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REVIEW

Zebrafish Embryos and Larvae: A New Generation of Disease Models and Drug Screens

Shaukat Ali, Danielle L. Champagne, Herman P. Spaink and Michael K. Richardson*

Technological innovation has helped the zebrafish embryo gain ground as a disease model and an assay system for drug screening. Here, we review the use of zebrafish embryos and early larvae in applied biomedical research, using selected cases. We look at the use of zebrafish embryos as disease models, taking fetal alcohol syndrome and tuberculosis as examples. We discuss advances in imaging, in culture techniques (including microfluidics), and in drug delivery (including new techniques for the robotic injection of compounds into the egg). The use of zebrafish embryos in early stages of drug safety-screening is discussed. So too are the new behavioral assays that are being adapted from rodent research for use in zebrafish embryos, and which may become relevant in validating the effects of neuroactive compounds such as anxiolytics and antidepressants. Readouts, such as morphological screening and cardiac function, are examined. There are several drawbacks in the zebrafish model. One is its very rapid development, which means that screening with zebrafish is analogous to "screening on a run-away train." Therefore, we argue that zebrafish embryos need to be precisely staged when used in acute assays, so as to ensure a consistent window of developmental exposure. We believe that zebrafish embryo screens can be used in the pre-regulatory phases of drug development, although more validation studies are needed to overcome industry scepticism. Finally, the zebrafish poses no challenge to the position of rodent models: it is complementary to them, especially in early stages of drug research. **Birth Defects Research (Part C) 93:115–133, 2011.** © **2011 Wiley-Liss, Inc.**

Key words: zebrafish; embryo; larvae; behavior; screens; toxicology; disease model

THE ZEBRAFISH EMBRYO IN DRUG SCREENING

The zebrafish (*Danio rerio*) is small, cheap to keep, fast to develop, and has high fecundity (Dahm and Geisler, 2006). Its early-stage embryos have a transparent body, making it relatively easy to collect numerous data points using high-quality imaging (including fluorescent imaging of transgenic lines). Annual maintenance costs for adult zebrafish are somewhat lower than those for rodents. However, this cost advantage is hugely multiplied when the test animal is a zebrafish embryo, because a female zebrafish can lay as many as 10,000 eggs per annum (Dahm and Geisler, 2006).

For these and other reasons, zebrafish embryos have been proposed as an *in vitro* animal model which could bridge the gap between simple assays based on cell or tissue culture, and biological validation in whole animals such as rodents (for reviews see Van den Belt et al., 2000; Shin and Fishman, 2002; Rubinstein, 2003; Goldsmith, 2004; Hill et al., 2005; Zon and Peterson, 2005; Dahm and Geisler, 2006; Reimers et al., 2006; Kari et al., 2007; Lieschke and Currie, 2007; Parng et al., 2007; Barros et al., 2008; Brittijn et al., 2009; Tsang, 2010).

The zebrafish embryo may be able to address the unmet need in biomedical research for low-cost, high-throughput whole-animal assays and models (Bull and Levin, 2000; Lieschke and Currie, 2007). *In vitro* assays offer the advantages of low cost, of being less prone to legal and ethical restrictions, and of having the ability to be scaled-up. By contrast, whole-animal assays provide data that are more easily extrapolated to humans and allow complex organismal functions (e.g., behavior and development) to be studied (Barnes, 1986).

After scaling up, it is possible, in principle, to reach high throughput (1000–10,000 assays per day; Verkman, 2004) or even ultra-high throughput (100,000 assays

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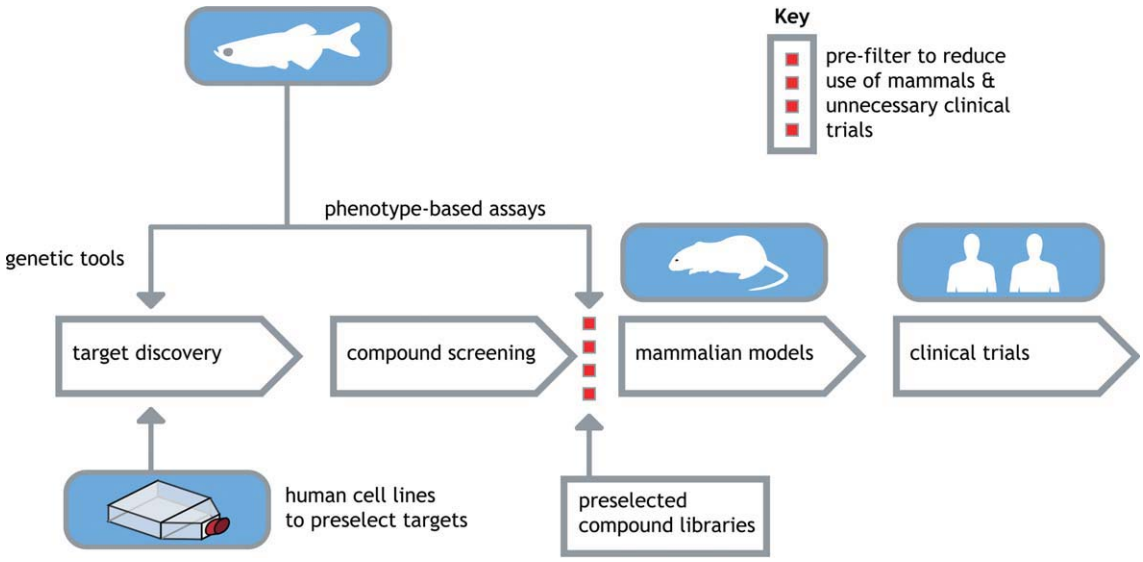

Figure 1. Drug discovery pipeline involving novel zebrafish models. This schematic illustrates a potential drug discovery pipeline showing the incorporation of novel approaches using cell-based and zebrafish assays into target discovery and zebrafish behavior-based assays into compound screen (Reproduced from Brittijn et al., 2009, with permission from UBC Press). The zebrafish model will never replace mammalian models in the drug development pipeline, particularly at later stages when the regulatory authorities demand studies in mammalians and clinical trials. Rather, the zebrafish model can serve as an invaluable screening tool in the pre-clinical phase, before rodent models, in the drug pipeline.

per day; Dove, 1999). Such large numbers of replicates increase the reliability of the statistics and allow rare (idiosyncratic) responses to be identified. Rare responses are most readily detected using "wild type" (pet shop) zebrafish with high genetic variability. Several zebrafish embryo assays can help to predict drug safety in humans (Berghmans et al., 2008; Redfern et al., 2008), and therefore zebrafish disease models have been developed (Flinn et al., 2008; Tanguay and Reimers, 2008; Brittijn et al., 2009).

We argue in this review that zebrafish embryos and early larvae can serve as invaluable screening tools in the pre-regulatory, preclinical phase of drug discovery. They can be used as a kind of filter that reduces the number of compounds passing through to testing on the much more expensive rodent models (Fig. 1). The zebrafish can never replace rodents in the later phases of drug discovery but may be complementary to rodent or cell-based assays at earlier stages. For a summary of some advantages and disadvantages of the zebrafish model, see Table 1.

What is a Zebrafish "Embryo"?

Strictly speaking, the fish embryo becomes a larva at hatching or when it begins exogenous feeding. It is then called a "larva" until it undergoes metamorphosis into a juvenile, finally being termed an "adult" when it is sexually mature (Falk-Petersen, 2005). In the zebrafish, hatching takes place between 48 and 72 hr post-fertilization (hpf). By convention, the zebrafish embryo takes the name of "larva" at 72 hpf, regardless of whether or not it has hatched (Kimmel et al., 1995). It remains a larva until the 30th day (Nüsslein-Volhard, 2002), when it undergoes metamorphosis and becomes a juvenile. At 3 to 4 months, it becomes sexually mature (Dahm and Geisler, 2006). In this review, we concentrate on embryos and few days old young larvae.

EMBRYO CULTURE PROTOCOLS

Zebrafish culture and breeding of zebrafish are discussed by Westerfield (2000); commonly used protocols are given online at http://zfin.org. In our laboratory, we keep adults at a maximum density of 100 individuals in glass recirculation aquaria (L 80 cm; H 50 cm, W 46 cm) on a 14-hr light : 10-hr dark cycle (lights on at 8:00 AM). Water and air are temperature controlled (28±0.5°C and 23°C, respectively). We feed adults twice daily with "Sprirulina" brand flake food (O.S.L. Marine Laboratory, and Burlingame, USA) and twice a week with frozen invertebrate larvae (Dutch Select Food, Aquadistri BV, The Netherlands).

Defined Embryo Buffer

Many laboratories use "egg water" (Westerfield, 2000) which is made up from Instant Ocean, a proprietary mixture of minerals. In some experiments, however, it may be useful to have a defined buffer, and in these cases we use 10% Hank's balanced salt solution. We make this with cell-culture tested, powdered Hank's salts, without sodium bicarbonate (Sigma Cat. No H6136-10X1L, Sigma-Aldrich, St Louis, MO) at a concentration 0.98 g/L in Milli-Q water

TABLE 1. Advantages and Disadvantages of the Use of Zebrafish in Biomedical Research

Feature	Benefit/Drawback
Advantages	
Easy maintenance	Low housing costs
Year round spawning	Research can run continuously
High fecundity (300–600 by single female at one time)	Low cost per assay
Optical transparency of early stages	Real-time (live) imaging of developmental processes and easy selection of precise developmental stages (in contrast to mammals)
Swimming begins at hatching (48–72 hpf) and more complex behavior (food seeking) at 5 dpf	Behavioral studies can be made on very early stages
Very rapid development	Large number of experiments possible in short time period
Fertilization is external	Embryos accessible noninvasively, can be continuously imaged; there is no placental barrier or maternal compartment to influence drug experiments
Minimal parental care	Reduced epigenetic parental influence on experimental outcome
Mutants available, genome sequenced, morpholino knockdowns possible	Genetic basis of teratogenesis can be investigated
Animal protection laws often less stringent for zebrafish embryos than for mammals	Fewer legal restrictions on research
Eggs develop in nonsterile, simple buffers	Easy to raise and maintain embryos
Genome has important similarities to human (e.g., nearly all mammalian genes have a zebrafish counterpart; high conservation of key developmental genes with the human)	Common molecular pathways can be studied
Very small size of early embryos	Only very low quantities of expensive test drugs and staining reagents needed; suitable for high throughput screening in 96 and 384 multiwell plates
Small egg size and external fertilization	Very precise control of drug delivery and dosage
Early embryo is permeable to small compounds	Suitable for drug testing
Disadvantages	
Last common ancestor with humans was 445 million years ago	Far more remote from humans than other animal models such as rodents (which have a 96 million year divergence time from humans)
Exothermic (cold-blooded)	Physiology not identical to humans
Anatomical differences with human (e.g., lack of heart septation, synovial joints, cancellous bone, limbs, and lungs)	Several human ethanol disorders are difficult or impossible to model in this species (e.g., cardiac septation defects)
Genome duplication	Many genes present as two copies, creating extra work to determine functional roles
Presence up to 48 hpf of the chorion	Interference with drug diffusion

Including information from Best et al. (2008), Tanguay and Reimers (2008), Grunwald and Eisen (2002), Gerlai et al. (2000), Gerlai (2003); Nei et al. (2001); Kimmel et al. (1995), Dahm and Geisler (2006), and Hisaoka (1958).

(resistivity = 18.2 MΩ·cm), with the addition of sodium bicarbonate at 0.035 g/L (Cell culture tested, Sigma Cat S5761) and adjusted to pH 7.46. A similar medium was previously used by Macphail et al. (2009) and Irons et al. (2010).

Embryo Care

Eggs are obtained by random pairwise mating of zebrafish. In our laboratory, we place three adult males and four adult females together in small plastic breeding tanks (Ehret GmbH, Emmendingen, Germany) the evening before eggs are required. The tanks (L 26 cm; H 12.5 cm, W 20 cm) have mesh across the bottom so that eggs will fall through and are protected from being eaten by the adults. The eggs are harvested the following morning and transferred into 92-mm plastic Petri dishes (50 eggs per dish) containing 40 ml fresh embryo buffer.

Eggs are washed four times to remove debris. Further, unfertilized, unhealthy and dead embryos were identified under a dissecting microscope and removed by selective aspiration with a pipette. At 3.5 hpf, embryos are again screened and any further dead and unhealthy embryos were

removed. Throughout all procedures, the embryos and the solutions are kept at 28±0.5°C, either in the incubator or in a climatized room. All incubations of embryos are carried out in an incubator with orbital shaking (50 rpm) under a light cycle of 14 hr light : 10 hr dark (lights on at 8:00 AM).

The reason why we screen the eggs so carefully is that substantial numbers of embryos may die during early stages. Such a "mortality wave" has been reported by Fraysse et al. (2006). In the report of the World Health Organisation/OECD on zebrafish assays (Organization for Economic Cooperation and Development, 1998) it was stated that "The mortality rate of the eggs is highest within the first 24 hpf. A mortality rate of 5% to 40% is often seen during this period." In our laboratory, we find a cumulative mortality and infertility rate of 9% and 5% respectively at 24 hr (Ali et al., 2011).

Culture Vessels

The containers used for the growing-on of zebrafish embryos for screening purposes vary widely between laboratories. Examples include 15-L aquaria (Gerlai et al., 2000), 92-mm Petri dishes with 60 embryos per dish (Fernandes and Gerlai, 2009), 96-well plates with one to three embryos per well (Macphail et al., 2009; Yang et al., 2009), $8 \times 6 \times 2$ cm^3 chambers with 10 embryos per chamber (Lockwood et al., 2004), five-gallon aquaria (Dlugos and Rabin, 2003), six-well plates with 10 embryos per well (Dlugos and Rabin, 2007), 24-well plates with 4 to 30 embryos per well (Carvan et al., 2004; McKinley et al., 2005; Ton et al., 2006; Berghmans et al., 2008), and glass beakers (Kashyap et al., 2007).

We and several other laboratories (Macphail et al., 2009; Peal et al., 2011; Truong et al., 2011) use ANSI/SBS format 96-well microtiter plates to isolate and track individual embryos. A single embryo can be cultured in each well, in a volume of 250 μL buffer. In principle, the embryo can survive at least 5 days without buffer refreshment. The 96-well format is also ideal for use in Viewpoint (France) and Noldus (The Netherlands) behavior analysis systems.

Microfluidic Devices

In microtiter plates, the buffer is refreshed periodically ("static renewal") or not at all ("static non-renewal"; see United States Environmental Protection Agency, 2002). It is not known what effect periodic aspiration and replacement of the buffer has on zebrafish larvae; it is conceivable that it causes stress to the young fish, although this has not been proven. Static replacement regimes may not be ideal for the zebrafish, a species which normally breeds in slow-flowing waters (Spence et al., 2008). For these and other reasons, microfluidic culture systems are being investigated.

One example is the static nonrenewal culture of zebrafish embryos inside Teflon tubing, each embryo being isolated in a drop of buffer (Funfak et al., 2007). Chronic exposure to drugs is possible in such a system, but the embryo is not accessible during the experiment. Furthermore, culture in Teflon tubing involves distortion of the image because of the curved surfaces and does not provide continuous buffer refreshment.

Another approach to the microfluidic culture of zebrafish embryos was developed by a student team and reported in an educational-themed issue of the journal *Zebrafish*. Unfortunately, no biological data were given in that paper, although the authors claimed that the zebrafish could survive for a few days in their single-well polydimethylsiloxane open Petri dish setup (Shen et al., 2009).

We have shown using a custom-designed lab-on-a-chip made of glass, in which zebrafish embryos can be cultured in a continuous flow-through ("dynamic renewal") of pressurized buffer (Wielhouwer et al., 2011). In such a system, the embryos are continuously accessible and isolated in parallel arrays to prevent cross-contamination. In our chip, the volume of each well was only 9 μL and we could conduct real-time imaging of the embryo at all stages. A buffer flow of 2 μL/(well min) was found to be optimal for zebrafish embryos (Wielhouwer et al., 2011).

COMPOUND DELIVERY TO THE EMBRYO

Until the embryo hatches at 48 to 72 hpf (Kimmel et al., 1995; Dahm and Geisler, 2006) it is surrounded by the chorion, which represents a barrier that can reduce drug diffusion (Mizell and Romig, 1997; Braunbeck et al., 2005). Therefore, if stages before hatching are to be treated with drugs, special consideration must be taken of the chorion.

Penetration of Drugs Through the Chorion

The chorion, which envelopes the embryo until hatching, substantially slows down the diffusion of small molecules into the embryo. It is perforated by "chorion pore canals" of around 0.5 to 0.7 μm diameter, and spaced at 1.5 to 2.5 μm intervals (center-to-center; Lee et al., 2007). The chorion pore canal has a viscosity 26× higher than egg water, and this has been shown the limit the diffusion of nanoparticles, (Lee et al., 2007). Small molecules such as ethanol can diffuse slowly through the chorion, especially if a high concentration is applied externally; they can then be quite rapidly cleared away if the embryo is washed several times with buffer (Fig. 2).

One way to overcome the barrier provided by the chorion is to digest it away with Pronase (www.zfin.org). An alternative is perivitelline injection (Mizell and Romig, 1997) which involves delivering the drug through the chorion into the underlying perivitelline space (i.e., the gap between the yolk sac/embryo, and the chorion). Perivitelline injection of volumes of drug as low as 40 nL can produce marked biological effects in zebrafish embryos (Mizell and Romig, 1997).

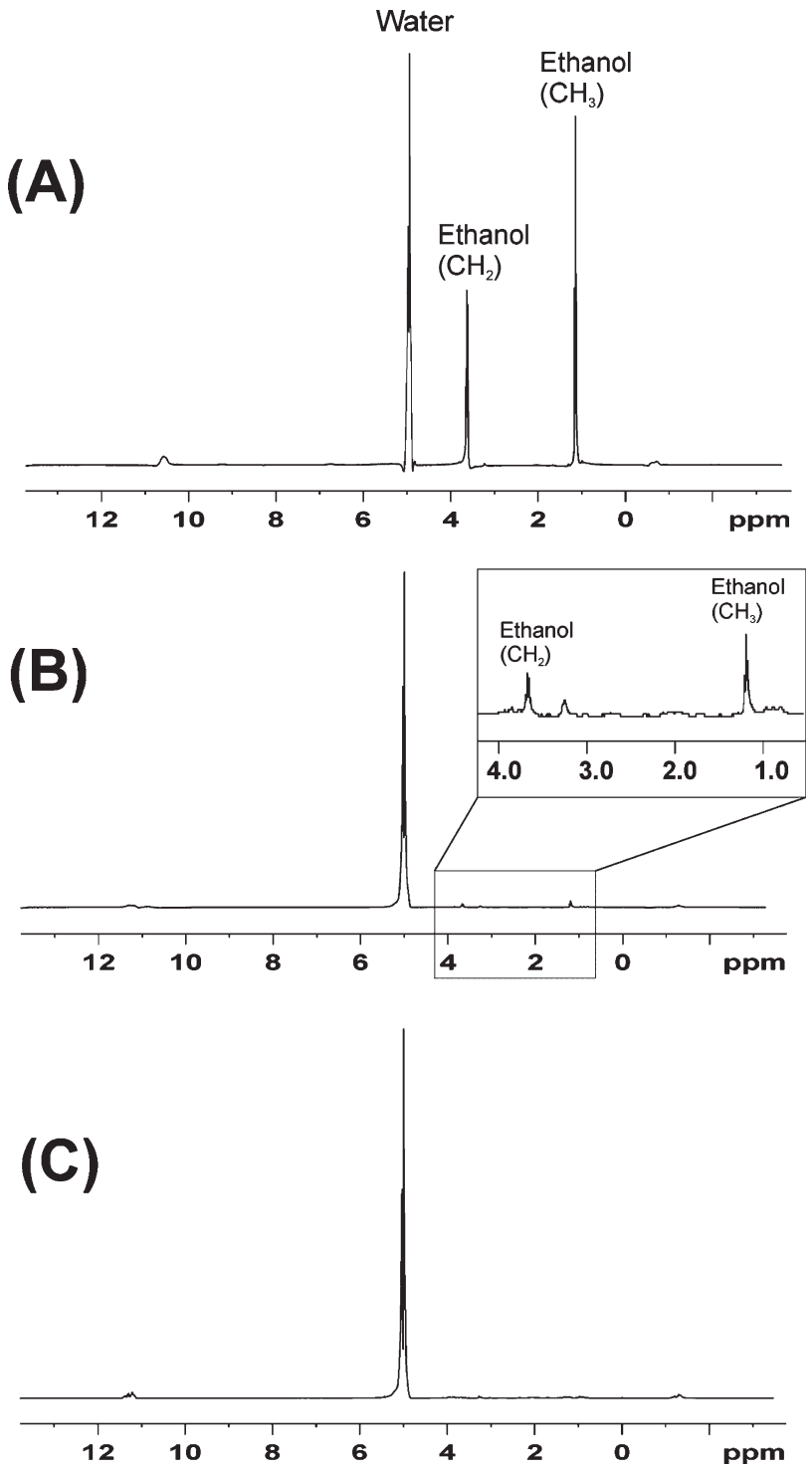

Figure 2. The chorion as a barrier to drug entry. HR-MAS ^{1}H MRS spectra of intact embryos treated with 10% ethanol for 1 hr and then spectra were measured (**A**) without subsequent washing, the ethanol level in the embryos had risen to 0.86% (**B**) after washing three times with buffer, the ethanol concentration in the embryos had fallen to 0.0003%. Spectrum in the inset of (B) is enlarged 30 times in *y* axis (**C**) after washing three times with buffer and subsequently allowed to grow for another 1 hr. the ethanol concentration in the embryos had fallen to 0.0% (Ali et al., 2011).

A technique for robotically microinjecting compounds into the embryonic tissue has also been developed (Wang et al., 2007).

Duration and Stage of Administration

In chronic exposure assays, it may be sufficient to add the test compound to the embryo buffer and leave it without replacement for the duration of the assay. In acute exposure regimes, however, the drug will have to be rinsed away after the exposure. Zebrafish develop very rapidly, and so screening with zebrafish embryo is a bit like "screening on a runaway train," as a colleague of ours has so vividly described it. In other words, it may be necessary to choose a very precisely defined time window for the drug exposure to ensure that all embryos are at the same stage of development. Unless this is done, errors arising from staging differences may be introduced into the data.

There is variation in the time at which embryos reach a particular stage (Kimmel et al., 1995). Therefore, adding a compound at a certain number of days or hours postfertilization is not guaranteed to produce a standardized stage of exposure. Because age is a poor guide to developmental maturity, it is much better to standardize the maturity of embryos using a developmental staging system, such as that reported by Kimmel et al. (1995). Unlike mammalian models, zebrafish embryos are easily staged because fertilization is external; they develop entirely outside the mother's body and are transparent (Table 1).

READOUTS AND READOUT TECHNOLOGIES

Readouts are the various types of data collected during, or at the end, of the assay. We give here a small selection of behavioral, fluorescent, morphological, and cardiac readouts and their associated technologies.

Behavioral Readouts

Despite obvious differences between zebrafish and humans, the zebrafish possesses a series of qualities that make it complementary to the mammalian models currently used in the behavioral sciences. This is because zebrafish share extensive homologies to other vertebrate species (including rodents and humans) in terms of their genome, brain patterning, and the structure and function of several neural and physiological systems, including the stress-regulating axis (Postlethwait et al., 2000; Rodriguez et al., 2002; Tropepe and Sive, 2003; Guo, 2004; Sison et al., 2006; Lieschke and Currie, 2007; Schaaf et al., 2008; Veldman and Lin, 2008; Guo, 2009; Morris, 2009; Pogoda and Hammerschmidt, 2009; Steenbergen et al., 2010; Burne et al., 2011).

Larval zebrafish are also particularly well suited for behavioral testing because of their maturity in terms of swimming capacity, and functionality of the motor, sensory, and stress-regulating systems, and ability to perform simple motor tasks and perceive relevant cues for the environment (Drapeau et al., 2002; Guo, 2004; Lockwood et al., 2004; Best and Alderton, 2008; Best et al., 2008; Emran et al., 2008; Kokel and Peterson, 2008; Guo, 2009; Irons et al., 2010; Kokel et al., 2010). These features make the zebrafish of interest for drug discovery in psychiatry where the discovery of new medicines is lagging behind relative to other clinical disciplines (Agid et al., 2007).

It is clear that zebrafish embryos will never develop a full range of complex, human-like disorders. However, they can be used to study certain biological markers (endophenotypes) of these disorders. A good example is dysfunction of the stress-regulating system referred to in humans as the hypothalamic-pituitary-adrenal axis. Dysfunction of this system plays an important role in the onset of several physiological disorders (e.g., hypertension) and also provides biological markers of depression (Holsboer, 2000; Pariante, 2003; Hasler et al., 2004).

Dysfunctions of the stress-regulating system are typically studied in rodent models using assays for stress/anxiety responses and cognition (Heim et al., 2004, 2008; Champagne et al., 2009; Meaney, 2010). Recently, the zebrafish has been used as an alternative model (Steenbergen et al., 2010), and several of the traditional rodent behavioral assays have been successfully adapted and pharmacologically validated for use in zebrafish (Gerlai et al., 2000; Lockwood et al., 2004; Levin et al., 2007; Best et al., 2008; Emran et al., 2008; Lopez-Patino et al., 2008; Guo, 2009; Cachat et al., 2010a,b; Champagne et al., 2010; Gerlai, 2010a, 2010b; Maximino et al., 2010a,b, 2011; Steenbergen et al., 2010; Stewart et al., 2010; Stephenson et al., 2011). Recent studies have also shown the feasibility of using larval zebrafish for high throughput behavioral-based drug screening (Kokel and Peterson, 2008; Kokel et al., 2010; Rihel et al., 2010). It is noteworthy that recent evidence also supports the feasibility and usefulness of adult zebrafish for medium throughput screening (Pather and Gerlai, 2009; Gerlai 2010a, 2010b).

Behavioral assays customized for zebrafish larvae often use multiwell plates (Gutman and Nemeroff, 2003; Muto et al., 2005; Berghmans et al., 2007; Burgess and Granato, 2007; Kokel and Peterson, 2008; Gerlai 2010a; Kokel et al., 2010; Rihel et al., 2010). Examples of customized behavioral (locomotor) assays for larval zebrafish conducted in multiwell plates include the acoustic startle test (Best et al., 2008), seizure liability test (Berghmans et al., 2007; Winter et al., 2008), visual safety assay (Richards et al., 2008), and the visual motor response test as discussed (see below; Emran et al., 2008; Macphail et al., 2009; Irons et al., 2010; Rihel et al., 2010).

Anxiety Assays

Light/dark preference test

The light/dark box and open field tests are well suited for zebrafish research as they are relatively simple, painless, and unconditioned tests that can readily assess spontaneous/natural tendency of an animal to explore or avoid a novel environment depending on the degree of aversiveness (Sousa et al., 2006). The light/dark box test is based on the innate aversion of brightly lit environments in rodents (Hascoet et al., 2001; Bourin and Hascoet, 2003). Several studies using rodent models have shown that the amount of time spent in the dark compartment represents a measure of light aversion (Hascoet et al., 2001; Bourin and Hascoet, 2003). Clinically effective antianxiety drugs (e.g. diazepam) can attenuate such avoidance behavior supporting a link between light-aversion behavior and anxiety in this paradigm (Hascoet et al., 2001; Bourin and Hascoet, 2003).

A similar version of the light/dark box test has been previously adapted for adult and larval zebrafish and shows that, if given a choice between bright and dark environments, both larval and adult zebrafish (Steenbergen et al., 2010) display dark-avoidance behavior and a significant preference for the bright area (Fig. 3). These results are in agreement with the natural ecology of this species. Thus, dark-avoidance behavior has been proposed to be an adaptive response for diurnal species like zebrafish, as they rely on vision and lit environments to capture prey and avoid predators in nature (Burgess and Granato, 2007; Emran et al., 2008; Macphail et al., 2009).

We have shown that treatment with antianxiety drugs reduces dark-avoidance behaviors in larval zebrafish in a manner similar to that observed in other species (Fig. 4). These findings support the hypothesis that dark-avoidance behaviors in zebrafish are part of a repertoire of anxiety-like behaviors; this validates the use of the light/dark preference test as a valid test for drug screening

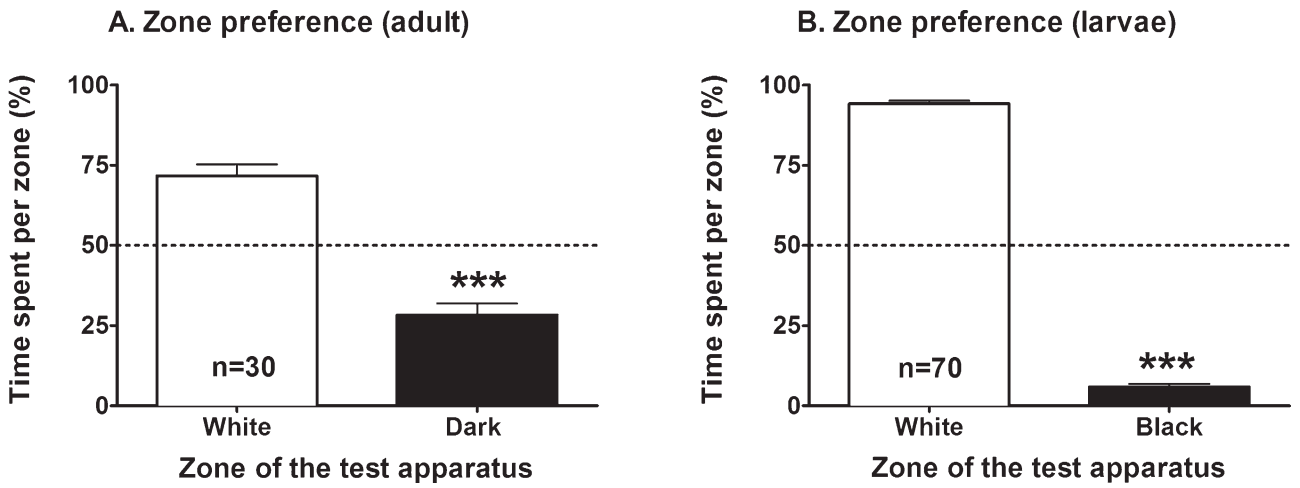

Figure 3. Light/dark preference test adapted for adult and larval zebrafish. Both adult (**A**) and larval (**B**) zebrafish display strong dark-avoidance behavior and show preference for the bright/white area in the light/dark preference test. Note that preference for the bright compartment is well above the 50% chance level for both adult and larval zebrafish. Statistical icons: ***$p < 0.001$. A is reproduced from Champagne et al., 2010 with permission from Elsevier B.V. B is reproduced from Steenbergen et al., 2011 with permission from Elsevier B.V.

(Steenbergen et al., 2010). While our findings are in agreement with some previous observations (Gerlai et al., 2000), they are in disagreement with others (Guo, 2004; Serra et al., 1999; Blaser et al., 2010; Grossman et al., 2010; Maximino et al., 2010a, 2010b, 2011). The reason for this discrepancy has not been yet resolved.

The open field test

This test measures the reactions of an individual to novel, large spaces. The individual faces a dilemma between finding food, mates, and other advantages in the space or being confronted with a danger (Prut and Belzung, 2003; Sousa et al., 2006). The aversive properties of the novel environment may inhibit or reduce exploratory behavior and promote thigmotaxis (wall-hugging or wall-following behavior; Treit and Fundytus, 1988; SharMa et al., 2009). Thigmotaxis has been seen in a wide range of species, including fish (SharMa et al., 2009).

We recently customized the open field test for use in both adult and larval zebrafish using a 24-well plate as an open field apparatus. Similar to adult zebrafish, we showed that larval zebrafish also exhibit thigmotaxis (Steenbergen et al., 2010) in response to a sudden transition from light to dark. Importantly, such behavior could be significantly attenuated with the anxiolytic drug diazepam (data not shown) in a manner similar to that which is observed in other species (Treit and Fundytus, 1988).

The pattern of exploratory behaviors reported above, which includes locomotor activity patterns, thigmotaxis, and habituation learning when facing a novel environment are not only well conserved between species, including rodents (Treit and Fundytus, 1988; Simon et al., 1994; Choleris et al., 2001; Sousa et al., 2006), fish (Lockwood et al., 2004; Lopez Patino et al., 2008; SharMa et al., 2009; Champagne et al., 2010), and humans (Kallai et al., 2005, 2007; Henry et al., 2010) but also emerge early in life and serve as a good predictor of adult patterns of behavior (Henry et al., 2010; Kavsek and Bornstein, 2010; Wong et al., 2010). The open field test has proven to be a good animal-to-man translational system (Perry et al., 2009; Henry et al., 2010). In humans, the open field test is referred to as the human behavior pattern monitor test and is successfully used in humans to discriminate between different psychiatric conditions, such as bipolar disorder, unipolar depression, and schizophrenia (Perry et al., 2009; Henry et al., 2010).

The visual motor response test

This test consists of frequent alternations between periods of light and dark (each period lasting 10–30 min) and results in behavioral patterns characterized by low (basal) locomotor activity under light exposure and transient but robust behavioral hyperactivity on sudden transition to dark (Burgess and Granato, 2007; Emran et al., 2008; Macphail et al., 2009; Irons et al., 2010; Rihel et al., 2010). Sudden transition to dark induces a visual startle response characterized by a sharp spike of fast-swimming activity ($\geq$20 cm/sec) lasting under 2 sec (Burgess and Granato, 2007; Emran et al., 2008; Macphail et al., 2009; Irons et al., 2010; Rihel et al., 2010). Locomotor activity (total distance moved) is elevated for the first 2 to 4 min and then gradually returns to baseline after 10 min.

Sudden transition to light also causes larval zebrafish to display a brief spike of fast-swimming activity of $\geq$20 cm/sec — less than that induced by sudden dark (Burgess and Granato, 2007; Emran et al.,

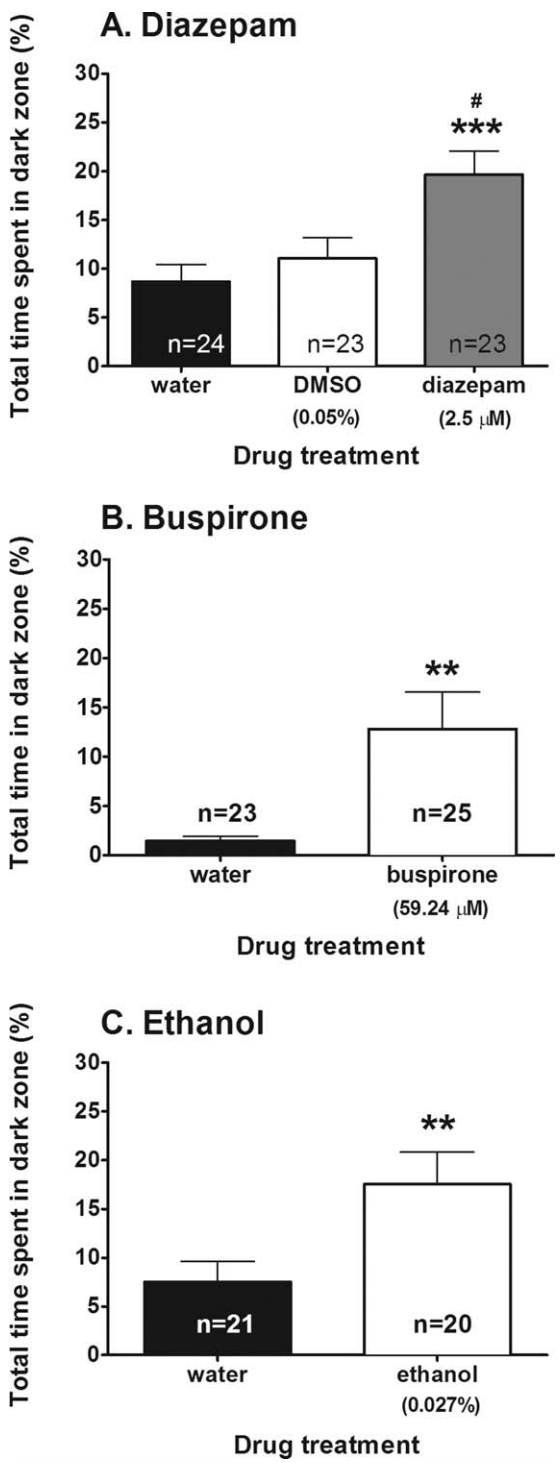

Figure 4. Impact of drug treatments on zone preference in the light/dark preference test in larval zebrafish. Analysis of the effects of anxiolytic drug treatments on the percentage of time spent in the dark compartment revealed that diazepam (**A**), buspirone (**B**), and ethanol (**C**) all exerted an anxiolytic effect in the light/dark preference test by significantly increasing the percentage of time spent in the dark compartment of the testing apparatus. Note that the organic solvent DMSO did not exert any significant effects on zone preference relative to control (egg water). Statistical icon: $**p < 0.01$ relative to control (egg water), $***p < 0.001$ relative to control (egg water), and $\#p < 0.05$ relative to DMSO. Abbreviations: DMSO = dimethyl sulfoxide. Adapted from Steenbergen et al., 2011 with permission from Elsevier B.V.

2008; Macphail et al., 2009; Irons et al., 2010; Rihel et al., 2010). Return to basal activity is attained within 1 min of light exposure. An intact visual system is required to perform this test as larvae of *chokh* mutant zebrafish (which lack eyes and therefore are blind) do not respond to light–dark transitions (Emran et al., 2008).

The visual motor response test has already been proven to be highly effective in the assessment of drug effects on relatively simple locomotor behaviors, which provided the first proof-of-concept for high-throughput screening in zebrafish larvae (Emran et al., 2008; Macphail et al., 2009; Irons et al., 2010; Rihel et al., 2010). We also show that this test can be used to assess the integrity of the nervous system in a zebrafish model of fetal alcohol syndrome (FAS; Fig. 5).

Fluorescent Readout Technologies

Owing to its small size and optically transparent embryos, the zebrafish is excellently suited for fluorescent imaging. In Figure 6, we give an example of how fluorescent screens can be incorporated into a drug development pipeline. In adult zebrafish, fluorescence imaging is facilitated by using albino mutants, the most popular being the Casper mutant (Pugach et al., 2009). The relative ease of making transgenic zebrafish using the Tol2 transposon technology (Suster et al., 2009) has led to many lines that express promoters fused to green fluorescent protein (GFP) variants. The most used GFP variant is enhanced GFP. However, with the availability of many new genes encoding color-shifted GFP variants and red-shifted fluorescent proteins from corals with higher quantum yields, many new transgenic zebrafish lines will be constructed in the near future.

For instance, the newly developed MTurquouise (Goedhart et al., 2010) is highly suited to be combined with SYFP2 (Kremers et al., 2006), MCherry (Shu et al., 2006), and the near infrared E2-Crimson

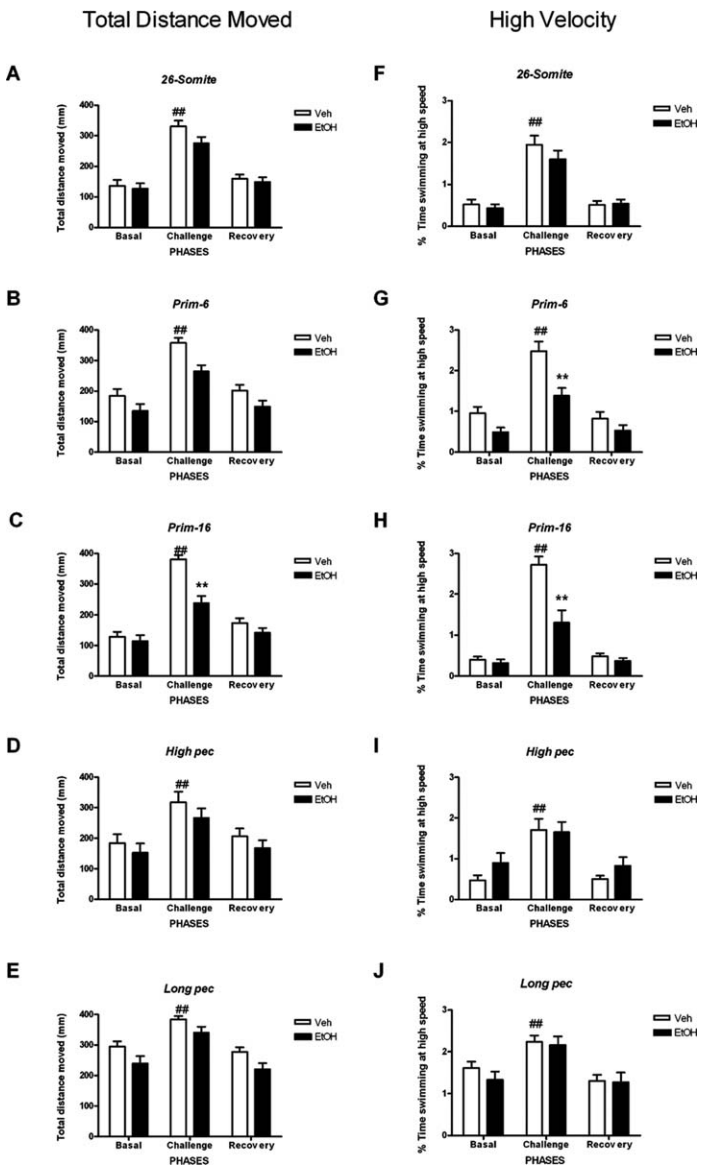

Figure 5. Behavioral performance in the visual motor response test. Analysis of the total distance moved (**A**–**E**) and percentage of time spent swimming at high velocity (**F**–**J**) were assessed in 5 dpf larvae exposed to the light–dark challenge test. The visual motor response test assesses behavioral responses to varying lighting conditions and is divided into four phases: (1) habituation phase (2-min habituation to light) is omitted for sake of clarity, (2) basal phase (4 min exposure to light, assesses basal activity), (3) challenge phase (4 min exposure to sudden darkness, triggers robust behavioral hyperactivity), and (4) recovery phase (4 min exposure to light, assesses return to basal activity). Behavioral analysis reveals that ethanol-treated embryos swam significantly less (reduced total distance moved) in the challenge phase (lights off) compared to the vehicle-treated controls only when ethanol exposure occurred at *prim-16* but not other stages (C). This finding is paralleled by a significantly reduced ability to maintain swimming velocity at a high speed (>20 mm/sec) (H). Furthermore, general decreases in total distance moved, regardless of the phases, are observed in ethanol-treated embryos at stages *prim-6* (B) and *long-pec* (E), suggesting general hypoactivity, a finding that is also accompanied by significant reduction in the ability to maintain swimming at high velocity for larvae ethanol-treated at stage *prim-6* (G) but not *long-pec* (J). Note that stages *26-somite* (A) and *high-pec* (D) appear spared from the impact of ethanol exposure on behavioral outcome. Each error bar represents ±SEM of $N = 37, 37, 32, 29, 27$ embryos for vehicle and 39, 28, 26, 16, 28 for ethanol treatment at *26-somite*, *prim-6*, *prim-16*, *high pec*, and *long pec* respectively. #Depicts differences within treatment group. *Depicts differences between treatment groups. Statistical icons: $^{\#\#}p < 0.01$, $^{*}p < 0.05$, and $^{**}p < 0.01$.

(Strack et al., 2009), providing the opportunity to simultaneously monitor four colors. In addition to autofluorescent protein genes, research in zebrafish also makes use of small molecular fluorescent probes. For instance, common use is made of Alexa dyes (Life Technologies) for whole mount in situ fluorescence hybridization (Clay and Ramakrishnan, 2005; Welten et al., 2006; Brend and Holley, 2009) or immunohistochemistry (Campos et al., 2011).

For analysis of the factors that influence drug administration, it has been shown that zebrafish embryos can directly take up artificial antisense DNA molecules, labeled with rhodamine, and added to the swimming water. These molecules could even been targeted to the nucleus (Spaink and Bagowski, 2009).

For the detection of fluorescent molecules, use can be made of confocal laser scanning microscopy (CLSM) and of camera systems with spectral unmixing (Kaijzel et al., 2007). The high quantum yield of some of the autofluorescent proteins even makes imaging at the single molecule level possible (Schaaf et al., 2009). For high throughput imaging, use can be made of microtiter plate CLSMs such as the Becton Dickinson (BD) Pathway analysis system (Becton, Dickinson and Company). Rapid advances in new imaging technologies will facilitate fast, high-resolution imaging methods such as sheet illumination, for which the zebrafish embryos are ideally suited (Keller et al., 2008, 2010).

Using two-photon fluorescence imaging using second or third harmonic generation technology, it is also possible to image directly matrix components, such as collagen, without the need of prior labeling (Campagnola et al., 2001; Bianchini and Diaspro, 2008; Hsieh et al., 2008). This enables direct imaging of processes such as remodeling of the extracellular matrix in a living embryo. In future research, we expect that fluorescence detection tools will be complemented by luminescence measurements. Standards for

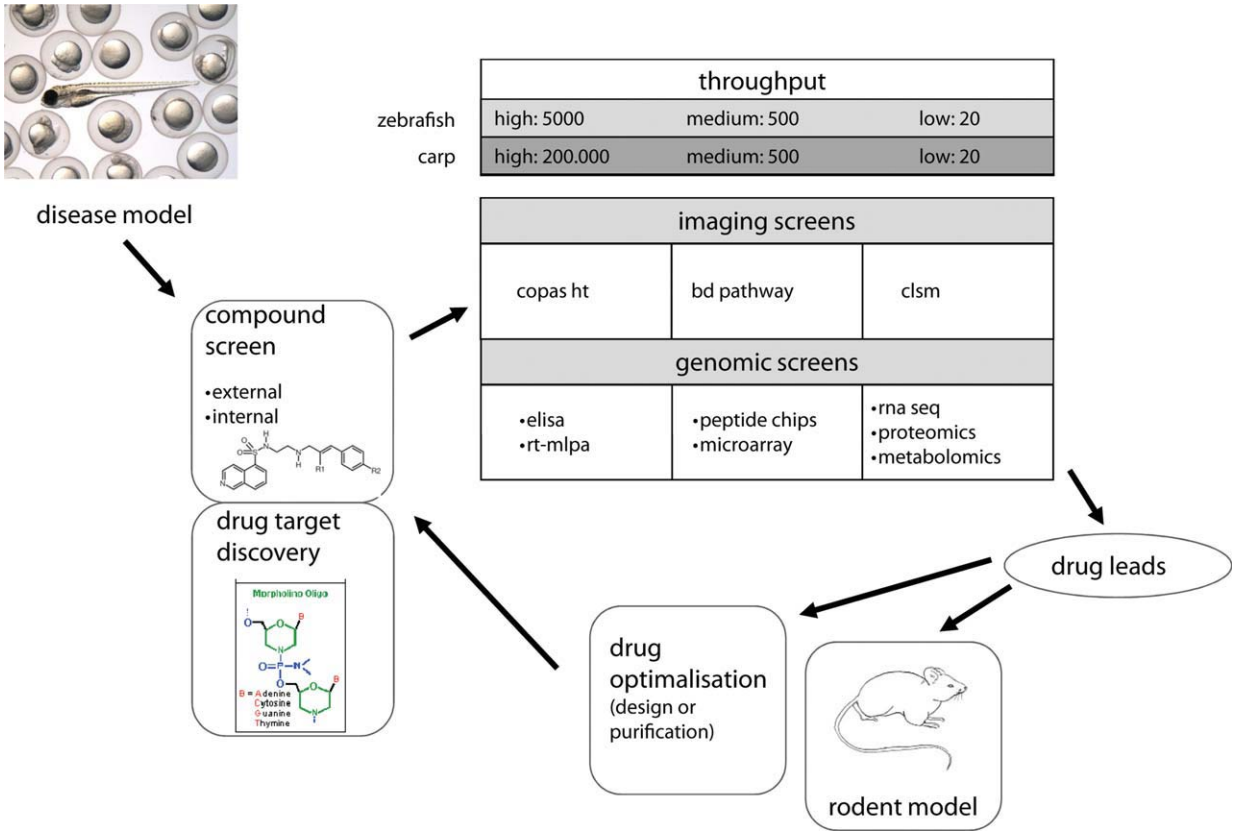

Figure 6. A general screen strategy using zebrafish embryos in the context of the use of other model organisms is shown based on the strategy used by the company, ZF-screens for discovery of new drugs against tuberculosis. Going though the figure clockwise from the left: the zebrafish embryos are injected at high throughput with pathogenic agents (microbes or cancer cells). They are subsequently treated with small molecular compounds, in the easiest embodiment (''external'') by adding them to the swimming water. Coinjection of drugs (''internal'') is also feasible. The effect of the compound can be measured using the Copas device (Carvalho et al., 2011) or at lower throughput and higher resolution using the BD pathway or CLSM. Other readouts are reverse transcription-multiplex ligation probe amplification, microarrays, and RNA deep sequencing (''RNA seq''). Disease screening at the protein level can make use of ELISA, peptide chips, or proteomics. Another cyprinid fish that can be used is the common carp that yields up to 200,000 eggs per spawning. Drug leads can be tested in rodents. Hits will be subjected to further optimization, for instance, by retesting chemical derivatives in the zebrafish. In the case of natural products, further fractions can be retested. Finally, targets for the identified drugs can be probed with antisense morpholino technology. This could be particularly powerful if the drug functions via upregulation of a signaling pathway; in such cases, it is expected that morpholinos can be identified that block the effect of the drug.

such applications have been set in the mouse model for instance in application in cancer studies (Henriquez et al., 2007). This will enable us to evaluate results from high throughput studies in embryos for their relevance in adult fish. For instance, the Gaussia luciferase probe (Sharma et al., 2011) will be useful for high throughput assays as it is secreted and therefore has the potential to be measurable in the swimming water of the fish. For quantification purposes, this tool will be useful in embryo high throughput screening, even when a combination with cellular imaging is not needed, for example in applications in infectious disease studies (Andreu et al., 2010).

Morphological Assessment

Embryos have been examined for a range of morphological parameters (Hisaoka, 1958; Arslanova et al., 2010; Brannen et al., 2010; Sawle et al., 2010; Yang et al., 2010; George et al., 2011; Hermsen et al., 2011). Large-scale mutagenesis screens often involve the assessment of a range of phenotypic traits by a researcher using a dissection microscope (Mullins et al., 1994; Haffter et al., 1996). In other cases, one may simply screen for a very few drug-specific defects. For example, in ethanol teratogenecity screens in zebrafish embryos, common readouts include: developmental retardation, pericardial and yolk-sac edema (Reimers et al., 2004; Giles et al., 2008), reduction in body length (Loucks and Ahlgren, 2009), branchial skeleton defects (Carvan et al., 2004), abnormal eye development (Stromland and Pinazo-Duran, 2002; Bilotta et al., 2004; Matsui et al., 2006; Dlugos and Rabin, 2007; Kashyap et al., 2007) as well as cognitive defects (Carvan et al., 2004; Reimers et al., 2006) and higher mortality (Loucks and Carvan 2004).

TABLE 2. Selected Zebrafish Models of Human Diseases and Syndromes

Human condition	Zebrafish model	Zebrafish genes	References
Cardiac arrhythmia: short QT syndrome	Reggae mutant (reg)	*zERG*	Hassel et al. (2008)
Cardiac arrhythmia: QT prolongation	Rate of atrial and ventricular contraction	–	Langheinrich et al. (2003)
Parkinson's disease	Oxidative stress, dopamine neuronal loss	*DAT*, *TH*, and *Dj-1*	McKinley et al. (2005), Bretaud et al. (2004, 2007)
Inflammatory bowel disease	Gut morphology, peristalsis	–	Fleming et al. (2010)
Epilepsy	Startle response	–	Berghmans et al. (2007)
Cerebral cavernous malformations	Ccm1 mutant	*Ccm1*	Liu et al. (2011)
Polycystic kidney disease	Bicaudal C and polycystic kidney disease mutant (Bicc1, Pkd2)	*Bicc1*, *Pkd2*,	Bouvrette et al. (2010)
Ullrich congenital muscular dystrophy	Collagen VI mutant (Col6a1)	*Col6a1*	Telfer et al. (2010)
Polycythemia vera	Janus kinase 2 mutant (jak2a)	$jak2a^{V581F}$	Ma et al. (2009)
Waardenburg syndrome type IV	Sex determining region Y mutant (sox10)	*fgf8, sox9a*, *sox9b*, and *sox10*	Dutton et al. (2009)
Variegate porphyria (porphyrias)	Montalcino mutant	*ppox*	Dooley et al. (2008)
Cancer	Transplantations of cancer cell lines (WM-266-4, SW620, FG CAS/Crk, CCD-1092Sk). Quantification of cancer cells in zebrafish	–	Haldi et al. (2006)

We do not duplicate here the disease models in zebrafish already listed by Kari et al. (2007b).

Readouts of Cardiac Function

Various methods have been reported for assessing cardiac function in zebrafish embryos and larvae. Microelectrodes can be used to record compound action potentials (Hassel et al., 2008). Heart rate can be recorded manually from live embryos or from live observation (Mittelstadt et al., 2008; George et al., 2011) and/or videotape recording (Langheinrich et al., 2003). Larvae can be immobilized using anesthetics during these recordings; MS-222 has been found to be suitable (Craig et al., 2006). Electrocardiogram recordings can be made noninvasively from 5 dpf embryonic zebrafish (Forouhar et al., 2004). For the analysis of cardiac conduction, the transgenic zebrafish line *Tg*(*cmlc2*:*gCaMP*)^{s878} can be used (Chi et al., 2008). This allows the mapping of the spread of calcium excitation in the heart during each cardiac cycle (Chi et al., 2010).

DISEASE MODELS

There is a growing list of disease models in the zebrafish (Table 2). For reviews, see Kari et al. (2007), Berghmans et al. (2005).

Human Fetal Alcohol Syndrome (FAS)

Alcohol consumption during pregnancy can cause FAS in humans (Rostand et al., 1990; Wattendorf and Muenke, 2005; Chudley et al., 2007; Moore et al., 2007; Spohr et al., 2007). Among the clinical features of this syndrome are retarded growth, craniofacial defects, and mental retardation (Jones and Smith, 1973; Clarren and Smith, 1978; Giles et al., 2008). The craniofacial defects include microphthalmia (Stromland and Pinazo-Duran, 2002) and pharyngeal arch abnormalities (Church and Kaltenbach, 1997). Like many teratogens, the effects of ethanol are dependent on the duration and stage of exposure (Gemma et al., 2007; Giles et al., 2008).

Zebrafish embryos have been used in several studies of ethanol teratogenesis, and the phenotypic outcomes include developmental retardation, pericardial and yolk-sac edema (Reimers et al., 2004; Giles et al., 2008), reduction in body length (Loucks and Ahlgren, 2009), branchial skeleton defects (Carvan et al., 2004), abnormal eye development (Stromland and Pinazo-Duran, 2002; Bilotta et al., 2004; Matsui et al., 2006; Dlugos and Rabin, 2007; Kashyap et al., 2007) as well as cognitive defects (Carvan et al., 2004; Reimers et al., 2006) and higher mortality (Loucks and Carvan, 2004). These phenotypes overlap with human FAS.

Most studies of ethanol toxicity in zebrafish use chronic exposure, often over several hours or days (Blader and Strahle, 1998; Bilotta

et al., 2002; Arenzana et al., 2006; Matsui et al., 2006; Kashyap et al., 2007; Loucks and Ahlgren, 2009). However, a recent study from our group used brief exposure of staged embryos to an ethanol pulse. This allowed us to identify *prim-6* and *prim-16* as being particularly susceptible for the induction of pharyngeal defects by ethanol (Ali et al., 2011).

Tuberculosis

Zebrafish embryos are increasingly popular as a model for infectious disease. Infections by bacteria can be monitored in real time with high sensitivity. Furthermore, there is now an extensive knowledge base on the immune system of zebrafish showing a remarkable conservation with the immune system of mammals (Meijer and Spaink, 2011).The zebrafish offers the advantage that in the embryonic stage a defense response can be studied in the absence of the adaptive immune system (which develops later), thereby allowing the identification of autonomous innate immune mechanisms.

For the study of the immune response to infection, there are many transgenic zebrafish lines in which particular subsets of immune cells are labeled with GFP color varieties. For instance, transgenic fish lines have been published in which neutrophil and macrophages can be imaged simultaneously using dual color detection methods (Ellett et al., 2011; Gray et al., 2011). The transgenic lines will be of great use to set up high throughput assays that monitor not only disease progression but also the effect of antimicrobial treatments. For various microbe infection systems, it has been shown that injection in the yolk of early stage embryos leads to disease systems that can be followed for several days after infection (Spaink and Dirks, 2011).

In one application, the infection with *Mycobacterium tuberculosis*, it was shown that disease progression after yolk injection in pregastrulation stage embryos recapitulates the infection phenotypes that are observed after blood injection in the larval stage (Carvalho et al., 2011). This has made it possible to design a high throughput robotic injection system that can be used to inject up to 2000 embryos per hour in a single setup (Carvalho et al., 2011). This injection system was also coupled to a flow through screening system based on fluorescence detection, resulting in a high throughput pipeline that can screen for bacterial loads. In this work, it was furthermore shown that various antibiotics can be screened at high throughput levels in zebrafish larvae, even using the human pathogen *M. tuberculosis*.

The great power of this approach is that it can be combined with genetics approaches that are already well established in the zebrafish model. A good example is given by the observation that coinjection of antisense morpholinos and microbes can completely alter the immune response to infection (Carvalho et al., 2011). This will lead to the identification of host factors that are important to infection and the identification and study of host factors important for regulating the response to drugs. Such host factors include putative enzymes that can break down antimicrobial compounds or tissue properties that influence the penetration of drugs into microbial infection sites. It should be noted that the screening of the results of microbial infection will be greatly assisted by a combination with genetic or proteomic screening methods (Fig. 6).

TOXICITY TESTING

The zebrafish is being increasingly used in toxicity testing (Van den Belt et al., 2000; Hill et al., 2005; Reimers et al., 2006; Parng et al., 2007), reviewed by Brittijn et al. (2009), Hill et al. (2005), Teraoka et al. (2003), and Kari et al. (2007). In the context of toxicity, the zebrafish finds application in drug safety testing and ecotoxicological screening. For further examples, see Table 3. Chronic exposure regimes have been used to assess the toxicity of lead and uranium (Labrot et al., 1999), colchicine (Roche et al., 1994), anilines (Zok et al., 1991), metronidazole (Lanzky and Halling-Sorensen, 1997), and agricultural biocides (Kumar and Ansari, 1986; Gorge and Nagel, 1990). Acute toxicity studies are fewer. Although there is no strict boundary between acute and chronic exposure regimes, one standard definition of ''acute'' toxicity, in the context of larval-fish assays, is 96 hr of exposure in static renewal or flow-through systems (United States Environmental Protection Agency, 1996). One could argue, given the rapid development of zebrafish, that 96 hr is in fact a long exposure, spanning many developmental stages.

Predictivity and Validation

The evolutionary divergence of zebrafish and mammals is around 445 million years ago (Peterson et al., 2004), and so it is by no means certain that we will necessarily share the same sensitivity to toxic substances. Therefore, there is a need for validation of the model (McGrath and Li, 2008). By validation, we mean evidence that drugs with specific effects in humans can produce similar effects in the zebrafish embryo. Such evidence allows us to assess the predictivity of the zebrafish model, that is, its success at flagging-up compounds that might have specific effects in humans. One study comparing the toxicity of 18 compounds between zebrafish and rodents found good correlation (Parng et al., 2002). The zebrafish embryo system has also been compared as a toxicology screen with the aquatic crustacean, *Daphnia magna* (Martins et al., 2007). Such studies are an important step toward the kind of comparative toxicity database represented by the well-known ''Registry of Cytotoxicity,'' which examines the predictive power of cell assays (Halle, 2003). For further examples, see Table 4.

TABLE 3. Examples of Zebrafish Embryo Assays for Compound Screening

Assay	Plate format	Readouts	Stage of exposure	Duration of exposure	References
QT-prolongation	–	Bradycardia and arrhythmia	3 dpf	90 min	Langheinrich et al. (2003)
QT-prolongation	35 mm Petri dish	Bradycardia and arrhythmia	3 dpf	80 min	Mittelstadt et al. (2008)
Inflammatory bowel disease	96-well plates	Gut morphology, peristalsis	3 dpf	3–8 days	Fleming et al. (2010)
Teratogenicity	24-well plates	Survival, morphology, cardiovascular function	4–6 hpf	5 days	Brannen et al. (2010)
Alzheimer's disease	96- or 384-well plates	Dead cells detection, gene expression, morphology	6–24 hpf	1–6 days	Arslanova et al. (2010)
Developmental toxicity	Finger bowls	Morphology (developmental defects)	1–3 dpf	1–4 days	Hisaoka (1958)
Angiogenesis	384-well plates	Quantification of angiogenic vessel growth	1 dpf	2 days	Tran et al. (2007)
Magnetic resonance imaging (MRI) signal intensity	Agarose wells	Measurement of compound concentration	1 dpf	1–3 days	Canaple et al. (2008)
Behavior	96-well plates	Locomotor activity	6 dpf	1 day	Berghmans et al. (2007)
Toxicological screening	96-well plates	Mortality rate, hatching rate, cardiac rate, and morphological defects	4 hpf	5 days	George et al. (2011)
Toxicological screening	24-well plates	Movement, hatching rate, heartbeat, and morphological defects, blood circulation	1–2 hpf	3 days	Hermsen et al. (2011)
Toxicological screening	24-well plates	Morphological defects	1 hr	4 days	Sun et al. (2010)
Toxicological screening	80-mm Petri dish	Mortality rate, morphological malformations, gene expression	1.5 hr	3 days	Sawle et al. (2010)
Cytotoxicity, genotoxicity and teratogenicity	24-well plates	Mortality rate, hatching rate, heartbeat, and morphological malformations	1 hr	3 days	Yang et al. (2010)
Developmental neurotoxicity	6-, 24- and or 48-well plates	Mortality, heartbeat, circulation, pigmentation, hatching, behavior, morphological defects	2 hpf	8 days	Selderslaghs et al. (2010)
Developmental toxicity	24-, 48-, and 96-well plates	Mortality, heartbeat, circulation, pigmentation, hatching, behavior, morphological defects	2 hpf	6 days	Selderslaghs et al. (2009)
Teratogenicity	2-ml vial	Mortality, morphological defects	1 hpf	2 days	Busquet et al. (2008)

For further examples of toxicity screening see Kari et al. (2007).

QT Prolongation

QT prolongation, a major cardiotoxic effect of drugs in humans, is beginning to be investigated in zebrafish. It has been shown that zebrafish embryos develop abnormal heart beat in response to some drugs that cause QT prolongation in humans (Langheinrich et al., 2003; Milan et al., 2003; Berghmans et al., 2008; Mittelstadt et al., 2008). The abnormalities induced

TABLE 4. Tests of the Predictive Power of Zebrafish Assays (i.e., their Degree of Correlation with the Results from Rodent Assays)

Disease condition	Study design	Results	Reference
QT-prolongation	100 small molecules screened	Bradycardia and atrioventricular node (AV) blockage	Milan et al. (2003)
QT-prolongation	Screening of 13 drugs	Blockage the human ether-a-go-go-related gene (HERG) channel resulted in arrhythmia	Langheinrich et al. (2003)
QT-prolongation	18 drugs screened	Induction of corrected QT interval (QTc) prolongation and dissociation between the atrium and ventricular rates	Mittelstadt et al. (2008)
Inflammatory bowel disease	Three compounds (nitric oxide synthase inhibitors)	Rescue of the disease phenotype in vivo (gut histology analysis)	Fleming et al. (2010)
Epilepsy	11 antiepileptic drugs	Locomotor activity decreased	Berghmans et al. (2007)

in zebrafish by these drugs include bradycardia, arrhythmia, and dissociation between atrial and ventricular rates. Many drugs prolonging the QT interval in humans interact with the human *ether-à-go-go* related gene (*hERG*). Zebrafish possess the homolog *zERG* (Langheinrich et al., 2003), and there appears to be a substantial degree of functional conservation between the human *hERG* and the zebrafish *zERG* (Langheinrich et al., 2003; Hassel et al., 2008; Scholz et al., 2009).

FUTURE PROSPECTS

We have outlined in this review a few of the biomedical models, screens, and tools becoming available from zebrafish embryo research. In the coming years, the challenge is to validate zebrafish assays and models against mammalian drug screens. Such data are necessary for the translation of results of zebrafish testing toward applications in human disease treatments. Uncertainty about the predictivity of the zebrafish model is a major cause of scepticism, from the pharmaceutical R&D community and regulators.

One can argue that drug treatments that are effective in zebrafish and rodent models will have the greatest chance to also be effective in the treatment of human patients. As shown in Figure 6, we anticipate that, during the entire screening pipeline, rapid switches can be made from zebrafish to rodent models. For instance, after discovery of lead compounds in zebrafish, and subsequent testing in rodents, we envisage another round of screening of new generation drugs, again in the zebrafish. Other fish species can be of great use. For example the carp, closely related to zebrafish, can be used when extremely many embryos are needed. Furthermore, the potential of zebrafish and other fish species for toxicology studies can be directly implemented in the very early stages of screening. This could lead to much faster and more efficient drug development.

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