MASTER ANIMAL USE PROTOCOL FORM FOR RESEARCH
University of California at Berkeley

Type all entries. See **Instructions** for details on completing each item. Answer all items on the form by inserting text in the various sections that follow. Submit the completed form to the Animal Care and Use Committee by email attachment (preferred method of submission) to acuc@uclink.berkeley.edu or by campus mail to Room 201, Northwest Animal Facility. For proposed uses of animals in teaching, use the "Animal Use Protocol Form for Instruction."

1. **PRINCIPAL INVESTIGATOR** Sharon L. Amacher **Email:** amacher@berkeley.edu

   Faculty Title: Assistant Professor **Department:** Molecular and Cell Biology

   Phones: Office: x-xxxx **Laboratory:** x-xxxx **Emergency**: (xxx)-xxx-xxxx

   Mailing Address: 142 LSA-MCB #3200

2. A. **ALTERNATE PRINCIPAL INVESTIGATOR** John Ngai **Email:** jngai@socrates.berkeley.edu

   Faculty Title: Professor **Department:** Molecular and Cell Biology

   Phones: Office: x-xxxx **Laboratory:** x-xxxx **Emergency**: (xxx)-xxx-xxxx

   Mailing Address: 142 LSA-MCB #3200

   B. **ALTERNATE LAB CONTACT** Jennifer Anderson **Email:** jen_ande@berkeley.edu

   Title: SRA III **Department:** Molecular and Cell Biology

   Phones: Office: x-xxxx **Laboratory:** x-xxxx **Emergency**: (xxx)-xxx-xxx

   Mailing Address: 142 LSA-MCB #3200

3. **HAZARDOUS AGENTS:**

   A. Will hazardous agents procedures be used in live animals or within animal facilities?
   ☒ Yes ☐ No. If **yes**, provide the information requested below.

   Radioisotopes: name of isotope(s) None
   Infectious Agents: name and biosafety level None
   Regulated Chemical Carcinogens: name and biosafety level 1-Nitroso-N-ethylurea (ENU; SARA Section 313), 1-Phenyl-2-thiourea (PTU; SARA Section 313)
   Other Hazardous Agents: name Cyclopamine
   Recombinant DNA: Biosafety level BL 1

   B. If remains or excreta of animals treated with hazardous agents will require special disposal, describe the method and who will perform:

[Begin typing here (box will expand as needed)]

4. **SUMMARY OF ANIMAL SPECIES, NUMBERS, AND SOURCE:** List all animal species and indicate numbers to be used per year. Give common name for standard laboratory species, and both common and scientific name for non-standard laboratory species. Indicate the source of each species to be used. See **Instructions** for additional information.

Species	Number per Year	Source
Zebrafish (Adults)	6,500	Laboratory-bred strains
Zebrafish (Embryos)	117,500	Laboratory-bred strains

Continued on page _____

5. **PERMITS**: Are permits required for handling, importation, collection or maintenance of animals?
☒ Yes ☐ No. If **yes**, list agencies that require permits, and permit status below:

A. Agency: Transgenic Animals require permits from CDFG; currently UCB maintains the CDFG permit for all aquatic transgenic species; our lab protocol has been approved by CLEB on campus
Permit Application: ☒ Approved ☐ Pending ☐ To be submitted

B. Agency: _____
Permit Application: ☐ Approved ☐ Pending ☐ To be submitted

Continued on page _____

6. **LOCATION OF ANIMAL PROCEDURES:** List all locations where animal procedures will be performed. See **Instructions** for details.

Species	Surgery/Recovery	Other Manipulation Restraint	Euthanasia
Zebrafish embryos	555 LSA		LSAB 26, 555 LSA
Zebrafish adults		LSAB 26	LSAB 26

Continued on page _____

7. **EXTRAMURAL SCIENTIFIC REVIEW:** Will all of the proposed uses of animals described in your AUP be subjected to extramural peer review for scientific merit? Yes ☒ No ☐

If no, indicate below those uses that will not be reviewed extramurally:

[Begin typing here (box will expand as needed)]

8. **PUBLIC DISCLOSURE:** Does this protocol describe any unpublished techniques, procedures or data for which copyright or patent may be sought, or the disclosure of which would place your research effort at a competitive disadvantage? Yes ☐ No ☒ If yes, indicate below the protocol section number(s) you wish considered for exemption from disclosure:

[Begin typing here (box will expand as needed)]

9. **APPLICANT'S CERTIFICATION** I certify that:

- This Protocol provides a complete and accurate description of all proposed uses of live vertebrate animals in this research activity. Any proposed revisions to animal care and use procedures will be promptly forwarded in writing to the ACUC for review and approval prior to implementation.
- I agree to abide by all applicable laws, policies and regulations, including the U. S. Animal Welfare Act, the National Research Council Guide to the Care and Use of Laboratory Animals, the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and all UCB policies and procedures, regulating the humane use of vertebrate animals in instruction and research.
- I will comply with all regulations governing the importation, collection and/or maintenance of wild species, including obtaining permits from all applicable regulatory agencies prior to the acquisition of animals.
- I have completed (or agree to complete) a Tier One Training seminar offered by the Office of Laboratory Animal Care. I agree to attend a Tier One Training seminar at least once every 5 years to assure that I remain current on laws, regulations, guidelines and policies pertaining to the use of animals.
- All procedures involving live animals will be performed under my supervision. All personnel having direct contact with live vertebrate animals, including myself, have been or will be trained in humane and scientifically acceptable procedures for animal handling, administration of therapeutic drugs and euthanasia to be used in this project. I accept responsibility for ensuring that all personnel working with live vertebrate animals are aware of, and will not deviate from, the ACUC approved procedures outlined in this protocol, that they will adhere to the regulations regarding the humane treatment of animals and that they will receive proper training as required by the ACUC. Personnel will be allowed adequate time to obtain necessary training for this project and will not begin any procedures with live animals until they have been successfully trained. Listed participants will perform only those procedures for which they have received adequate training.
- I understand that personnel with substantial animal contact, as defined by the Campus Occupational Health Service, will be required to participate in a medical surveillance program. I agree to inform all persons working with animals under my supervision regarding the availability of this program (contact the ACUC office if you need additional information).

Sharon L. Amacher	5/17/04	
Typed name: Instructor	Date	Signature (no *per* signatures)

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(For ACUC use only)

Comments or Conditions of Approval:

Animal Care and Use Committee	Date
Approval Period	Protocol Number

NARRATIVE DESCRIPTION OF ANIMAL RESEARCH

The following items (#10-13) are to be answered in narrative form by inserting text in the sections that follow. For each item, provide the detailed information described in **Instructions**. If not applicable, indicate N/A. ATTACHMENTS A, B, C, and D are to be completed on the forms themselves. *Please note that an incomplete or poorly prepared description will delay ACUC review of your protocol.*

10. **RESEARCH GOALS**

We are interested in how cells become sequentially specified to more precisely defined fates during vertebrate embryonic development. We use genetics, molecular biology, and embryology to investigate mesodermal patterning and segmentation in the zebrafish embryo. We aim to (1) characterize the genes involved these processes, (2) establish how these genes interact in genetic pathways and networks, (3) understand how these genes function to regulate cell fate and cell movements during development. Our genetic screens identify genes that function during normal embryonic development. We anticipate that our work will eventually provide animal models, diagnostic tools, and therapeutic approaches for human genetic diseases, cancer, and birth defects.

11. **JUSTIFICATION FOR ANIMAL USE**

A. Rationale for Use of Animals

The goals of this project are to understand how a group of seemingly homogeneous mesodermal precursor cells become different from one another. We study some of the earliest cell fate decisions, that of notochord, floor plate, and muscle cells, and there is no cell line available that would allow us to study this process in vitro. We study cell behaviors and gene expression over time as the living embryo matures; these experiments are impossible to duplicate in non-animal alternatives. Almost all of our procedures are done in embryos before the nervous system has matured and experiments are terminated before hatching and long before feeding would normally begin. In the few procedures when slight pain or distress might be felt (egg and sperm collection, ENU mutagenesis, and fin clipping), every effort is made to minimize pain and distress.

B. Rationale for Choice of Species to be used

The zebrafish has become widely accepted throughout the world as a particularly useful model system to study vertebrate development at the cellular, genetic, and molecular level. There are a number of reasons for this assessment: (1) the fish are easy to maintain and readily reproduce under laboratory conditions, (2) adult fish can be subjected to mutagenesis and mutations can be screened in the second 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 humans), (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 the mesoderm can be readily observed, (5) direct access to developing embryos make it possible to introduce foreign genetic material and to perform cell labeling and other experimental perturbations, and (6) the zebrafish is a small animal so that large numbers, required for genetics, can be kept and studied in a relatively small space. The number of embryos proposed for the research are necessary for (1) embryological observation, morphological characterization, and microsurgery experiments, (2) gene expression analyses, and (3) identification of heterozygous mutant carrier adults. The number of adults proposed for the research are necessary for (1) maintenance of wild-type and mapping strains, (2) generation of mutagenesis stocks, and (3) maintenance and breeding of mutant and transgenic lines.

For laboratory research, complete item 12. For field research, skip to item 13. For laboratory and field research, complete both items 12 and 13.

12. **DESCRIPTION OF LABORATORY RESEARCH**

A. Description of Proposed Procedures*
*See **Instructions** for level of detail required.

The zebrafish research community has prepared its own detailed user manual that describes the standard procedures used by zebrafish researchers. This was originally done because of the special requirements of the zebrafish and how we use them as contrasted with use or other vertebrates (particularly birds and mammals) at research institutions. The zebrafish standard operating procedure manual is The Zebrafish Book (1995). The

Zebrafish Book covers all of the experimental procedures proposed, including invasive ones carried out with embryos, either done within the facility (e.g. DNA or vital dye injection into early embryonic cells) or in the laboratory (e.g. cell labeling and microsurgery). In addition, all usual facility operations are described in The Zebrafish Book and include care and maintenance of adults, breeding and obtaining gametes and embryos (including parthenogenetic embryos), raising larvae, cryogenic preservation of sperm, fin clips, mutagenesis, strain record keeping, sending fish to and receiving fish from other laboratories, quarantine and other procedures related to disease control, and euthanasia. We have provided a copy of The Zebrafish Book to the OLAC basement staff. In addition, The Zebrafish Book is available on the internet: go to http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg and choose the "The Zebrafish Book" under “News and Information” on the sidebar menu.

Procedures to obtain eggs, sperm, and embryos.

Natural spawnings. To obtain fertilized eggs, we place one male and one female (or a combination up to 5 fish) in a mouse cage the evening before fertilized eggs are needed. Spawning occurs throughout the year on a diurnal cycle; eggs are laid and fertilized upon exposure to morning light. In order to identify heterozygous carriers and to obtain enough embryos for experiments, it is often necessary to set up several matings. The fertilized eggs are collected on the morning of spawning, and the fish are then returned to their holding tanks. Zebrafish are fertile for about 1 1/2 years, and single fish are bred in this non-stressful manner no more often than once weekly. Depending upon the strain, adult fish may be bred in this manner between 3 and 30 times. In addition, the Aquatic Habitats Holding System is specially designedto make it possible to collect fertilized eggs directly from the tanks without moving the fish into separate containers; if this method is accepted by OLAC staff, we may use it in some instances.

Procedure to obtain unfertilized eggs. Females are anesthetized in 0.004% MS-222 (tricaine) until gill movement is slowed. The abdomen of the fish is then gently squeezed with the index finger in a rostral to caudal direction to express the eggs. The fish is then placed into a mouse cage containing system water to recover. Excessive pain and distress will be judged by the failure to recover normal swimming movements; these animals will be euthanized. We will monitor and evaluate the fish frequently following the procedure (continuous for the first few hours), and then at least twice per day thereafter. Mortality is <5% (usually no animals are lost); we will advise OLAC and ACUC if we observe higher rates of mortality. Eggs are obtained from females in this manner no more often than once every three weeks, and the procedure rarely performed more than 5 times in a fish’s lifetime (usually only once or twice).

Procedure to obtain sperm. Males are anesthetized in 0.004% MS-222 (tricaine) until gill movement is slowed. The fish is then held ventral upward and the abdomen is gently stroked in a rostral to caudal direction to release sperm, which is collected in a glass capillary. The fish is then placed into a mouse cage containing aquarium water to recover. Excessive pain and distress will be judged by the failure to recover normal swimming movements; these animals will be euthanized. We will monitor and evaluate the fish frequently following the procedure (continuous for the first few hours), and then at least twice per day thereafter. Mortality is <5% (usually no animals are lost); we will advise OLAC and ACUC if we observe higher rates of mortality. Sperm is obtained from males no more often than once every three weeks, and the procedure is rarely performed more than 5 times in a fish’s lifetime.

Procedure for generating transgenic zebrafish

Linearized plasmid DNA is injected using a pressure injector at the 1-4 cell stage. In some experiments, SceI meganuclease is co-injected to increase efficiency of transgenesis (this technique has been shown to substantially reduce the number of transgenic founders that need to be raised). Experimental glass pipets used to inject DNA are pulled under a tungsten heat source. Cell injections are performed long before any skin has formed, so no preparation of skin is required. We expect none of the constructs injected to be toxic or detrimental to the transgenic strains. The only exception to this are sequences under the control of inducible promoters; in this case, some of the constructs may have detrimental effects in transgenic progeny, but only under inducible conditions. Final approval of our transgenic zebrafish SOP and inspection of our animal facility by a Biosafety officer from EH&S was completed on 3/23/2003.

The DNA will be derived from any of the following sources (or combinations thereof): genomic zebrafish DNA from genomic libraries; zebrafish cDNA from various cDNA libraries constructed in our laboratory or by reverse-transcription PCR of zebrafish RNA; a gene encoding green fluoresecent protein (GFP) and its variants (e.g. EGFP) from the jellyfish Aequorea Victoria to use as a visual in vivo marker; a gene encoding a red fluorescent

protein, dsRED, and its variants, from Discosoma coral to use as a visual in vivo marker; the bacterial lacZ gene encoding the enzyme beta-galactosidase to use as a histochemical marker; a yeast upstream activating sequence (UAS) and yeast GAL4 coding sequence to use for GAL4-inducible expression; plant Phytochrome A (PhyA), Phytochrome B (PhyB), and PIF3 coding sequences, a ~500 bp internal ribosome entry site (IRES) from the encephalomyocarditis virus (ECMV); polyadenylation signal sequence from SV40 (<200 bp); transcriptional promoter sequence from the Xenopus EF1-alpha gene; transcription promoter sequence from the alpha-actin gene; various bacterial plasmid sequences; human beta-cytoplasmic actin fused in frame to GFP (Clontech vector #6116-1), a 30 bp sequence encoding a 10 amino acid amino acid palmitylation/myristylation signal sequence (MGCIRSKRKD) (tethers proteins to the cell membrane); partial coding sequences from human rac and cdc42 that encode dominant-negative proteins.

The zebrafish inserts will include presomitic transcription factors (her1, her7, Suppressor of hairless) and membrane receptors (ephrins, EphR, Notch, Delta) [including coding regions, 5' flanking sequences, and/or 3' flanking sequences], as well as other sequences normally expressed in zebrafish. These sequences will be constructed together with various "marker" genes such as lacZ or GFP, either as a direct fusion protein (to visualize protein localization in vivo), or in conjection with the ECMV IRES, which facilitates the translation of multiple proteins from a single mRNA molecule (e.g. her-1 transcription factor-IRES-marker protein). Other inserts, derived from human or mouse include sequences from actin regulatory molecules such as rac, cdc42, and beta-actin. In other experiments, we will will use published constructs containing plant phytochrome and PIF3 coding sequences fused in frame to yeast GAL4 coding sequence (Shimizu-Sato et al., 2002) and a separate construct containing yeast UAS regulatory sequence driving expression of various zebrafish cDNAs; red-light activates the phytochrome-GAL4 and PIF-GAL4 proteins, which then activate expression of the zebrafish cDNA. In some cases, constructs may also include virally-encoded transcriptional promoter and polyadenylation sequences or varying amounts of bacterial plasmid vector sequences.

Procedure for ENU mutagenesis:
Once or twice a year, ten to twenty males are treated with 0.8-1.0 mM 1-nitroso-N-ethylurea (ENU) for 1 hour at 28.5ºC to induce mutations in their sperm. The mutagenesis procedure is performed in a chemical hood. After treatment, the fish are gently netted and passed through three containers of fresh aquarium water to rinse off any residual ENU. The fish recover in the hood for approximately 8 hours, with noise, vibration, and lighting kept to a minimum. After the recovery period, they are returned to the animal facility. The ENU-treated males are bred over the next two weeks to pass potential mutations to the next generation, after which they are euthanized. No special disposal of animal remains or excreta is required. ENU is a highly unstable molecule and is completely inactivated by treatment with high pH and sodium thiosulfate for 24 hours. Our SOP for the potential carcinogen ENU was approved by EH&S on 9/19/2001.

1) Drug Administration: The invasive procedures done on embryos will be performed at very early developmental stages before the nervous system has matured. Because embryos lack the structures (sensory neurons) necessary to detect pain, they are very unlikely to be susceptible to painful stimuli. On the other hand, the developing muscle cells in the embryos twitch spontaneously, causing the embryos to move. To prevent such movements, which make observations of cells more difficult, embryos older than 17 hours will be anesthetized in Tricaine, also called MS 222, added to the water. Tricaine is the best one available for lower (aquatic "cold-blooded") vertebrates. The dosage is age-dependent. Anesthesia is administered by immersing the animal in the anesthetic to facilitate handling of the fish, e.g. during procedures to obtain gametes from adults which involves handling of the fish but produces minimal discomfort even if the fish were alert. There is no permanent impairment.

In some experiments, embryos are incubated in 100 μM cyclopamine (CAS # 4449-51-8) to block the Hedgehog signaling pathway. Embryos are treated at low density (25 embryos/35mm dish) beginning prior to gastrulation (0-6 hours) until they are sacrificed at approximately 24-36 hours. Gloves are worn during the procedure. We are in contact with Biosafety officers at EH&S, who are advising us regarding disposal procedures and animal use regulations for this chemical.

In some experiments, embryos are incubated in 100 μM DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; Sigma D5492; CAS #208255-80-5) to block the Notch signaling pathway. Our protocol and dose are based upon published zebrafish experiments [Geling et al. 2002, *EMBO* **3**, 688-694]. Embryos are treated at low density (25 embryos/35mm dish), with treatment beginning between the blastula stage (3 hours) and mid-segmentation stage (<18 hours) and continuing for varying amounts of time, usually 1-12 hours. The

embryos are sacrificed no later than 24-36 hours. Gloves are worn during the procedure. We are in contact with Biosafety officers at EH&S, who are advising us regarding disposal procedures and animal use regulations for this chemical.

In some experiments, embryos are treated with 75-100 μM (~0.001 – 0.0015%) 1-phenyl-2-thiourea (PTU; CAS # 103-85-5) to block pigmentation by inhibiting the tyrosinase-dependent steps in the melanin biosynthetic pathway. The dose we use is reported to be optimal as it is effective at blocking pigmentation and has no effect on zebrafish hatching and survival [Karlsson et al. (2001), *Mar Biotehnol* **3**, 522-527). Embryos are treated at low density (25 embryos/35 mm dish or 100 embryos/100 mm dish) beginning at approximately 18-24 hours until they are sacrificed at approximately 36-48 hours (prior to hatching). We are in contact with Biosafety officers at EH&S, who are advising us regarding disposal procedures and animal use regulations for this chemical.

2) Surgery
Embryological procedures: Most cell injections and microsurgery procedures that we perform are done before or at the gastrula stage, well before the animals have developed nervous systems or epidermis (skin). Injection of vital dyes, mRNAs, plasmid DNA, antisense morpholino-modified oligonucleotides (MOs), and linearized plasmid DNA at the one to two-cell stage is described in detail in The Zebrafish Book. Embryos either develop normally (vital dye injections) or sometimes abnormally (RNA, DNA, and MO injections), but in all cases, embryos are sacrificed before nervous system maturation. EH&S confirmed (on 6/19/2003) that we need only to register "transgenic" experiments that stably introduce DNA into the genome, thus our transient expression experiments involving injections of mRNA, plasmid DNA, and MOs need not be registered with their office. Experiments involving stable integration of DNA into the genome must be registered and our transgenic zebrafish SOP was approved by EH&S on 3/23/2003. In microsurgery (transplantation) experiments (also described in detail in The Zebrafish Book), vital dye-labeled cells are mixed with unlabelled ones before or during gastrulation; these mosaic embryos develop completely normally, just as unoperated controls. The dye allows one to follow a few labeled cells in the context of the entire (unlabeled) embryo. Cell injections and microsurgery are performed before any skin has formed, thus no preparation of skin is required. The embryo medium contains antibiotics (Penicillin (100,000 units per liter) and Streptomycin (100 mg per liter) to prevent bacterial growth. Experimental glass pipets used to inject dye or DNA or to transfer cells are pulled under a tungsten heat source and then fire polished to smooth and sterilize. Anesthesia is not necessary (see above #1). Experiments are performed in rooms and solutions that are heated to 28.5 C, the standard temperature used to raise zebrafish. Embryos are typically sacrificed before 24 hours of development, well before hatching and feeding begins (2-5 days).

Caudal fin clip procedure: The only surgical procedure we will perform on zebrafish adults is a caudal fin clip to collect tissue for DNA isolation and PCR analysis. The procedure allows us to genotype and screen for individuals carrying mutations in specific genes. The zebrafish caudal fin reliably regenerates in about two weeks when the procedure is done correctly. The surgical area will be cleaned are sterilized with 95% ethanol, and surgical tools (very sharp microscissors) will be dipped in 95% ethanol and flame-sterilized before use. Gloves will be worn during the procedure. Fish are anesthetized in 0.004% MS-222 (tricaine) until gill movement is slowed. While the fish is held still in a net with the tail accessible, no more than half the caudal fin (halfway between the tip of the fin and the point where the scales end) is quickly clipped with sterilized scissors. The procedure is extremely rapid and should cause no bleeding. Fish are kept in individual holding tanks containing aquarium water for post-surgical observation. Excessive pain and distress will be judged by the failure to recover normal swimming movements; these animals will be euthanized. We will monitor and evaluate the fish frequently following the procedure (continuous for the first few hours), and then at least twice per day thereafter. Mortality is <1% (usually no animals are lost); we will advise OLAC and ACUC if we observe higher rates of mortality. Fin-clipped fish will be kept separate from non-clipped fish until fin regeneration is well underway or is complete.

3) Pain and Distress:
The procedures that we perform on adult zebrafish that could cause pain, discomfort, and/or distress are the caudal fin clip procedure and the procedure to obtain sperm and unfertilized eggs. Indications of pain and distress may include escape behavior or frantic movements, increased respiration (rapid opercular movement), blanching of color, and failure to recover normal swimming movements. From past experience, we expect that there is only very minimal discomfort during and after these procedures, as the fish resume normal behaviors immediately after the anesthetic wears off. The person performing the procedure will visually monitor and evaluate the fish frequently following the procedure (continuously for the first few hours), and then either s/he or our trained laboratory fish technicians will evaluate the fish at least twice per day thereafter. If excessive pain and distress is

suspected, the animal will be euthanized. For fin clip and sperm and egg collection procedures, fish are anesthetized in 0.004% MS-222 during the procedure to decrease overall stress and are recovered by placing them in fresh aquarium water. Adult fish may also experience slight discomfort when treated with ENU during the mutagenesis procedure; noise, vibration, and lighting is kept to a minimum during the procedure and for several hours afterward to help minimize any potential stress.

4) Food/Water Restriction: N/A

5) Restraint: N/A

6) Need to keep animals in a laboratory: In some experiments, cells must be followed by microscopy in the living embryo. In these cases, embryos are kept in beakers or dishes of system water or embryo medium in a room heated to 28.5 C, the standard temperature used to raise zebrafish. Embryos are rarely observed past 30 hours of development, which is well before the time of hatching (2-3 days) and feeding (4-5 days). Embryos are maintained at low density and the medium is changed once daily.

7) Immunological Procedures: Currently, we have no plans to use immunological procedures. In the future, we may have custom polyclonal antibodies made a vendor approved by the ACUC office. We will submit the bacterially-produced and purified antigen, or a commercially synthesized peptide, to the company and they will prepare and purify the antibodies. None of the laboratory personnel named in this AUP will handle the rabbits and/or rats used to make antibodies. Prior to ordering custom antibodies, we will contact the ACUC to verify that the vendor chosen is acceptable. We will also notify the ACUC when we submit grants that propose the use of custom antibodies and when graduate students file theses that involve the use of custom antibodies.

A. Method of Euthanasia or Other Disposition of Animals

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Zebrafish will be euthanized by methods consistent with the 2000 Report of the AVMA Panel on Euthanasia. Fish will be anesthetized (0.004% MS-222 or another OLAC-approved drug) or euthanized (0.2% MS-222 or another OLAC-approved drug) before freezing. Carcasses will be disposed by an approved OLAC method. Currently, the procedure is that frozen fish will be incinerated.

Excess healthy non-transgenic adult fish that are no longer needed (e.g., homozygous wild-type fish [non-carriers of the mutations we study] or non-breeding fish) will be reserved in one or more holding tanks to be used as a clean source of feeder fish for other laboratory animals. A trained member of our laboratory will provide excess zebrafish to OLAC staff upon request (if fish are available). We anticipate that the number of fish provided will not exceed 1500 adult fish per year. Under no circumstances will members of other laboratories be permitted to enter our facility to collect these fish.

B. Proposed Animal Housing

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1) No new space is requested.

2) Zebrafish are housed in Suite 26 of the OLAC LSA Basement Facility in a recirculating fish facility constructed by Aquatic Habitats (a division of Aquatic Ecosystems).

C. Special Animal Care Requirements

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1) Unusual Husbandry Needs: none requested

Currently, there are no health concerns for our mutation-bearing zebrafish lines. No special housing or husbandry procedures are needed for their care. Most of the mutations we study are homozygous lethal mutations, meaning that homozygous mutant fish die during early embryonic stages. To date, none of the mutations has any dominant effects, thus the heterozygous adult carriers of these mutations show no differences from their wild-type siblings and require no special care. We do have a few homozygous viable mutation bearing lines, but the mutations do not appear to create any disadvantages in the laboratory setting. If in the future we obtain homozygous viable or dominant mutations which do affect adult fish, we will consult with OLAC veterinary staff to determine how best to care for these lines (i.e., separation of mutant fish from wild-type siblings, extra feedings, etc.).

2) Provision of Animal Care by Non-OLAC Personnel: The fish will be housed in a recirculating water system, specially designed and constructed by Aquatic Habitats. This type of system allows the fish to be housed in much denser numbers per tank than a typical flow-through system. The molded FDA-approved polycarbonate tanks come in three sizes: 9L (for housing 50-75 adults), 2.75L (for housing about 20-30 adults) and 1L (for housing about 5 fish). Research Assistants trained by the P.I. or another qualified individual in the laboratory provide weekday and weekend care for the fish. Animals are reared at 28.5 C on a light: dark cycle of 14 hours: 10 hours. The fish are fed and checked at least twice daily, and we have extensive checklists to ensure that facility upkeep and feeding are completed. In addition, the Aquanode package included in the system will monitor pH, temperature and conductivity. OLAC veterinarians have reviewed our SOP with us and approved it.

D. Breeding

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The wild-type and mapping strains have been laboratory-bred and selected over many years to provide an appropriate genetic background for the proposed experiments. These strains are not available commercially. Most of the animals used in experiments are embryos produced by crossing laboratory-bred animals. The approximately 117,500 embryos proposed for the research are necessary for (1) embryological observation and morphological characterization, (2) gene expression analyses, (3) identification of heterozyous mutant and transgenic carrier adults and (4) maintenance of wild-type and mapping strains. Approximately 6,500 adults proposed for the research are necessary for (1) maintenance of wild-type and mapping strains, (2) generation of mutagenesis stocks and transgenic lines, and (3) maintenance and breeding of mutant lines. Most of the mutant lines that are used bear recessive lethal mutations, thus the line must be maintained and studied by isolating heterozygous carriers every generation. Thus, in order to isolate a given number of heterozygous carriers, twice that number of fish must be raised in a typical outcrossed stock. Surplus offspring and retired breeders will be humanely euthanized. The P.I. will supervise breeding. About 25 mutation-bearing lines will be maintained as two or three outcrossed stocks yearly; each of these stocks is typically generated by breeding 4 adults (2 females and 2 males), for a total of about 2500 fish. Wild-type and mapping stocks are typically generated twice or three times yearly by in vitro fertilization methods; eggs are expressed from 20-40 females and mixed with sperm collected from 20-40 males. In order to have enough breeding pairs to both generate embryos for experiments and for maintaining mutant lines, we estimate that approximately 1300 breeding adults will be maintained. All of the wild-type and mapping stock adults are considered breeders. Census records are kept in a FileMakerPro database developed by the University of Oregon Zebrafish Facility. See Appendix A for more details on animal numbers.

E. Transportation of Animals

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Embryos are transported out of the facility in order to perform microsurgery and microscopic observations on the powerful microscopes that we have in our laboratory. These are terminal experiments and the embryos are not returned to the animal facility. Embryos will be transported from the facility to the laboratory in beakers or plastic dishes at a density of no greater than 100 embryos per 50 ml media. Embryos will be protected from temperature extremes due to the extremely short transit time (about 5 minutes). Embryos will be transported at times at which they are not yet feeding (before 4 days of development).

Adult fish are transported rarely out of the animal facility. Once or twice a year, twenty males are transported to the laboratory to be mutagenized with 1-nitroso-N-ethylurea (ENU). They will be transported in plastic Rubbermaid containers with lids (density of ~10 fish per liter), and moved on carts the very short distance between the basement facility and the laboratory (555 LSA). They will be transported in between the normal feeding times and returned without missing a feeding. The animals will be protected from temperature extremes due to the extremely short transit time (about 5 minutes). The mutagenesis procedure must be performed in a chemical hood. The procedure takes about half a day, then the fish are returned to the animal facility for recovery. These males are then bred over the next two weeks to pass potential mutations to the next generation. The ENU-treated males are then euthanized. I would be happy to perform the mutagenesis in the basement facility if chemical hood becomes available for the procedure.

7. **DESCRIPTION OF FIELD RESEARCH**

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A. Animal Capture

B. Animal Restraint/Handling

C. Animal Marking and Radiotelemetry

D. Description of Proposed Procedures
E. Release, Euthanasia or Other Disposition of Animals
F. Recapture of Animals
G. Health Precautions for Personnel
H. Housing of Captive Wild Animals
I. Transportation of Animals

ATTACHMENT A: Justification for Numbers of Animals to be Used ~ MUST be completed

ATTACHMENT B: Methods and Sources Used to Consider Alternatives to Potentially Painful Procedures ~ MUST be completed for ALL potentially painful procedures

ATTACHMENT C: Non-duplication of Research ~ MUST be completed

ATTACHMENT D: Qualifications of Personnel ~ MUST be completed

ATTACHMENT A: Rationale for Choice of Numbers of Animal to be Used

Provide a justification for the numbers of animals proposed to be used. For each study described in this Master Animal Use Protocol, list the numbers of control and experimental groups and justify any proposed replications within individual experiments. Justify the proposed group sizes, ***preferably based on statistical estimates*** of sample size*, although you may also include experience from similar previous experiments, literature review, or another method that justifies the number of animals proposed. In cases where statistical significance is not expected (e.g., breeding colonies, teaching labs, pilot studies, antibody production, tissue harvesting, etc.), explain why the proposed numbers of animals are the minimum necessary to achieve the desired outcome. If the protocol includes a number of separate experiments or has an otherwise complex design, provide a table* to help the ACUC members better understand the breakdown of and rationale for the total numbers of animals needed.

(* Information about simple statistical methods for determining minimum sample sizes in various types of experiments is provided on the ACUC website at http://www.acuc.berkeley.edu/. A sample table that can be used as is or easily modified to provide a breakdown of animal numbers in complex experiments can be downloaded in MS Word format from the ACUC website at http://www.acuc.berkeley.edu/.)

The focus of our research is embryonic development, so the primary use of adult zebrafish is to generate embryos for study, either by natural spawning or by in vitro fertilization. In order to ensure that we always have breeding adults of our wildtype and mapping strains, our mutant-bearing lines, and our transgenic lines, we must raise a new generation of fish 2-3 times per year. The estimated number is necessary to ensure a robust breeding colony and allows a resting period of one week between natural spawning and 3 weeks between each sperm and egg collection and guards against complications such as sex ratio bias. In some situations (initial outcross from mutagenized female or from founder transgenic), the number needed is greater due to mosaicism in the germline. **Please note** that we cannot control the number of embryos a pair of fish will produce; thus, embryos are almost always produced in excess of the number needed for experiments. Unused embryos are euthanized as described in this AUP.

	# Adults for stock maintenance	# Embryos generated to identify mutant carriers	# Embryos generated in crosses for experiments
Wildtype AB strain	900[a]	NA	20,000[h]
Mapping strains (2 strains)	400[b]	NA	2,500[i]
Mutagenesis stock	2000[c]	(Haploid embryos only)[f]	NA
Mutation-bearing lines (~25 total)	2500[d]	75,000[g]	15,000[j]
Transgenic lines (~4 total)	700[e]	NA	5,000[k]
TOTALS	**6500** Adults	**117, 500 Embryos**	

STOCK MAINTENANCE

[a] 3 stocks per year, 300 fish per stock (one main AB line). This line is heavily used in both natural crosses and by in vitro fertilization. Since fish can be crossed only once a week and must rest approximately 3 weeks after sperm and egg collection procedures, we need a large breeding colony to provide enough embryos for experiments. A larger stock also provides a cushion in cases where the stock has a sex ratio bias.

[b] 2 stocks per year, 100 fish per stock (two mapping wild-type strains, SJD and WIK)

[c] 2 stocks per year, 1000 fish per stock. Each stock provides approximately 300-500 females whose haploid progeny we examine for new mutations. We typically isolate an interesting new mutation in 1 of

every 50 females; this would equate to 6-10 new mutations per stock.
[d] 2 stocks per year, 50 fish per stock (25 mutant lines)
[e] 2 stocks per year, 50-75 fish per stock (4 lines). Additional numbers reflect the fact that the first generation of any line must contain more individuals to account for mosaicism in the germline. Thus, we estimate an additional 100-300 fish per year to account for the extra individuals required for newly established lines.

CARRIER IDENTIFICATION

[f] Mutagenesis stocks are screened for new mutations by examination of the haploid progeny of females whose unfertilized eggs initiate development after addition of UV-treated sperm (which activates the eggs but contributes no genetic material). Although we can screen haploid embryos for new mutaions, they have developmental syndromes and fail to survive past 3-4 days, thus we do not count them here.
[g] Most of our ~25 mutant-bearing lines are recessive lethal, which means that we must identify heterozygous carriers every generation. Heterozygous carriers are needed to provide embryos for experiments and for the next generation. In an outcrossed population, about half the fish will be carriers; thus 1 of 4 blind sibling intercrosses will identify 2 new carrier adults. Thus to identify 10 carriers from each stock, 20 pairwise crosses must be set up. If each pair typically lays 75 eggs, 1500 embryos will be generated to find enough heterozygous carriers from each stock. With 25 mutant lines and 2 stocks per year, this totals 75,000 embryos. Homozygous mutant embryos and their control wild-type siblings generated from heterozygous parents can be used in experiments, provided that the embryos are not needed before they can be identified by mophological criteria.

EXPERIMENTS

[h] Wild-type embryos are typically needed for experiments 4 days a week. An average cross may give 100 embryos per day, or 400 embryos per week, whether or not all the embryos are used in the experiment. For 50 weeks (~ 1 year), this totals 20,000 embryos/year.
[i] Mapping strain embryos are needed for experiments once every other week. Using the same logic as above, this totals 2,500/year.
[j] Fish from various mutant-bearing lines are crossed to provide embryos for experiments 3 days a week. Using the same logic as above, this totals 15,000/year.
[k] Fish from our transgenic lines are crossed to provide embryos for experiments once a week. Using the same logic as above, this totals 5,000/year.

ATTACHMENT B: Methods and Sources Used to Consider Alternatives to Potentially Painful Procedures

- USDA regulations require the ACUC to ensure that: *"The principal investigator has considered alternatives to procedures that may cause more than momentary or slight pain or distress to the animals, and has provided a written narrative description of the methods and sources, e.g., the Animal Welfare Information Center, used to determine that alternatives were not available." (9CFR §2.31,d,ii)*

  The best way to satisfy this requirement is to perform and describe a literature search. A useful source of information on techniques for conducting such a search, and list of possible databases to be searched, is found at the website for the Animal Welfare Information Center, or AWIC, (http://www.nal.usda.gov/awic/). Information is also available on the ACUC website at www.acuc.berkeley.edu or by calling our office at 642-8855.

- Use a separate ATTACHMENT B form for each search conducted; you may copy or duplicate this page as needed. It may be necessary to conduct a separate literature search for each potentially painful procedure proposed.
- The narrative description of the search results should be an analysis of the information found rather than a simple bibliographic listing. Blanket statements such as "no information found" or "no alternatives available" are not acceptable.
- **For each literature search, USDA Policy 12 (http://www.aphis.usda.gov/ac/policy/policy12) requires that the following information be provided:**

Database(s) consulted:	Pubmed
Date of the search:	5/21/04
Years covered by the search:	1966-2004
Key words or search strategy:	Fin amputation ("Fin clip" gave zero references)
Description of search results and applicability to your proposed research (provide a narrative description such that the ACUC can readily assess whether the search topics were appropriate and the search was sufficiently thorough):	
The search was performed to investigate whether there are any published reports of alternatives to "fin clips" for genotyping zebrafish adults. The search produced 31 references. Six references pertain to unrelated observations in human and are published in mostly medical journals (e.g., hand infections from fish bone). Two are reviews of the fish fin regeneration literature. Twenty references describe the process of limb regeneration in zebrafish or related species. These references were checked to see if any alternative methods were used; the studies used a protocol similar to the one described in this AUP. Some of the references do confirm that no more than half the fin should be taken to ensure proper regeneration and appropriate nerve supply. Finally, one reference describes limb regenerates in the salamander, one describes msx gene expression in the tail of the swordtail, and one describes heart regeneration in zebrafish.	

Database(s) consulted:	Pubmed
Date of the search:	5/21/04
Years covered by the search:	1966-2004
Key words or search strategy:	Zebrafish and in vitro fertilization
Description of search results and applicability to your proposed research (provide a narrative description such that the ACUC can readily assess whether the search topics were appropriate and the search was sufficiently thorough):	

This search was performed to check for alternatives to expression of eggs and sperm from anesthetized adult zebrafish (a procedure that causes only minimal discomfort) for use in in vitro fertilization experiments. The search produced 11 references, three of which were irrelevant (e.g. primate nuclear transfer facilities, etc.). Three references described standard in vitro fertilization methods, one described fertilization by sperm injection into eggs, and one described a sperm freezing protocol. In all these methods, eggs and sperm were collected via methods similar to those described in this AUP. Three of the references described methods of in vitro cultured zebrafish sperm or testicular cell lines: one described a primary culture system (where isolated testis was cultured to produce sperm in vitro) and two very recent 2004 papers describe new zebrafish germ cell lines that will be evaluated further by us for use in generating cultured sperm. Since cell lines often obtain abnormal chromosome numbers and organization over time, it will be important to evaluate whether these new lines have stable karyotypes over time. One exciting potential use for these lines may be in the generation of transgenic animals.

- **Methods other than a literature search:**

Years of relevant experience:	[years of relevant experience]
Professional conferences/meetings attended:	[List of professional conferences/meetings attended (include date attended)]
Names of other experts consulted:	[Names of other experts consulted]
List of service provided to grant review committees, panels or editorial boards:	[List of service provided to grant review committees, panels or editorial boards]
List of journals subscribed to and/or read:	[List of journals subscribed to and/or read]

ATTACHMENT C: Non-Duplication of Research

- Federal regulations (9CFR §2.31,d,iii) also require assurance from the PI that the work proposed in this protocol does not unnecessarily duplicate previous experiments. Provide a statement indicating that studies such as these have not been done, and indicate the sources used to make that determination. While a database search remains the best method to assure non-duplication, methods other than a literature search may be cited (see below).
- **If the proposed studies are similar to those done previously, please include a justification for the need to duplicate any earlier research in your description of search results below.**

Database(s) consulted:	Pubmed
Date of the search:	5/21/2004
Years covered by the search:	1966-2004
Key words or search strategy:	Genetic screen and mesoderm
Description of search results and applicability to your proposed research (provide a narrative description such that the ACUC can readily assess whether the search topics were appropriate and the search was sufficiently thorough):	
A literature search using the Medline database indicates that no other laboratory is taking a systematic genetic approach to study mesodermal segmentation and/or mesodermal specification in zebrafish. The search above gave 32 references dating between 1989-2000. Nine of the references describe genetic screens and/or gene identification in flies, which do not have a notochord and whose mechanism of segmentation appears different from that in vertebrates. One reference described a mesodermal gene in sea urchins. Seven of the references simply describe the isolation (by RT-PCR, cDNA library screens, or yeast two-hybrid screen) and expression of a homolog of a known gene. Although these screens are useful once you have an idea of the signaling pathways and molecules involved in developmental processes, they are biased towards known factors. Six of the references describe biased strategies to find genes that are differentially expressed in different tissues or up- or down-regulated after different treatments or are expressed in a tissue-specific manner; genes isolated in this way must be regulated at the level of transcription and further functional experiments are required to determine which of the genes are necessary for the given process. One describes a gene-trapping approach in mouse ES cells; in order to test function of genes isolated this way, mice must be generated that carry the mutated gene. Six of the references describe zebrafish genetic screens (performed in 1996) that identified several genes involved in a variety of embryonic processes, including mesoderm specification and segmentation. Our segmentation screen, being more sensitive (we assay the fish by morphology and by gene expression patterns) has isolated additional somite mutations not found in these prior screens, validating our approach and showing that we are not duplicating the work of others. To our knowledge, we are the only laboratory performing such screens (Zebrafish Development and Genetics meeting, 2002). Two references describe more recent genetic screens in zebrafish, one of which is our paper describing one of the mutants isolated in our screens and the other describes angiogenesis mutants.	

- **Methods other than a literature search:**

Years of relevant experience:	[years of relevant experience]
Professional conferences/meetings attended:	[List of professional conferences/meetings attended (include date attended)]
Names of other experts consulted:	[Names of other experts consulted]

List of service provided to grant review committees, panels or editorial boards:	[List of service provided to grant review committees, panels or editorial boards]
List of journals subscribed to and/or read:	[List of journals subscribed to and/or read]
List of seminars/lab meetings presented/attended:	[List of seminars/lab meetings presented/attended]

ATTACHMENT D: Qualifications of Personnel
LIST OF ALL PERSONNEL USING LIVE VERTEBRATE ANIMALS
ON THIS MASTER ANIMAL USE PROTOCOL

ator:	Sharon L. Amacher	**Campus Phone:**	**3-1608**
	amacher@berkeley.edu	**Protocol Number:**	R255-0604C
nitted via PI's required)		**Department:**	MCB

licies and guidelines require that personnel handling and/or conducting procedures on live vertebrate animals must be appropriately qualified and trained, prior al activity. The Tier One Training seminar offered by the Office of Laboratory Animal Care (OLAC) meets all the requirements for training as set forth in these licies and guidelines. It is the responsibility of the PI to ensure that all personnel listed on the PI's animal use protocol receive the required training in animal ning, which must be documented in writing below, may be obtained directly from the PI or by attending a Tier One Training seminar presented by OLAC. **Note: attend a Tier One Training seminar at least once every 5 years to assure that they remain current on laws, regulations, guidelines and policies.**

equire training in more specific animal use procedures, please contact the Office of Laboratory Animal Care at 642-9232. Use the table below to provide all personnel you are requesting to be added to your Master Animal Use Protocol. **Please be specific and include all relevant details. Submit the the Animal Care and Use Committee by email attachment from the PI's single-user email account (preferred method of submission) to r.edu or by campus mail to Room 201, Northwest Animal Facility (MC 7160).**

on your Master Animal Use Protocol:
e and contact information, summarize his/her specific role in the study, and indicate number of years of experience with all listed procedures species.
person has received the required training in animal care and use provided by Tier One Training.
edure where the individual's experience is less than one year, certify training in relevant species-specific techniques, either through OLAC or qualified designate.

E-mail, Mailing t from PI's) urned if	**Species to be used and procedures to be performed** (e.g., care, handling, pre-treatments, anesthesia, surgery, monitoring, post-procedural care, euthanasia in the stated species)	**Years of Experience** (itemize number of years of experience with each listed procedure in the stated species)	**How Tier One Training was received** (e.g., Tier One Training seminar given by OLAC on [date], provided by PI on [date])	**If additional training has been or will be provided by qualified study personnel (who must be listed in the protocol) or others, please describe the training received and by whom** (e.g., hands-on assistance with [name, title], direct supervision of PI, OLAC training session (specify, e.g. "Rodent Handling Course"), coursework (specify)
P.I.	Zebrafish:		Tier One Training seminar given by OLAC on 5/22/2001.	Previous zebrafish experience as postdoctoral fellow in the laboratory of Dr. Charles B. Kimmel, University of Oregon
	Natural spawns	10 years		
	Sperm and egg collection	10 years		
	ENU mutagenesis	10 years		
)	Euthanasia	10 years		
	Gastrula or pre-gastrula cell injections and microsurgery	10 years		
	Zebrafish:		Provided by P.I. on 8/1/2001	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.
	Natural spawns	9 years		
	Sperm and egg collection	3 years		
du	ENU mutagenesis	2 years		Previous zebrafish experience (6 years) as graduate student in Cooper/Hille lab, University of Washington
)	Euthanasia	9 years		
	Gastrula or pre-gastrula cell injections and microsurgery	3 years		
	Zebrafish:		Provided by P.I. on 2/1/2002	Hands-on assistance and direct

	Natural spawns	2.25 years		supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Clarissa Henry, postdoc; Kariena Dill, graduate student
	Sperm and egg collection	2.25 years		
ı	ENU mutagenesis	2 years		
)	Euthanasia	2.25 years		
	Gastrula or pre-gastrula cell injections and microsurgery	2.25 years		
	Zebrafish:		Provided by P.I. on 7/1/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Clarissa Henry, postdoc; Tina Han, postdoc; Kariena Dill, graduate student Previous zebrafish experience (3 years) as postdoc in Currie lab, MRC London
	Natural spawns	4 years		
	Sperm and egg collection	1 year		
ɔdu	ENU mutagenesis	1 year		
)	Euthanasia	4 years		
	Gastrula or pre-gastrula cell injections and microsurgery	4 years		
	Zebrafish:		Tier One Training seminar given by OLAC on 2/11/2003	Hands-on assistance and direct supervision by Sharon Amacher, P.I.
	Natural spawns	3 years		
	Sperm and egg collection	3 years		
edu	ENU mutagenesis	2 years		
)	Euthanasia	3 years		
	Gastrula or pre-gastrula cell injections and microsurgery	3 years		
	Zebrafish:		Provided by P.I. on 2/1/2000	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Clarissa Henry, postdoc
	Natural spawns	4 years		
	Sperm and egg collection	4 years		
lu	ENU mutagenesis	2 years		
)	Euthanasia	4 years		
	Gastrula or pre-gastrula cell injections and microsurgery	4 years		
	Zebrafish:		Provided by P.I. on 2/1/2000	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Clarissa Henry, postdoc; Kariena Dill, graduate student
	Natural spawns	4 years		
	Sperm and egg collection	4 years		
du	ENU mutagenesis	2 years		
)	Euthanasia	4 years		
	Gastrula or pre-gastrula cell injections and microsurgery	4 years		
	Zebrafish:		Provided by P.I. on 2/3/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Clarissa Henry, postdoc; Tina Han, postdoc; Kariena Dill, graduate student
	Natural spawns	1.25 years		
	Sperm and egg collection	1.25 years		
lu	ENU mutagenesis	1 year		
)	Euthanasia	1.25 years		
	Gastrula or pre-gastrula cell injections and microsurgery	1.25 years		
	Zebrafish:		Provided by P.I. on 2/3/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Clarissa Henry, postdoc; Tina Han, postdoc; Kariena Dill, graduate student
	Natural spawns	1.25 years		
	Sperm and egg collection	1.25 years		
org	ENU mutagenesis	1 year		

)	Euthanasia	1.25 years		
	Gastrula or pre-gastrula cell injections and microsurgery	1.25 years		
	Zebrafish:		Provided by P.I. on 9/2/03	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Clarissa Henry, postdoc; Benjamin Martin, graduate student
	Natural spawns	3 months		
	Sperm and egg collection	3 months		
lu	ENU mutagenesis	no experience		
)	Euthanasia	3 months		
	Gastrula or pre-gastrula cell injections and microsurgery	3 months		
	Zebrafish:		Provided by P.I. on 8/1/2002	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I. Previous training by Brian Meux, Lab Assistant III before he left the lab
	Natural spawns	1.75 years		
	Sperm and egg collection	1.75 years		
n	Euthanasia	1.75 years		
)				
	Zebrafish:		Provided by P.I. on 1/21/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III Previous training by Brian Meux, Lab Assistant III before he left the lab
	Natural spawns	1.25 years		
	Sperm and egg collection	1.25 years		
l.com	Euthanasia	1.25 years		
)				
	Zebrafish:		Provided by P.I. on 2/3/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Clarissa Henry, postdoc
	Natural spawns	1 year		
	Sperm and egg collection	1 year		
m	Euthanasia	1 year		
)	Gastrula or pre-gastrula cell injections and microsurgery	1 year		
	Zebrafish:		Provided by P.I. on 5/7/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; Tina Han, postdoc
	Natural spawns	No experience		
	Sperm and egg collection	No experience		
.edu	Euthanasia	No experience		
)	Gastrula or pre-gastrula cell injections and microsurgery	No experience		
	Zebrafish:		Provided by P.I. on 5/7/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I; David Daggett, postdoc; Kariena Dill, graduate student
	Natural spawns	No experience		
	Sperm and egg collection	No experience		
du	Euthanasia	No experience		
)	Gastrula or pre-gastrula cell injections and microsurgery	No experience		
ıpan	Zebrafish:		Provided by P.I. on 5/7/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; David Daggett, postdoc
	Natural spawns	No experience		
	Sperm and egg collection	No experience		
ɔy.edu	Euthanasia	No experience		
)	Gastrula or pre-gastrula cell injections and microsurgery	No experience		
	Zebrafish:		Provided by P.I. on 5/7/2003	Hands-on assistance and direct

	Natural spawns	No experience		supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I.; David Daggett, postdoc
	Sperm and egg collection	No experience		
du	Euthanasia	No experience		
)	Gastrula or pre-gastrula cell injections and microsurgery	No experience		
	Zebrafish:		Provided by P.I. on 5/7/2003	Hands-on assistance and direct supervision by: Jennifer Anderson, SRA III; Sharon Amacher, P.I
	Natural spawns	No experience		
	Sperm and egg collection	No experience		
berkeley.edu	Euthanasia	No experience		
)	Gastrula or pre-gastrula cell injections and microsurgery	No experience		