A Virtual Tour of the *Guide* for Zebrafish Users

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PHS-funded and AAALAC-accredited facilities are required to use the *Guide* as the basis for setting up a zebrafish care and use program. The authors describe how they accomplished this task at the University of Oregon Zebrafish Facility.

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Although fish have long been used in biomedical research and testing, only in the last 30 years has the zebrafish (*Danio rerio*) become an important research tool[1]. The explosion of developmental biology, neurobiology, and genetics research[2-4], as well as environmental science, teratology, carcinogenicity testing, and reproductive and behavioral studies[3-6], have contributed to the rise in popularity of the laboratory zebrafish.

Several zebrafish qualities contribute to their suitability as models for biomedical research. First, they are easy to maintain in large numbers, readily reproducing under laboratory conditions. Second, adult fish can be subjected to mutagenesis and haploid embryos screened for mutations in the first generation. Third, the zebrafish embryo has few cells relative to other vertebrates, making it a "simple" model for more complex vertebrates; moreover, the embryo is transparent and develops very rapidly and externally, permitting ready observation of the events involved in differentiation of tissues such as the nervous system. Fourth, direct access to the developing embryos permits such experimental manipulations as introducing foreign genetic material and labeling of cells. Finally, their small size allows the large numbers of zebrafish required for genetics studies to be easily maintained[7].

Many of the institutions using zebrafish for research, testing, or teaching are funded by the Public Health Service (PHS) and/or accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Therefore, these institutions should use the *Guide for the Care and Use of Laboratory Animals*[8] (*Guide*) as a basis for designing, implementing, and evaluating the program for zebrafish care and use. Because PHS *Policy on the Humane Care and Use of Laboratory Animals*[9] (PHS *Policy*) defines animal as "Any live, vertebrate animal used or intended for use in research, research training, experimentation, or biological testing or for related purposes," institutions that receive PHS funds or support must have a defined policy Assurance that describes the institution's compliance with the PHS *Policy* and the *Guide*.

Although the *Guide* and PHS *Policy* do not provide specific guidelines for the use of zebrafish, the Office of Laboratory Animal Welfare (OLAW) states, "Many of the principles embodied in the *Guide*, although not specifically addressing cold-blooded vertebrates, generally can be adapted to animal care and use programs for various kinds of amphibians, reptiles, and fishes[10]." Detailed descriptions of the care and use of zebrafish are clearly beyond the scope of this article, but we will demonstrate in broad terms—based on our experiences with zebrafish at the University of Oregon—how the principles in the *Guide* can be applied to the use of zebrafish in animal research programs, referring to each chapter in the *Guide*, with the exception of the physical plant description.

The 1996 revision of the *Guide*, which emphasizes performance-based standards, allows institutions to develop and define their own goals, the methods for achieving those goals, and the means for evaluating them. This approach is particularly useful for zebrafish users who have few engineering standards to follow[8].

Although zebrafish are not covered by United States Department of Agriculture (USDA) regulations, the University of Oregon IACUC has chosen to adopt a single standard of care when dealing with ani-

mals that are not covered by both the PHS *Policy* and USDA regulations. For instance, we use USDA's 12-hour rule for defining a study area rather than PHS's 24-hour rule for animal facility. Also, for painful or distressful procedures, we require the principal investigator who works with zebrafish to perform an alternatives search (USDA requirement).

Institutional Policies and Responsibilities

PHS-funded and AAALAC-accredited zebrafish facilities must have an Institutional Animal Care and Use Committee (IACUC) to oversee the animal program, facilities, and animal procedures, and to ensure that the institution's program is based on the *Guide* and PHS *Policy*. The *US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training*[11] form the basis of the *Guide* and can be used by IACUCs to evaluate their program and individual animal use protocols. For example, regarding the minimization of discomfort, distress, and pain, Principle IV states, "Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals." Because little is known about zebrafish pain, distress, and discomfort, the University of Oregon IACUC uses this principle when evaluating potentially painful or stressful procedures.

PHS *Policy* Section IV and Chapter 1 of the *Guide* describe general IACUC functions and responsibilities. The protocol review process, personnel training, and occupational health and safety (OHS) are examples of some unique applications we have developed for zebrafish users.

Protocol Review

Sections IV.C and IV.D in PHS *Policy* and Chapter 1 in the *Guide* describe those components that need to be addressed in the animal care and use protocol or activity. Because many of our zebrafish researchers perform identical animal use procedures, have similar reasons for using zebrafish, use a centralized zebrafish facility for husbandry and care, and have standard procedures for minimization of pain, distress, discomfort and injury, the University of Oregon has adopted a template *Zebrafish Protocol Form*[7]. This form still requires the Principal Investigator to fill out all the animal care and use procedures that are not described in the template protocol or the *Zebrafish Book*[12], and are unique to the study.

Personnel Qualifications and Training

All personnel who work with zebrafish must be adequately trained in the techniques described in the protocol or included in general zebrafish husbandry and care. Training or instruction must be made available to all researchers, technicians, students and other personnel involved in zebrafish care or use. Most training at our zebrafish facility is task-specific and oriented either to the individual or to small groups.

Occupational Health and Safety of Personnel

As required by PHS *Policy* and as stated in the *Guide*, "An occupational health and safety program must be a part of the overall animal care and use program. The program must be consistent with federal, state, and local regulations and should focus on maintaining a safe and healthy workplace[8]". An OHS program should be based on the *Guide*, *Occupational Health and Safety in the Care and Use of Research Animals*[13], and *Biosafety in Microbiological and Biomedical Laboratories*[14]. Essential to the OHS program are hazard identification and risk assessment, personnel training, personal hygiene, facilities, procedures and monitoring, personal protection, medical evaluation, and preventive medicine.

Those who work with zebrafish at the University of Oregon are required to participate in the OHS program. As part of our training OHS program, we use a one-page handout designed specifically for those working with fish that conveys information about zoonoses, personal hygiene, and other hazards associated with animal exposure. Participation in the OHS program is linked to the animal use protocol[7].

Aside from food poisonings, the overall incidence of transmission of disease-pro-

Known and potential fishborne zoonoses.

***Mycobacterium* spp.**
Organisms in the genus *Mycobacterium* are nonmotile, acid-fast rods. There are multiple atypical (non-tuberculosis) species of *Mycobacterium* (*M. marinum*, *M. fortuitum*, *M. chelonae*, *M. abscessus*) that are recognized pathogens of laboratory zebrafish. Humans can be infected by contamination of lacerated or abraded skin with aquarium water or fish contact. A localized granulomatous nodule may form at the site of infection, most commonly on hands or fingers. The granulomas usually appear approximately six to eight weeks after exposure to the organism. They initially appear as reddish bumps (papules) that slowly enlarge into purplish nodules. The infection can spread to nearby lymph nodes. More disseminated forms of the disease are likely in immunocompromised individuals. It is possible for these species of *Mycobacterium* to cause some degree of positive reaction to the tuberculin skin test.

***Aeromonas* spp.**
Aeromonad organisms are facultative anaerobic, Gram-negative rods, which can produce septicemia in infected fish. The species most commonly isolated is *A. hydrophilia*. It is found worldwide in tropical fresh water and is considered part of the normal intestinal microflora of healthy fish. Humans infected with *Aeromonas* may show a variety of clinical signs, but the two most common syndromes are gastroenteritis and localized wound infections. Again, infections are more common and serious in the immunocompromised individual.

Other Bacteria and Protozoa
Below is a list of additional zoonotic organisms that have been documented in fish or aquarium water. Human infections are typically acquired through ingestion of contaminated water resulting in gastroenteritis symptoms or wound contamination.

Gram-negative Organisms: *Plesiomonas shigelloides, Pseudomonas fluorescens, Escherichia coli, Salmonella* spp., *Klebsiella* spp., *Edwardsiella tarda*

Gram-positive Organisms: *Streptococcus, Staphylcoccus, Clostridium, Erysipelothrix, Nocardia*

Protozoa: *Cryptosporidium*

ducing agents from fish to humans is low. In general, humans contract fishborne disease through ingestion of infected fish tissues or aquarium water, or by contamination of lacerated or abraded skin. To minimize the possibility of exposure to one these agents, zebrafish handlers should wash their hands regularly, wear gloves, seek medical attention promptly if exposure is suspected, and remember to inform their physician that they work with fish should they become ill.

Animal Environment, Housing, and Management

Physical Environment

Microenvironment and macroenvironment: The tanks or aquaria that contain the animals make up the microenvironment, which predominantly comprises water and the small air volume enclosed by the tank cover. The room housing the fish tanks constitutes the macroenvironment. There are many ways to link the micro and macroenvironments, depending on the arrangement of the laboratory and water system.

Automatic water exchange can be by either continuous or discontinuous flowthrough, or by recirculation. A flowthrough system introduces clean water into the tank and discards outflow water. A recirculating water system processes the outflow water from the tanks by one or more filtration steps to remove undesirable materials and compounds and restore desirable ones. This treated water is then recirculated to the tanks. Tanks maintained by manual water changes should be fitted with small filtration units that will continually remove undesirable materials from the water.

Housing: Zebrafish are usually kept in transparent glass, Plexiglas (acrylic), or polycarbonate tanks or aquaria, permitting easy observation of the animals. Tank drains should be screened to keep fish in the tank. Ideally, the drains should be designed so that they require no cleaning until replacement. Zebrafish space requirements for physiological, behavioral, and social interactions are affected by other variables such as water quality, food and feeding regimen, size, and age, and therefore may require optimization in each facility (see below).

Zebrafish kept together for breeding should have some means to escape from more aggressive fish, in the form of either more space or plantlike materials to be used as hiding spaces. To prevent zebrafish from eating their eggs, the tank bottom should be fitted so as to make the eggs (~1.0–1.5 mm diameter) inaccessible to the fish, yet easy to collect—for example, a layer of marbles, an array of closely spaced rods, mesh, or a box containing marbles or covered with mesh.

Space recommendations: Zebrafish space requirements are usually given as numbers of fish per volume of water. As schooling fish, zebrafish can be kept at fairly high densities. We use the following general guidelines. Starting with 20 eggs or embryos per 100 ml water, the 20 fish can be kept in volumes ranging from 400 ml as young larvae to 3 liters as the fish approach juvenile stage. Recommended density for growing juvenile fish and holding adults is five fish/liter. Growing fish are considered to require more space than breeding adults, which require more space than nonbreeding adults, but this requirement has not been critically tested for zebrafish. In small breeding tanks, a pair of fish can be kept overnight in 1.5 liters or as many as six fish in 2.3 liters of water.

Depending on experimental parameters, such criteria as survivability, growth rate, and fecundity can be used to assess not only the adequacy of the space being provided, but also the genetics of the fish, the food quality, frequency of feeding, and the water quality. For eggs and embryos the most appropriate criteria seem to be survival (80–95% is good) and final size obtained (1.0–1.5 cm is good) over a standard time period (0–21 days post fertilization in our facility). During the first few days fish larvae should be kept in shallow water, so that they can gulp some air to inflate their swim bladder[15]. For juvenile fish, a useful standard consists of rapid growth to a breedable adult size, with a minimum variation in size. For adult fish, lack of mortality and the ease and reproducibility of breeding success define successful husbandry.

Temperature and humidity: Zebrafish can tolerate a fairly wide temperature range. They have been kept for fairly long periods at 22–30˚C (71.6–86.0˚F) and can survive temperatures of 18–32˚C (64.4–89.6˚F). A widely used standard temperature for developmental studies is 28.5˚C (or 83.3˚F). Our facility is frequently kept at this temperature. Heat-shock experiments have been carried out at temperatures above 30˚C (or 86˚F). A gradual drop in temperature to 22–23˚C (or 71.6–73.4˚F) to lower the zebrafish metabolic rate is acceptable in emergencies, such as water system mechanical failures.

Water temperature can be monitored by daily thermometer readings or by electronic thermister readings. Automatic temperature monitoring is preferable because problems can be detected immediately. Thermostats are usually used to control the water temperature, either by heating the water directly or by heating the room air. To maintain a given water temperature and compensate for evaporative cooling, one should expect to heat the air a degree or two warmer.

Ventilation: Proper ventilation for a fish tank or facility depends primarily on sufficient oxygen (O_2) supply and adequate carbon dioxide (CO_2) removal. To facilitate optimal O_2/CO_2 exchange, the surface area and turbulence of the water in tanks can be increased, bringing the O_2-poor, CO_2-rich water to the surface so that the diffusion of the gasses is driven by concentration differences. This can be accomplished in individual fish tanks, centrally in a pump/filtration area, or both, with aeration and gas exchange equipment.

An adequate dissolved oxygen (DO) reading is 6.0 p.p.m. (mg/l). Fish that remain close to the surface of the water and appear to be gulping air are probably not getting enough O_2. Whether a given tank contains adequate oxygen depends on many variables: number and size of fish, tank surface area, aeration in the tank,

temperature (oxygen is more soluble at lower temperatures), and rate of water exchange if there is central aeration. If possible, the fish rooms should be kept at slightly positive pressures to exclude potential pathogens.

Illumination: Illumination is important for breeding and minimizing stress and disease. For these purposes, standard fluorescent lamps are sufficient. Eliminating certain wavelengths may inhibit algae growth, but this is not as critical for fish health as the intensity (between 5 and 30 ft cd or 54–324 lux at the surface of the water) and the circadian light cycle (14 h light, 10 h dark). Unlike other fish, zebrafish do not require a seasonal change in their day length to bring them into a breeding state.

Fluorescent lighting is relatively inexpensive and easy to maintain. Fixtures similar to stack lights in libraries or asymmetrical wall-washing lights used in commercial displays will provide better directed light and thus better illumination levels.

Noise: Zebrafish react to loud sudden noises. Their sensitivity to various vibrations or sounds like talking or music is uncertain. Although they do not display any obvious reactions to such sounds, whether or not they are stressed has not been well investigated. Fish raised in a particular environment may adapt to the stimuli commonly present there. Fish raised in one environment may become stressed on being moved to an unfamiliar one. Common design considerations and management decisions are usually based on human health and comfort.

Behavioral Management

Structural environment: As shallow-water schooling fish, zebrafish seem more or less indifferent to environmental enrichments, with the possible exceptions of such environmental irregularities that become a focus of egg-laying during mating.

Social environment: Although their schooling habits indicate they are somewhat sociable, zebrafish may not require social interactions. Indeed, zebrafish have been kept in isolation for extended periods, after which they have still bred successfully.

Activity: Species-typical behaviors include swimming, feeding, mating, and social interactions. Human interaction with fish can be stressful. Habituation to the presence of humans may reduce or eliminate the stress.

Husbandry

Food: The precise nutritional requirements of zebrafish have not been determined. There are two nutritional models for zebrafish—warm-water and cold-water commercial aquacultural fish. Neither is perfect. Zebrafish are warm-water cyprinaformes. The commercial fish with well-studied dietary requirements are cold-water cyprinaformes or rather distantly related warm-water fish.

Zebrafish are fed different foods depending on their age. Young zebrafish require small-sized food items that can be swallowed whole. Larger juveniles can swallow larger items but also require more food. Newly hatched zebrafish can eat paramecia (800 μm × 80 μm), as well as a variety of prepared foods, infusoria, and rotifers. As they grow larger, zebrafish hatchlings can add to their diet larger items such as vinegar eels, microworms, or larger prepared foods. Eventually they are large enough to eat *Artemia* nauplii (newly hatched brine shrimp), which have a high protein content and can be hatched on demand in large numbers, but the eggs can be very expensive. Alternative foods in this size include cultured *Moina* and larger sizes of prepared foods.

Juvenile AB fish in our facility thrive on live foods, an observation that is supported by results of other investigators. Those studies indicate that some less inbred pet store zebrafish do as well on either live or prepared foods, whereas the AB fish still do better on live foods than prepared foods. Hence, facility-specific growth rate and survival tests should be used to optimize results. Adult-size fish can be fed adult prepared foods (tropical fish flake foods, tropical fish micropellets, and ground trout meal) and live adult brine shrimp. Alternative live foods include *Artemia*, *Moina*, and *Drosophila* larvae.

To minimize contamination, prepared foods should be purchased in small amounts, stored only a few months, and kept frozen or refrigerated in sealed containers. All food containers should be labeled with date of production (if known), date of receipt, and date opened. Live foods should be cultured on the premises, derived from environments considered unlikely to harbor pathogens of freshwater fish (*e.g.*, brine shrimp cysts), or treated after collection to decontaminate (*e.g.*, bleach-decapsulated brine shrimp cysts). Live foods collected directly from the wild in fresh waters should not be used as food for laboratory zebrafish.

Water: Critical for successful zebrafish maintenance are control and monitoring of water quality. An understanding of the complex chemical cycles and the many dynamic components involved can help in manipulating or controlling parameters through chemical additions and filtration processes. Among the chemical cycles are the nitrogen, carbon, oxygen, and carbonate and buffering cycles. These processes partially overlap and can interact extensively. One reason for choosing zebrafish as a model system is their broad tolerance of water conditions.

Water quality parameters can be monitored by hand or by instruments. Procedures can use colorimetric reagents, electrodes of various kinds, or various combinations of the two. Which parameters should be monitored and what they indicate are complex issues that involve a good understanding of your particular water system. Among the most useful water parameters to monitor are concentrations of ammonia, $[NH_3/NH_4^+]$; nitrite, $[NO_2^-]$; nitrate, $[NO_3^-]$; calcium, $[Ca^{2+}]$; and magnesium, $[Mg^{2+}]$; temperature; pH; buffering (or alkalinity); salinity (either as dissolved solids or conductivity); and levels of dissolved oxygen (DO). In addition, it is sometimes important to determine the chlorine concentration, $[Cl_2]$—as distinct from the chloride ion concentration, $[Cl^-]$, which is a component of salinity—and the total dissolved gases (to determine whether or not the water is supersaturated).

Wedemeyer[16], Moe[17], and Spotte[18] should be consulted for a comprehensive review of water-quality parameters.

Monitoring should be sufficiently frequent to detect problems. Some parameters should be checked several times daily, others once a day, some only once a week or less. For example, mechanical systems, such as pumps, should be continuously monitored. Some water-quality parameters that change rapidly, like DO and salinity, should be monitored daily. Other parameters, such as NH_3/NH_4^+, NO_2^-, NO_3^-, and calcium, can be monitored weekly. These monitoring frequencies will depend on each facility's set-up and experience. Several companies produce systems that can automatically monitor, log data, trigger alarms at certain thresholds, and dial-out to people who are "on call."

As outlined above, water systems can be flowthrough or recirculating, and centralized or distributed. A flowthrough water system maintains good water quality by exchanging old water for new. Although all water systems exchange some water, recirculating water systems rely more heavily than flowthrough systems on maintaining good filtration processes. A well-operated recirculating water system will provide greater control over the fish environment and less sensitivity to external environmental variables. Centralized water systems maintain water quality using a relatively small number of pieces of equipment serving many fish tanks. More distributed systems will involve fewer tanks being served by a given set of equipment, whereas more centralized systems generally make more efficient use of labor in maintenance and can cost less. Because centralized water systems circulate essentially the same water through many or all tanks, a water quality parameter affecting many tanks can be measured with one test in a single location. By contrast, in a distributed water system, the test would have to be repeated for each independently regulated body of water. On the other hand, distributed water systems provide greater biosecurity because water is not shared among as many tanks. Even though water in a recirculating system is typically exposed to UV light to kill bacteria in the water, this treatment should not be expected to provide 100% protection against any organism. The choice of which water-quality parameters to monitor depends on the system design (*e.g.*, in a flow-through water system using dechlorinated tap water, frequent monitoring of chlorine levels is essential).

Sanitation: A good sanitation program prevents buildup of waste products and potential cross-contamination between tanks. Holding and breeding tanks, and their tops, can be washed with disinfectant or sterilized by autoclaving. Racks and rooms can also be sanitized periodically as necessary. Nets used with fish can be sterilized by autoclaving or disinfected by bleaching. Tank surfaces need regular cleaning to permit easy viewing of the fish.

Waste disposal: Tank wastes, including uneaten food, fish feces, and dead, decayed fish, must be removed from the tank by hand (siphoning), by the tank's filter, or by a centralized (room-scale or rack-scale) water system filter. Filters, if used, must also be cleaned to remove filtered material from the water system. Tank wastes, as well as euthanized fish and wastewater, will usually be disposed of through municipal sewer lines. Important considerations include preventing contamination of the local environment either with disease pathogens (perhaps being used intentionally in the laboratory) or with foreign organisms—recalling that zebrafish are native to watersheds in and around India and Myanmar.

Pest control: Pests most often encountered in fish facilities are arthropods: roaches, silverfish, and lice. Control consists predominantly of prevention by eliminating pest food, hiding places, and access. Minimize fish food on the floor or the lid of the tanks through careful feeding methods that result in little spillage, as well as good sanitation in all areas. Control pest access by tightly closing doors and by blocking conduits and other less obvious paths. Eliminate insect hideouts by treating spaces behind walls and between wall studs with boric acid and removing unnecessary paper and cardboard. Sodium borate treatment is fairly effective against insects and is unlikely to harm the fish.

Emergency, weekend, and holiday care: An emergency or disaster plan should address all reasonably foreseeable situations. With electrical power outages and mechanical equipment failures high on the list of failure scenarios, minimal precautions include having backup equipment and an electrical generator.

Weekend and holiday care should provide at least minimal feeding for adults and feeding and care for the baby fish. Adult fish can tolerate a few days without food but require daily feeding for optimal egg production. Because larval fish require normal care daily, it may be advisable to schedule fewer fish in the nursery during holidays to reduce labor requirements.

Population Management

Identification and records: Identification labels placed on zebrafish tanks should contain information about the genetic background, stock number, the date of fertilization, and the researcher's name. In larger facilities, these records may be kept in computer database files. A downloadable version of such a database can be obtained from the University of Oregon Zebrafish Facility website (http://darkwing.uoregon.edu/~zfish/). Some facilities also use barcodes to automate and reduce data entry error. More sophisticated systems have their files on a dedicated server powered by an uninterruptible power source (UPS), with automatic backup.

Genetics and nomenclature: Genetic nomenclature guidelines have been established for zebrafish mutations and wild-type lines. These guidelines are briefly explained on the Zebrafish Information Network (ZFIN) nomenclature page (http://zfish.uoregon.edu/). Zebrafish genetic composition is determined from the records of the parents that generated the fish, phenotypic classifications of the fish and their siblings, and genetic and molecular tests to determine if fish carry particular recessive traits.

Veterinary Medical Care

Animal Procurement and Transportation

Specific zebrafish mutants and wild-type lines can be obtained from stock centers, other laboratories, or commercial dealers. The ZFIN website lists many suppliers.

Stock centers and academic laboratories generally provide the expected mutations and wild-type strains. Their facilities vary somewhat in their health surveillance and quarantine procedures. Some will ship out bleached eggs (facilitating the quarantine procedures of the receiving institution). An academic institution's shipment response time can vary as a result of limited availability of rarely requested mutant strains. In contrast, purchases from commercial dealers are usually genetically poorly defined "wild types."

Zebrafish are frequently transported between laboratory facilities. Transporting fish over the short distances on a campus can be as simple as placing adult fish in a bag in a box or, more commonly, in a small covered plastic box (Tupperware® or polycarbonate filled with fish system water). Eggs or larvae can be transported in covered petri plates, beakers, or tissue culture flasks.

Transporting zebrafish long distances to other facilities is more complicated. The packing requirements are more stringent. Adult fish should be double-bagged in a good-quality plastic fish shipping bag at a density of about 10 fish to a half gallon of water. The bag should be about two-thirds full of air or oxygen. Food should be withheld for a day before shipping so that fish will produce less ammonia while confined. Amquel (a commercially available ammonia sequestrator) can be added to bind up any ammonia that is produced. Zebrafish eggs and larvae are usually packed 200–300 to a 250- to 500-ml tissue culture flask filled ~50–90% with water. Ideally, the sender bleaches the eggs and places them in sterile water. Methylene blue (0.5 mg/liter or 0.5 p.p.m.) can also be added to the water to reduce fungal growth. The packing box should be insulated and any extra space filled with packing chips.

For international shipments, additional paperwork may be necessary, including a letter to customs agents, a customs invoice, and perhaps fish import permits. Forms that designate certain customs agents and specific airports can facilitate the shipment handling, as well. Unlike domestic shipments, international shipments are often timed to be in transit during the weekend.

Preventive Medicine

Quarantine, stabilization, and separation: All newly acquired zebrafish should be quarantined to avoid introducing disease into a colony. The health status of incoming fish is rarely known, and the stress of shipping can often cause underlying diseases to become apparent. The preferred quarantine method is absolute, wherein only surface-sanitized embryos are introduced into a facility. An isolated location and water system should be designated as the quarantine area. This can range from a simple individual aquarium with a self-contained filter unit to an entire separate room with many tanks and a flowthrough water system. Newly arrived fish should be equilibrated over an hour or two by floating the bag in the new tank and periodically introducing a small amount of the new tank water into the bag. If the shipment water is in poor condition, it may be necessary to transfer the fish immediately. Once introduced into their new tank, the quarantined fish remain there for three to four weeks. During this time, the fish are observed closely for signs of disease and treated, if necessary. The new fish are then bred and the embryos are surface-sanitized with a mild bleach solution (35 mg/liter sodium hypochlorite for 5 min). Only these sanitized embryos are introduced into the main aquarium facility. Upon confirmation that the fish strain has been successfully established from these embryos, the adults in the quarantine area are euthanized.

Surveillance, diagnosis, treatment, and control of disease: Fish should be observed daily for signs of illness or injury. Signs of disease in fish include color changes (paleness, redness, hemorrhage), changes in shape (weight loss, bloating, skeletal deformity, masses/swellings, exophthalmia), external lesions (ulcerations, fin erosion, gas bubbles, protruding scales), behavioral alterations (rapid breathing, loss of equilibrium, lethargy, erratic movements, gathering at water surface), and increased mortality. When moribund or dead fish are noted, a clinical investigation is warranted and a veterinarian trained in aquatic animal medicine should be consulted. Water quality and husbandry conditions should be reviewed. Depending on the problem, diagnostic workups can include skin scrapings, fin biopsy, gill biopsy, necropsy, bacteriology, virology, and histopathology. Histopathology is a particularly useful diagnostic technique, because the tiny zebrafish can be fixed and sectioned whole, permitting the examination of all primary organs on a single microscope slide. A useful tool for monitoring recirculating water systems is the sentinel fish. Kept in a separate tank that is fed with water from the dirty or return water sump, these fish are sampled periodically for disease investigation (**Fig. 1**). The Zebrafish International Resource Center Pathology Service is one

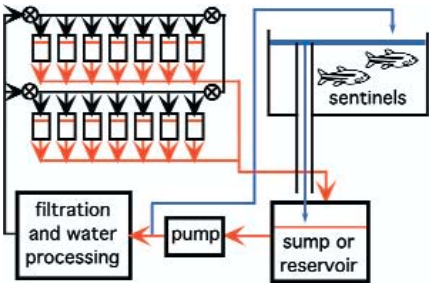

FIGURE 1. Schematic of a sentinel fish tank on a water system. A mixture of potential pathogens are collected from all the tanks served by the water system in the water flowing into the return water sump. This untreated water (red) is then pumped out of the sump to be filtered and UV-irradiated before it is returned to the tanks (black). A side stream (blue) is directed into the sentinel tank before any filtration or irradiation treatment. The sentinel fish thus provide a good, easily sampled system for detecting any pathogens being shed by any of the fish served by the water system.

pathology service that offers complete diagnostic services and consultations on zebrafish health and husbandry (see http://zfin.org/zf_info/stckctr/health.html).

Most treatments for zebrafish disease have been adapted from the tropical pet fish industry or food fish aquaculture. Among the many considerations involved in initiating a treatment protocol are the diagnosis, the number of fish being treated, the drug and route of administration, and the potential for toxicity to biological filtration. Before starting a generalized treatment, one should always test a drug on a few fish.

Surgery

The most common surgical procedure done on zebrafish is a caudal fin clip to collect tissue for DNA isolation and PCR analysis. The technique permits screening for fish carrying mutations in a particular gene. With practice one can do the procedure very rapidly and cause no bleeding. No presurgical cleansing of the caudal fin should be necessary, but gloves should be worn and the surgical area should be clean. Before doing surgery, small (500 ml) individual tanks containing clean fish water for anesthesia recovery and holding should be set up. Individual fish should be identified and isolated until the PCR analysis is complete. Another surgical manipulation of zebrafish is tattooing.

Pain, Anesthesia, and Analgesia

Although it is generally accepted that fish do experience pain, it can be difficult to assess. Signs of pain or distress could include escape behavior or frantic movements, increased respiration (rapid movement of opercula), and blanching of color. To decrease overall stress, fish are anesthetized for all procedures that could cause pain and distress or that require temporary immobilization. The most common anesthetic agent used is MS-222 (tricaine methanesulfonate) in an aqueous solution. Fish are induced rapidly following immersion in a solution containing MS-222 (100-200 mg/L)[12,19] and are recovered by returning them to fresh, well-aerated water. Because most procedures performed on zebrafish are very rapid, the need for a maintenance phase of anesthesia is usually not necessary. Maintenance anesthesia doses would be lower (50-100 mg/L)[19]. During induction, spontaneous ventilation should be monitored closely and can be used as an indicator to the depth of anesthesia.

Euthanasia

Zebrafish should be euthanized by methods consistent with the *2000 Report of the AVMA Panel on Euthanasia*[20]. The method chosen depends on the researcher, facility, or the intended use of the fish after euthanasia. An overdose of MS-222 is the most common method, using a slightly more concentrated solution (*e.g.*, 200–500 mg/liter) than is typical for anesthesia. Fish are left in the MS-222 solution for 5–10 min following the cessation of opercular movement. A second method of euthanizing zebrafish is immobilization by submersion in ice water followed by cranial concussion and decapitation using an in-sink garbage disposal. Another method, useful when tissues must be preserved, is anesthesia with MS-222 followed by quick freezing in liquid nitrogen.

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References

1. West, R. Big splash. *Oregon Quarterly* **Spring,** 22-26 (1996).
2. Klontz, G W. in *Animal Models for Biomedical Research* Vol. 4 27-30 (National Academy of Sciences, Washington DC, 1971).
3. Laale, H.W. The biology and use of zebrafish, *Brachydanio rerio* in fisheries research—a literature review. *J. Fish Biol.* **10,** 121-173 (1977).
4. Vascotto, S.G., Beckham, V. & Kelly, G.M. The zebrafish's swim to fame as an experimental model in biology. *Biochem. Cell Biol,* **75,** 479-485 (1997).
5. Travis, J. Gone fishing! Scientists use mutant zebrafish to learn how vertebrate embryos develop. *Sci. News* **150,** 360-361 (1996).
6. Bunton, T.E. The use of an aquatic bioassay in carcinogenicity testing. *Lab Anim. (NY)* **19,** 46-47 (1990).
7. Animal use application to the Institutional Animal Care & Use Committee (IACUC) Univeristy of Oregon: Zebrafish Form, http://zfin.org/zf_info/zfbook/chapt1/animzeb.pdf.
8. Institute of Laboratory Animal Resources, National Research Council. *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington DC, 1996).
9. Office of Laboratory Animal Welfare. Public Health Service Policy on Humane Care and Use of Laboratory Animals (Washington DC, 1996).
10. Potkay, S., Garnett, N., Miller, J.G., Pond, C.L. & Doyle, D.J. Frequently asked questions about the Public Health Service Policy on Humane Care and Use of Laboratory Animals. *Contemp. Top. Lab. Anim. Sci.* **36,** 47-50 (1997); http://grants.nih.gov/grants/olaw/references/faq_labanimals1997.htm#6
11. Interagency Research Animal Committee. US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (1985).
12. Westerfield, M. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish* Danio*(*Brachydanio*)* rerio (University of Oregon Press, Eugene, 2000).
13. Committee on Occupational Safety and Health in Research Animal Facilities, Institute of Laboratory Animal Resources, National Research Council. *Occupational Health and Safety in the Care and Use of Research Animals* (National Academy Press, Washington DC, 1996).
14. US Department of Health and Human Services, Centers for Disease Control and Prevention & National Institutes of Health. *Biosafety in Microbiological and Biomedical Laboratories* 4th edn (US Government Printing Office, Washington DC, 1999).
15. Goolish, E.M. & Okutake, K. Lack of gas bladder inflation by larvae of zebrafish in the absence of an air-water interface. *J. Fish Biol.* **55,** 1054-1063 (1999).
16. Wedemeyer, G. *Physiology of Fish in Intensive Culture Systems* (Kluwer Academic Publications, Boston, 1996).
17. Moe, M.A. Jr *The Marine Aquarium Reference: Systems and Invertebrates* (Green Turtle Publications, Plantation, 1989).
18. Spotte, S. *Captive Seawater Fishes: Science and Technology* (Wiley-Interscience, New York, 1992).
19. Mashima, T.Y. & Lewbart, G.A. Pet fish formulary. *Vet. Clin. North Am. Exotic Anim. Prac.* **3,** 117-130 (2000).
20. 2000 Report of the AVMA Panel on Euthanasia. *J. Am. Vet. Med. Assoc.* **218,** 669–696 (2001).