74] Alternative strategies have been developed in the recent years where targeted double-stranded DNA breaks are introduced using a combination of endonucleases and sequence-specific DNA binding protein motifs. The mutagenesis relies on errors incorporated during DNA break repair within the cell. The zinc finger nuclease (ZFN)-based method uses a zinc finger triplet protein custom-designed to bind a specific sequence in the gene of interest, thus targeting the attached nonspecific nuclease domain to the genetic locus.[75−77] DNA constructs containing the gene-specific zinc finger nuclease are injected into 1-cell-stage zebrafish embryos. The sequence-specific nuclease creates breaks that lead to repair in multiple cells of the embryo, in the process introducing errors in the genome. The progeny of the founder generation are then screened for mutations in the target gene by sequencing. The type of error could be a substitution, deletion, or insertion and could vary from event to event. This approach has the advantage that it generates a number of different mutations (alleles) of the same gene in a single experiment. Commercial services for designing and creating ZFNs against genes of interest are available from Sigma Aldrich. Consortia within the zebrafish community have also developed screening systems to design ZFNs against specific genes upon request.[78,79] Online tools for designing gene-specific ZFNs[76] are also available (Table 1). However, the ZFN approach to creating mutations in genome is rapidly being taken over by other more versatile new technologies such as those based on the transcription activation like effector (TALE) domains, proteins derived from the bacteria *Xanthomonas*, to create TALENs (TALE nucleases).

While zinc finger recognition motif is 3-nucleotide long, TALE protein domains bind to single nucleotides,[80] and this means a broader targeting range allowing almost any sequence to be targeted.[81] In 2011, two groups simultaneously demonstrated that TALEN-based technology could induce efficient[82] and heritable mutagenesis in zebrafish embryos.[83] Further modifications of the TALE domain[84]and the nuclease domain dimerization[85]have improved the efficiency of TALEN-based mutagenesis in zebrafish in later studies.

Recent studies have used coinjection of TALENs with single strand DNA oligonucleotides harboring homology regions flanking short sequence changes, for example, loxP sites, such that the change or insertion is introduced into the zebrafish genome by strand-based repair mechanisms.[84]A similar strategy, but with longer homologous arms, enabled the insertion of large lengths of DNA, for example, the insertion of GFP-coding sequences target genes using homologous recombination.[86]Be-Because small genomic mutations that can disrupt coding sequences are not useful for noncoding RNA genes, Gupta and colleagues engineered two nuclease targeting sites on the same chromosome, and they were able to delete segments as large as 69kb at modest efficiencies in the zebrafish genome.[87] TALE protein design software and the plasmid toolkits needed for creating gene-specific TALENs are now available from Addgene, the plasmid distribution resource (Table 1).

CRISPR (clustered interspaced short palindromic repeats) associated systems (Cas) are bacterial defense mechanisms that utilize fragments of foreign DNA to guide the Cas endonuclease to target the destruction of the foreign DNA.[88] This system has been adapted in vitro such that in the presence of a synthetic guide RNA, Cas9 would cleave and induce error-prone repair, thus creating mutations in the target gene.[89] Very recently, Hwang and colleagues have used the CRIPR-Cas system to generate mutations in a number of zebrafish genes.[90]

As described above, a number of different strategies exist to create loss of function in genes to study the functional role of genes in zebrafish biology. The antisense RNA-based (or chemically modified oligonucleotides) knock-down strategies offer quick but transient solutions, while genomic level mutagenesis or deletion strategies are useful for studying the effects of sustained loss of function in embryos and adults.

4.3. Transgenic Studies

Genetically engineered organisms or transgenic animals are created to ectopically express genes of interest. In zebrafish, transient expression is quick and easy through microinjection of the coding RNA or cDNA into a single-cell stage embryo. However, the exogenous expression is sustained only until the RNA/cDNA is stable in the embryo, which is not more than a few days. Moreover, it leads to mosaic expression, which complicates analyzing the effects of the overexpression. To overcome these limitations and to achieve sustained and controlled expression, it is best to create zebrafish transgenic lines. Transgenic lines are created by the microinjection of the DNA construct into the one-cell stage embryos. However, unlike transient expression, the gene of interest is cloned into "expression cassettes" that contain all of the DNA elements essential for ectopic expression of the gene. Transposon-based DNA sequences such as Tol2 and *sleeping beauty* are used to enable precise and efficient integration of the DNA segment into the genome.[91] Upon microinjection of the construct along with RNA coding for the transposase enzyme, the integration of the DNA construct into the host genome results in a permanent zebrafish line containing the transgene. Tol2-based expression vectors kits have been generated with Gateway-cloning compatible promoters, reporters, and other commonly used sequences.[92]

4.3.1. Expression Studies. Expression of a gene in excess or in ectopic regions can inform on the functional potential of the genes. Expression cassettes for such applications would typically contain a promoter sequence followed by the cDNA with a 5′ ribosome entry site and 3′ polyadenylation signals. The simplest of such constructs contains a ubiquitously active promoter (e.g., promoter of β-actin or TATA binding protein) upstream of the gene of interest, such that the cDNA is expressed in all cells of the embryos throughout development

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Table 2. Tools for Transgenic Studies

Type	Description
	Tissue Specific Expression
Promoter/Gene specific	Drives the expression of particular gene in tissues specific manner using either tissue-specific promoters or RNA elements.[149,150,151,152]. 3'UTR of certain genes have also been used to impart tissue specific expression or localization of the transcript[153].
Gal4-UAS	Yeast transcriptional activator (Gal4) and the upstream activation sequence (UAS), an enhancer element that controls transcription, are used in combination to drive expression of reporter genes or the gene of interest (GoI). The Gal4 coding sequence is cloned downstream of a tissue specific promoter (as described) and the GoI or reporter is cloned downstream of UAS. This modular system allows for a variety of different tissue-specific Gal4 zebrafish lines to be combined with different UAS-reporters and UAS-GoI providing quick and flexible expression systems.[154,98]
	Inducible Expression
Heat Shock responsive promoter	Heat shock proteins are known to be transcriptionally activated in response to change in temperature or during stress. Promoters of heat shock proteins have been used to obtain heat-shock inducible gene expression of GoI[155].
Estrogen Receptor responsive promoters	Estrogen receptor translocates from the cytoplasm to the nucleus upon binding to its ligand, estrogen or tamoxifen a synthetic derivative of estrogen. A modified ligand binding domain of ER is fused to transcription factors to generate a ligand inducible transcription cassette [156].
Tetracycline regulated gene expression	Tetracycline controlled transcriptional activation or repression is brought about using an engineered bacterial tetracycline binding repressor protein (TetR) [106].
Tissue specific Inducible expression	Combinations of inducible gene expression systems with tissue specific promoters can provide spatial and temporal control of gene expression.[157,158].
	Lineage tracing
Cre lox	A sequence specific recombinase that has been used to permanently and genetically mark cells.[143] This enables the fate mapping of the cells in the embryo and study cellular movement in the animal [159]. The expression of the cre enzyme can be induced by heat shock for example, by cloning it downstream of the Hsp promoter elements [160].
Inducible Cre	The activity of the Cre enzyme can be made inducible by creating fusions with the estrogen receptor domain as discussed previously[146]
	Reporters
Qualitative reporters	Fluorescent proteins such as GFP[161],[150], RFP [162],[163],[164] or Cherry [165] are cloned and expressed downstream of a desired promoter such that the reporter expression reflects the promoter activity.
Quantitative reporter	Luciferase isolated from fireflies and other marine organisms is a light-emitting enzyme that can be used as a quantitative readout 166,167.
	Cellular Ablation
Nitroreductase	Bacterial Nitroreductase (Ntr) catalyzes the reduction of the metrodinazole prodrug into a toxic byproduct which can be used to inducibly ablate Ntr expressing cells.[168,157,169].
Tetanus toxin	TetTox-Expression of tetanus toxin in cells cause targeted ablation of the cells.[170]
Diphtheria toxin	Diphtheria toxin A-chain (DTA) expression in select cells can cause effective ablation of the target cell population.[171]
Killer Red	A photosensitizer protein from the hydrozoa class that generates reactive oxygen species when activated with light. The oxidative damage can be used to kill the cell expressing Killer red. [172]

and adulthood.[93] Other than these ubiquitous promoters, a number of other well-characterized promoters and enhancers are now available for transgenic expression such as the *cardiac myosin light chain2* promoter driving expression in the heart, etc.,[94,95]and the *kinase insert domain receptor like (kdrl/flk)* promoter that expresses selectively in the endothelial cells.[96] Cell-type specific control of gene expression is possible by using promoters or enhancers of developmentally regulated genes, such as *sox10*, which expresses only in neural crest cells during a short time window in development[97] (Table 2).

A versatile and flexible system for tissue-specific expression of genes extensively in drosophila and now very successfully adapted into zebrafish is the Gal4−UAS system. This is a yeast-based two-component expression made of an activating DNA binding transcription factor, Gal4, and a DNA element upstream activating sequence (UAS) that is the target-binding site of Gal4. Transgenic lines may be created where the gene of interest is cloned downstream of the UAS element; upon mating these with any Gal4 lines, the gene of interest will be expressed in the tissues where the Gal4 protein is expressed. The power of the Gal4−UAS system is its flexibility; one single transgenic line for the gene of interest can be used in a number of different combinations with existing Gal4 lines to achieve a large array of inducible and tissue-specific expression patterns[98]without creating individual expression lines for each tissue. Zebrafish lines expressing Gal4 in a number of different tissues and cell types are currently available. Individual promoters have been cloned upstream of Gal4 cDNA to express the protein in a tissue-specific manner.[99]However, promoters of only a handful of genes have been identified and their sequence elements characterized. In the absence of such well-characterized promoters, alternative strategies have been used to achieve cell type-specific expression. The "trap" strategy exploits the transposon-based random genomic insertion strategy for identifying new regulatory elements. A "trap" cassette is microinjected into the zebrafish embryo, leading to its random integration into the genome. If the cassette integrates within the sphere of influence of a promoter or enhancer, the reporter will be expressed in a pattern reminiscent of the endogenous gene.[100,101] In the case of the Gal4 enhancer trap lines, the Gal4 gene is expressed where the enhancer is active. This strategy has generated a large collection of zebrafish lines where Gal4 is expressed in discrete cell populations. Thus, these lines, when crossed with the UAS-gene, can ectopically express the gene of interest in a variety of tissues/cell populations.[102,103] Various modifications of Gal4 have been developed to achieve stronger and more specific transcriptional activation.[102]

Gene overexpression can be temporally controlled by using promoter systems that are responsive to the environmental stimuli such as small molecules, heat, light, etc. The promoter of the heat shock protein 70 (Hsp70) gene is responsive to sudden changes in temperature, and by giving mild heat shock for short periods of time it is possible to induce the expression of genes cloned downstream of Hsp promoters without damaging the embryo itself.[104] In an innovative use of the zebrafish Hsp70 promoter, Halloran and colleagues achieved temporal as well as spatial control of induction by focusing a

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sublethal laser microbeam in zebrafish embryos to create a localized "heat shock".[104]

Ligand inducible expression systems depend on the presence of a ligand binding receptor-like module and a promoter element responsive to the receptor binding. One such example is the 2-tier tetracycline system that utilizes a modified version of tetR (tetracycline repressor), a bacterial protein that binds to the tertracycline response element (TRE) in response to tetracycline. The system has been engineered to provide great versatility. The Tet-ON system is able to activate genes in the presence of tetracycline, while the Tet-OFF system represses genes when exposed to tetracycline.[105] Both systems have been successfully used in zebrafish to control gene expression[106] (Table 2). Similar inducible systems have been created using nuclear hormone receptors and their ligands; for example, tamoxifen, the estrogen antagonist, activates transcription using a modified estrogen receptor.[107] Other examples of similar inducible systems include the mifepristone (progesterone antagonist) inducible expression system[108,109] and the ecdysone inducible expression system.[110]

A combination of the ligand−receptor−response element system with light has led to the development of light inducible expression systems that can achieve both temporal as well as spatial control of expression. Ecdysone is the insect molting hormone, which induces its nuclear receptor Ecdysone receptor (EcR) to activate transcription from the Ecdysone Response Element (EcRE). Lin and colleagues created a caged Ecdysone that is activated only when light uncages the Ecdysone molecule. When cultured cells constitutively expressing the Ecdysone receptor complex are treated with the caged Ecdysone, downstream gene expression can be induced in individual cells or cell populations with light.[111] Gene expression can be spatially and temporally controlled with similar photo-uncageable versions of isopropyl-b-D-thiogalacto-pyranoside.[112]

Plants have a number of light-responsive systems in place to be able to best utilize the changing light in the environment to regulate their metabolism, growth, flowering, etc. Using two proteins from *Arabidopsis thaliana*, Cryptochrome 2 (CRY2) and the bHLH transcription factor CIB1 (CRY-interacting bHLH1), Kennedy and colleagues created a fast and reversible protein interacting machine.[113] This system was further adapted and shown to be effective in light-regulated transcriptional control in zebrafish.[114]

The Crabtree and Wandless groups have developed an alternative to achieving inducible protein expression. They engineered mutant versions of the FK506 binding protein (FKBP) such that these fusion proteins are highly unstable and are degraded rapidly in the normal mouse embryo. However, exposure to a small molecule modulator SLF stabilizes the protein, rendering it active in the cell.[115,116] Modifications of this strategy might be effective in zebrafish to achieve temporal protein expression to study their role in development or disease.

4.3.2. Protein Activity Modulation. Inducible activation of protein activity is possible at the level of the protein itself. Wu and colleagues created a Rac1 mutant that was fused to the photoreactive LOV (light oxygen voltage) domain from phototropin.[117] This steric block on Rac1 interactions could be released only upon photoactivation. Using this construct, the authors were able to study the role of Rac1 in neutrophil movement in live embryos.[118]

Chemical-based allosteric regulation of protein activity was stimulated by the use of azobenzene photoswitches. Azobenzene is a molecule that switches reversibly between the trans and cis forms in response to different wavelengths of light. The cis and trans forms have significantly different planar geometry and end-to-end molecular distance. This has made it ideal for coupling with biomolecules and for controlling the protein conformation.[119] Beharry and colleagues have demonstrated its utility in zebrafish by using fluorescent peptides conjugated to azobenzene such that the fluorescence becomes apparent upon photoactivating the molecule with light of appropriate wavelength.[120] In an innovative use of the azobenzene switches, Volgraf and colleagues covalently tethered the ionotropic glutamate receptor (iGluR) with its ligand via an azobenzene linker converting it into a light activated channel (LiGluR).[121] The iGluR are ligand-gated nonselective cation channels of great importance in neuronal synapses. Activation of these channels can control neuronal activity, and experiments that manipulate their activity can lead us to understand the relationship between neurons and the behavior they control. Using a Gal4−UAS-based system to express LiGluR in a specific neuronal subtype Wyart and colleagues induced specific forward swimming behavior, thus linking these neurons to this behavior.[122] Similar photoswitchable neuronal nicotinic acetylcholine receptors (nAChR) also have been created that helps us understand the role of nAChR neurons in different behavioral responses.[123]

Channelrhodopsin 2 (ChR2) is a naturally occurring light-gated cation channel found in green algae, while halorhodopsin (NpHR) is a light-gated chloride channel from halobacteria, Natronomonas. The Deisseroth group demonstrated that ChR2, when expressed in voltage-sensitive cells such as neurons, can be used to photomodulate their activity.[124] This field, now known as optogenetics, has revolutionized the way behavior can be studied at a molecular level.[125] Following this, the authors used NpHR to hyperpolarize and thus deactivate neuronal stimulation to study the roles of discrete neurons in distinct behavioral patterns.[126] Many more such studies in neurons have followed since then.[127] Further innovations on this theme have followed, such as the use of ChR2 and NpHR in zebrafish heart in combination with light-sheet microscopy to localize the pacemaker and study its development in the embryo.[119]

The powerful rhodopsin-based optogenetic strategies have a limitation that they are applicable only to genetically engineered animals. Transient receptor potential (Trp) channels are naturally present on a variety of cell surfaces in the animal and are responsible for the sensory stimulus such as vision, taste, temperature, and touch. Chemobehavioral screens in zebrafish larvae by Kokel and colleagues led to the identification of Optovin, a photoactivatable ligand of Trp1.[128] Using Optovin, the authors were able to stimulate motor activity in adult zebrafish with severed spinal cord, thus illustrating that this compound will be valuable in studying and manipulating neuronal activity in wild-type animals.

4.3.3. Reporters and Markers. Gene expression is dynamic, and changes temporally, spatially, as well as in response to the environment. Thus, quantifying and visualizing gene expression is crucial to understanding cellular behavior and gene function. Often expression of genes is restricted to discrete populations of cells, and the gene expression may also be used as a "marker" for the particular cell type.

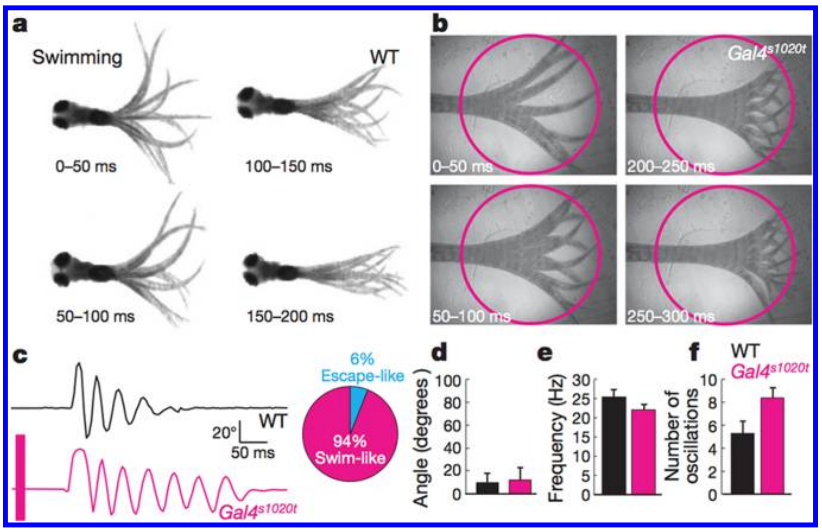

Figure 5. Optical stimulation of specific spinal neurons leads to distinct locomotor behaviors. (A) Spontaneous swim (superimposed frames). (B) Light stimulation (circle) of Gal4^{s1020t}/UAS:LiGluR evokes a "spontaneous swim"-like behavior. Here, the LiGluR is expressed in a subset of ventral spinal cord neurons including the Kolmer–Agduhr cells. (C) Comparison of deflection angle traces corresponding to (A) (top, black) and (B) (bottom, magenta, bar for stimulation). No difference in angle (D) or frequency (F), but more oscillations (E) in light-induced swims. Reprinted with permission from ref 122. Copyright 2009 Macmillan Publishers Ltd.

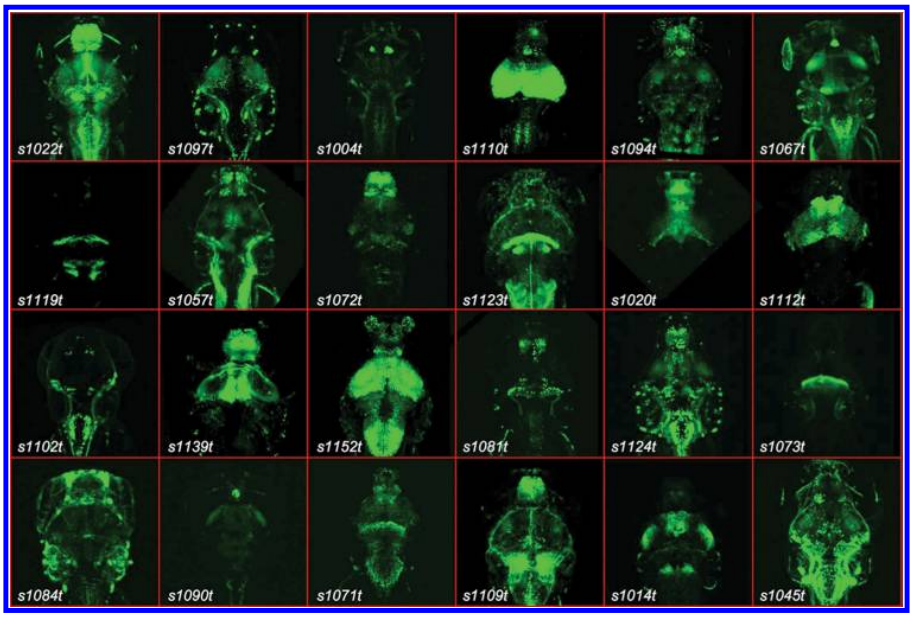

Figure 6. Gal4 expression patterns from an enhancer trap screen with the trap line number indicated. All are dorsal images of 5- or 6-dpf larvae. All larvae carry the indicated Gal4 insertion and *UAS:Kaede*. Reprinted with permission from ref 139. Copyright 2009 Scott and Baier.

Endogenous transcripts in embryos may be visualized using RNA in situ hybridizations. Chemically modified antisense RNA probe against the gene of interest is used to localize the sense RNA in the embryo. The presence of the RNA probe is detected using enzymatic reactions or fluorescent label.[129] Large-scale RNA in situ hybridization screens have been performed by a number of groups to systematically catalogue the expression patterns of zebrafish genes in the embryonic stages.[130,131] Higher resolution expression catalogues have also been made of specific regions of the embryo; for example, Gong and colleagues have created a gene expression atlas of the central nervous system using probes generated from large zebrafish gene clones made in bacterial artificial chromosomes (BACs).[132] MicroRNAs (miRNAs) are especially challenging to detect using RNA in situ hybridizations in embryos due to their low expression levels and their small size. Locked nucleic acid (LNA), as discussed in the previous sections, is modified nucleic acids with increased stability.[65] LNA-based probes are an efficient alternative for miRNA in situ hybridizations.[133,134] Although RNA in situ hybridization is a faithful indicator of gene expression, it is an end point detection technique. Because the embryo is a dynamic environment, to obtain a true picture of the events, it is essential to visualize gene expression in the live embryo.

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Transgenic zebrafish lines have been engineered that use fluorescent reporter proteins to visualize gene expression in live embryos such that the expression of the fluorescent protein will reflect the expression of the endogenous gene.[135,136] As many functionally important genes express in discrete cell types, gene expression reporter lines can be used to mark and follow the fate of particular cell populations (Table 2). Gene expression reporters are created by the fusion of DNA for the reporter protein such as GFP or red fluorescent protein (RFP) to the promoter sequence of interest. However, as described previously, enhancer traps are a very powerful means for identifying markers for interesting cell types. A typical reporter-based enhancer trap cassette would contain a reporter gene encoding GFP/RFP downstream of minimal regulatory elements. Upon microinjection into the embryos, the GFP/RFP will express as the cassette integrates within the sphere of influence of an enhancer and will reflect the expression regulated by the enhancer. This is a very powerful tool for marking specific cell types in live embryos such that cell behavior, for example, migration, differentiation, etc., may be followed in real time. Many laboratories have independently developed DNA cassettes that efficiently trap promoters and enhancers. These cassettes as well as the zebrafish lines generated with trapped enhancers or promoters are available upon request from the respective laboratories.[44,137,138] There have also been Gal4 enhancer trap screens, where the expression of the trapped enhancer is visualized using a UAS-GFP or UAS-*Kaede*, for instance. The Gal4 trap screen performed by Scott and Baier focused mainly on the nervous system[139] (Figure 6). Using *Kaede*, a photoconvertible fluorescent protein, allows discrete cells to be marked by focused light source such that these cells may then be followed through development to observe their behavior. Short-term tracking of cell fate and movement may also be done using other photoconvertible molecules such as Dendra2. Dendra2, an engineered version of the Dendronephthya fluorescent protein, is photoconvertible and can change color from neutral-to-red or green-to-red upon irradiation with different wavelengths of light.[140] Dempsey and colleagues have used the Dendra2 protein to track cellular movement and proliferation during early embryogenesis[141] (Figure 7). Yoo and Huttenlocher utilized the dendra2 protein to track neutrophil movement during injury.[142]

One of the limitations of promoter/enhancer-based reporter constructs is that the reporter protein is expressed only as long as the driver is active in the cells. Thus, these lines are not useful for following the fate or movements of a cell type over a longer period of developmental time where gene expression is extremely dynamic. Cre is a sequence-specific recombinase that recombines between two 34 base pair sequences known as loxP sites,[143] and by controlling the expression of this enzyme in a tissue-specific manner mouse developmental biologists have been able to irreversibly mark cells so that their fate may be followed through time.[144] Yoshikawa and colleagues have created a zebrafish line expressing GFP in all cells. However, the GFP cDNA is cloned between two loxP sites such that when the Cre enzyme cloned downstream of an Hsp promoter was induced by heat shock, the loxP recombination caused the deletion of GFP from genomic locus of a small number of randomly selected cells. These cells now express RFP from the same ubiquitous promoter and are permanently marked red and can be traced through developmental time in the zebrafish embryo.[145] Hans and colleagues modified the system further to fuse the Cre with the estrogen receptor protein domain (Ert). As described earlier, the Ert system allows one to induce gene expression only when Tamoxifen is present. The authors expressed this tamoxifen inducible Cre-Ert fusion downstream of a tissue-specific promoter, enabling them to spatially and temporally control the induction of recombination in a very precise and distinct population of cells in the brain at a particular time of development for fate tracking.[146] Other such innovative combinations of strategies have also been used in zebrafish in many different contexts to achieve controlled expression and genetic marking of cells.

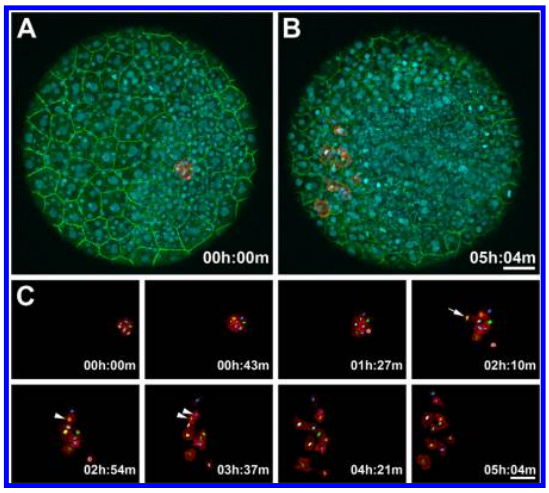

Figure 7. Tracking cellular movement and division during development using photoconvertible dendra2 fusion proteins. (A) Whole embryo at the beginning of imaging at late gastrulation stage showing unconverted membrane dendra2 (green), photoconverted membrane Dendra2 (red), and histone-cerulean fusion highlighting nucleus (blue). (B) Whole embryo imaging during the end of time-lapse imaging. (C) Close-up of the cells marked by the photoconverted Dendra2. Arrow indicates a cell coming into view from below the focal plane. Arrowhead depicts a cell division event. Reprinted with permission from ref 141. Copyright 2012 Dempsey et al.

4.3.4. Cellular Ablation. Just as creating a loss-of-function allele of a gene enables study of its function in the organism, cellular ablation allows us to address the functional role of the cell in a system. A number of precise genetic methods exist that can ablate cells in a regulated manner. When these are combined with Gal4 enhancer trap lines, it is possible to select a small population of cells that may be ablated in a temporally controlled manner.

As described previously, Wyart demonstrated that in the Kolmer–Agduhr cells, a neuronal subset is sufficient to generate a forward swimming behavior in zebrafish larvae. They went on to selectively ablate the Kolmer–Agduhr neurons to understand if these cells are necessary for the said behavior[122] (Figure 5). Andersson and colleagues created a model for pancreas degeneration by expressing the bacterial nitroreductase (Ntr) enzyme in the pancreas under the insulin promoter.[147] Upon exposure to the nontoxic prodrug Metronidazole (Mtz), Ntr converts it into a cytotoxic agent, thus providing an inducible cellular ablation tool. Using this strategy, the authors caused cellular ablation in the pancreas and then screened for compounds that would enhance the pancreatic regeneration identifying a number of interesting molecules.

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Table 3. Disease Models in Zebrafish

Disease	Description of the zebrafish model
Cancer	
Acute lymphoblastic leukemia (ALL)	c-myc induced acute lymphoblastic leukemia (ALL) in zebrafish.[204]
	TEL-AML-1 transgenic zebrafish expresses the product of the chromosomal rearrangement found in a subtype of ALL[205].
Acute myelocytic leukemia (AML)	Inducible overexpression of chromosomal rearrangement product AML-ETO1oncogene in zebrafish [200,206].
Hepatocellular carcinoma	Overexpression of c-Myc and kras V12 [207,208].
Embryonalrhabdomyosarcoma (ERMS)	Zebrafish model of RAS-induced Embryonal rhabdomyosarcoma (ERMS)[209]
p53 associated cancers	Variety of models for p53 related cancers have been generated in zebrafish [210]
Infectious Diseases	
Mycobacterium marinum infection.	Used as a surrogate for *M. tuberculosis* infection. Genes identified as important in the *M.marinum* model shown to be crucial in *Mycobacterium tuberculosis* infection in humans [211,212].
Aeromonas salmonicida	*A. salmonicida* infection model shows LECT2 induction [213].
Salmonella typhimurium	Injection model for host-pathogen interaction studies in zebrafish embryos [214].
Nervous necrosis virus (NNV)	Injection of NNV in embryo results in lesions in brain similar to natural host infection [215].
Infectious pancreatic necrosis virus (IPNV)	In zebrafish hematopoietic precursors cells and terminally differentiated red cells showed toxicity when infected with IPNV and IHNV [216].
Viral hemorrhagic septicemia virus (VHSV)	A vaccination model generated by injection of viral load of VHSV in adult fish [217].
Neuropsychiatric & degenerative disorders	
Attention deficit hyperactivity disorder (ADHD)	Loss of function of *lphn3.1* gene causes a reduction and misplacement of dopamine-positive neurons in the ventral diencephalon and a hyperactive motor phenotype in zebrafish [218].
Alzheimer's disease	Mutation in presenilin-1 gene affects the amyloid beta-peptide (A beta) secretion in zebrafish[219].
Addiction	Molecular and physiological effects of nicotine exposure have been studied in zebrafish[220].
Huntington's Disease	Zebrafish has been used to understand the function of the Huntington's disease protein, Huntingtin, in normal cells [221,222]
Parkinson's Disease (PD)	Knock down of Parkin (Park2) showed dopaminergic cell loss in zebrafish reminiscent of PD[199].
	PINK1 gene, implicated in PD has been studied in zebrafish[223].
	Knock down of LRRK2 gene express Parkinson like phenotypes, including loss of dopaminergic neurons in diencephalon and locomotion defects in zebrafish [224].
Schizophrenia	Down-regulation of Disrupted-in-schizophrenia 1 (DISC1) and Neuregulin1causes loss of olig2-positive cerebellar neurons in zebrafish [225].
Spinocerebellar ataxias (SCAs)	Expressing a dominant-negative SCA13 mutant subunit decreases the excitability of Kv3.3- expressing; fast-spiking neurons impair locomotor control and cause cell death in zebrafish[226] .
Seizure	MO-knockdown of leucine-rich, glioma inactivated 1a (*lgi1a*) gene leads to morphological abnormality and hyperactive swimming behavior modeling seizure [227].
Other degenerative disorders	
Amyotrophic lateral sclerosis (ALS)	Overexpressing Sod1 gene and G93R mutation in zebrafish causes motoneuron loss, muscle atrophy, paralysis and premature death modeling ALS [228].
Congenital muscular dystrophy	Mutations in laminin alpha2 (lama2) gene shows degenerative muscle phenotype in zebrafish[229].
Duchenne muscular dystrophy(DMD)	*sapje* mutant zebrafish have mutations in the dystrophin gene and have been used as a model for DMD
Dystroglycanopathies	Knockdown of Fukutin or FKRP, implicated in Dystroglycanopathies leads to a notochord defect and muscle in zebrafish[230,231].
Cardiovascular diseases	
Congenital heart disease (CHD)	CHD has been modeled by over expression of Nip3a protein through in zebrafish embryo [232].
Cardiomyopathy	Zebrafish *silent heart* (*sih*) mutation affects *tnnt2 gene*, resulting in sarcomere loss and myocyte disarray[233].
Hematological disorders	
Anemia	The zebrafish mutants *merlot* (mot) and *chablis* (cha) exhibit severe hemolytic anemia [234].
Microcytic anemia	Zebrafish *chianti* (*cia*) mutant results in hypochromic, microcytic anemia[235].

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Table 3. continued

Disease	Description of the zebrafish model
Hematological disorders	
Hypochromic anemia	MO-knockdown of transferrin-a in wild-type embryos reproduced the anemia phenotype [236].
Erythropoietic protoporphyria (EPP)	Zebrafish *dracula* (*drc*) mutant shows light-dependent hemolysis and liver disease [237].
Vascular diseases	
Aortic coarctation	Zebrafish mutation *gridlock* (*grl*) has blocked blood flow into trunk regions of the body[238].
Cerebral cavernous malformations (CCMs)	Mutation in ccm3 gene leads to abnormality in cranial vasculature integrity and development [239].
Hereditary hemorrhagic telangiectasia (HHT)	Zebrafish mutant violet beauregarde (vbg) shows abnormal circulation pattern and has arterio ventricular malformations [240].
Liver Diseases	
Hepatomegaly	Mutation of the class C vacuolar protein sorting gene vps18 results in hepatomegaly, which was associated with large, vesicle-filled hepatocytes [241].
ExtrahepaticCholedochal cysts	Mutation in nf2 gene leads to formation of cysts in the common bile duct [241].
Kidney Diseases	
Pronephric Cysts	The mutant *double bubble* (*dbbm153*) developed pronephric cysts [242].
Immunological Disorder	
T-cell dysfunction	Zebrafish rag1 mutants are highly susceptible to *Mycobacterium marinum* infection[211].
WHIM syndrome	Expression of WHIM mutations in zebrafish neutrophils induces neutrophil retention in hematopoietic tissue, impairing neutrophil motility and wound recruitment [243].
Metabolic Disorders	
Diabetic retinopathy	Both the IPL (Inner plexiform layer) and INL (inner nuclear layer) were significantly reduced in retinas of the glucose treated zebrafish[244].
Fatty Liver Disease	Zebrafish mutant foiegras (fgr), ducttrip (dpt), hi559, red moon (rmn) showed hepatic steatosis, hepatomegally and also lethality [245].
Obesity	Zebrafish model of diet-induced obesity (DIO)[246].
Chemically induced disease models	
Bipolar disease	Zebrafish larvae treated with GABA antagonist pentylenetetrazol (PTZ), model the symptoms of bipolar disorder[247].
Epilepsy	Pentylenetetrazole (PTZ) treated zebrafish larvae exhibit behavioral, electrographic, and molecular changes reminiscent of epilepsy[202]
Fetal alcohol syndrome	Ethanol treated larvae showed developmental defect and uncoordinated movement [248,249].
Hemolytic anemia	Small molecule 3-[5-methyl-furan 2-yl]-propionic acid N'-phenyl-hydrazide generate hemoglobin aggregates and Heinz bodies in red cells in zebrafish[250].
Steatohepatitis	Using thioacetamide (TAA) steato hepatitis model has been created in zebrafish embryo, up-regulation of many marker genes such as, ACC, adiponectin, PTL, CEBP- alpha and beta, SREBP-1 was observed [251].

In the third example of cellular ablation using genetics, Teh and colleagues expressed the phototoxic fluorescent protein Killer Red in zebrafish using enhancer trap Gal4−UAS system.[148] Killer Red protein generates a large amount of reactive oxygen species (ROS) when exposed to white light. Thus, using this light-activated cellular ablation technique, the authors were able to study the effects of cellular death in heart and hindbrain in developing embryos.

4.4. Systems Biology

To identify patterns and changes in the patterns of regulation, biologists are increasingly moving toward system biology approaches as compared to the traditional study of individual genes or pathways. Early studies using cDNA sequencing[173] and microarray profiling focused on obtaining gene expression profiles of different developmental stages[174,175] and tissue types such as retinal pigment epithelium.[176] More recently, transcriptome profiling of embryonic stages using whole RNA sequencing has revealed a large number of coding[177] and noncoding transcripts.[27,28] Proteome profiles of embryonic development[178] and multiple tissues of the adult zebrafish such as brain and kidney[179−181] are also available. Better detection and analysis techniques in mass spectrometric separation and proteomics have begun yielding profiles of modified proteins such as phosphorylated and glycosylated proteins[182−185] and the cellular metabolomes[186,187] of zebrafish developmental stages and tissues. These data surfeit can now be harnessed to build regulatory networks in the organism. Such network maps can be further tested by perturbing the system in multiple ways and studying the downstream effects. As nutrigenomics[188] and pharmacogenomics[189,190] studies are beginning to uncover, genomic level variation has profound effects on the body's response to nutrition, drugs, and other environmental stimuli. These studies in humans are usually limited to the study groups and patient samples available. With the sequencing of genetically diverse, wild strains of zebrafish and the complete "omics" profiles of the tissues, comparative and correlative studies of genomic level changes with varieties of pharmacological and nutritional stimuli will also become possible.[191]

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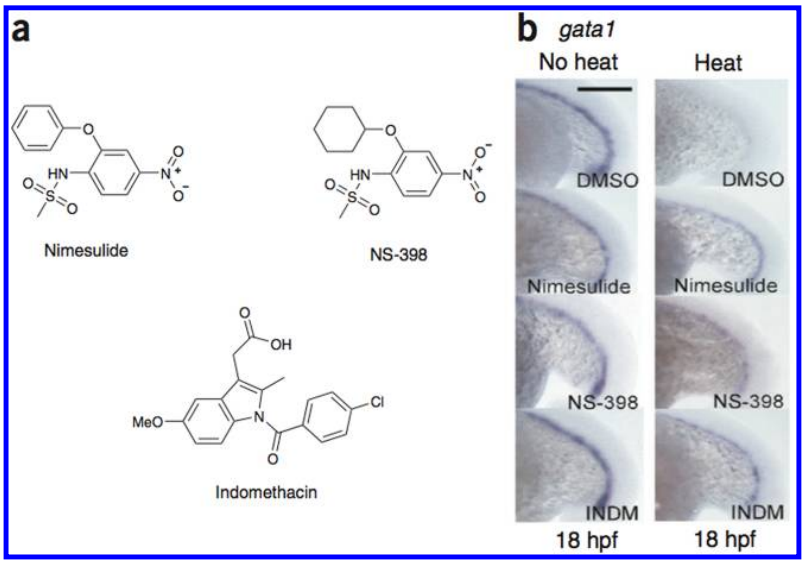

Figure 8. Chemical reversal of a disease phentoype in a zebrafish model for Acutemyelocytic leukemia. (a) Structures of cyclooxygenase (COX) inhibitors found to reverse a hematopoietic differentiation phenotype in the AML model. (b) Gata1, a marker of differentiation of hemaotopoietic stem cells, is induced upon heat shock in the zebrafish AML model. This effect is reversed by treatment with COX inhibitors. Reprinted with permission from ref 201. Copyright 2009 Macmillan Publishers Ltd.

4.5. Disease Models for Human Diseases

For making the most of the available and ever growing information on the role of genomic changes in human diseases, it is essential to have well conserved model organisms that allow for nuanced functional analysis of these genetic elements implicated in diseases. The disease phenotype, although a manifestation of the genetic change, cannot be often explained with the piece-meal understanding of gene regulatory networks that we have today. Thus, using an animal model with the same mutation or similar modification of function allows us to model all of the aspects of the disease in the context of the cell as well as the whole organism. Chemical screens that incorporate this complexity allow the discovery of drugs that may revert or alleviate the symptoms through unexpected mechanisms such as by modulating a downstream effector, by targeting more than one step in the process, or by activating a compensatory mechanism.

The conservation of genes and their functions between human and zebrafish enables modeling of human diseases and disorders in zebrafish efficiently.[13]The large-scale forward genetic screens of the 1990s resulted in mutations in a number of genes now implicated in congenital diseases in humans[192]- (Table 3). One such classic case is the *sapje* mutant isolated for its locomotion and abnormal muscle phenotype.[193]The mutation was later mapped to the dystrophin gene.[194]Duch-Duchenne muscular dystrophy (DMD), a degenerative disorder of the muscle, is caused by mutations in the dystrophin gene, a structural component of the muscle architecture. *Sapje* has been successfully used for chemical screens, yielding compounds that rescued the degeneration phenotype in zebrafish embryos by mechanisms independent of dystrophin protein synthesis.[195] *Weh* (*Weissherbst*) was identified as an anemic mutant in the ENU screen; however, the animal appeared to have an iron overload.[196] The mutation was mapped to a gene, which was then christened ferroportin.[197]This led to the discovery of the human ferroportin and its role in iron overload disease in humans[198](Table 3).

In contrast, reverse genetics approaches have been used to model human diseases for which genes have been implicated or suspected. Flinn and colleagues created a model for Parkinson's disease by knocking down the most commonly mutated gene in this disease, Parkin using morpholino oligonucleotides.[199] From ultrastructural studies of the morphant fish, they were able to identify crucial functions that may explain other types of late onset Parkinson's disease (Table 3).

Transgenic animals may also be used to model diseases. Acute myeloid leukemia (AML) is an adult-onset leukemia caused in part by a chromosomal translocation leading to the formation of an oncogenic fusion protein known as AML1-ETO. Yeh and colleagues created a transgenic zebrafish with inducible overexpression of AML1-ETO, and the dissection of AML1-ETO induced molecular changes that have led them to possible mechanisms of the leukemic pathophysiology.[200] They further used this animal model to perform a chemical screen and discovered compounds that were able to rescue the phenotype not only in the zebrafish model, but also in established human cell culture models for this disease[201] (Figure 8).

Yet another strategy for creating disease models is by using chemical treatments. Pentylenetetrazol (PTZ) treatment of zebrafish embryos elicits behavioral and cellular changes similar to epileptic seizure.[202] Such models can be instrumental in understanding the molecular mechanisms of complex diseases and also as tractable models for drug discovery.

Although not all human infectious diseases can be modeled in the fish, a number of them have been studied in the zebrafish model such as *Salmonella typhimurium* and infectious pancreatic necrosis virus (IPNV). *Mycobacterium marinum*, a close relative of *M. tuberculosis*, has been extensively studied in fish. A zebrafish forward genetics screen in the *M. marinum* model identified a number of mutations that modified the organisms' susceptibility to the bacteria.[203] Upon analysis, polymorphisms in one of these genes were also found to correlate with susceptibility to *Mtb* in humans.

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5. IMAGING

New developments in microscopy and image analysis are revolutionizing the way we address fundamental questions in biology. It is now possible to follow cellular movements in ever-higher precision and visualize molecular events inside the cells with ever increasing temporal and spatial resolution. Zebrafish embryos are optically transparent and develop ex utero, that is, outside the mother's body unlike mammals. This facilitates live imaging from the earliest point in development. With advanced chemical and genetic techniques to label molecules and cell types, zebrafish is well placed for intracellular and intercellular events in the context of the whole organism.

5.1. Intracellular Environment

Genes control cellular behavior by changing the intracellular environment. These changes may manifest as changes in the oxidative states or local concentrations of macromolecules such as proteins and RNA or small metabolites or signalers such as calcium and nitric oxide. New live imaging techniques that visualize these changes in high resolution can shed important light on such small but very significant dynamics in the intracellular environment, which are missed by global measurements of homogenates and lysates. Applying these techniques to transparent zebrafish embryos allows for studying these processes in a live animal, lending such studies more relevance.

5.1.1. Protein Sensors. Fluorescent labeling of protein domains has been used extensively to study protein folding and protein−protein interactions in purified preparations as well as in cells. A similar fluorescence resonance energy transfer (FRET)-based approach may be used variously to study the changes in calcium,[252] voltage,[253] hydrogen peroxide,[254] and protein activity[255] within cells in zebrafish. All of these biosensors rely on the principle that changes in intracellular environments change protein conformations, and these can be measured using FRET in proteins labeled with a donor and an acceptor fluorophore.

Calcium plays very important roles in signaling and regulation in cells. Protein sensors such as Cameleon[256] and GCaMP[257] have been created by combining domains of GFP fluorescent proteins and calcium binding calmodulin protein. These rely on the conformational change in calmodulin upon binding to calcium. These sensors have been used to study the changes in calcium levels in active neurons in zebrafish larvae.[258,259] Tsutsui and colleagues have used domains from a voltage-sensitive protein in combination with fluorescent proteins to create a FRET-based voltage sensor they call "mermaid", to understand the mechanism of action of cardiotoxic drugs on the zebrafish heart[253] (Figure 9). The prokaryotic hydrogen peroxide (H_2O_2) sensing protein OxyR and yellow fluorescent protein fusions have been fused to create an H_2O_2 sensor known as HyPer.[254] HyPer transgenic zebrafish were used to discover the role of H_2O_2 gradients at the wound margins in zebrafish fin in attracting leukocytes to the site of injury.[260] All of the above-mentioned sensors rely on excitation-based fluorescence to detect energy transfer, and this may be a limitation in following development over a period of time as repeated excitation may cause photobleaching and photodamage. An alternative is aequorin, a photoprotein derived from jelly fish, which is luminescent in the presence of calcium. Gilland and colleagues have used an Aequorin-based bioluminescent detector to discover rhythmic waves of calcium ions during gastrulation stages in zebrafish embryos.[261]

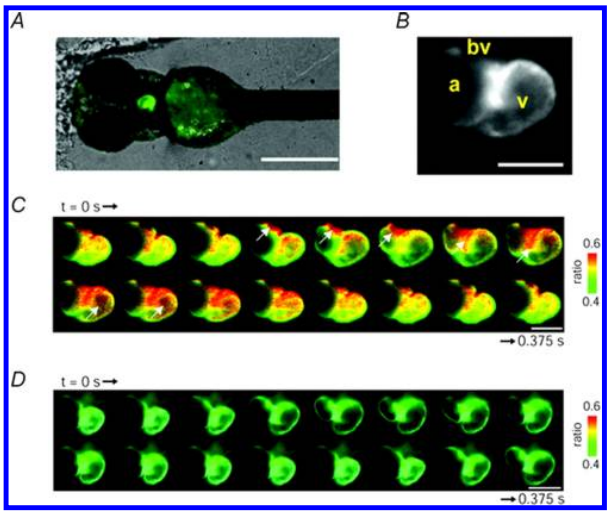

Figure 9. Voltage dynamics of a beating heart in zebrafish visualized by fluorescence resonance energy transfer (FRET)-based protein sensor, mermaid. (A) A transgenic zebrafish expressing mermaid in the heart. (B) A representative donor channel fluorescence image. a, atrium; v, ventricle; bv, blood vessel. (C) Pseudocoloured fluorescence emission ratio (acceptor/donor) images representing a single cycle of heart contraction. (D) Pseudocoloured ratio of a voltage nonresponsive mutant mermaid expression zebrafish heart. Reprinted with permission from ref 253.Copyright 2010 Journal of Physiology.

Programmed cell death or apoptosis is a cellular behavior very important in development as well as disease, and detection of apoptosis in live animals would enable the study of disease models in greater detail. Annexin V is a protein that binds to phosphatidyleserine, a phospholipid that gets exposed on the outer surface of the lipid bilayer of apoptosing cells. By fusing Yellow fluorescent protein to a secreted version of Annexin V (sec-A5-YFP), Van Ham and colleagues were able to generate a live detector of apoptosis in zebrafish embryos.[262] Time lapse imaging of transgenic zebrafish embryos expressing the sec-A5-YFP enabled them to capture the clearing of apoptotic cells by macrophages in the larval brain.[263]

5.1.2. Chemical Sensors. As discussed in the previous sections, protein sensors are usually introduced into the zebrafish as transgenes, and transgenic lines need to be established before detection experiments may be done. In contrast, chemical sensors allow rapid experimental design and execution. In most cases, the compounds that act as sensors are either injected straight into the tissue of interest, or zebrafish larvae may even be bathed in the compound for uptake.

Chemical sensors have been developed for sensing ions, metabolites, signaling molecules, as well as nutrients. NBD (nitrobenzoxadiazolyl) conjugated glucose and cholesterol fluorescent probes have been used to assay uptake of these compounds in mutant zebrafish embryos.[34] Using these probes, chemical screens have been performed on live cells as well as zebrafish embryos to identify drugs that interfere with absorption and metabolism of glucose[264] and cholesterol[265] (Figure 10).

Glycans, monosaccharides and polysaccharides, and glycosylated proteins are very important components of the cellular machinery, and the glycome patterns change with developmental stages and disease states. However, tracking these molecules is challenging because they are not amenable to the usual genetic labeling techniques. Different groups have

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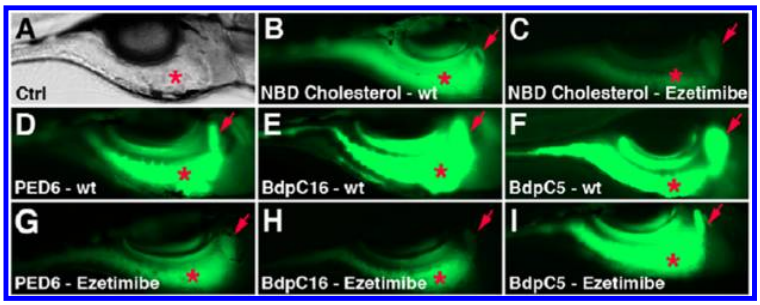

Figure 10. Visualization of the effects of ezetimibe on the absorption of different kinds of lipids using fluorescent lipid sensors in live zebrafish embryos. (A) Bright field image of a 6 day postfertilization wild type zebrafish larva, lateral view. Fluorescent sensors for (B,C) cholesterol, (D,G) phospholipid, (E,H) saturated long chain fatty acid, and (F,I) short chain fatty acid have been used to study the effect of ezetimibe on lipid absorption. Gallbladder (red arrow) and intestinal (red asterisk). Reprinted with permission from ref 265. Copyright 2010 Clifton et al.

developed labeling methods to fluorescently tag cell surface glycans for in vivo tracking of the dynamic glycosylation patterns in zebrafish embryos.[266,267]

Nitric oxide (NO), a small volatile compound, is a very important intracellular and intercellular messenger. NO is involved in processes such as vasodilation, inflammation, embryonic development, and many disease conditions. DAF-2, diaminofluorescein-based probe, has been used to detect cellular changes of NO in developing zebrafish embryos.[268]

5.2. Extracellular Currents

In a more innovative use of chemistry in biology, Nallathamby et al. developed silver nanoparticles that served as single nanoparticle photonics probes to measure nanoenvironments in the developing zebrafish embryos using localized surface plasmon resonance spectra.[269] Currents of macromolecules in the liquid environment of the embryos play an important role in determining left and right axes[270] as well as other growth patterns, and such tools will make it possible to measure such properties in living organisms.[269] Njagi and colleagues used implanted carbon fiber microelectrodes as electrochemical sensors and differential pulse voltammetry to measure the serotonin levels as low as 1 nM in the intestine of zebrafish embryos.[271] Altered levels of serotonin in the intestine affect the contractility of the gut and are implicated in irritable bowel syndrome.[272]

5.3. Whole Animal Imaging

Early bright field in vivo imaging studies aided by injection of dyes into single or groups of cells led to the creation of fate maps that showed that cells from different areas in the embryo "know" a priori their eventual fate in the embryonic body plan.[273,274]

Recently, Keller and colleagues developed a form of digital scanning laser light sheet fluorescent microscopy to quantify localization and movement of every cell in a gastrulating embryo (Figure 11). This has led to a map of mass cell movement, and a new understanding of how these cell movements led to specification of different germ layers in the zebrafish embryo.[275] Techniques such as optical tomography have also been used successfully to develop highly detailed fine maps of embryos and to visualize phenotypes in mutants.[276,277] Zheng and colleagues have been able to simulate magnetic resonance imaging (MRI) and CT scan of the brain and eye of zebrafish embryos and larvae in fine detail using a 633 nm laser.[278]

Zebrafish are completely transparent only until about 10 days or so of development. As the pigment cells spread and the skin

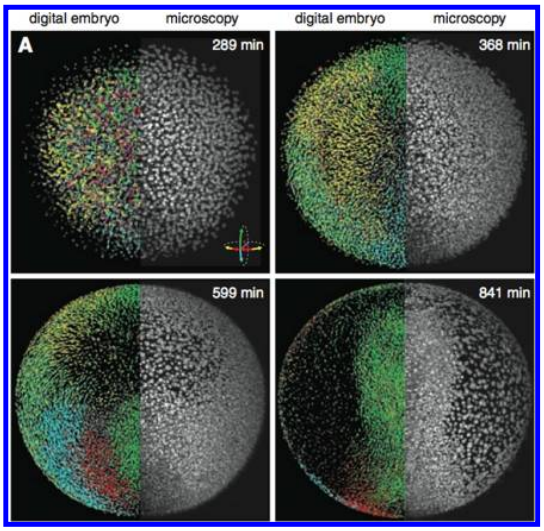

Figure 11. Cell tracking and detection of cell divisions in live zebrafish embryo using digital scanned laser light sheet fluorescence microscopy. Microscopy data (right half of the embryo: animal view) and digital embryo (left half of the embryo) with color encoded migration directions as shown in the inset in the top left-hand figure. Reprinted with permission from ref 275. Copyright 2008 AAAS.

thickens, they become less amenable to imaging experiments. For many zebrafish disease models, it is relevant and crucial to be able to study the biology of the adult. Imaging techniques for visualizing processes unique to these stages need to be developed to address this lacuna. In 2003, Fisher and colleagues developed the use of X-rays in adult zebrafish to analyze skeletal defects in mutant fish.[279] Micro CT projection of X-ray images is able to visualize the skeletal structure as well as soft tissue and internal organs of adult fish in great detail (Figure 12). Recently, the Penn State Zebrafish Atlas project, in conjunction with Micro Photonics Inc. and Numira Biosciences Inc., have developed an atlas of different stages of the juvenile and different views of the adult zebrafish based on both micro CT projections as well as histology (http://zfatlas.psu.edu). Such visualization can be useful for detecting internal organ pathology in mutants and disease models in a noninvasive way as has been already demonstrated in chick and mouse.[280] Stoletov and colleagues have used high-resolution confocal imaging to visualize tumor cell morphology and the pattern of invasion in transgenically labeled fluorescent adult zebrafish.[281] Magnetic resonance imaging (MRI) has been used to image

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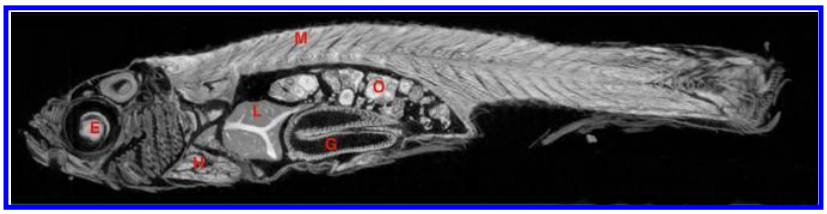

Figure 12. Internal organs of an adult zebrafish visualized using micro CT projection of X-ray images by Numira Biosciences Inc. E, eye; H, heart; G, gut; L, liver; M, muscle; O, ovary.

opaque tissue in frog embryos using a modified substrate of the lacZ reporter enzyme.[282] Similar strategies might be also effective in visualizing internal organs of adult zebrafish.

Despite the above development, the transparent zebrafish embryos remain the most accessible for imaging studies. White and colleagues developed a zebrafish compound mutant line that lacks all pigment cells, rendering the adults completely transparent (Figure 13). The *casper* mutant has been used to observe the in vivo migration of cells from a solid tumor during metastasis.[283] Other transparent zebrafish lines such as "sheer" and "absolute" are also available in pet stores and on the Internet, and these may also be used for adult imaging.

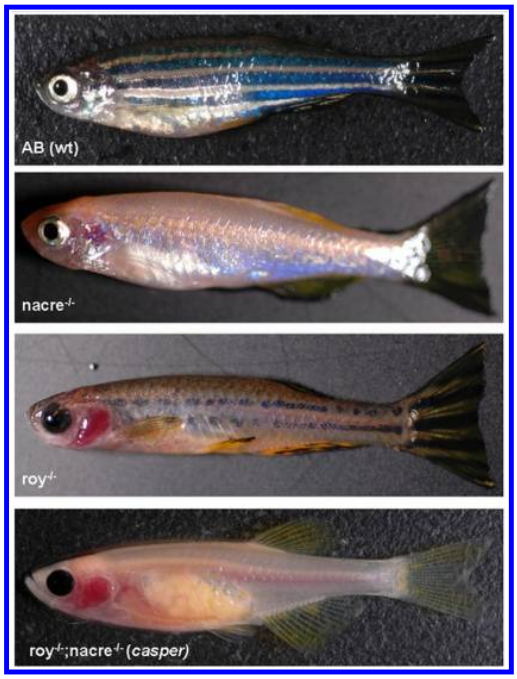

Figure 13. Transparent adult zebrafish "casper" generated from a combination of pigmentation mutants. Reprinted with permission from ref 283. Copyright 2008 Elsevier.

6. CHEMICAL GENETICS

Genetic mutations have been instrumental in dissecting the role of genes in a number of processes; this can be equated to disabling components of a machine, one at a time, and observing the consequences to determine its function in the whole. Chemical genetics instead employs small molecules to disable components of pathways.[284] This approach has been successfully applied to cultured cells and has been responsible for advances in our knowledge of a number of different aspects of biology, most notably the role of cytoskeleton in cellular events, cell signaling, protein−protein interactions, and gene transcription.[285,286]

Even as a new entrant, zebrafish is ideally suited for the chemical genetics approach. Zebrafish embryonic development is a remarkably rapid, stereotypic process that occurs ex utero and thus allows for an in vivo assay that is both complex and biologically relevant. Because the embryos normally are submerged in water and relatively permeable to small molecules in the milieu, exposure to chemicals is simple. The optical clarity of the embryos enable complex phenotypes to be detected by microscopy. The small size, inexpensive experimental setup, and easy amenability of the embryos cater to the needs of a high-throughput, in vivo, small molecule screening platform for chemical genetics and drug discovery.

6.1. Development-Based Screens

Embryonic development is a complex but stereotypical sequence of events that are regulated by a combination of intercellular signaling and intracellular transcriptional machinery. These same machinery are used in various different contexts to achieve the regulatory needs of the organism at different stages of development and adult life. Thus, embryonic development offers a complex in vivo assay system for identifying biomodulatory functions of chemicals.

The simplest of such screens involves screening for a variety of gross developmental abnormalities. However, such screens can be designed to identify molecules of specific function and clinical relevance. An example is the small molecule screen we performed to identify a bone morphogenetic protein (BMP) signaling modulator. Most human signaling pathways including the BMP pathway are highly conserved in zebrafish. Genetic screens of the past have generated a number of different mutants in this pathway with well-defined phenotypes all perturbing the dorsoventral patterning of the embryo. Thus, BMP pathway has emerged as the most important player in determining the dorsal-ventral axis of the embryo. One of the authors and colleagues performed an unbiased small molecule screen for compounds that elicit dorsoventral patterning defects in zebrafish embryo and discovered the first small molecular inhibitor of bone morphogenetic protein (BMP) signaling, now known as Dorsomorphin[287](Figure 14). A common problem with pharmacological strategies for identifying modulators of closely related pathways is promiscuity. However, because most such pathways would have distinct developmental phenotypes in an embryo, the specific activities may be easily distinguished in an embryonic assay. Thus, Dorsomorphin was selected for specific dorsoventral patterning defects and does not show cross reactivity with other closely related pathways such as the TGF b superfamily members.[287] Deregulation of BMP signaling is implicated in a number of diseases such as *fibrodysplasia ossificans progressiva*, anemia of inflammation, and prostate

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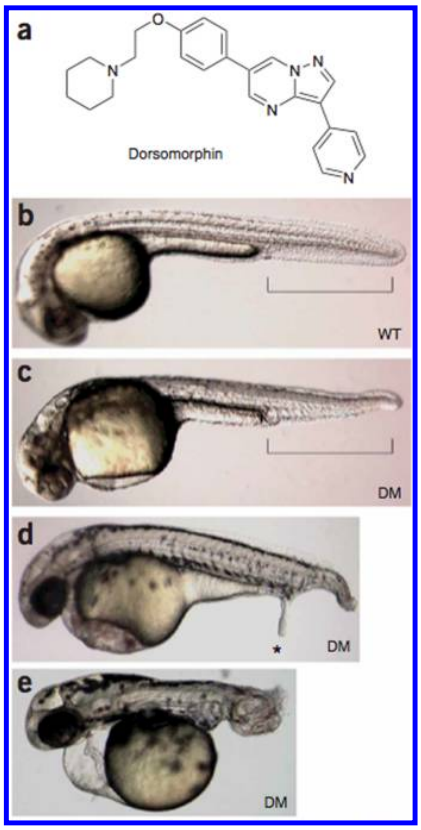

Figure 14. Identification of a bone morphogenetic protein (BMP) signaling inhibitor using zebrafish developmental assays. (a) The structure of the BMP inhibitor dorsomorphin, (b) 36 h old vehicle treated embryo, and (c–e) 48 h old zebrafish embryos exposed to 10 uM dorsomorphin starting from 8 h (c), 6 h (d), and 2 h (e). Reprinted with permission from ref 287. Copyright 2008 Yu et al.

carcinoma;[288,289] further studies with more potent analogues of dorsomorphin[290] have shown promise in mouse models.[291,292] A similar strategy was employed to identify a novel agonist of retinoic acid (RA) because RA induces anterior–posterior patterning defects in zebrafish embryos.[293] Das and colleagues went on to screen a variety of RA analogues in zebrafish to identify new derivatives with different specificities to RA receptors.[294] A variety of important signaling pathways are involved in early embryonic development of zebrafish such as Hedgehog, Notch, FGF, Wnt, and many others, and an embryonic screening strategy can be useful for identifying chemical modulators of many of these pathways.

6.2. Pathway-Based Screens

A straightforward strategy to identify activators or inhibitors of a pathway is to develop reporters based on the promoter sequences from one of the effectors of the pathway. The advantage of using whole organism screens such as in zebrafish is that many cultured cell types may not have all of the machinery needed to activate a particular pathway. In the organism, the reporter will be visualized in the tissues that the pathway is naturally active, and thus the supporting machinery is present. Dual specificity phosphatase 6 (Dusp6) is a target as well as a feedback negative regulator of fibroblast growth factor (FGF) signaling. Molina and colleagues cloned the cDNA coding for unstable GFP (d2EGFP) downstream of the Dusp6 promoter. Zebrafish transgenic lines generated by injecting this DNA construct expressed green fluorescence in tissues known to have a high FGF signaling.[295] Via a chemical screen using this fluorescence-based reporter assay, they were able to identify a number of specific inhibitors of FGF signaling.[295,296]

Hedgehog (HH) signaling plays a very important role in the development of vertebrates, and the major player sonic hedgehog is expressed in the notochord of the developing embryo. HH ligands bind to their receptors and activate transcription through the Gli transcription factors. Yang and colleagues cloned a number of repeats of the Gli-binding DNA sequence upstream of GFP to create a zebrafish transgenic reporter line for HH signaling.[297] They hypothesized that antagonists of HH receptors will share structural features with their agonists and synthesized a small library of compounds based on the scaffold of an agonist, SAG. A screen with this library yielded compounds that inhibited HH signaling in vivo, and the authors were able to demonstrate that the antagonist and agonist functioned through modification of the HH coreceptor, Smoothened.

Chemical genetic screens can be designed to reveal interacting players of important developmental pathways; these are usually done in mutant backgrounds such that the screen will identify suppressors of mutant phenotypes. Such a strategy was adopted by Peterson and colleagues for the *gridlock* mutant in zebrafish, a mutation in the Notch effector *hey2*. The *gridlock* mutant models the human condition known as coarctation of aorta, which in the fish results in blockage of blood flow to the trunk. They screened for small molecules that restore circulation to the trunk and tail and identified compounds that rescued the phenotype through distinct molecular mechanisms, thus uncovering novel molecular pathways involved in artery-vein specification[238,298] (Figure 15). Paik and colleagues adopted a similar strategy to identify molecular players in hematopoiesis using a mutant in cdx4 gene, which develops severe anemia due to aberrant hematopoiesis.[299] Their suppressor screen looked at recovery of expression of gata1, a marker for erythrocytes, by RNA in situ hybridization and identified two distinct molecules that could recover the expression of gata1 in red blood cells.

Chemical genetic screens complement genetic screens and can be valuable in discovering not just novel inhibitors of molecular pathways but also new molecular players in biological processes.

6.3. Disease-Relevant Screens

As discussed in the previous sections, modeling human diseases is possible and has been very successfully done in zebrafish. Many of these models have yielded biological insights into the disease but also have been used in chemical screen for small molecule modulators of the phenotypes. However, screens for compounds that perturb the wild-type physiology may also shed light on biology of great relevance to diseases.

6.3.1. Organ Systems. Hematopoietic stem cells (HSCs) are responsible for repopulating our blood (erythroid) and immune system (myeloid) continuously, and therapeutic intervention that depletes or removes blood lineages such as chemotherapy is usually accompanied by HSC transplantations. HSC expansion and differentiation is tightly controlled by a number of interacting signaling and transcriptional networks. To identify chemical modulators of these processes, North and colleagues performed a chemical screen on zebrafish embryos for compounds perturbing the number of HSCs. The HSCs

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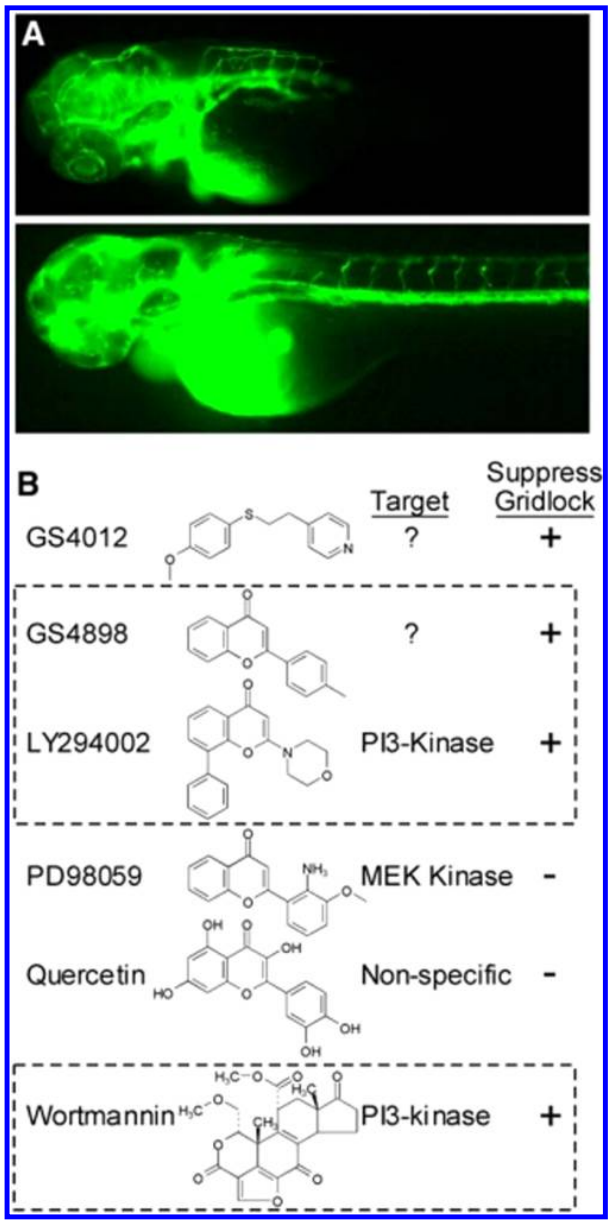

Figure 15. Chemical suppression of a genetic phenotype. (A) Fluorescence microangiogram of *gridlock* mutant zebrafish embryos untreated (upper figure) and treated with chemical suppressor (bottom figure) to visualize blood circulation. (B) Chemical compounds that suppressed the *gridlock* mutant phenotype and rescued circulation. Reprinted with permission from ref 298. Copyright 2006 Elsevier.

were assayed by RNA in situ hybridization against known molecular markers in zebrafish.[300] A number of their "hits" turned out to be modulators of prostaglandins (PGE2), inflammation mediators. This study thus revealed a role for prostaglandins in HSC formation and also identified a number of novel potential therapeutic targets for modulating HSC numbers.[300]

Polycystic kidney disease (PKD) is a common human disease, and autosomal dominant mutations in pkd2 are causative of this disease. Cao and colleagues used a pkd2 mutant line to screen for modifiers of the phenotype and identified a class of histone deacetylase inhibitors, thus implicating aberrant histone acetylation in the pathophysiology of this disease and suggesting possible therapeutic avenues[301] (Figure 16).

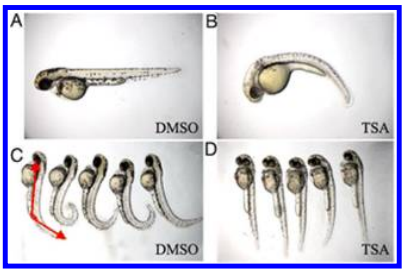

Figure 16. Suppression of pkd2/hi4166 mutant phenotype by trichostatin (TSA). (A,B) Treatment of wild-type embryos with control DMSO (A) and with TSA (B) from the shield stage leads to slight ventral curvature. (C) pkd2 mutant embryos exhibit a dorsal curvature of body. (D) TSA can suppress the body curvature of *pkd2/hi4166* mutant embryos. Reprinted with permission from ref 301. Copyright 2009 Cao et al.

Rovira and colleagues used a transgenic zebrafish expressing GFP in the pancreas to screen for compounds that may enhance endocrine differentiation in vivo.[302] Using a library of small molecules with known mechanisms of action, they identified a number of compounds that could cause precocious differentiation of cells into pancreatic endocrine cells.

Myocardial hypertrophy and congestive heart failure in humans is marked by an increase in the circulating levels of cardiac natriuretic peptides. These peptides are also induced in the developing heart in zebrafish. One of the authors and colleagues developed a quantitative luciferase-based reporter system for the natriuretic peptide expression in zebrafish, which could reproduce faithfully the pathological responses of these genes to disease and to the drugs prescribed for the heart condition.[167] Using such reporters, it will be possible to screen for new therapeutic candidates and to identify novel components of the pathological pathways of heart failure.

6.3.2. Metabolic Diseases. Many diseases are characterized by the breakdown of normal physiology often controlled by complex networks of pathways that are not yet completely understood. Identifying small molecular modulators of such processes would not be feasible through a targeted drug discovery approach. Yet it is of immense value to discover drugs that restore homeostasis, even when the mechanisms of action are not precisely defined. Discovery of such drugs requires screening assays that can faithfully replicate the in vivo complexity.

Tight regulation of nutrients and hormones in the plasma is essential to maintain a healthy physiology. Precise levels are achieved through complex regulation of processes such as absorption, mobilization, and synthesis. Despite the differences in the habitat, these processes and the genes involved are highly conserved between humans and zebrafish.[303] Assays have been developed in zebrafish for digestive physiology, hormone activities, and rates of metabolism, and some of these have been adapted for high-throughput chemical screens for modulators of these processes as discussed below.

Phospholipids play an important role in cell signaling as well as lipid metabolism. Fluorescently tagged or quenched

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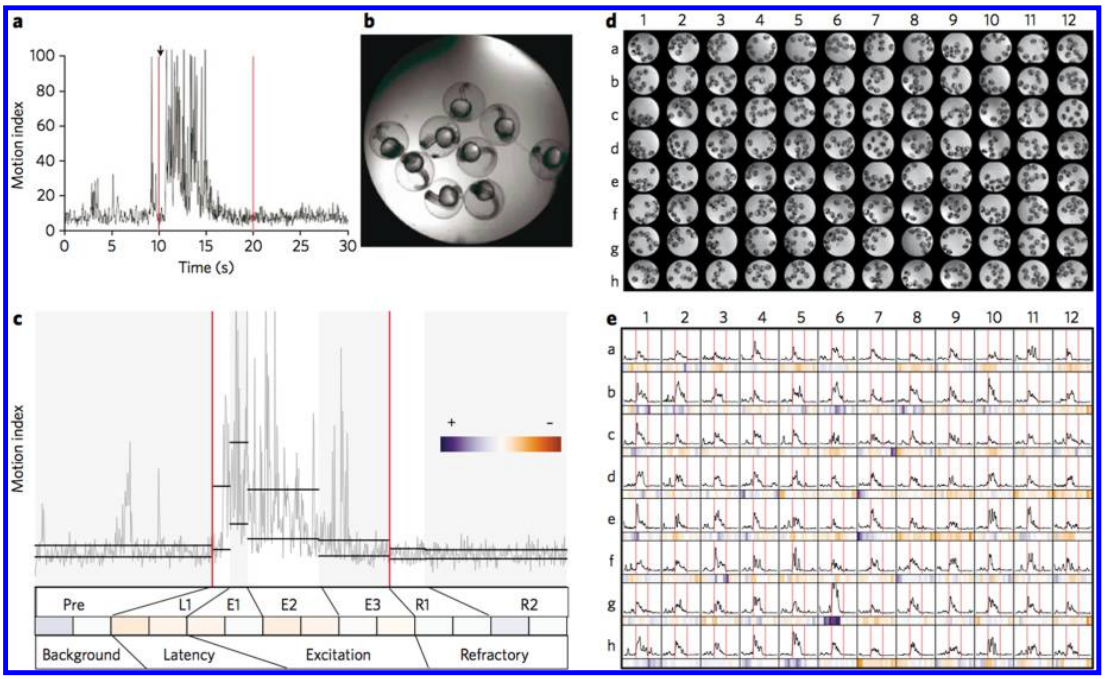

Figure 17. The photomotor response (PMR) and high-throughput behavioral barcoding. (A) Representative plot of the aggregate motor activity (in arbitrary units) over time from all embryos in a single control well during the PMR assay. Before the first light stimulus (red line at 10 s), brief asynchronous movements in individual embryos appear as narrow spikes with short (<1 s) duration. A latency phase, lasting for approximately 1 s (arrow), exists between the first light pulse and the onset of the excitation phase. The PMR excitation phase is clearly distinguished from background as a large, sustained increase in motor activity. After the excitation phase, embryos enter a refractory phase in which a second light pulse (red line at 20 s) does not elicit a response. (B) Nine zebrafish embryos in a single well of a 96-well plate. (c) PMR plot from a representative untreated control well. The motion index profile for each well was partitioned into seven periods: PrE, l1, E1, E2, E3, r1, r2. For each period, the motion index values for each well were summarized in two features, Q1 (lower line, first bar in barcode) and Q3 (upper line, second bar in barcode), which are the first and third quartiles, respectively. This 14-number summary formed the behavioral barcode. Colors in the heat map represent deviation from the average control phenotype: purple, higher activity; orange, lower activity. (D) Composite photograph of a 96-well microtiter plate before behavioral analysis. Each well contains 8−10 unhatched zebrafish embryos. (E) PMR profiles and the respective behavioral barcodes of 96 untreated control wells. Reprinted with permission from ref 314. Copyright 2010 Macmillan Publishers Ltd.

phospholipids have been used to study the digestive physiology of zebrafish.[34,304] The phospholipids were chemically modified such that their substrate properties were not inhibited while their processing could be followed live in transparent zebrafish larvae through the changes in their fluorescent properties. Fluorescently labeled NBD-cholesterol was used in an in vivo screen to identify novel chemical modulators of digestive physiology.[265]

Blood sugar levels are tightly regulated in the human body, and perturbations in this regulation result in diabetes and associated conditions. The glucose regulation pathways are well conserved in zebrafish, and as in humans the transcript levels of phosphoenol pyruvate carboxykinase (PEPCK) change inversely with respect to blood glucose levels.[305] Elo and colleagues use real time quantitative PCR for PEPCK to monitor the blood glucose changes in zebrafish larvae and demonstrate that this assay can be used for in vivo screens for antidiabetic compounds.[305]

6.3.3. Cancer. Solid tumor models in zebrafish are not very common. However, models have been created for melanoma[306] and rhabdomyosarcoma[209](Table 3). Xiang and colleagues used a simple zebrafish embryonic cell proliferation assay to identify potential tumor growth inhibitors of the cyclin-dependent kinase inhibitor class and later demonstrated that these compounds were effective in retarding the proliferation of cultured tumor cells.[307]

6.3.4. Regeneration. Zebrafish can regenerate a number of different organs including the heart,[308] liver,[309] pancreas,[147] and the fin.[311] Mathew and colleagues performed a chemical screen on larval fin regeneration and identified a hitherto unknown role for glucocorticoids in the process of regeneration.[310] Recently, it has also been demonstrated that it is feasible to perform chemical screens on adult models of fin regeneration.[311] Zebrafish adult heart can regenerate unlike mammalian hearts; however, high-throughput screens on adult heart regeneration are not practical. In lieu of an adult screen, Choi and colleagues created transgenic lines with the FUCCI live cell cycle sensors.[308] With these fish, they screened and identified small molecules that could enhance or inhibit cell proliferation in the growing embryonic heart. They found that these compounds could enhance/inhibit regeneration in damaged hearts in the adults. This approach circumvents the need for adult screens to identify compounds that may be active in the adult.

6.3.5. Behavior. Modeling diseases where we understand the biology and the molecular mechanisms is more straightforward than for complex processes such as behavior. Our understanding of basic brain functions that govern behavior is, at best, rudimentary. The use of animal models has revolutionized neurobiology and consequently disease biology.[312] Behavior modulating drug discovery has until now relied heavily on serendipity, and it is only recently that

technology and our understanding of the biology of human behavior has allowed us to systematize drug discovery for neuropsychiatric disorders. Zebrafish, with its complex behavior and experimental amenability, is fast becoming popular for use in dissecting the neural circuitry and molecular pathways important for regulating behavior.[313]

Kokel and Peterson developed a simple behavioral assay that could be quantified and barcoded. Young zebrafish embryos when exposed to bright light exhibit a vigorous stereotypic motor activity, which the authors name photomotor response[314] (Figure 17). By systematizing and classifying each segment of the behavior, they were able to generate a behavioral barcode for each fish.[315] Using this barcode, they screened through thousands of compounds to identify molecules that modulate any or all of the parameters. A similar screen was performed by Rihel and colleagues on the sleep/wake behavior of zebrafish embryos.[316] Both groups were able to classify and categorize the "hits" they got according to the different behavioral parameters and the structural features of the compounds. Extending their findings, they were able to predict the targets of unknown compounds with similar behavior.[314,316] More recently, Wolman and colleagues used visual and acoustic stimuli to create models of nonassociative learning in larval zebrafish. They used these assays to screen through a chemical library and identified known as well as unanticipated compounds with effects on learning and habituation.[317]

These screens lay the foundations for systematic in vivo high-throughput screening to identify neuroactive compounds. As described in the previous sections, pentylenetetrazole (PTZ) induces seizure-like behavior in zebrafish and has many similarities to epileptic seizures, and mutagenesis screens have identified genes involved in the behavior.[318] Similarly, mutants have been identified that modulate the sensitivity of adult zebrafish to addiction.[35,36] Thus, there are a number of complex behaviors in an organism as simple as zebrafish that may allow us to screen for compounds that may prove to be valuable in neurological and psychiatric disorders.

More recently, various private companies such as Evotec AG, PHYLONIX, UniServices Ltd. Zygogen LLC, ZF BioLabs, and InDanio Bioscience Inc. have begun offering disease model generation and screening services, making the benefits of the system easily accessible to scientists who are new to zebrafish.

6.4. Bioactivity Screens

Drug discovery efforts are constantly on the lookout for new compounds to test in various commercial and laboratory screens. The natural world, plants, fungi, and microorganisms, is a vast untapped resource of novel and complex small molecules. Many of these have biological activity in their own context. A one-stop strategy for selecting compounds with bioactivities relevant to vertebrates is to perform developmental screens using zebrafish embryos.[319,320] This has been demonstrated by Crawford and colleagues who were able to identify extracts from African plants that exhibited antiangiogenic activity and further isolate the active components from them using fluorescent transgenic reporters in zebrafish embryos for screening.[321]

Zebrafish embryos also allow for easy and informative structure activity relationship (SAR) studies to be conducted on synthetic derivatives based on biologically important scaffolds. As described previously, early developmental screens yielded a specific BMP inhibitor known as dorsomorphin.[287] Hao and colleagues recently demonstrated that this compound elicited angiogenic phenotypes in treated embryos and went on to perform SAR on a number of dorsomorphin derivatives to determine whether this phenotype was dependent on BMP signaling or VEGF signaling. Their studies revealed that angiogenic phenotypes could be ascribed to the off-target effects of dorsomorphin on VEGF, and they identified dorsomorphin analogues that did not cause these phenotypes.[322]Such screens to identify specific bioactivity of new small molecules or better define activities of structurally related compounds would accelerate the discovery process for novel drugs with natural origins.

7. TESTING

Discovery of drugs is only the first step toward developing viable therapeutic options. The drug and its interaction with the organism is of critical importance for the drug to be effective, efficient, and specific. Discovery of potential drugs is followed by testing them in animal models for absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox), before they are taken into the next phase of development. Mouse has been the system of choice for such studies because of its physiological similarity to humans and its experimental amenability even though it means an expensive and time-consuming investment. All potential drugs identified through cell-based or in vitro screens are routinely tested in mice for toxicity. Some of the common toxic side-effects that lead to drugs being withdrawn during clinical trials are life-threatening cardiac arrhythmia, hepatotoxicity, and teratogenicity.

Drug-induced cardiac arrhythmia usually manifests as QT interval prolongation in an electrocardiogram and is the result of off-target inhibition of the HERG K+ channels in the heart. Zebrafish heart electrophysiology is closely related to that of the human heart, and knock-down of HERG (KCNH2) channel in zebrafish leads to bradycardia in a dose-dependent manner.[323] Milan and colleagues performed a screen on zebrafish larval heart rate with known drugs and found that most of the drugs known to cause QT prolongation side-effects in human patients caused bradycardia in the embryos.[323] Thus, a zebrafish screen can predict possible heart side-effects for drugs before they go on trial.

As our understanding of diseases deepens and medical technologies improve, the possible treatment options for diseases have become more varied and customized. Therapeutic options have expanded from being just small molecule-based drugs to peptides,[324] peptidomimetics,[325] antibodies,[326] oligonucleotides,[327,328] and nanoparticles.[329] Hand-in-hand with the development in the therapeutics are studies to selectively target the therapeutic agent into the target tissue in the patient. When developing new and uncharacterized therapeutics, the challenge is to have a reliable system for testing toxicity, efficacy, and tissue-targeting for a large number of derivatives. Zebrafish provides an efficient first-level screening model for such studies.

Zebrafish has been used for individual studies on the toxicity of various kinds of nanoparticles,[330,331] and in one such study copper-based nanoparticles were found to be highly toxic to the organism.[332] Gross morphology as well as fluorescent-based assays have been utilized to develop a high-throughput screening platform for general and tissue-specific toxicity screening of nanoparticles.[333]

To develop tissue-targeting therapeutic strategies, it is imperative that animal models are used to predict targeting

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and dispersal in a patient. Zebrafish with its transparency offers a much more versatile alternative than mammals for visual screens of tissue-specific localization and tissue-specific effects.[334−336] Nanoparticle-mediated delivery of anticancer drugs to tumor tissue has been successfully tested in a zebrafish model.[337]

Noninvasive in vivo imaging is becoming an important diagnosis methodology in fields ranging from oncology to neurology. Development of such imaging-based diagnosis has to be done in animal models, usually mice and rat. However, zebrafish is a very valuable model for such studies because of its transparency. Initial stage testing of efficiency and fidelity of the diagnosis may be performed using visual screens in zebrafish. Techniques for automated high content high-throughput imaging of zebrafish have also been developed,[338] and these can be utilized for high-throughput screening of diagnostic probes .

8. ENVIRONMENTAL MANAGEMENT

Aquatic pollution is one of the most deleterious, as toxins in water have the potential to reach and affect organisms at every level of the food chain and thus can have far-reaching implications on the environment and human life. Water can be contaminated with pathogenic microorganisms or chemical contaminants. In this section, we shall focus primarily on the chemicals that pollute drinking water and public water bodies.

The major sources of chemical pollution in water are the domestic waste from cities, agricultural runoff from farmlands, and effluents from industries. Even though in most countries there are laws to ensure effective decontamination of wastewater before release into common water bodies, contaminated water remains a major problem the world over. To influence public policy and to develop treatments, efficient scientific methods for identification of pollutants and a clear understanding of their effect on human biology are important.

8.1. Monitoring Pollutants

The types of toxic chemicals in the water may vary significantly depending on the source of the contamination. However, there are a number of chemicals that are found commonly in contaminated water (water.epa.gov/drink/contaminants/). Some of these common pollutants are highly toxic bioactives such as dioxins, heavy metals, and hormones. Human beings, being at the top of the food chain, are exposed to a much higher concentration of these chemicals than found in the water sources because aquatic species accumulate them through bioconcentration. Development of sensitive assays in animal models such as zebrafish that occur naturally in water bodies can serve as "sentinels" for continuous monitoring of water quality. Previously, assays have been established for the detection of genotoxic compounds and estrogenic chemicals in fish species such as those based on observation of nuclear morphology in fish erythrocytes[339] or comparing sex ratios in medaka fish.[340] However, these assays are laborious and are not completely reliable or easily scalable to high-throughput monitoring of water bodies in the environment. Thus, there is a need for real-time methods for detection and reporting of toxic contaminants in water that are both fast and reliable.

8.1.1. Genetic Sensors. In previous sections, we have discussed ligand-induced gene expression in transgenic zebrafish. Similar principles may be applied to generate pollutant-detecting transgenic reporter lines. DNA sequence elements in the genome have evolved to respond to levels of biologically relevant organic and inorganic chemicals. In the presence of aryl hydrocarbons such as the pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the aryl-hydrocarbon receptor (AhR) binds to the AhR response elements (AhRE) on DNA and activates transcription of downstream genes.[341] Mattingly and colleagues cloned the cDNA encoding green fluorescent protein (GFP) downstream of the AhRE and generated a zebrafish transgenic line.[342] These zebrafish now respond to high levels of TCDD in the water by expressing the fluorescent protein, which can be detected with simple fluorescent microscopy. Similar transgenic lines exist that can activate fluorescence in response to sex hormones such as estrogen[343] and teratogens such as retinoic acid.[344] Blechinger and colleagues exploited the inducibility of the heat shock protein (hsp70) transcription via the metal response element (MRE) in response to heavy metals, to develop a GFP-based reporter that reports on cadmium contamination in water[345] (Figure 18).

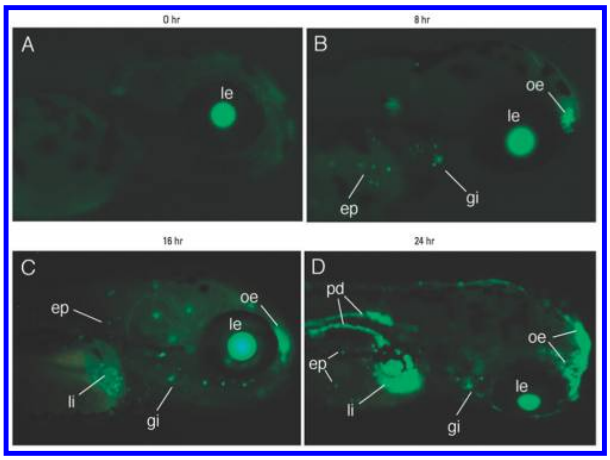

Figure 18. Heat-shock protein promoter-based detection of cadmium in water. (A−D) Green fluorescent protein (GFP) is expressed in various tissues in the zebrafish larvae in response to a 3 h exposure to cadmium followed by chase in clean water for (A) 0 h, (B) 8 h, (C) 16 h, and (D) 24 h. ep, skin epithelium; gi, gill; le, lens; li, liver; oe, olfactory epithelium; pd, pronephric ducts. Reproduced with permission from ref 345. Copyright 2002 U.S. Department of Health and Human Services.

Although easy to visualize with the appropriate microscopy, fluorescence signals are not quantitative. To circumvent this problem, luciferase (an enzyme that releases light in the presence of its substrate luciferin) may be used as a reporter to detect any of the above family of compounds. Using the hypopigmented *golden* line of zebrafish, Carvan and colleagues have established luciferase as well as GFP-based reporter lines to detect aryl hydrocarbons, heavy metals, sex hormones, and electrophilic oxidants.[346] Being partially transparent, these fishes can be used in the adult stage for whole animal imaging and can act as sentinels in water bodies to sense and warn of potential contaminants. Moreover, using multiple colored luciferase reporter genes now available, it would be possible to develop a single point quantitative assay for multiple toxins in the zebrafish.[347]

8.1.2. Chemosensors. Genetic sensors require creation of a number of different transgenic zebrafish lines, which is time-consuming and labor intensive. Instead, if the endogenous

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enzymes present in the fish are co-opted as reporters, even wild fishes may be used as sentinels for pollution.[348] A possible candidate for such reporters is the enzyme cytochrome P450, an enzyme that catalyzes the oxidation of a variety of organic substrates. Cytochrome P450 is induced by toxins such as TCDD and is an indicator of pollutants in the water. The induction of the enzyme can be assayed by exposure of zebrafish to ethoxyresorufin, a substrate for the enzyme, which catalyzes the production of a fluorescent product.[349] In another approach, Yang and colleagues have developed a rhodamine-based chemosensor of mercury that enables real time and stoichiometric monitoring of mercury accumulation in cells as well as in zebrafish larvae[350] (Figure 19). There are various other such chemical sensors of metals that have been developed to understand biological roles of these ions.[351]

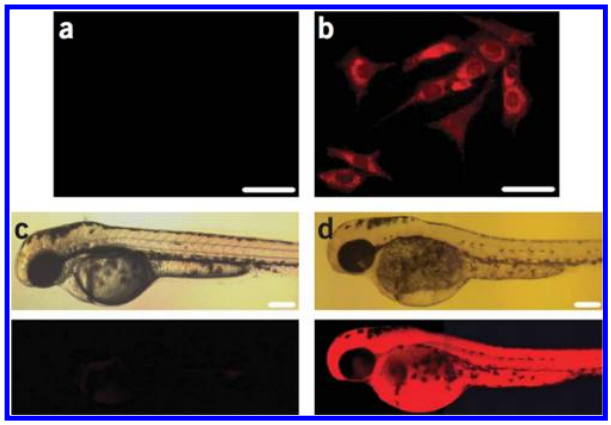

Figure 19. A rhodamine-based sensor for mercury detects mercury in cultured cells and zebrafish embryos. (a) Untreated and (b) mercury treated cultured muscle cells stained with Hg probe. (c) Zebrafish embryos stained with the Hg probe. (d) Zebrafish embryos treated with mercury and stained with Hg probe. Reprinted with permission from ref 350. Copyright 2007 Macmillan Publishers Ltd.

8.2. Detection of Novel Contaminants

With new manufacturing chemistry and new products, novel chemical contaminants get introduced into the environment with increasing frequency, some of which are highly toxic or dangerous to life forms. Designing specific assays or sensors is not possible for pollutants that are as yet undefined. To define such new toxins, general toxicity and teratogenicity assays are useful.

Toxicity may be assayed at the cellular or at the whole organism level. Organism-level viability assays are based on gross toxic effects on the organism and may identify highly toxic compounds that are strong enough to cause death. However, to identify compounds that are processed within the body to give rise to toxic byproducts that damage individual organs or affect the physiology or behavior of the organism in more subtle ways, it is essential to develop assays with complex readouts in the whole organism. Zebrafish embryonic stages offer an alternative for detecting and warning against toxins in the water, that may yet be unknown or uncharacterized.[352] Moreover, in contrast to screens with adult fish, embryonic zebrafish screens are more sensitive to lower concentrations of toxins because of critical patterning events that take place at younger stages. Many regulatory guidelines, such as the "EU Directive 2010/63/EU on the protection of animals used for scientific purposes", also exempt early embryonic stages (before free-feeding sets in) from protection.[353]

Zebrafish embryonic toxicity assays have been developed as an alternative for adult fish acute exposure test to detect toxins[354] in many countries but have been legally authorized for testing only in Germany.[355] This ISO certified toxicity test [ISO 15088:2007] is being used for testing wastewater treatment plant effluents in Germany.

It is worth noting that most of the sensors described in this section would need specialized equipment that can read fluorescence or luminescence to be able to detect toxins. However, sensors that elicit easily readable phenotypes such as pigmentation defects or morphological features such as tail fin, eye deformity, etc., would be much more useful as sentinels and would be of practical use in monitoring pollution by the general public as well as regulatory bodies.

8.3. Toxicology of Pollutants

The need for understanding the biological effects of the pollutants is 2-fold: one to trace the molecular pathways resulting in health problems and developing treatments for them, and the second to mobilize public support and convince the regulatory bodies of the need for action. Zebrafish offers a pliable model for toxicological studies as the results may be easily extended to humans due to the conservation of genetic information and physiological processes.

Certain compounds such as components of the musk affect the reproductive hormones in zebrafish and provide leads for the effects on mammals.[356] Other conditions such as hypoxia that is a consequence of dropping biological oxygen demand (BOD) in water also have effects on sex organs and sex determination in zebrafish.[357] Other endocrine effects also have been observed in fish exposed to polybrominateddiphenyl ethers, a common component of a number of everyday materials.[358]Bisphenol A (BPA) is found in various plastic materials and is recognized to be toxic to living organisms. Using in vivo studies on zebrafish, McCormick and colleagues discovered that *mycobacterium* species metabolizes BPA into derivatives that are even more toxic to the fish.[359]

Another aspect in pollutant toxicology where zebrafish has proved useful is in assaying and defining enantioselectivity of chiral pesticides. Zebrafish development can be used as an assay to assess the differential toxicity and molecular effects of different enantiomers of chiral pesticides.[360,361]

Thus, zebrafish as a model organism has multiple uses in combating the menace of pollution. As an aquatic model, zebrafish are well suited to act as sentinels of pollution, and as a well-studied genetic model organism they are highly informative in toxicology studies.

9. CONCLUSIONS

Zebrafish is a relatively "young" model organism. However, in the three decades or so of research, it has made important contributions to our understanding of vertebrate developmental biology and adult human disorders. In this Review, we have highlighted the biological advances that have been facilitated by this model organism as well as the new and exciting areas of research and tool development where zebrafish can be potentially important.

While new advances in zebrafish research await new tools, reagents, and approaches from the chemist, the system offers the chemical biologist with a highly accessible, amenable,

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complex, informative, and versatile vertebrate model to chart new territories in chemistry and biology.

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