UNIVERSITY OF OREGON | Office of the Vice President for Research and Innovation

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Basic Information

1. * **Select research team:**
   [redacted] (ZIRC) Research Team

2. * **Select admin office:**
   IACUC

3. * **Title of protocol:**
   Zebrafish International Resource Center

4. * **Short title:**
   Zebrafish International Resource Center

5. * **Summary of research:**
   To serve as a central repository for zebrafish genetic stocks and research materials We will maintain healthy stocks of fish and frozen sperm of identified genotypes and make them widely available to the research community We will continue to expand our collection by obtaining carriers of mutations and transgenes from the research community and breeding them to produce new generations We will freeze and store sperm from all these lines We will develop genotyping protocols where applicable and make them available online through the ZIRC website (http //zebrafish org/zirc/home/guide php) We will acquire the most widely used wild type lines and maintain them in a manner that preserves their genetic purpose We will receive and store antibodies and gene probes that are used to identify and analyze wild type and mutant stocks Upon request, we will ship fish and materials to research laboratories throughout the world We will provide information online about stocks, materials, ordering procedures, and protocols, and we will provide links to corresponding pages in ZFIN, the zebrafish model organism database (http //zfin org) By providing these services in an efficient manner, ZIRC saves laboratories time and expense that can be better used for their research goals

   To provide pathology and consultation services We will provide diagnostic services and health status testing for laboratory zebrafish We will use histopathology, bacteriology, and necropsy to analyze specific or suspected disease problems (http //zebrafish org/health/index php) We will provide routine sentinel and quality control

testing of zebrafish from healthy laboratory colonies for early detection of problems and to fulfill institutional animal care and use committee (IACUC) health monitoring requirements. We will provide consultations to aid fish facilities in maintaining healthy fish populations and to deal effectively with disease. We will continue to characterize the significant diseases of zebrafish and develop methods to detect and control disease in laboratory colonies. We will maintain and update our online manual for the prevention, diagnosis, and treatment of diseases affecting zebrafish.
http://zebrafish.org/zirc/health/diseaseManual.php

We will develop a platform, based on a panel of PCR assays, to screen for the most prevalent pathogens of laboratory zebrafish. We will use genomic sequence information from known zebrafish pathogens to design and test PCR primers for species-specific detection. We will validate the sensitivity of these molecular assays with standard health screening by histopathology. We will use these PCR assays and other diagnostic tools to establish and implement screening procedures for in-house detection and monitoring of zebrafish pathogens. We will establish a platform, through the ZIRC website, for sharing the ZIRC pathogen monitoring program and tools for diagnostic testing with the research community, and we will provide consultation and diagnostic services, using this new platform, to identify pathogens in research laboratories, through our Pathology Service (Resource Aim 2).

6. * Principal investigator:

[redacted]

7. * What is the intention of the animal protocol?

Experimental Research

Experimental Research Protocol Addition

1. * **Will the protocol include breeding?**
   - (●) **Yes** ( ) No

Protocol Team Members

1. **Identify each additional person involved in the design, conduct, or reporting of the research:**

Name	Role	Involved in Animal Handling	Authorized To Order Animals	E-mail	Phone
[redacted]	Researcher	yes	yes	[redacted]	[redacted]
[redacted]	Researcher	yes	yes	[redacted]	[redacted]
[redacted]	Researcher	yes	yes	[redacted]	[redacted]
[redacted]	Researcher	yes	yes	[redacted]	
[redacted]	Researcher	yes	yes	[redacted]	[redacted]
[redacted]	Researcher	yes	yes	[redacted]	[redacted]
[redacted]	Researcher	yes	yes	[redacted]	
[redacted]	Researcher	yes	yes	[redacted]	
[redacted]	Researcher Lab Animal Technician	yes	yes	[redacted]	[redacted]
[redacted]	Researcher	yes	yes	[redacted]	
[redacted]	Researcher	yes	yes	[redacted]	[redacted]
[redacted]	Researcher	yes	yes	[redacted]	[redacted]
[redacted]	Researcher Co-Investigator	yes	yes	[redacted]	[redacted]

Name	Role	Involved in Animal Handling	Authorized To Order Animals	E-mail	Phone
[redacted]	Researcher	yes	yes	[redacted]	[redacted]

2. External team member information:

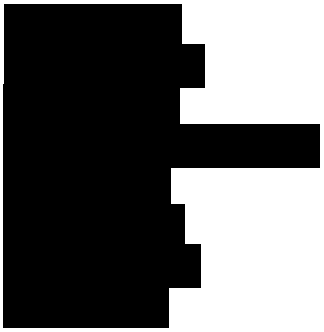
Document Name	Date Modified
There are no items to display	

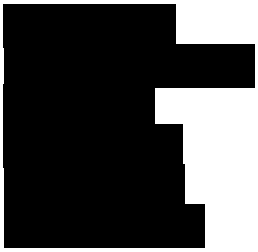
Funding Sources

1. Identify each organization supplying funding for the protocol:

	Funding Organization	Sponsor's Funding ID	Grants Office ID	Documents
View	National Institutes of Health (NIH)	P40 OD11021-16	26509	
View	National Institutes of Health (NIH)	P40 OD11021-16	27593	
View	National Institutes of Health (NIH)	P40 OD11021-16	27932	
View	National Institutes of Health (NIH)	P40 OD11021-16	31695	
View	National Institutes of Health (NIH)	P40 OD11021-16	31255	
View	National Institutes of Health (NIH)	P40 OD11021-16	30463	
View	National Institutes of Health (NIH)	P40 OD11021-16	24999	
View	National Institutes of Health (NIH)	1 R24 RR023998-01A1	25538	
View	National Institutes of Health (NIH)	National Institutes of Health (NIH)	TBA	
View	National Institutes of Health (NIH)	P40 OD11021-16	30582	
View	National Institutes of Health (NIH)	P40 OD TBD	GRANT13626680	

2. Indicate the protocol team members who have a financial interest in this research:





Scientific Aims

1. * **Scientific aims of the research:**

To serve as a central repository for zebrafish genetic stocks and research materials. We will maintain healthy stocks of fish and frozen sperm of identified genotypes and make them widely available to the research community. We will continue to expand our collection by obtaining carriers of mutations and transgenes from the research community and breeding them to produce new generations. We will freeze and store sperm from all these lines. We will develop genotyping protocols where applicable and make them available online through the ZIRC website (http://zebrafish.org/zirc/home/guide.php). We will acquire the most widely used wild-type lines and maintain them in a manner that preserves their genetic purpose. We will receive and store antibodies and gene probes that are used to identify and analyze wild-type and mutant stocks. Upon request, we will ship fish and materials to research laboratories throughout the world. We will provide information online about stocks, materials, ordering procedures, and protocols, and we will provide links to corresponding pages in ZFIN, the zebrafish model organism database (http://zfin.org). By providing these services in an efficient manner, ZIRC saves laboratories time and expense that can be better used for their research goals.

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2. * Significance and benefits of the research:

A major goal of biomedical research is to understand how genes regulate developmental and physiological processes. By studying genes, including the regulation of their expression and the functions of their products, we can obtain a deeper and richer understanding of biological processes and gain important insights into vertebrate, including human, health and disease. In recent years, we have witnessed an explosion in our understanding of how genes regulate biological processes, largely based on work from a few model genetic organisms including mouse, fruit fly, nematode worm, yeast, and zebrafish. Each system has specific and complementary strengths. Our understanding of human development, hereditary medical conditions and disease has been tremendously augmented by the research performed with these genetic model organisms. The zebrafish is the newest of them. Because the basic genetic principles of embryonic development are very similar for vertebrates, insights gained from the research with zebrafish have implications for human health and disease. Moreover, research on this organism meets the intent of the Animal Welfare Act and the "3 R"s because higher vertebrate models can now be replaced with this ‘lower’ vertebrate.

In the past decades, there was a dramatic increase in the number of laboratories using this organism to study the basic mechanisms of vertebrate development. Laboratories have generated several thousand transgenic fish lines, identified over 20,000 genetic mutations, and due to the completion of the zebrafish genome sequencing project, plans are underway to produce a mutation in every gene of the zebrafish genome (ca. 25,000 genes). Even more recently, genome editing methods have become available that enable scientists to target previously not mutagenized genes by conventional forward genetic methods. Most of the genetic stocks are distributed among more than 1000 laboratories in more than 40 countries. To make room for new mutants, laboratories must discontinue some of their current stocks. Although mutations can be preserved as frozen sperm, not all laboratories are proficient with this technique. Thus, discontinued stocks may be permanently lost unless a central site serves as a repository to preserve and redistribute them for future research.

The Zebrafish International Resource Center acquires and maintains wild-type,

transgenic, and mutant zebrafish stocks and makes them available to the international biomedical research community.

Experiments

1. * Define the experiments to be used in this protocol:

Name	Species	Is USDA	Total	Pain Category	Common Procedures	Variable Procedures	Variable Description
Transition experiment	Zebrafish	no	70330	B: 0, C: 0, D: 70330, E: 0			

2. If the experiments include survival surgery, will any single animal undergo more than one survival surgery? (include any animal that underwent surgery prior to use on this protocol)

○ Yes ● **No**

Zebrafish International Resource Center Create and Edit

1. **Display order:**

2. * **Experiment name:**
   Transition experiment

3. * **Species:**
   Zebrafish

4. **Describe the experiment:** (including animal characteristics such as age, weight, and sex)

5. **Justify the purpose of this experiment:**

6. **Select procedures:** (applied to all animals in the experiment)

Name	Type	Version
There are no items to display		

7. **Describe any variations to the selected standard procedures:**

8. **Procedure timing:**

9. * **Total number of animals used in this experiment:** (including all the animals to be produced)

70330

10. Number of animals by pain category: (include each animal only once in the highest pain category)

B: 0
C: 0
D: 70330
E: 0

11. Identify husbandry exceptions:

Exception Type	Description and Justification
There are no items to display	

12. Supporting documents:

Document Name	Date Modified
There are no items to display	

Procedure Personnel Assignment

1. Team member training:

First Name	Last Name	Training

Zebrafish International Resource Center Strains

Strains

1. Identify background strains:

Species	Is USDA Species	Strain	Genetically Modified Strain	Phenotype

There are no items to display

Animal Justification

1. Adjust the number of animals to be used or produced for this protocol as needed:

Species	USDA Covered Species	Pain Category	Animals Identified in Experiments	Adjusted Animal Count
Zebrafish	no	Pain Category B	0	0
Zebrafish	no	Pain Category C	0	0
Zebrafish	no	Pain Category D	70330	70330
Zebrafish	no	Pain Category E	0	0

2. If you adjusted the number of animals for this protocol, explain why:

3. * Provide the rationale for using animals in this protocol:

ZIRC is supported by the Office of Research Infrastructure Programs (NIH) with the specific purpose to function as a genetic repository of zebrafish (*Danio rerio*) research lines and not any other organism. Thus, there are no reasonable alternatives to the use of zebrafish at ZIRC.

A database search was not conducted as alternatives do not actually exist. As a resource center, ZIRC is relatively unique (replicated only twice worldwide) and serves a very specific function that supports the assurance statements of all zebrafish researchers in general. Resource Centers exist for other species, however, ZIRC is supported by NIH/ORIP to function as the genetic repository for zebrafish specifically. Two other zebrafish Resource Centers exist, with which ZIRC cooperates to support assurance statements:

EZRC - the European Zebrafish Resource Center and

CZRC, the China Zebrafish Resource Center.

ZIRC's Role for the Assurance Statements, the 3 R's.

The ZIRC serves other research groups to replace other vertebrate model organisms with zebrafish for some of their research goals. In addition, because many lines are maintained in a centralized, efficiently operating facility, ZIRC also helps to reduce the overall number of zebrafish maintained globally for research purposes. Cryopreservation research at ZIRC is also aimed at reducing the number of animals that have to be maintained as live stocks at ZIRC or elsewhere. Thus, ZIRC activities are aimed to support two of the "R"s in the Assurance Statement XI.A (Alternatives): Replace and Reduce. ZIRC's husbandry and health research activities and publications are in addition aimed to refine and optimize maintenance standards and therefore support the third "R" also.

Several regional Stock Centers (Taiwan, China, Japan, Germany) support the operations of ZIRC, because we will be able to focus better on the specific research programs of NIH and US based researchers. Importantly, shipping live fish to countries with complicated local regulations will become less frequent. Because Stock Centers are better equipped to exchange and work with cryopreserved samples, we will exchange frozen samples between Stock Centers in bulk, facilitating the regional distribution of live fish strains and minimizing potential discomfort or waiting times of animals in transit. In the long run, we aim to mirror the (cryopreserved) genetic stock inventories between ZIRC, EZRC, and CZRC to further reduce the numbers of animals that need to be maintained alive worldwide. The cooperation between zebrafish Stock Centers thus refines animal handling, husbandry (including shipping) and health and reduces overall animal numbers.

In general, there are no reasonable alternatives to the use of vertebrate animals such as zebrafish (*Danio rerio*) for research supported by the Resource Center. Some studies, like cellular differentiation, for example, can be conducted in cell culture using established cell lines. However, cell functions and interactions in culture are not completely normal. It is impossible to know with much precision whether proper patterning of tissues occurs in vitro; the developmental context of the embryo, including surrounding cells and extracellular material, is lost; many physiological processes depend upon tissues or organs and their interactions. If we wish to understand the mechanisms that regulate these processes, rather than just the mechanics of cellular differentiation, we need to study these processes in live animals where the effects of specific mutations can be assessed. By studying processes in the animal, we will learn how they are regulated in their normal physiological context. Such research can be done

only with live animals.
Zebrafish are simple, small vertebrates that are easily reared in the lab. Development is rapid and the embryos are optically transparent so that we can directly observe the growth of individual cells, tissues, and organs. Many mutant strains are available and more are being continuously created and identified in laboratories throughout the world. Because the basic principles of body patterning and physiology appear similar in all vertebrates, insights gained from work on embryonic zebrafish will have implications for human health and disease. Moreover, research on this organism meets the intent of the Animal Welfare Act because use of many higher vertebrates can now be replaced by use of this so-called lower vertebrate.
Another potential alternative is the use of invertebrates, and much progress has been made in our understanding of genetics and development using these organisms. However, it is thought that vertebrates have evolved at least a few new tricks for organizing and forming their organs and bodies (see for example Easter et al., 1985) and, thus, vertebrates need to be studied, too. The fish is especially well suited for the proposed research because it sits close to the evolutionary step between invertebrates and vertebrates and it teaches us about both kinds of animals. Finally, it is important to remember that, because humans are vertebrates too, the study of fish will teach us something about ourselves.

4. * Justify the number of animals to be involved in this protocol: (the Adjusted Animal Count above)
The requested animal numbers for ZIRC are based on actual shipping and breeding database entries (animal census) for the years 2018-2020, inflated by 10% (as buffer). In contrast to regular laboratories, colony breeding and distribution is the key part of ZIRC's mission, whereas "research activities" have somewhat lower priority. Hence, we list breeding colony and distribution animal numbers also in the research section.

5. * Justify why each proposed species was chosen for this protocol:
Zebrafish have become widely accepted throughout the world as a particularly useful organism to analyze how vertebrate development is regulated at the cellular, genetic, and molecular levels. There are a number of reasons for this assessment: (1) the fish are easy to maintain in large numbers and readily reproduce under laboratory conditions; (2) adult fish can be subjected to mutagenesis and mutations can be screened in the first generation by analyzing haploid embryos; (3) the zebrafish embryo has few cells relative to other vertebrates, thus making it a "simple" model for more complex vertebrates such as ourselves; (4) the embryos are optically clear and develop very rapidly and externally (not inside the mother or an eggshell) so that the events involved in the differentiation of tissue, such as the nervous system, can be readily observed; (5) direct access to the developing embryos make it possible to introduce foreign genetic material and to perform

cell labeling and other experimental manipulations; and (6) the zebrafish is a small animal so that large numbers, required for genetics, can be kept and studied.

6. Identify each source of animals for this protocol:

Aquatic Animal Care Services Huestis Facility (AqACS)

Sinnhuber Aquatic Research Laboratory(SARL)

The European Zebrafish Resource Center (EZRC)

Zebrafish International Resource Center (ZIRC)

In-house breeding colony

ZIRC imports novel zebrafish lines from any submitting zebrafish laboratory at the UO and external insitutions, if the line has scientific value for the research community. Specific PIs to be determined.

7. Supporting documents:

Document Name	Date Modified
21-15.pdf(0.01)	10/11/2022 8:58 AM

Alternatives Searches and Duplication

1. Record all searches for alternatives for each procedure that causes pain or distress:

Search Date	Searched Databases	Keywords

There are no items to display

2. Identify any other references used to find alternatives: (such as periodicals, publications and consultation)

ZIRC is supported by the Office of Research Infrastructure Programs (NIH, ORIP) with the specific purpose to function as a genetic repository of zebrafish (Danio rerio) research lines and not any other organism. Thus, there are no reasonable alternatives to the use of zebrafish at ZIRC.

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frozen samples between Stock Centers in bulk, facilitating the regional distribution of live fish strains and minimizing potential discomfort or waiting times of animals in transit. In the long run, we aim to mirror the (cryopreserved) genetic stock inventories between ZIRC, EZRC, and CZRC to further reduce the numbers of animals that need to be maintained alive worldwide. The cooperation between Stock Centers thus refines animal handling and husbandry (shipping) and reduces overall animal numbers.
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3. * The principal investigator asserts that the activities described in this proposal do not unnecessarily duplicate previous experiments:

● **Yes** ○ No

Breeding

1. * **Describe the objectives and justifications for this breeding activity:**

   ZIRC's goal is to propagate genetically modified and wild-type zebrafish strains and to provide these upon request to research laboratories in form of embryos or adult fish. To this end ZIRC has created a cryogenic repository of zebrafish sperm samples for (currently) 46,344 unique zebrafish alleles. When fish are imported typically enough samples are (>20 samples) are cryopreserved for each fish line that are thawed upon request by a researcher and used for *in vitro* fertilization of AB eggs. The ZIRC then provides either embryos directly from this cross, or raises the fish to adulthood and ships genetically identified carriers. When cryopreserved sample counts reach a low threshold (typically 5 samples) a sample is thawed, *in vitro* fertilized with AB eggs, and raised to adulthood to amplify the resource by rearing enough genetically identified males to collect additional sperm samples for cryopreservation. ZIRC also maintains a few dozen genetic lines that either can not be cryopreserved effectively with current methods such as the wild-type lines AB, TU, WIK, NHGRI-1, and SAT, or that are frequently enough requested by researchers to justify that they are more efficiently housed as lice lines rather than frozen sperm. Lastly, ZIRC houses extra stocks of AB fish for cryopreservation- and diet/feeding research that support reduction and refinement goals for fish health and maintenance

2. * **Describe the methods you will use to identify the offspring:**

   ZIRC expends significant effort to ensure that its fish lines are properly identified for the genetic modifications they carry, and that they are healthy.
   To this end ZIRC predominantly uses molecular assays: For example for genetic identification fish are reared, fin clipped, and genetically identified using molecular assays (PCR protocols). Visual observation of embryonic and adult phenotypes, behavior, and gene expression is also used, depending on the particlar line: For example normal versus mutant morphology or pigmentation, or expression of transgenic reporter genes such as GFP.
   Visual characterization is initially used to (daily) monitor fish health in the colony for example by observing appearance (coloration, lesions) and behavior. Other diagnostic tools are used either as follow-up to the initial visual observation or for the quarterly sampling of random and selected colony specimen that are subjected to histochemistry, molecular assays (PCR), necropsy and bacteriology as needed for colony health assessment.

3. * Describe the genotyping methods you will use:

1. Fin clipping for DNA isolation and subsequent PCR analysis
Fin clips are performed under MS-222 anesthesia (178 mg/L in buffered fish water) with a sterile, disposable blade on a clean piece of parafilm to avoid genetic cross contamination between specimen. Personnel is wearing sterile gloves. Infections have not been observed at the amputation site with this technique.
After fin clipping, fish are placed in transparent 1-gallon static water tanks in the main fish room, and are observed for successful and full recovery from MS-222 anesthesia immediately after fin clipping. Typically 2 animals are stored in a crossing cage with a central divider. Fin clipped fish are placed in a dedicated area of the facility and are observed for any signs of pain, distress, or discomfort (or other humane endpoints listed previously) at least once a day until genotyping results are obtained and identified fish can be placed into recirculating water tanks (2-4 days). Water is changed every other day after a feeding with flake food. If genotyoing exceeds 4 days, animals are placed individually in 1-gallon tanks on the recirculating water system (over the weekend and until results are available). Post-operative analgesia is not needed because wound healing is typically extremely fast, and fish do not show signs of discomfort, pain, or distress. If humane endpoints or unexpected outcomes are observed fish will be euthanized by IACUC approved methods (rapid chilling and/or MS-222 overdose).
DNA is extracted from clipped fin tissue and is used for molecular analysis by PCR as described in publications, on ZFIN or NCBI genetic/genomic records, or by protocols specifically developed at ZIRC.

Visual Phenotype in experimental animals or their offspring is used to identify individuals or parent(s): Genotyping information is provided by ZFIN, submitting laboratory, or publication. We carry >12,600 distinct lines that harbor 46,344 alleles among them. Embryonic, larval, or adult phenotypes (or gene expression) will be assessed at appropriate stages, according to published, or submitter-provided information to determine the genotype of parents (recessive traits) or individual fish (dominant). In addition, ZFIN records will be looked up and used to identify genetic carriers, as needed (or as available) for each individual fish line. Molecular genotyping is more efficient, therefore morphology/phenotype will only be assessed if molecular characterization protocols do not exist, or if additional confirmation is needed (e.g. for transgenes that have been detected by PCR, but require additional functional verification of the gene).

Identification of mutation carriers.
Carriers of mutations with obvious visual defects are identified by their phenotype. Individuals carrying visual dominant traits can be easily distinguished from their wild-type siblings. Similarly, fish homozygous for viable recessive mutations with visible phenotypes can be readily distinguished and separated from their heterozygous and

wild-type siblings. Identification of carriers of zygotic recessive lethal mutations is more complex because it involves the analysis of putative carrier progeny derived from single-pair incrosses. A genetic incross scheme is even more complicated when performed to identify carriers of maternal recessive lethal mutations, because it is carried out through two generations. Thus, phenotypic analysis of both zygotic and maternal recessive lethal mutations requires a large number of tanks to accommodate single-pair crosses and to maintain incrossed pairs individually during the time required to phenotype their progeny. In addition, phenotypic analysis of these lines is labor-intensive and depends on single-pair breeding, which can be a limiting factor for stocks that do not breed well or have a skewed sex ratio towards males or females. For these reasons, we identify many of the recessive lethal mutations whose lesions have already been characterized molecularly by genotyping.

Genotyping is independent of breeding, and it allows identification of a large number of mutation carriers in a relatively short time. On the other hand, however, some genotyping procedures rely on expensive reagents such as restriction enzymes and, for some lines, the overall cost of genotyping exceeds significantly the cost of mutant identification by phenotyping. In addition, lines propagated solely on the basis of genotyping may accumulate background mutations that could eventually interfere with or obscure the specific phenotype of the line. For this reason, some mutant lines that are propagated strictly by genotyping are also occasionally analyzed visually to confirm their phenotype. Alleles predominantly identified by genotyping include point mutations generated in the TILLING projects and those recovered in a large-scale mutagenesis screen performed in the [redacted] laboratory as well as retrovirus-induced insertional mutations from [redacted] laboratories.

Lines with “difficult” phenotypes. There are some lines that the Resource Center is not capable of maintaining because the assays required to identify mutant phenotypes cannot be readily performed at ZIRC because specialized equipment (which ZIRC does not have) is required for phenotypic identification of these lines. Examples of such “difficult” lines include mutations that can be identified only by histology or circadian rhythm analysis or mutations affecting embryonic blood pressure. This limitation will change once the mutations are mapped and/or characterized molecularly, allowing identification of mutation carriers by genotyping.

Identification of transgene carriers.

Homozygous and heterozygous transgenic fish expressing fluorescent reporter proteins are identified visually. Transgene carriers that express reporter genes during early development are typically identified as embryos or larvae derived from large group incrosses. In the case of lines with maternal transgene expression, transgene carriers are identified during later stages of development when the maternal component is no

longer detected. Otherwise, embryos lacking the transgene but containing fluorescent reporter products deposited by the mothers could be falsely identified as transgene carriers. Early embryonic stages can be used for transgene identification in lines with the maternal transgene expression only if the screened embryos derive from an outcross in which transgenic males, but not females, are used.
Transgenic lines that do not express visually detectable products are identified based on functional assays. For example, progeny of lines with hsp70 promoter-dependent transgene expression are heat-shocked and then the effects of ectopic transgene expression on morphology are analyzed.
Finally, transgene carriers for a number of lines are also identified by genotyping. Genotyping assays work well for transgenic lines for which the genomic location of the functional transgene has been molecularly characterized. This allows for designing protocols in which transgene- and flanking region-specific primers are used.
Unfortunately, for a majority of transgenic lines maintained at ZIRC, the genomic sites of transgene integration are not known and genotyping is based only on transgene-specific primers. This may lead to false positives if the line contains fragments or non-functional copies of the transgene. For this reason, transgene carriers identified by transgene-specific primers also need to be confirmed, if possible, by identification of transgene function.

4. Describe the breeding scheme:

Tracking alleles in different genetic backgrounds.
Each line imported to the Resource Center is assigned a unique ZIRC Line identification number (ZL) to track the history of the line. ZL numbers are background-specific, and a new number is assigned to a line each time a mutation or a transgene is transferred onto a new genetic background. In our “Lines and Stocks” database, ZL numbers are linked to a stock number (assigned to each new generation) as well as to an allele designation.

Types of lines maintained at ZIRC.
The large variety of strains maintained at ZIRC can be grouped into three categories:
• Wild-type lines
• Mutant lines
• Transgenic lines
Each line type is described in more detail in the following sections:

1) Wild-type lines.
ZIRC maintains several wild-type strains. They include AB, Tübingen (TU), and WIK. Tüpfel longfin (TL) is a double mutant line with a recessive pigment (leo^t1)- and a dominant long fin (lof^dt2) mutation. It is maintained at ZIRC as if it was a wild-type line because it has been used in that manner historically. In addition, two hybrid lines created from AB and TU, namely SAT and NHGRI-1, are also maintained. The SAT line was

provided to ZIRC by the [redacted] laboratory, and NHGRI-1 was obtained from the [redacted] laboratory.

Propagation of wild-type “in-house” stocks.
To maintain the polymorphic character of wild-type lines, the propagation of AB, TU, TL, WIK, SAT and NHGRI-1 “in-house” (IH) stocks is accomplished by combining the same number of embryos (e.g. 40) from each of a minimum of 25 small-group incrosses consisting of 3 females and 2 males. Each generation is screened against embryonic lethal mutations between days 1 and 5, and for swim bladder development at day 5. By applying the selection criteria established at the Institute of Neuroscience and maintaining the line’s genetic diversity, we are following the principles originally established to limit inbreeding in AB.
For SAT and AB, two lineages of IH stocks are maintained in parallel. These lineages are maintained at intervals that are regularly spaced to produce new generations of stocks. In the case of AB, any surplus fish are used for shipping, outcrossing, and egg production for sperm thaws. Should any of the lineages underperform or “crash”, they could be reconstituted from the other one.

“Shipping” stocks.
ZIRC IH wild-type stocks are not distributed to the research community. Dedicated “shipping adult” stocks are created from IH stocks, usually at the same time and in the same manner as new IH stock generations, in order to fulfill customer orders for adult fish. Embryo orders are fulfilled using medium-to-large group crosses of 15-50 IH fish.

AB Wild-type Line Propagation.
Two lineages, named AB-1 and AB-4, are currently maintained in the ZIRC fish facility. Lineage 1 is the original ZIRC AB line acquired from the Institute of Neuroscience (IoN) prior to 2009 and lineage 4 was imported from the IoN zebrafish facility in July 2017.

One aspect of AB line maintenance that has changed is how in-vitro fertilization (IVF) is used as a propagation method. Previously, IVF and natural breeding were alternated with each generation of IH stocks in order to maintain natural spawning capacity when set up for breeding, and to provide gametes when stripped. The latter procedure involved pooling sperm from multiple males then rotating through multiple sperm pools to fertilize individual clutches of eggs. However, this round-robin approach has the potential to disseminate recessive background mutations unchecked as compared with natural breeding, so we discontinued round robin IVF for IH stocks.
In order to preserve the quality of gamete stripping in our populations, IVF has been integrated into a different process of AB line maintenance: 1) We now maintain AB populations designated specifically for the collection of stripped eggs for IVF with thawed sperm samples, and we now generate these populations through alternating group crosses and IVF. Males that do not produce sperm are euthanized since these males do not typically convert to sperm producers over time. Females that release eggs during stripping are isolated from those that do not. When a new IH stock is being generated,

we track the small-group crosses that had been stripped successfully and unsuccessfully for eggs. Several criteria are considered when we select clutches that will contribute to the next IH generation, including clutch size, fertility, and embryonic morphology; however, all else being equal, naturally spawned clutches from females that previously yielded eggs when stripped are prioritized to contribute to the next generation.
To better monitor the quality of the AB population, an added level of screening has been incorporated into AB-1 IH lines as of August 2017. Beyond selecting for normal embryo development, we are now also tracking the progeny of single-pair crosses until they reach approximately 4 months of age. This strategy was initially developed to address pigment mutations that began showing up in AB stocks with increasing frequency yet were not detectable in 5-6 dpf larvae when we only screened for swim bladder development. 4-month screening may prove beneficial in other regards as well: We currently observe female bias in the AB-1 population (62% females on average), which is not ideal due to our heavy reliance on AB for sperm cryopreservation. Since the sex of single-pair offspring can be determined at the 4 month evaluation, we can now choose families with balanced sex-ratios to generate future stocks. Screening fish at 3-4 months requires an added husbandry effort but helps eliminate pigment mutations, non-lethal malformations of fish morphology, and skewed sex ratios.

We are also trying to decrease disease susceptibility and increase the fertile period in the AB population. Previously, 5-month old AB siblings were bred every five months, and new generations were established sequentially from the preceding ones. We are now trying a new breeding scheme that back crosses the 5 month-old offspring of an AB stock to their 10 month-old parents to generate a new generation. While this does not slow down inbreeding significantly over the sequential breeding approach, we select for sustained breeding at 10 months of age. Furthermore, because health monitoring typically happens around 8 months, we also select for better disease resistance.

2) Mutant lines. The Resource Center maintains a large number of mutant lines carrying a variety of mutations. These include:
• Point mutations
• Deletions
• Translocations and complex chromosomal rearrangements
• Insertional mutations

Recessive zygotic lethal mutations maintained at ZIRC were predominantly generated in the original Tübingen and Boston screens as well as in the retrovirus-mediated insertional mutagenesis carried out in the [redacted] laboratory. The mutant lines that have been more recently generated by the TILLING consortia or large-scale mutagenesis screens performed in the [redacted] laboratory as well as in the [redacted] and [redacted] laboratories have a significant number of recessive viable mutations. The Resource Center also maintains lines with maternal-effect mutations received from the Mullins lab. Recently, targeted CRISPR/cas9-generated knock-out mutations (e.g., y656) have also been imported from numerous laboratories and are propagated at the ZIRC.

3) Transgenic lines. The majority of the transgenic lines propagated and maintained at the Resource Center can be classified into the following groups:
• Lines with transgenes that contain exogenous promoters driving the expression of a reporter gene (e.g. Tg(-1.8gsc:GFP)ml1)
• Lines generated for ectopic gene expression (e.g. Tg(hsp70l:XlFgfr1)pd3)
• Enhancer traps (e.g. Et(-1.5hsp70l:Gal4-VP16)s1005t)
• Gene traps (e.g. Gt(T2KSAG)j1104a)
• Chromosomal position markers (e.g. Tg(XlEef1a1:EGFP)j1642a)
• CRISPR/cas9-generated transgenic lines (e.g. Tg(mlanYFP)^xt17Tg)

Propagation of mutant and transgenic lines.
Only unambiguously identified carriers of mutations and transgenes are used to propagate a line. Typically, we set up group crosses to produce new stocks. Based on our experience, these crosses are more productive if the number of females exceeds the number of males. The group crosses are set up in tanks of different sizes, based on the number of identified and available carriers, as well as on the demand for the line.
Crosses set up most commonly at ZIRC for line propagation include:
• “Big Breeder” crosses set up in large tanks that hold up to 50 fish
• 1.5-gallon tank group crosses that contain 15 females and 10 males
• Small group crosses set up in crossing cages with 3 females and 2 males
To maximize the number of mutation and transgene carriers in each new generation, incrosses rather than outcrosses are preferred for line propagation. Incrosses also ensure that homozygous individuals are generated. The demand is very high for homozygous fish, such as those carrying viable recessive mutations, or transgenes with reporter gene expression under control of UAS (e.g. Tg(UAS:EGFP)kca33).
Line propagation solely by incrossing may lead to inbreeding which significantly reduces the vigor and fecundity of the line. For this reason, after three generations of incrossing, an outcross is used to propagate the line. Some lines, such as those carrying maternal dominant lethal mutations, can only be propagated by male outcrosses. Outcrossing is also frequently used for lines carrying multiple alleles generated in large-scale screens in an attempt to “clean up” these lines by crossing out background mutations. Based on the finding that crossing-over events are suppressed in zebrafish males as compared to females (Singer et al., Genetics, vol. 160, 649-657, 2002), only females are used for outcrossing these lines. This is to assure a more effective elimination of background mutations residing on the same chromosome as the induced mutation is.
The AB wild-type line is used for outcrossing mutant and transgenic lines. To accommodate outcrosses, some AB stocks are exclusively dedicated and strictly used only for mutant and transgenic line propagation.
In-house biosafety. The Resource Center bleaches all embryos before each new stock is submitted to the nursery. This is a precautionary measure to minimize transmission of disease. To minimize pathogen transmission further, individual AB fish that are exposed to specific mutant lines during outcrossing are either saved for future breeding with the same line only or euthanized.

Maintenance of zebrafish stocks.
Genetic lines are evaluated individually to determine the optimal number of animals per stock as well as their specific tank requirements. Lines frequently requested or used (such as the wild-type AB line) are bred in large quantities (approximately 1000 animals per stock) to satisfy demand. Mutant and transgenic fish for which moderate or infrequent requests are received are maintained in lower quantities (either from 100 to 150 individuals per unidentified stock or 25 identified fish per stock). Depending on the number of fish, tanks of two sizes are used for growing and housing fish after they leave the nursery:
• 20-gallon tanks can accommodate up to 300 fish. We use these tanks for lines that are in great demand (e.g. wild-type fish, pigmentation mutants used to assess the effectiveness of mutagenesis protocols, and other frequently requested mutant and transgenic lines)
• 5-gallon tanks can accommodate up to 120 fish. We use these tanks to house AB stocks specifically for production and egg stripping. In the case that stocks consist of 840 fish, this can provide 7 different tanks to be used 7 weeks in a row without having to rest fish between egg stripping or breeding events.
• 1-gallon tanks can house maximally 25 fish. We use these tanks to house unidentified as well as identified mutant/transgene carriers that are in low demand.

Double mutant/transgenic lines.
The Resource Center does not generally maintain double mutant/transgenic lines, but instead distributes carriers of single mutations and transgenes. ZIRC customers are advised to generate complex genotypes themselves. We also do not provide crosses between different genetic strains. In both cases, the labor involved outweighs the benefits. However, we may revisit these policies in the future as more mutations are characterized molecularly and their identification and maintenance is facilitated by genotyping. Exceptions to this policy are lines such as TL that contains mutations in lof and leo or the “transparent” Casper line (mitfaw2/w2;roya9/a9). Additional exceptions are enhancer trap lines expressing either Gal4 or Gal4VP16. To identify functional enhancer trap lines visually, we cross unidentified individuals to carriers of reporter transgenes such as Tg(UAS:GFP) or Tg(UAS:Kaede). Similarly, to identify reporter lines visually, we cross unidentified individuals to lines with functional enhancer traps. 25% of the progeny in these crosses express the fluorescent reporter and are therefore easily identified as double transgenic carriers.

Criteria for maintaining a line as a living stock or as frozen sperm.
Tank space in the Resource Center is limited, therefore we need to decide which lines are maintained as live stocks and which should be made available only as frozen sperm. An email poll of the research community and the records of our order requests are both used to compile a list of the most “popular” lines maintained at the Resource Center as live stocks.
Each newly imported line is evaluated with regard to its perceived scientific popularity.

The lines deemed popular are maintained as live stocks and are made available to the research community as fish and/or embryos. These lines are then evaluated every 6 months to determine whether they should be “retired” (and be made available only as embryos from frozen sperm) or continued as living stocks. Our current guideline is to maintain lines alive if they are requested twice or more within 6 months. Lines with fewer requests are “retired”. The fish destined for “retirement” are typically euthanized, but not before they are a year old. The turn-over of frozen and live lines has significantly improved the efficiency and efficacy of ZIRC line management. Developing the criteria, workflow, database support and interaction between work-groups took several years of streamlining.

Line Management: Live fish and frozen samples turn-over
Cryopreservation of imported lines. Zebrafish lines are imported to ZIRC either as live males directly into the quarantine room, or as frozen sperm samples that are transferred to vapor-phase liquid nitrogen freezers. The goal for both import pathways is to generate and provide as many quality samples as possible for these lines for redistribution to the research community. However, the methods for achieving the goal are different for each import method. In the case of live lines submitted from outside facilities, the males are cryopreserved by testis dissection and the sperm cells are pooled from all males of the same line. Briefly, following the Quarantine Room procedures, males are separated from females after importation, housed in 1-gallon tanks at a maximum density of 9 males per tank, and maintained at this density for 2-4 weeks (conditioning). To obtain sperm samples, each male is humanely euthanized, a fin clip is acquired for genotype confirmation, sperm is stripped, testes are dissected, and the males are fixed for pathology. The pooled cryogenic samples are designated as C4 (samples from males genotyped elsewhere) and can then be distributed directly to the research community.
Thawing samples for production and regeneration. When customers order an allele online (http://zebrafish.org/fish/lineAll.php) that is available either alive, frozen, or frozen star (frozen*), ZIRC’s sales manager ([redacted]) schedules the order for the first available week that works for the recipients and ZIRC schedule (shipping pipeline/production). A production schedule is prepared. Typically, this is the week following the receipt of an order if it was placed before Thursday at noon, and if international documents do not need to be procured and prepared.
All live and frozen samples are reserved in the database by the caretakers on the production list a week before the scheduled shipment. If the allele is maintained live in the facility, the responsible fish caretaker will determine the number of fish that are required for breeding to complete the order. After spawning, embryos are collected, sorted, and bleached. The number of embryos ordered are counted out into embryo medium and placed in flasks for shipments on the scheduled shipping day.
If an allele is available as a frozen sample, meaning there are more than 3 samples available for shipment, then the sample needs to be thawed two days prior to the date of shipping. Before thawing, the available category(ies) of sample(s) (C5, C4, C3, C2, C1) are reviewed in the database and the appropriate sample for thawing is determined. For shipping, C5 samples have highest priority, followed by C2/C4 and finally C1 type

samples. (C3 samples are used for in-house purposes only).
The samples for production are collected on Friday afternoon and placed in a specific cryovial box set aside for the thawing events of the following week. Samples are thawed and in vitro fertilized with AB eggs. The resulting embryos are sorted and bleached at 24hpf. When C5 and C4 samples are shipped, a minimum of 30 embryos and a maximum of 200 embryos are counted and packaged, depending on the fertility rate of the thawed sperm sample. When C2 samples need to be shipped, a minimum of 80 embryos and a maximum of 200 embryos are shipped to the customer. The difference in the provided numbers between C2 and C5/C4 is due to the probability of obtaining the particular/requested allele in the sample and by the process of cryopreservation and the expected post-thaw quality of the sample.
For the Distribution and Regeneration of Sanger F1 lines: Only 3 sperm samples are available per line, which normally, would fall into the frozen* category. With the significant improvement of the cryopreservation, thawing and IVF protocols, we now obtain sufficient embryos from these thaws to fullfil two goals: Expedient distribution after a request has been received and amplifying the resource. Therefore, in order to shorten the turn-around time between receiving an order and shipping, and to ensure proper preservation of the line at ZIRC we established a new strategy: C1 samples are split after IVF and a number of embryos is set as side for rearing at ZIRC and future amplification of the samples. The second portion is shipped to the researchers.
However, if less than 3 frozen samples exist for a requested allele (i.e. a frozen* availability), then the line must be regenerated before the line can be provided to the research community. In this case, the customer will be given the option of receiving an unidentified pair of adults or if the males are needed for cryopreservation, then unidentified adult females are offered. If researchers prefer to receive identified adults, we will generate and ship embryos from a C2 thaw once sufficient samples have been frozen. This will typically require a 5-6 months waiting period after regeneration of the line.

Cryopreservation of reamplified lines.
Since 2012, the majority of lines submitted to ZIRC have been imported as frozen sperm samples of multi-allelic lines for which we received only 3 samples from the submitter. After a request for an allele has been received, samples are thawed, and a stock is raised at ZIRC. Once the males from that stock have reached maturity (typically around 5 moths of age), they are conditioned for an additional 4-6 weeks before gametes are obtained. We amplify our cryogenic repository stocks with two methods. 1) The first approach is to pool sperm from a group of unidentified males (C2 sperm samples) The second method is carried out either by collecting sperm from genotyped males, following the ID-Freeze procedure, or by first obtaining a fin-clip at the time of cryopreservation and then performing the genotyping procedure to identify the alleles in the samples. Both methods result in the generation of C5 sperm samples, and thaws of each procedure generate embryos that can be distributed directly to the research community. When the number of males for these multi-allelic lines is low (below 15), testis dissection is performed to generate the desired minimal number of samples determined for the line

(typically 20 samples). When there are more than 15 males, males are only stripped for their sperm, as the sperm density is high enough and testis dissection is not required to obtain adequate cell densities for the pooled samples.
Mono-allelic lines, as well as lines with 4 or fewer alleles identified, are similarly cryopreserved using the process for pooled ID-Freeze sperm (see above). Again, once mature, males are conditioned for 4-6 weeks depending on their age and size, and then sperm is collected, pooled and cryopreserved for each line. Typically, testis have to be dissected from all males to obtain the desired cell density and sample number (between 15 and 30 depending on the number of alleles present). This procedure is followed when there are fewer than 15 males available. When 16 or more males are identified for cryopreservation, which is most often the case, the males are simply stripped of sperm and kept alive to fulfill future orders for live adults or embryos for the line while the fish are of good breeding age.
The consistent turn-over of lines between the cryopreserved and live states is designed to provide sufficient samples to always respond swiftly to user requests - before the resource is entirely depleted, to provide live lines to replenish the frozen resources, and also to identify previously unconfirmed genotypes in the repository. Owing to the streamlined turn-over strategies, ZIRC has reduced the turn-around time between incoming orders and shipments from several months to few (1-2) weeks for most of its frozen lines.

5. Identify any other protocols to which you will supply animals bred from this protocol:

Housing and Use

1. Identify each vivarium location where animals will be housed or used:

	Name	Species	Hours
View	ZIRC 118	Zebrafish	Greater than 24 hours
View	ZIRC 103	Zebrafish	Greater than 24 hours
View	ZIRC 128	Zebrafish	Greater than 24 hours

2. Identify each non-vivarium location where animals will be housed or used:

	Name	Species	Hours	Reason
View	ZIRC 105	Zebrafish	Less than 12 hours	For euthanasia, with subsequent organ removal, DNA extraction, or specimen fixation and processing, e.g. for experimental or diagnostic processing.
View	ZIRC 109	Zebrafish	Less than 12 hours	For anesthesia and gamete stripping, e.g. for sperm cryopreservation or in vitro fertilization of eggs with sperm.
View	ZIRC 126	Zebrafish	Less than 12 hours	For euthanasia, with subsequent organ removal, DNA extraction, or specimen fixation and processing, e.g. for experimental or diagnostic processing.
View	ZIRC 127A	Zebrafish	Less than 12 hours	Anesthetized embryos or adults for microscopy

Disposition

1. **Disposition plans for the animals when this research is complete:**

(check all that apply)

The animals will be euthanized according to the procedures described in this protocol.

2. **If other, provide an animal disposition description:**

Cooperative Research

1. * **Is this a cooperative research project (are there principal investigators from more than one institution involved)?**
   ● **Yes** ○ No

2. * **Is any of the animal work and animal housing being conducted at the cooperating institution?**
   ○ Yes ● **No**

3. * **Describe the nature of the collaboration.**
   [redacted], Professor Emeritus at OSU is supported by the ZIRC grant in his role providing diagnostic support for ZIRC's veterinarian [redacted] and to conduct health research with her and ZIRC.

Zebrafish International Resource Center

Supporting Documents

1. Attach supporting files:

Document Name	Date Modified
There are no items to display	