Case Western Reserve University
Institutional Animal Care and Use Committee
Protocol Form

Instructions

The form is modular in construction. A Core form (this document) must be completed fully. Attachments may need to be completed depending on your responses to the checklist in Section V of the Core.

Submit the signed Core, the completed Attachments, and any additional documentation to the CWRU IACUC Office, Room WG-77, School of Medicine, Telephone (216) 368-3815, Fax (216) 368-4805, http://iacuc.cwru.edu.

- ✓**Enter your responses in the white cells only.**
- ✓**Submit only completed Attachments.**
- ✓**On some systems, users may need to "control + click" links.**

Rationale

Revisions were made to facilitate form completion and protocol review. Information on IACUC policies are provided in the form at relevant locations. Questions designed to elicit the specific information that reviewers need to understand the science, procedures, outcomes, justifications, and expected clinical condition of the animals have been added.

Your Input

We recognize that a new form, no matter how well designed, is an inconvenience to investigators and reviewers, but expect that the net result will be positive. We are committed to improving the form, and ask that you help us in this task by providing constructive criticism.

The last section of the core document is provided for your comments and criticisms on the forms and the review process.

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CWRU IACUC WG-77 School of Medicine (216) 368-3815	Date:	Species: Protocol Number: Designated or Full Review:

Notice Of Intent To Use Vertebrate Animals

Protocol Version 2.6 Form Date 10/2006

I. Contact Information

Principal Investigator:	**Dr. Jennifer O. Liang**				
Project Title:	**Biology 363/463: Experimental Developmental Biology**				
Department:	**Biology**				
Building:	**Millis**	Room:	**126**	Location code:	**7080**
E-mail:	**jol@case.edu**				
Phone:	**216-368-5428**	Fax:	**216-368-4672**	Pager:	**216-570-5412**
Animal Emergency Contact*:	**Jennifer Liang**			Phone:	**216-368-5428**
E-mail:	**jol@case.edu**			Pager:	**216-570-5412**
Name of External Funding Agency or Source:				Or Internal Funding Source†:	**Biol. Dept.**
New Submission?				Or Renewal of Protocol Number:	**2003-0220**

* This individual will be contacted when animal morbidity or welfare requires immediate action.

II. Animal Use Summary

List the total number of animals of each species required for the entire protocol: **600 adult zebrafish (Danio rerio)**

Type of use: [ ] Research [X] Teaching [ ] Other (specify):

Check all that apply to the use of animals in this submission

X	Breeding		Survival (Chronic) Study		Chemical Mutagenesis
X	Tissue Collection		Transgenic/KO Production		Infectious Agents
X	Genetic Mapping		Viral Therapeutics		Surgery
	Nutritional Studies		Protein/DNA Therapeutics		Multiple Surgeries
	Aging		Drug Therapeutics		Inducement of Disease State
	Exercise Physiology		Nude Mouse Implants		Inducement of Stress
	Behavioral Tests		Neuromuscular Blockade		Prolonged Restraint
	Antibody Production		Ascites Production		Tumor Implants
	Terminal (Acute) Study		Irradiation		Cell Implants
	Other (specify):				

Primary Reviewer	Title	Signature	Date
Veterinary Reviewer	Title	Signature	Date
IACUC Executive	Title	Signature	Date

III. Investigator Assurances

- I agree to abide by the policies of the CWRU Institutional Animal Care and Use Committee (IACUC) and all applicable federal regulations.
- I will adhere to the protocol as described and as modified.
- I will submit any modifications of the protocol to the IACUC for review.
- I will notify the IACUC of changes in the location of the animal research.
- I will assist the IACUC in verifying compliance with the regulations.
- I will notify the IACUC of any unexpected results that affect the welfare of the animals. I will report any unanticipated pain or distress, morbidity or mortality to the attending veterinarian and the IACUC.
- I understand and agree that emergency veterinary care including euthanasia will be administered to animals exhibiting unbearable pain, distress or illness. An effort to contact me or my representative (the animal emergency contact identified on page 1) will be made by the veterinary staff prior to any emergency treatment.
- I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All other personnel involved in animal use in this project have been or will be trained in proper procedures in animal handling, administration of anesthetics and analgesics, aseptic technique, post-operative monitoring, and euthanasia.
- I declare that the information provided in this application is accurate. If this project is to be funded by extramural source(s), I certify that this application accurately reflects all procedures involving laboratory animal subjects described in the proposal.
- I declare that the studies described here do not unnecessarily duplicate previous work by myself or others.

Do you have a financial interest* in the funding source named that could be perceived as a conflict of interest?		Yes	X	No

**A financial interest is a "significant financial interest" which must be disclosed if income from one company is expected to exceed $10,000 in one year, or represents 5% or more ownership interest (total ownership interest of the faculty member, spouse and dependent children).*

If yes, was this interest reported on the most recent conflict of interest disclosure form?		Yes		No
Is there any possibility that the data collected in this study will be submitted to, or reviewed by, the Food and Drug Administration?		Yes	X	No

For further information about FDA policies go to http://ora.ra.cwru.edu/research/ORC/iacuc/Case_IACUC_GoodLaboratoryPractices.cfm or contact Tami McCourt at txm9@case.edu.

Are the results of the studies in this protocol to be used in any of the following circumstances? Please check all that apply.

New drug application	
New animal drug application	
Research or marketing permit	
Notice of claimed exemption for a new animal drug	
Notice of claimed investigational exemption for a new drug	
A biological product license	
An investigative device exemption	
Permit approval of a medical device	
Product development protocol for a medical device	

Signature of Principal Investigator **Date**

IV. Departmental Assurance:

This research protocol has been evaluated based on the following criteria:

1. Scientific design of the study and adequacy of methods.
2. Scientific value of the study.

Name and Signature of Department Chairman **Date**

V. Justification:

1) What is the objective of these experiments? *Please respond in language that can be understood by a layperson. This means minimal use of technical terms and a brief explanation of any specialized terms which you must use.*

This course has three goals:

Experimental Science

Much of the excitement of being a scientist comes from discovering something new about how the world works. In this part of the course, the students will have the opportunity to carry their your own experiments, building on what they learned in my lecture course "Biol 362/462: Principles of Developmental Biology". Experiments will focus on the mechanisms that pattern the embryo during vertebrate development and how these mechanisms are explored using molecular, cellular, and genetic approaches. Students will learn how to generate a hypothesis, design and carry out experiments to test their hypothesis, and gather data and form conclusions. To do this, they will have access to reagents and wildtype, mutant, and transgenic fish from my zebrafish research laboratory. My goal is for the students to gain insight into the scientific process.

Communicating work to others

A large part of being a successful scientist is communicating findings and ideas to others. How effectively one communicates can affect whether a grant gets funded, whether a paper gets published, or whether one earns a prize at the Case SOURCE Symposium. In this part of the course, students will improve their presentation skills by preparing and presenting a poster on one (or more) of the experiments they have done during the course of the semester. Emphasis will be on how to present data and ideas clearly to a wide audience, how to answer questions, and how to demonstrate the excitement and importance of the work that has been done.

Mentoring

Another important part of being a researcher/educator is mentoring others and training the next generation of scientists. Faculty mentor graduate students, graduate students mentor undergraduates, and undergraduates mentor….students in the Cleveland Municipal School District (CMSD). There is at present very little experimental science in the CMSD. To correct this, high school teachers from the CMSD have been participating in the Cleveland Math and Science Partnership. We are going to contribute to this effort through a service-learning component in this course. As defined by the Case Office of Student Community Service, "Service learning integrates experiential learning and community service in an academic context. Through activities and experiences mutually negotiated between academic and community partners, service learning addresses identified needs, enhances the curriculum, and fosters civic responsibility." Our service learning component will enable you to use what you have learned through your experiences in this course to improve learning in local high schools and at the same time give you a valuable opportunity to gain mentoring experience.

2) How is society likely to benefit, or what new knowledge will be gained from these studies?

This course will provide valuable training for the next generation of biomedical scientists

3) Why must this species be used? *Justify the selection of this animal model. The IACUC is mandated to encourage the substitution of species more acceptable to society. Describe the characteristics of the species, stock, strain, or mutant which are important for your investigations.*

The same characteristics that make zebrafish (Danio rerio) a powerful system for basic research also make it an excellent tool for teaching: embryos are easy to obtain in large numbers, development can observed in vivo using simple dissecting microscopes, and mutants can be used to demonstrate principles of development and genetics. Thus, zebrafish are ideal for teaching major concepts in developmental biology and for giving students an active role in their own learning.

4) Briefly summarize the design of the animal experiments. *Include all procedures using animals and manipulations of animals. Give your best estimate of how many animals will undergo each procedure or manipulation described. For complicated experimental designs, a flow chart, diagram, list or table is strongly recommended to help the IACUC understand what is proposed. Additional documents may be attached to the form.*

First Section: Experimental Science

Students will carry out experiments within the following outline. Students work in small groups of 2-4 to minimize the number of embryos used. The techniques used each in laboratory are listed briefly beneath each laboratory title. A more detailed description of each technique is included at the end of this section.

<u>Laboratory 1: Stages of zebrafish development</u>
This laboratory will use natural matings and microscopy of live embryos

The goal of this laboratory is to learn the stages of zebrafish development, and to use dissecting and compound microscopes.

A. Tools
You will use several tools for the experiment today:

Dissecting stereomicroscope and a compound microscope. We will go over how to use these before we start. If you have not used one of these before and need some help, please let me know and I will give you an introduction. We will be using these for almost every experiment this semester

A pair of fine forceps. Students will have their own pair to use throughout the semester. They are quite fragile and expensive, so please take care of them. Use them only when working with the embryos. These are fairly dull, and you may prefer to sharpen them with a stone. You can use these to dechorionate (take the shell off) the embryos and to push them around if you are careful.

Embryo loops. These are for orienting and pushing the embryos around. These are made with fishing line, capillary tubes, and super glue. You should make several of these for yourself.

Slides. Live embryos must be mounted on slides in methyl cellulose before you can view them on the compound microscope. We have two kinds of glass depression slides that you can use. See the attached protocol for how to mount the embryos.

B. Staging zebrafish embryos
You have been given a petri dish with four-six stages of embryos. Your challenge is to separate embryos at different stages of development into different dishes, and to determine their stage.

Dissecting microscope
First look at them under the stereo dissecting microscope (low power). Put the petri dish on the stage, and turn on the light underneath the stage. Adjust the mirror so you get even illumination across the field of vision. This is called brightfield microscopy.

As you look at the embryos, think about the following issues, and write about them in your laboratory report.

Use the attached staging sheets to stage your embryos. Note characteristic features of each stage that help you identify it.

What are the limitations of an observational approach to developmental biology? What are some of its advantages? Think about developmental structures and processes that are easy and hard to observe.

Play with the setting of the light and the mirror on your microscope. Are their settings that make it easier or harder to see specific structures? For example, try turning the mirror so light is not going directly through the embryo. This is pseudo dark field microscopy. Is it easier or harder to see the somites? Can you count how many somites your embryos have?

An important skill for the course will be the ability to work with the embryo underneath the dissecting microscope. There are several things you can do even with the simple tools you have today. Most of the stages you are looking at still have their chorion-can you take it off (dechorionate the embryo) with your fine forceps? Does dechorionating make it easier of harder to see structures within the fish? When do you first see movement of the embryo? What kinds of movements can you see? When does it start to respond to touch?

Compound microscope

Next take one of your embryos and look at it under different lenses of the compound microscope. You can use one that is dechorionated, or one that is still in the chorion (but choose one with a clean chorion). Follow the directions for mounting the embryos using methyl cellulose.

Adjust the compound microscope for bright field microscopy (see microscopy intro), and observe the embryos at different magnifications using the different objectives on the compound microscope. How does what you see differ from what you could see using the dissecting microscope?

Now adjust the microscope for dark field microscopy. In this method, Put a coin on top of the light source-a big coin for a low power objective, and small coin for a high power objective. This will create a ring of light that is illuminating your embryo-this is called dark field microscopy. How does this

change the brightness and contrast of the image you see? Are their structures that are easier to see in dark field versus bright field, and vice versa?

Laboratory 2: Sex determination
This laboratory uses natural matings and microscopy

Zebrafish do not have sex chromosomes: all of their chromosomes are autosomes. It is not unusual for a clutch of zebrafish to develop as 90% female or 90% male.

What factors control whether a zebrafish becomes male or female? Circumstantial evidence suggests that environmental conditions during the first few weeks of life influences whether zebrafish will become males or females.

Based on your reading and our class discussions, come up with a hypothesis about how sex is determined in zebrafish.

Design an experiment to test your hypothesis, including any required positive and negative controls.

An example of an experiment designed by previous students:

Hypothesis: Sex is determined by temperature during early development

A. Cross adult wildtype fish to obtain several clutches of zebrafish embryos at an early developmental stage (preferably 2- 4 cell stage).

B. Sort these embryos into three groups of 60 embryos each.

C. Place these embryos at three different temperatures 82.5 (standard temperature), 79, and 86 degrees Fahrenheit (these are all viable temperatures for zebrafish). Raise the embryos at these temperatures for one week.

D. At approximately 7 days post fertilization, place the embryos in the fish facility, and raise them in parallel.

E. At three months post fertilization, when the fish are mature, examine each group of fish and determine the sex of each individual to determine if there are different sex ratios in each group.

Laboratory 3: Cardiovascular function in *casanova* mutants
This experiment uses natural matings and microscopy

During normal development, heart precursors are specified in two lateral domains on either side of the trunk. As development proceeds, these two domains converge towards the midline , fuse, and eventually form the heart tube and the mature heart. It has been demonstrated in several vertebrates that the migration of heart precursors requires endoderm.

Zebrafish *casanova* mutants lack endoderm, and as a consequence, the heart precursors remain in two domains at the left and right sides of the trunk. Despite this, some heart formation occurs, as the presumptive heart cells begin to beat (Alexander et al, 1999). **Based on your reading (Alexander et al., 1999) and our class discussions, generate a testable hypothesis about how cardiovascular function is affected in *casanova* mutants.**

My job will be to carry out the natural matings to produce embryos for the experiment, and to provide practical advice about what can be done within the confines of the available reagents and our time in class. To make it easier to assess cardiovascular function in vivo, I have crossed the casanova mutation into the actin:GFP transgenic line. Because the heart of these fish is fluorescent green, it makes it easy to watch the heart beating by fluorescence microscopy.

Class period 1: Carry out a pilot experiment to test the effectiveness of your experimental plan, paying special attention to your plan for gathering data.

Class period 2: Carry out your experiment and gather data.

Here is an abstract describing the experiments carried out by the 2006 class of Biol363:

Since the heart is the first organ to develop in the vertebrate embryo, it is a valuable research tool to understand organogenesis. Zebrafish are a useful model organism for studying cardiovascular development because they are vertebrates, easy to raise in large numbers, and translucent. Zebrafish *casanova* (*cas*) mutants do not form endoderm and exhibit cardia bifida, the formation of two bilateral hearts. In these experiments, we used fluorescent and bright field microscopy to examine defects in live *cas* mutant embryos. We found a systemic lack of blood flow, suggesting that the vascular system did not fuse with the hearts. This finding is consistent with the presence of cardia edema (fluid build-up around the heart). We also observed differences in heart rate between the left and right *cas* hearts, as well as between the *cas* and wildtype hearts. The *cas* mutant hearts beat less frequently than the wildtype hearts. Comparison of the left and right hearts in *cas* embryos showed that the two hearts beat asynchronously. Analysis over short periods revealed that one heart beat more consistently and quickly, but the sidedness of the "strong" heart varied. We conclude that although the *cas* mutant hearts beat, their function is severely impaired because they cannot support blood flow and do not beat regularly.

Laboratory 4: *squint/one eyed pinhead*
This laboratory uses natural matings of adult fish, and fixation of embryos followed by in situ hybridization

My laboratory has found that the zebrafish neural tube does not close in zebrafish lacking the Nodal signal Squint (Sqt) or the Nodal receptor component One eyed pinhead (Oep). However, we still do not know why the neural tube does not close in these mutants. Complicating the analysis, both *sqt* and *oep* mutants have many defects, including cyclopia, lack of mesoderm, lack of endoderm, and reduction or absence of ventral brain and floor plate. Further, the phenotype of homozygous *sqt* mutants is extremely variable. Some *sqt* mutants are indistinguishable from wildtype fish and live to adulthood, while others have severe developmental defects.

I want you to design an experiment that uses whole mount RNA in situ hybridization to give insight into the neurulation phenotype in these mutants. I will do my best to provide the fixed embryos and probes that you need for your experiment.

Here are a few potential hypotheses that you may choose from. Work with your small group to design an experiment to test one of these hypotheses or a hypothesis of your own.

Hypothesis #1: Failure in neural tube closure is due to a lack of cell adhesion between cells of the neural tube.

Hypothesis #2: Failure in neural tube closure is due to a lack of head mesoderm.

Hypothesis #3: Sqt and Oep are required for neurulation only in the anterior region of the embryo.

You will perform in situ hybridization on fixed batches of embryos according to your plan.

Laboratory 5: Dorsal/Ventral Patterning
(Adapted from protocols written by Nora Wininger and Galina Nikolskaya)
This laboratory will use natural matings of adult fish, embryo injections, and fixation of embryos

The secreted protein BMP is required to establish ventral fates. Chordin binds to BMP and therefore blocks ventral fates on the dorsal side of the embryo. In this laboratory, you will determine how depleting Chordin protein alters the ventral and dorsal fates of zebrafish embryos. One-cell stage embryos will be injected with a high dose and a medium dose of *chordin* antisense morpholino. Antisense morpholinos bind to a sequence in a selected mRNA and prevent the translation of that specific mRNA, so no protein product coded by that mRNA is made. Therefore, *chordin* morpholinos block the synthesis of Chordin protein. Dorsal morphology can be assayed by scoring the morphology of the notochord and head (both dorsal fates) and ventral morphology can be assayed by scoring the amount of blood in the tail (a ventral tissue).

Before you make your hypotheses, read Schier's "Axis formation and patterning in zebrafish" and Nasevicius & Ekker's paper "Effective targeted 'knockdown' in zebrafish". Gilbert's "Developmental Biology" textbook may also provide useful background information.

Based on the background readings form hypotheses to address the following questions:
1. What do you expect will happen to the dorsal and ventral fates in the *chordin* morpholino injected embryos?
2. How will the phenotypes of the high dosage *chordin* morpholino-injected embryos differ from the embryos that receive a medium dosage?

Day one

1. Each student will receive 15 one-cell stage (about 15-30 minutes post-fertilization) zebrafish embryos from flh: GFP transgenic line. 1/3 of the embryos will be microinjected with high dosage of *chordin* antisense morpholino (chordin-MO), 1/3 with a medium dosage, and 1/3 will serve as controls. I recommend a high dose injection of 4.5 ng and a moderate dose injection of 1.5 ng. However, you can pick your own dosage amounts using the Ekker paper to help you out. The morpholino should be injected into the yolk of the embryos. Water MO solution injections will be used for the control group. I will help everyone individually with the injections. Make sure to label dishes and slides well.
2. You will be scoring the presence of dorsal and ventral structures of each embryo next week. Dorsal structures that can be scored include notochord (which will be the easiest to visualize using the fluorescence dissecting microscope since the embryos are GFP transgenic fish), floor plate, brain, hypochord, hatching gland, and somites. Score the notochord and at least one other structure. As for the ventral side, a large bulging of the tail is seen in embryos without Chordin protein due to extra blood.
3. Determine the stage you want the embryos to be fixed at. I will fix them after lab day one.

Day two

Examine the fixed embryos under a dissecting microscope to note the morphological features of all three groups. You may use a compound microscope for higher magnification. Make sure that there is enough water in the petri dishes or slides so that the embryos don't dry out. Once again, make sure to label well. Use a fluorescence-dissecting microscope to visualize the notochord in all groups.

Which dorsal and ventral structures are present in each of the three groups?
How do the phenotypes differ across groups?
Do all of the embryos within one group have the same phenotype?
If not, what may have caused a difference in phenotypes?
Do you think that all of the Chordin protein has been depleted in the high dosage groups?
Do you think that all of your injections were successful? If not, what may have gone wrong?

Although each of you will be working individually, I suggest forming groups and discussing your results together.

Second Section: Communicating work to others

No fish will be used for this section of the course

Third Section: Mentoring

Simplified experiments similar to those described for the experimental science sections will be used in this part of the course. If the experiments used for mentoring deviate from the experimental approached described above, we will submit a addendum to this protocol for IACUC approval.

Because of the risk of zoonitic agents in the live fish, I will encourage the Case students to use only fixed, stained fish for their experiments with the high school students. If the Case students decide that they want to use live fish, they will develop a plan for these experiments and submit it for review and approval by IACUC.

Techniques used in the course

1. Natural matings

Adult fish will be used only for natural matings to obtain embryos for students to observe or for experiments. Male/female pairs are moved from their home tank using a fish net into a 1L breeding tank. As some fish can be aggressive, artificial foliage is included as a refuge. Fish are maintained in the mating tank for no more than one day. If fish need to be maintained for a longer time outside the aquatic system, they are changed into a new tank every day. They are returned to their home tank unharmed. To minimize stress, and maximize egg production, fish will not be set up to mate more than once a week. Approximately 600 adult fish will be used.

2. Microscopy

Live embryos and larva obtained from natural matings will be observed by light, fluorescence, and confocal microscopy. This procedure does not harm the fish in any way. Once the fish become mobile, they may be anaesthetized using the procedures described below (See Attachment C) so that can be more easily observed. The anesthesia is completely reversible and does not harm the fish in any way. Wildtype (WT) and mutant embryos will be observed to demonstrate the genetic bases of various developmental processes. Approximately 1000 embryos will be used for this purpose.

3. Fixation

Fixation of embryos and larva. WT and mutant embryos and larva will be fixed for use in biochemical and molecular biological experiments such as in situ hybridization and antibody staining. Embryos that have not yet developed a nervous system will be fixed in 4% paraformaldehyde directly. Those that are older will be euthanized before fixing. Approximately 1000 embryos will be used for this purpose.

4. Embryo injection. Embryos will be injected at the one cell stage with molecules that affect their development. The purpose of this experiment is to uncover the roles of signaling pathways that regulate

development. For example, the students might inject mRNAs or morpholinos that affect different parts of the pathway that regulates the dorsal ventral axis of the embryo. Approximately 1000 embryos will be used for this purpose.

5) Justify the number of animals. *The IACUC is mandated to minimize the number of animals used. Include a statistical justification, if possible. Alternatively, a listing of experimental procedures and the estimated number of animals required for each may be submitted. Prior experience is not adequate justification in itself. Tools for power analysis and other statistical methods for estimating sample size are available at* http://StatPages.org.

Very few embryos and adults will be used specifically for this course. In most cases, we are using adult fish that are already present in our fish facility, and embryos produced as part of our maintenance of our fish stocks. For instance, we routinely fix extra embryos produced in our research projects for later use in the course. Only the minimal number of fish needed for the students in the class will be used. In addition, I have chosen experiments that cause no pain to be used in my course. Live embryos and fish are used only in procedures that cause no pain or distress. For instance, embryo injections are done at a stage when there is no nervous system. **A**dult fish will be used only in natural matings.

A list of the experimental procedures and an estimated number of fish that will be used for each is included in the previous section.

6. SEARCHES FOR ALTERNATIVES: For any procedure that is likely to cause more than slight or momentary pain or distress, Federal Regulations require that you document your justifications with data from **two or more databases**. At least one source **must** be a set of searches of a relevant electronic database such as Pubmed. The second source can be another electronic database, a recent authoritative article, information obtained at a conference, or a consultation with a qualified expert. Searches must address

REPLACEMENT: Search for alternatives including in vitro models, in silico methods, invertebrate models and vertebrate models which will be more informative.

REDUCTION: Search to demonstrate that the proposed studies do not unnecessarily duplicate previous work.

REFINEMENT: Search for procedures which would cause less pain, or distress, or would result in better animal welfare. Housing, environmental enrichment, animal identification, anesthesia and analgesia and euthanasia procedures can be refined, in addition to things normally thought of as procedures, such as surgeries, tissue or fluid collection, etc.

Assistance with the design and use of electronic database searches can be found on the IACUC website (casemeds.case.edu/ora/iacuc) or by contacting Mike McGraw 368-3218 mpm3@case.edu.

6a. Indicate the databases searched by checking the appropriate boxes below.

A	**X**	AGRICOLA Data Base (Nat'l. Ag. Libr.) http://www.nal.usda.gov/ag98/ag98.html	
B	☐	ANIMAL WELFARE INFO Center (AWIC) http://www.nal.usda.gov/awic/index.html	
C	☐	BIOSIS Data Base http://www.biosis.org/	
D	☐	CAB Abstracts Data Base http://www.lib.edina.ac.uk/cab/	
E	☐	CURRENT RESEARCH INFORMATION System (CRIS) http://cris.csrees.usda.gov/	
F	☐	MEDLINE Data Base http://www.medline.cos.com	**Note:** MEDLINE & PUBMED pull data from an identical source and thus are regarded as a single search.
G	☒	PUBMED http://www.ncbi.nlm.nih.gov/PubMed/	
H	☐	SCIENCE CITATION Index http://cite.ohiolink.edu/isi/CIW.cgi	
I	☐	TOXNET Web Interface http://toxnet.nlm.nih.gov/	
J	☐	Mouse Genome Informatics http://informatics.jax.org/	
K	☐	Authoritative article from Lab Animal or Similar Journal/Magazine	
L	☒	Other (Specify): `http://www.google.com`	

6b. For electronic database searches, indicate the database, species, procedure, and date of search.

Species	Danio rerio	Danio rerio	Danio rerio	Danio rerio	Danio rerio
Procedure	microinjection	euthanasia	teaching	anesthesia	statistical methods
Database	Pubmed	http://depts.washington.edu/iacuc/policies/index.html	Google	Agricola	Pubmed
Search Strategy /Key words	"microinjection"[MeSH] AND "zebrafish"[MeSH]	read website	Google: Key words "zebrafish" and "education	w=Anesthesia & w=Fish	("Statistics"[MESH] AND "zebrafish"[MESH] AND ("Animal Experimentation"[MESH] OR "Models, Animal"[MeS

Search Strategy /Key words	"microinjection"[MeSH] AND "zebrafish"[MeSH]	read website	Google: Key words "zebrafish" and "education	w=Anesthesia & w=Fish	("Statistics"[MESH] AND "zebrafish"[MESH] AND ("Animal Experimentation"[MESH] OR "Models, Animal"[MeSH]))
Last Search	12/11/06	12/11/06	12/11/06	12/11/06	12/11/06
Years Covered			all		2006

6c. If a consultation is meant to replace one database in the searches, complete the following table.	
Consultant's Name	
Consultant's Qualifications	
Date of Consult	
Content of Consult	

7. Pain or distress search narrative. Provide a brief written narrative summarizing the results of the search(s) indicated above and the consideration of alternatives to all procedures which may cause more than momentary or slight pain or distress.

7a. Replacement: Describe any alternatives to live vertebrate animals (mathematical models, computer simulations, in vitro biological systems, lower-order animal) and provide justification for using the proposed methods in an alternative method, model or procedure was found. Please indicate if no reduction alternatives were found.

Our purpose is to study the vertebrate development, so the use of non-vertebrate animals is not possible. The zebrafish has become widely accepted throughout the world as a useful vertebrate model system. The embryos are transparent and develop rapidly outside the mother, making possible to viewing differentiation of many tissues in the live organism. Access to the developing embryos makes it possible to do experimental manipulations (injection of different molecules, whole mount in situ hybridization, in vivo imaging) that reveal mechanisms underlying development. Many developmental mutants are freely available. Fish are small and easy to maintain and breed in large numbers in laboratory conditions. None of the other vertebrate model systems have all of these characteristics, which are essential for a laboratory course. Searches for alternative animal models found only mammalian systems and chicks (which are higher vertebrates than zebrafish) and non-vertebrates. I am developing a web site (http://www.case.edu/artsci/biol/lianglab/classroompage.html) that will ultimately serve as a source of digital data, so that students can do some of their research through "virtual" experiments instead of using live fish.

7b. Reduction: If any studies were found that are similar to the proposed studies please explain why the proposed experiments are different, necessary or do not duplicate what has already been reported. Please indicate if similar studies were not found.

The adult fish used only for natural matings, so they are not directly used in experiments. Typically, we raise 60 fish in each new stock, and 2/3 of these will be carrying the given mutation. This gives us 40 fish, or up to 20 mating pairs. However, the sex of zebrafish is determined by unknown environmental factors, and so there is often an uneven mix between males and females, and we may get as few as five

The searches of Pubmed and Agricola did not identify any techniques for zebrafish other than the ones we are currently using. Although the University of Washington IACUC website is not officially a database, I knew from Nan Kleinman that they were doing some work on zebrafish euthanasia. I found additional documentation for our method of euthanasia on this website. In addition, I am continuing to compile resources that will help students in the laboratory learn fundamental skills. For example, I used searches of developmental biology websites to identify new movies of zebrafish development that will help teach the stages of zebrafish development and zebrafish anatomy without using live fish.

8) Can these animals be used for other purposes after the study? *Reuse of animals is encouraged. If your animals can be reused in some manner, please state how and by whom.*

We almost always use the fish in our laboratory for multiple purposes. Adult fish are used mainly for natural matings, and can be used as often as once a week for this purpose. A typical adult fish in our facility might be set up to mate 20-40 times during its natural lifetime. Natural matings not only produce embryos for our experiments, but also maintains the health of the adult fish. In another example, adult fish that carry a specific mutation are usually identified by setting them up in natural matings, and examining the phenotype of their progeny. Embryos produced during this process are often fixed and used later for the course.

VI. Animal Use Checklist

Please complete the checklist and any required attachments.

Yes/No	
No	Will you be collecting body fluids prior to euthanasia? If *Yes*, complete Attachment A: Antemortem Fluid Collection.
No	Will you be using animals to produce antibodies? If *Yes*, complete Attachment B: Immunizations.
Yes	Will anesthetics, analgesics, sedatives, paralytics or tranquilizers be used? If *Yes*, complete Attachment C: Anesthesia, Analgesia Or Paralysis.
No	Will surgery be performed on the animals and the animals allowed to recover? If *Yes*, complete Attachment D: Survival Surgery.
No	Will surgery be performed on the animals and the animals *not* allowed to recover? If *Yes*, complete Attachment E: Non-Survival Surgery.
No	Will animals be subjected to Pain Class E procedures? If *Yes*, complete Attachment F: Justification Of Pain Class E Animal Use.
No	Will animals be subjected to restraint other than regulation caging? If *Yes*, complete Attachment G: Animal Restraint.

No	Will infectious agents, hazardous chemicals, recombinant DNA (creation of knockout or transgenic animals) or radioactive materials be introduced into animals? Will animals which present a biohazard be imported? If *Yes*, complete Attachment H: Infectious Agents, Biohazards And Recombinant DNA.
No	Will tumor cells (including hybridomas) be introduced into animals? If *Yes*, complete Attachment I: Tumors In Animals.
No	Will chemicals, non-infectious biological substances, cells (nontumorous) or tissues be administered to animals other than for anesthesia or production of antibodies? If *Yes*, complete Attachment J: Exogenous Substance Administration.
No	Will animals be irradiated? If *Yes*, complete Attachment K: Irradiation.
Yes	Will animals be housed for more than 12 hours in locations other than those listed in Part VII? If *Yes*, complete Attachment L: Principal Investigator Satellite Facility Registration.
Yes	Will diet be restricted or modified or the environment manipulated? If *Yes*, complete Attachment M: Manipulation of Environment.
No	Will there be operant conditioning or other behavioral tests? If *Yes*, complete Attachment N: Behavioral Tests.
No	Will procedures not fitting the above descriptions be performed on the animals? If *Yes*, complete Attachment O: Other Procedures.
No	Will animals be studied in the field? If *Yes*, complete Attachment P: Field Studies.
No	Will species of animals be handled that may possibly have rabies (e.g. dogs)? If *Yes*, complete Attachment Q: Rabies Pre-Exposure Immunization Consent Form.
No	Will you obtain rodents from private colonies? All shipments must be approved prior to shipment by the CWRU veterinarian. The mice may be subject to Quarantine, prophylactic treatment for pathogens, and/or rederivation by the transgenic core. Nonstandard vendor forms are available for download on the web at: http://iacuc.cwru.edu/forms/nonstandardvendor.doc

VII. Pain Classification

Classify individual procedures as C, D or E in the chart below. Investigators should assign procedures to pain classes honestly. There is no penalty to the investigator for placing a procedure in a higher pain class.

Class C:

Pain or distress will be absent or minimal and momentary

This category includes but is not limited to

- *Breeding.*
- *Gavage.*
- *Tissue collection after euthanasia.*
- *Most injections, if the injection does not introduce an agent which causes pain or distress.*
- *Blood collection from a peripheral vein.*
- *The use of Freund's complete adjuvant in immunizations in accord with IACUC policy* http://iacuc.cwru.edu/policy/freunds.html.
- *Nutritional studies if they do not lead to debilitation of the animal.*
- *Hypoxic or hypobaric chambers.*
- *Mutants with little or no pain or debilitation.*
- *Subclinical infections.*
- *Procedures which cause only minor discomfort.*

Class D:

Pain or distress will be present and appropriate anesthetics, analgesics or tranquilizers will be provided.

This category includes but is not limited to

- *Terminal tissue or organ harvest under anesthesia.*
- *Pithing or exsanguination under anesthesia.*
- *Surgeries such as biopsy, gonadectomy, exposure of blood vessels, chronic catheter implantation, bronchoalveolar lavage, laparotomy or laparoscopy when performed with appropriate anesthesia and analgesia.*
- *Surgical invasion of body cavities, orthopedic procedures, dentistry or other hard or soft tissue damage when performed with appropriate anesthesia and analgesia.*
- *Blood collection by invasive procedures such as intracardiac or periorbital collection from species without a true orbital sinus such as rats and guinea pigs when performed with appropriate anesthesia.*
- *Administration of chemicals or agents which produce chronic pain or distress which will be relieved with analgesics.*
- *Mutants with debilitating effects which are alleviated by analgesia or appropriate intervention.*
- *Clinical infection alleviated by appropriate treatment.*
- *Blood collection from the periorbital sinus of mice when performed with appropriate anesthesia.*
- *Tumor studies in rodents within the IACUC-recommended limit* http://iacuc.cwru.edu/policy/tumor_inoculation.html.

Class E:

Pain or distress will be induced and will not be relieved because relief would interfere with procedures, results or interpretation of the results.

This category includes but is not limited to

- *Administration of chemicals or agents which produce pain or distress which is not relieved with analgesics.*
- *Mutants with chronic pain or debilitation which is not relieved with analgesics or by appropriate intervention.*
- *Procedures producing pain or distress unrelieved by analgesics such as toxicity studies, microbial virulence testing, radiation sickness studies and research on stress, shock or pain.*
- *Negative conditioning via electric shocks or other methods of stress which would cause pain*

or distress in humans.

- *Prolonged restraint without appropriate measures to alleviate distress.*
- *Clinical infection without treatment.*
- *The administration of Freund's complete adjuvant by means not in accord with IACUC policy* http://iacuc.cwru.edu/policy/freunds.html.
- *Studies where the death of an animal is an experimental endpoint.*

Investigators should consider that pain relief may be incomplete despite best efforts to provide analgesia. In such cases, it may be appropriate to categorize the procedure as Class E. Where appropriate, the experience of humans subject to the same conditions can be used as a guide to classification.

USDA Regulations and Guidelines Pertaining to Pain Classification:
http://iacuc.cwru.edu/resources/policy11.pdf

List the estimated number of animals in each pain class for the complete duration of the protocol, using the guidelines above.

		Number In Pain Class			
Species, Strain and Procedure		**C**	**D***	**E*†**	**Total**
Adult zebrafish (Danio rerio) Natural breeding (B-breeding)	purchased	**0**			
	bred	**600**			
Zebrafish embryos, microscopy	purchased	**0**			
	bred	**1000**			
Zebrafish embryos, fixation	purchased	**0**			
	bred	**1000**			
Zebrafish embryos, injection	purchased	**0**			
	bred	**1000**			
	purchased				
	bred				
	purchased				
	bred				
	purchased				
	bred				
	purchased				
	bred				

	purchased				
	bred				
	purchased				
	bred				

* If animals are present in Class D or E, consultation with a lab animal veterinarian is required.
† If animals are present in Class E, complete Attachment F: Justification for Pain Class E Animal Use.

For Class D and E animal procedures, identify the laboratory animal veterinarian consulted and the date they were consulted.

VIII. Housing Of Animals And Location Of Procedures

1) Where Will The Animals Be Housed*?

Wolstein Animal Facility
Main ARC Facility – Health Science Animal Facility (HSAF)
Metro Health Medical Center/Rammelkamp
Wearn Animal Facility
Veterans' Administration

Athymic (Nude) Mouse Facility/CRC
Biomedical Engineering /Wickenden
ABSL3/P 3
Ultra-Barrier Facility

X Other. If checked, complete Attachment L.

*It is recommended that social animals be housed in groups whenever possible.

2) Are there special requirements for housing (for example, microisolators for rodents, special conditions for immunocompromised animals, biohazard or infectious agent containment)**?**

A fresh water Aquatic Habitats aquatic facility with ~500 individual tanks (1L, 2.75L, and 9L) has been built in 126 Millis. The facility has a recirculating water system. Building tap water is first purified by a reverse osmosis system, and then conductivity (salt concentration) and pH are adjusted by an automatic dosing system. Water quality (pH, temperature, conductivity) is constantly measured by an electronic monitor. To prevent the spread of disease, water passes through only one tank, and then is pumped through a carbon filter, particle filters, and a UV sterilizer before it returns to a tank. In addition to the main facility, we have a quarantine rack that has its own independent water system. Water quality is monitored in this rack by a dedicated portable pH/conductivity meter, and maintained by Kristine Ilagan, a fish technician with over one year experience. Water levels in the main system and quarantine rack are maintained by an automatic fill system controlled by float valve switch. Ammonia and nitrite are monitored manually. If parameters are outside of normal ranges, water is changed and manipulated to correct the problem. Water parameters are recorded every morning.

All fish are fed twice daily, am and pm. The am feed is composed of live food raised within the aquatic facility: brine shrimp for adults and juveniles and paramecium for babies (<2 weeks). The afternoon feed is composed of dry flake fish food for adults and juveniles and paramecium again for babies. When feedings are completed, they are logged.

Health of fish is monitored daily by observation during the feeding process. If a fish appears sluggish and skinny, it will be moved to a quarantine tank to be monitored and fed. If no improvement is seen the fish will be euthanized and recorded. If this behavior is seen in numerous fish, or if physical wounds, fungus, parasites and the like are observed, fish will be moved onto the quarantine rack and administered a formaldehyde treatment 25ppm. This treatment will continue every other day along with water changes. If this occurs in the main facility, all fish will be treated since the water recirculates within the facility.

3) Where will animal procedures be performed? Give the Building and Room Number. (If procedures will be performed in multiple places, please list each.)

Zebrafish facility, Millis 126
Teaching laboratory, Millis 326

IX. Euthanasia

The IACUC follows the recommendations of the AVMA Panel on Euthanasia. This guide to acceptable methods of euthanasia is available for download http://iacuc.cwru.edu/resources/euthanasia.pdf*.The document is readable and easy to navigate. We recommend that you examine the document to find the best approved methods for your needs. Justification is required for methods not in accord with these recommendations. For guidelines on euthanasia of neonatal rodents, see* http://iacuc.cwru.edu/policy/fetal_rodent_euthanasia.html*. Guidelines to the principles of humane euthanasia:* http://iacuc.cwru.edu/guide/guide4.html#anasia*.*

1) Describe the method of euthanasia.

Chemical Methods:

Carbon dioxide.
Describe the method.

Anesthetic agent. If using an anesthetic agent, provide the following information:

Drug:

Dosage:

Route of administration:

Physical Methods:

Exsanguination under anesthesia.
Describe:

Cervical dislocation under anesthesia or after CO2.
Describe:

Cervical dislocation without anesthesia.
Describe:

Decapitation without anesthesia.
Describe:

Provide scientific justification for decapitation without anesthesia.

X Other Methods:

Describe other methods in detail for each species. Justify the methods if they are not in accord with the AVMA recommendations.

Zebrafish will be euthanized by immersion for 20 minutes in a cold water bath. The 2000 Report of the American Veterinary Medical Association Panel on Euthanasia does not approve this method for euthanisia of fish species in general, but does not address the specific case of tropical species, which have little or no ability to adapt to the cold. This deficiency was addressed in 2002 by the University of Washington IACUC committee:

> "Because tropical fish species, (i.e. zebrafish, medaka, and platyfish), have minimal to no physiologic adaptation mechanism for adjusting to cold (4˚C) water, cooling to 4˚C should be considered an acceptable method of euthanasia since the rapid decrease in temperature from 26˚C (or higher) to 4˚C induces rapid loss of consciousness and is lethal to these species."

The full text of this report can be found at:
http://depts.washington.edu/compmed/iacuc/policies/fish_euthanasia.html.

Therefore, a cold water bath fulfills the requirements of the Panel on Euthanasia that a method of euthanasia cause rapid loss of consciousness, and minimize pain and distress.

2) How will the carcasses be disposed of? *(The ARC provides for disposal of animal carcasses in room EB09A in the HSAF.)*

They will be collected in Millis 126, frozen, and then incinerated at the ARC

X. Animal Identification

Identification of animals should be according to approved methods. Ear punches, tattoos, ear tags, and implanted microchips are acceptable for rodent identification. For IACUC policy on toe clipping, see http://iacuc.cwru.edu/policy/toe_clipping.html. For general guidelines on animal identification, see http://iacuc.cwru.edu/guide/guide3b.html#popman.

How will individual animals be identified?

Animals are not individually identified. Different stocks of fish, including wildtype strains and strains carrying specific mutations or transgenes, are identified by a label attached to their tank with birth date, stock name and number, the number of fish housed and the name of the person in the laboratory responsible for the stock. When the fish are removed from their tank as for breeding, the temporary tank is always labeled with the number of the tank of origin and the strain and sex of the fish.

XI. Environmental Enrichment

Enrichment is required for dogs and primates.
Information on enrichment: http://iacuc.cwru.edu/enrichment/enrichment.html

Dogs

Provide a plan for exercise and socialization for dogs. If you are seeking exemption from the institution's requirement to provide dogs with the opportunity for exercise or human contact, provide justification. *Dogs housed in cages or runs providing less than twice the minimum required floor space must be provided with daily access to an exercise pen during routine cage/pen cleaning. Dogs housed alone in a room must receive daily positive human contact.*

Primates

Provide a plan for enrichment for primates. If you seek exemption from the requirement to provide enrichment, provide a scientific justification.

X **Other Species (specify):** **Danio rerio**

Choose one of the following options for environmental enrichment of other species:

1. ARC-provided environmental enrichment program

The ARC will provide environmental enrichment to other species: Nestlets will be provided to singly-housed mice and nylabones provided to singly-housed rats. Environmental enrichment of at least one of the following will be provided to other species on a random/rotating basis: Alfalfa blocks, pineapple rings or plastic balls to rabbits and guinea pigs, rubber or rawhide chews or plastic balls to ferrets and cats.

X **2. No ARC provided enrichment: Investigator will provide environmental enrichment.**

Special care instructions forms and cage, room or rack labels must be provided for cages not subject to ARC-provided environmental enrichment. Please describe plan for enrichment that you will provide.

Fish are housed in clear tanks with anywhere from 2-40 fish. They can interact with one another within the same tank and can see fish in neighboring tanks. Fish are set up regularly in natural matings.

3. No ARC provided enrichment: No environmental enrichment.

Special care instruction forms and cage, rack or room labels must be provided for cages not subject to ARC-provided environmental enrichment. Provide a scientific justification if animals must be exempt from environmental enrichment.

XII. Personnel

Identify all personnel handling animals in this study, and identify the procedures they will perform, and their training or level of experience in performing the proposed procedures. ***Include the principal investigator, co-investigators, research associates, postdoctoral fellows, students, research associates, research assistants and technicians.***

Personnel forms are only needed for new personnel to the University or personnel who have not completed a Facility Access and Occupational Health form for over 3 years.

Duplicate this page when necessary*.*

Name And Degree	Species you will work with on this protocol	Procedures you will perform on this protocol	Briefly describe your experience or training in these procedures	Signature
Jennifer Liang, PhD	**Danio rerio**	All procedures	9 years experience with all aspects of the zebrafish system, and in training others	
Kristine Ilagan, B.A.	**Danio rerio**	All procedures	Over 1 year experience with all of the procedures	
Debra Vadja	**Danio rerio**	Natural matings, microscopy, fish feeding	Over 1 year experience	

XIII. Occupational Health And Safety

1) Will all personnel who will have contact with animals in this project participate in the Occupational Health Program? http://iacuc.cwru.edu/policy/occupational_health.html.

Yes	X	No. If *No*, explain why not.

The personnel listed by name above are already participating in this program through my research animal protocol and have thus taken all of the online training and filled out a FAHT form.

The Case students in the course will be added to the protocol at the beginning of the semester. In addition, they will fill out an FAHT form and release form stating that they understand the procedures that are required to work safely with the fish. A copy of this form is attached to this protocol.

As I stated above in section V, part 4, I will encourage the Case students to use only fixed tissues in their outreach to the high schools. If any of the Case students decide they want to use live embryos in their outreach, they will write an addendum to this protocol describing their experimental plan, and how the health and safety of the high school students will be protected. This addendum will be submitted for IACUC review and approval.

2) Are there any additional measures such as special vaccines or additional health screening which could benefit research, husbandry, or veterinary staff in this project? Vaccination for tetanus need not be mentioned.

No

XIV. Transportation

Transportation of animals must conform to all institutional policies and federal regulations. The "Guide for the Care and Use of Animals" http://iacuc.cwru.edu/guide/guide4.html#apt *is a good source of information on the regulations governing transport of animals. If animals will be transported on public roads or out of state, describe efforts to comply with USDA regulations* http://iacuc.cwru.edu/resources/cfr.html. *If animals will be transported within or between buildings, appropriate containment must be used. (Appropriate containment prevents pathogen spread and inadvertent observation. Examples of appropriate containment include cages of rodents placed inside a cardboard box or under a drape, and draped anesthetized animals on a cart.)*

If you will transport animals, describe the manner of transportation.

Adult fish and embryos will be placed in a tank and transported between the floors of Millis on a freight elevator

XV. Conditioning And Quarantine

Conditioning is required for mammals other than rodents. Purpose-bred rabbits, ferrets, cats, dogs and hoofed stock require a minimum of 7 days of conditioning. Random-source cats and dogs require a minimum of 14 days. Primates may require in excess of 90 days depending on source and use. The IACUC policies on conditioning and quarantine of animals are available as a pdf file for download at: http://iacuc.cwru.edu/policy/cqm.pdf.

Describe plans for conditioning and/or quarantine for your animals. If the required conditioning and quarantine is to be waived, provide scientific justification.

Fish received from an outside source are kept in a designated quarantine rack. This is a self-contained recirculating system holding 60 tanks.

To release this strain of fish from quarantine, embryos are disinfected by soaking them for 5 minutes in 170 ml of system water containing 0.1 ml of 5.25% bleach. The embryos are then transferred to 170 ml of system water and soaked for an additional 5 minutes. This process is then repeated once, after which the embryos are transferred to new petri dishes containing approximately 40 ml system water. These embryos are then raised to adulthood in the main (non-quarantine) part of the facility.

XVI. Suspended Caging

The use of suspended caging for rodents is discouraged by the IACUC.

Provide scientific justification for the use of suspended caging.

XVII. Investigator Input on the Form and Review Process

Please use the space below to provide constructive criticism on how the form and the review process could be changed to serve you better.

-- end of Core --

Attachment A: Fluid Collection

table of contents (attachments are to be completed only as required by the checklist)

Please describe the procedures for fluid (blood, urine, bile, lymph, etc.) collection prior to death. IACUC policies on the collection of blood are available at http://iacuc.cwru.edu/policy/blood_collection.html. NIH guidelines for the collection of blood from rats and mice can be downloaded from http://iacuc.cwru.edu/policy/nihpolicies/nihsurvivalbleeding.pdf.

Describe procedures for fluid collection, using a separate attachment for each procedure or species.

1) Species and Procedure:

2) Fluid:

3) Volume per collection:

4) Frequency of collection:

5) Total number of collections:

6) Site and method of collection:

7) Will the animals be anesthetized or sedated during the procedure? Yes or No.

If *Yes*, complete Attachment C.

8) Describe the method of restraint.

-- end of Attachment A --

Attachment B: Immunizations

table of contents (attachments are to be completed only as required by the checklist)

IACUC guidelines for immunizations and the use of complete Freund's adjuvant are available at http://iacuc.cwru.edu/policy/freunds.html. For example, footpad injections are strongly discouraged, complete Freund's adjuvant beyond the initial injection is strongly discouraged, and attention to potential contaminants that may lead to inflammation is required.

Describe the immunization procedure, using a separate attachment for each species or route of administration.

1) Species:

2) Antigen(s):

3) Adjuvant for initial injection:

4) Adjuvant for subsequent injections:

5) Injection site(s):

6) Volume per injection site:

7) Number of sites:

8) Preparation of injection site (e.g. clipping, disinfection):

9) Total volume of adjuvant and antigen:

10) Frequency of immunization:

11) Will the animals be anesthetized or sedated during the immunization procedure?

☐ No ☐ Yes (If *Yes*, complete Attachment C)

12) Fluid or tissue collected:

☐ Blood (Complete Attachment A)

☐ Ascites fluid (Complete Attachment A)

☐ Tissue (Specify):

-- end of Attachment B --

Attachment C: Anesthesia, Analgesia Or Paralysis

table of contents (attachments are to be completed only as required by the checklist)

For CWRU Guidelines for appropriate anesthesia and analgesia for different animal species, see http://iacuc.cwru.edu/policy/analganesth.html. For IACUC guidelines on the safe use of ethyl ether as an anesthetic, see http://iacuc.cwru.edu/policy/ethyl_ether.html. For general guidelines to anesthesia and analgesia, see http://iacuc.cwru.edu/guide/guide4.html#paa. Investigators are strongly encouraged to consult with an ARC veterinarian about the most appropriate methods of anesthesia, analgesia and the appropriate use of tranquilizers and paralytics. The ARC phone number is 368-3490.

Describe the conditions of anesthesia, using a separate Attachment for each species or procedure.

1) Species and Procedure: Zebrafish anesthesia

2) Drugs used for restraint, tranquilization, sedation.

Drug(s): Zebrafish anesthesia

Dosage(s): MS222/Tricane/3-amino benzoic acidethylester

Route(s) of Administration: Absorption through skin

3) Drugs used for anesthesia.

Pre-Anesthetic:

Drug(s): Zebrafish anesthesia

Dosage(s): MS222/Tricane/3-amino benzoic acidethylester

Route(s) of Administration: Absorption through skin

Induction:

Drug(s):

Dosage(s):

Route(s) of Administration:

Maintenance:

Drug(s):

Dosage(s):

Route(s) of Administration:

4) Expected duration of anesthesia:

X <30 minutes

30 - 60 minutes

1 - 2 hours

2 - 4 hours

> 4 hours, specify:

5) How will depth of anesthesia be monitored?

Fish will slow down or stop moving, however heart rate will continue at a slow pace and will be monitored visually throughout the process.

Anesthesia will be used only for immobilizing embryonic/larval fish so that they can be photographed. Therefore there is no painful procedure associated with the anesthesia.

6) If analgesics will be used, but the method of their use is not described elsewhere, please do so here, giving drug, dose, route and frequency.

7) Will a paralytic agent be used?

X No Yes. If *Yes*, complete the following:

Drug(s):

Dosage:

Route of administration:

What is the purpose of using a paralytic agent?

Describe how ventilation will be maintained and how pain will be assessed.

Justify the use of a paralytic.

-- end of Attachment C --

Attachment D: Survival Surgery

table of contents (attachments are to be completed only as required by the checklist)

It is required that you consult with a veterinarian trained in Laboratory Animal Medicine. ARC veterinarians may be contacted at 368-3490.

NIH guidelines for survival surgery on rodents: http://iacuc.cwru.edu/policy/nihpolicies/surguide.htm.
NIH guidelines for oocyte collection from Xenopus: http://iacuc.cwru.edu/policy/nihpolicies/oocyte.htm.
IACUC guidelines for surgery on rodents: http://iacuc.cwru.edu/policy/rodent_surgery_guide.html
IACUC guidelines for surgery on non-rodents: http://iacuc.cwru.edu/policy/survsurgnonrodents.html *General guidelines to surgery*: http://iacuc.cwru.edu/guide/guide4.html#surg

Describe survival surgery, using a separate attachment for each procedure and species.

1) Species and Procedure:

2) Location of Procedure:

3) How many animals will undergo this procedure per year?

4) Will more than one survival surgery be performed on an animal? ☐ Yes ☐ No

If *Yes*, provide scientific justification for multiple survival surgeries. *USDA guidelines for justification of multiple survival surgery are available at* http://iacuc.cwru.edu/policy/policy14.pdf.

5) Are some animals likely to require an **elective** subsequent surgical intervention to correct or modify the original procedure? ☐ Yes ☐ No

If Yes, describe the type and necessity of surgical repair anticipated and estimate the percentage of animals needing reoperation. The IACUC Policy for Elective Surgical Repair and submission form is available at http://iacuc.cwru.edu/policy
NOTE: ARC Veterinary Staff must be notified in advance of all surgical procedures including emergency repairs via the submission of a **Procedure Data Sheet.**

6) Describe the presurgical procedure in detail.

If food will be withheld, give duration.

If water will be withheld, give duration.

If analgesics will be given, describe route and dosage.

7) Describe the aseptic procedures (fur clip, disinfection, sterilization of instruments, and maintenance of asepsis between surgeries).

8) Describe the surgical procedures, including a description of access to the anatomic site.

9) Expected duration of surgery:

- [ ] <30 minutes
- [ ] 30 - 60 minutes
- [ ] 1 - 2 hours
- [ ] 2 - 4 hours
- [ ] > 4 hours, specify:

10) List major support equipment used or available for use (e.g., anesthesia machines, electrocautery, suction, fluoroscope, ventilator, heating pad, CO2 analyzer, defibrillator, EKG monitor).

11) Administration of antibiotics or other drugs (specify):

Drug:
Dosage:
Route:
Frequency:

12) Post-operative care:

Observation frequency and duration:

Analgesics

Drug:
Dosage:
Route:
Frequency:

Antibiotics

Drug:
Dosage:

Route:

Frequency:

13) Describe other supportive care in detail, including fluids or special diets and the frequency and duration of treatment.

14) Who will provide post-operative care?

15) How long will the animals be maintained after surgery?

16) If the surgery will normally result in an impairment of the physical or physiological function of the animal, or if analgesia is expected to be incomplete despite best efforts, describe the expected severity and effect on the animals' welfare.

17) What unintended complications may occur as a result of this surgical procedure (e.g., hemorrhage, wound infection, physical impairment, etc.)?

18) Describe how complications will be managed and the criteria for termination of the experiment by euthanasia. *(Examples of appropriate criteria that should be considered include a weight loss limit as a percentage of initial or expected body weight, allowable duration of anorexia, the presence of health problems refractory to medical intervention, and severe psychological disturbances.)*

19) For cannulae, acrylic implants, venous catheters and other devices which will extend through the skin for longer than 12 hours, explain what wound management measures will be taken to minimize infections at the site of penetration.

--end attachment D--

Attachement E: Non-Survival Surgery

table of contents (attachments are to be completed only as required by the checklist)

In non-survival surgery, the animal is euthanized at the end of the procedure without recovery from anesthesia. The procedures for anesthesia must be described in Attachment C.

Describe non-survival surgery, using a separate attachment for each procedure and species.

1) Species and Procedure:

2) Location of Procedure:

3) Describe the presurgical procedure.

If food will be withheld, give the duration.

If water will be withheld, give the duration.

If anesthetics will be loaded, give drug, route and dosage.

Describe the aseptic preparation.

4) Describe the surgical procedures, including a description of access to the anatomic site.

5) How long will the animal be maintained under anesthesia prior to euthanasia?

6) How will humane euthanasia be enacted, and how will death be determined?

-- end of Attachment E --

Attachment F: Justification For Pain Class E Animal Use

table of contents (attachments are to be completed only as required by the checklist)

Procedures which may cause more than momentary or slight pain must, in their planning, involve consultation with a veterinarian trained in Laboratory Animal Medicine. ARC veterinarians may be contacted at 368-3490.

The definition of experimental endpoints are particularly important for Class E Animal Use. The NIH provides guidelines for the selection of humane experimental endpoints: http://iacuc.cwru.edu/policy/nihpolicies/endpoint.htm.

1) If pain relief will be withheld from animals in pain or distress, provide scientific justification. *Provide the scientific rationale for this decision and provide references. This information is required in our annual USDA report and may be quoted directly from this protocol form.*

2) Describe the expected clinical signs of pain or distress. *For example, signs of extreme distress in rodents include hunched posture, disheveled coat, reduced food consumption, emaciation, inactivity, difficulty in ambulation, respiratory problems, and solid tumor growth. Indicate the expected severity and duration, the frequency the animal will be monitored and when the pain will be eliminated or managed by euthanasia, drugs or withdrawal of painful stimulus.*

3) If death will be the experimental endpoint, you must justify why an alternate endpoint (such as weight loss, clinical signs, tumor size, etc.) prior to death cannot be used.

4) List the number of animals of each species to be used in Class E procedures for each year.

	Number Of Animals			
Species and Procedure	**Year 1**	**Year 2**	**Year 3**	**Total**

-- end of Attachment F --

Attachment G: Animal Restraint

table of contents (attachments are to be completed only as required by the checklist)

Short periods of restraint such as those required for routine veterinary or husbandry procedures do not require documentation or IACUC approval.

- What is the maximum length of time any one animal would be restrained within a 24-hour period?

2) Method of restraint:

Manual.

Chemical (i.e., tranquilizers and/or anesthetics agents. Complete Attachment C).

Restraint device or cage. Describe the device or cage.

3) Animals should be gradually trained to accept extended restraint periods. Please describe your training program.

4) Justify the need for prolonged restraint, and address why alternatives to prolonged restraint are inadequate.

-- end of Attachment G --

Attachment H: Infectious Agents, Biohazards, Recombinant DNA

table of contents (attachments are to be completed only as required by the checklist)

If the project requires approval for any of the agents below, a copy of the approved safety form from the appropriate safety committee must be provided before activation of the protocol, with the exception of transgenic and knockout mice, as work can proceed on these while the application is pending.

1) Identify the agent in the appropriate category. (Pathogen)

☐ Infectious agents. Identify:

For forms, http://www.cwru.edu/finadmin/does/web/Forms/PDFdocs/IACUCPathProt.pdf

☐ Hazardous chemicals, including chemical carcinogens. (Carcinogen) Identify:

For forms, contact G. David McCoy 368-3233, gdm@po.cwru.edu.

☐ Recombinant DNA. (IBC) Describe:

Production of transgenic and knockout mice needs to be approved for Recombinant DNA, in accord with NIH policy. For forms, http://ora.ra.cwru.edu/maim.ibc

☐ Importation of Biohazardous Animals. (Non-Standard Vendor) Describe:

The importation of animals which present a biohazard must be approved by the CWRU IBC. For forms, http://ora.ra.cwru.edu/main_institutional_biosafety_committee_page.htm

☐ Radioactive Materials. Describe:

For forms, http://www.cwru.edu/finadmin/does/web/RadSafety/application.pdf

2) Specify the containment methods to be followed in protecting other research animals and personnel from any of the agents listed above. Describe the procedures required for the safe handling and disposal of contaminated animals, caging, bedding, food and materials associated with this study. Describe methods for removal of radioactive waste and monitoring of radioactivity, if applicable.

3) Describe the expected physical or physiological consequences to the animals due to administration of pathogens, hazardous chemicals, carcinogens, transgenes, or mutation(s).

4) Describe any special care or monitoring that the animals will require.

5) How long will the animals be maintained after introduction of the agent?

6) Describe the criteria for interventional euthanasia. *(For example, signs of extreme distress in rodents include hunched posture, disheveled coat, reduced food consumption, emaciation, inactivity, difficulty in ambulation, respiratory problems, and solid tumor growth.)*

-- end of Attachment H --

Attachment I: Tumors In Animals

table of contents (attachments are to be completed only as required by the checklist)

IACUC guidelines for tumor inoculation: http://iacuc.cwru.edu/policy/tumor_inoculation.html

The NIH Office of Animal Care and Use (OACU) and the CWRU IACUC discourage the use of the ascites method for propagation of monoclonal antibodies. If the ascites method must be used, you must provide a justification (question 6, below), and procedures should be in accord with the NIH guidelines: http://iacuc.cwru.edu/policy/nihpolicies/ascites.htm
Resources and information on in vitro production of monoclonal antibodies and ascites production are available at: http://iacuc.cwru.edu/policy/mabs.html, http://www.nal.usda.gov/awic/pubs/antibody/, http://grants1.nih.gov/grants/olaw/references/dc98-01.htm

1) Host animal (species and strain):

2) Is this a human primary tumor cell? Yes No

Human primary cell lines require a pathogen safety form.

3) Is the tumor derived from a rodent or has the tumor been passaged through rodents? Yes No

Tumor cell lines derived from or passaged through rodents must be tested by Rat or Mouse Antibody Production or PCR to detect rodent infectious agents (MAP or RAP) before being implanted into rodents. Tissue culture Mycoplasma testing does not meet the testing requirements.

4) If derived from rodents or humans, has the material been tested for pathogens? Yes No

A copy of RAP, MAP or PCR test results must be attached to this protocol.

5) Describe criteria for interventional euthanasia (see the guidelines at the links above).

6) If the size of the tumor will exceed 10% of the body weight of the animal provide scientific justification.

7) If the ascites method is used for the production of monoclonal antibodies, justify why *in vitro* production would not be adequate.

Describe the priming, inoculation(s), and number of taps and monitoring of animals during ascites production. It is highly recommended that the NIH guidelines http://iacuc.cwru.edu/policy/nihpolicies/ascites.htm be followed.

-- end of Attachment I --

Attachment J: Administration of Exogenous Substances or Tissues

table of contents (attachments are to be completed only as required by the checklist)

For tumor studies and ascites production, please use Attachment I instead.

Use a separate Attachment for each recipient species

1) Recipient species:

2) Identify the substance and its source. Describe whether the substance is biological and give its source (species, preparation, synthesis, purification, etc as appropriate). If the substance is a proprietary compound or agent that cannot be explicitly described, address the issues listed in Attachment J, Item 3 (Proprietary Substances). If the substance is of biological origin, state what steps have been taken to render it free of adventitious infectious agents to man and the recipient species or provide documentation that the substance has been tested (via PCR, MAP test, RAP test, etc.)? Animals given biologic material with an unknown infectious potential will be maintained in quarantine for the duration of the study.

3) Proprietary Substances:

a) Provide written assurance that the substance, at the levels/doses to be used in the animal protocols, is not toxic or otherwise harmful to animals/humans involved in the proposed research. This assurance should briefly describe how the substance has been subjected to standard *in vitro* tests for toxicity or mutagenicity.

b) If evaluation of *in vivo* toxicity or potential harmfulness of the proprietary substance is the goal of the proposed animal experimentation, indicate how the protocol will estimate the maximum tolerated dose (MTD) and no effect dose level (NOEL) for that substance in the animal subjects.]

4) Administration.

Dosage:

Volume:

Route:

Frequency:

Animals will be anesthetized or sedated for administration ☐ Yes ☐ No

If *Yes*, complete Attachment C

5) How long will animals be maintained after administration of the substance, and what are the experimental endpoints?

6) Will physical or physiological effect(s) (e.g., inflammation, decreased blood pressure, increased heart rate, etc.) likely result from this treatment? If *Yes*, describe in detail expected physical or physiological effects, including expected severity, duration and frequency. For introduction of cells and tissues, what will be done to prevent graft rejection?

7) Describe the plan for monitoring the animals, including frequency and length of observation, and the criteria for termination of the experiment through interventional euthanasia. *(Examples of appropriate criteria that should be considered include a weight loss limit as a percentage of initial or expected body weight, allowable durations of anorexia, allowable tumor size or total tumor burden expressed as a percentage of body weight, the presence of health problems refractory to medical intervention, and severe psychological disturbances. Other criteria appropriate for the species under consideration should also be considered.)*

-- end of Attachment J --

Attachment K: Irradiation

table of contents (attachments are to be completed only as required by the checklist)

Complete a separate Attachment for each procedure or species.

1) Describe the method of irradiation of the animals.

Species:

Radiation Source:

Dose:

Frequency of Irradiation:

Anatomical site irradiated:

2) Expected physical and physiological effects:

3) Describe the experimental endpoints, and how long the animals will be maintained after irradiation.

4) Give the criteria for interventional euthanasia of animals too sick to continue in the study.

5) Describe the plan for monitoring the animals, including the length and frequency of observation, and clinical signs which will be monitored.

-- end of Attachment K --

Attachment L: Principal Investigator Satellite Facility Registration

table of contents (attachments are to be completed only as required by the checklist)

All locations where animals are kept longer than **12 hours** *or where rodent survival or nonsurvival surgeries are performed must be approved by the IACUC. All locations for housing and surgery must meet legal requirements for these functions. The IACUC must inspect these housing and surgical areas prior to use, and every 6 months thereafter. A copy of this attachment must be posted in the surgery or housing area. Investigators must supply standard operating procedures for all animal housing areas.*

1) Location of surgery

Building: Room number(s):

2) Surgical procedures performed:

3) Survival/ Nonsurvival

4) Location of alternative housing.

Building: Millis Room number: 126B

5) Justify your need for alternative housing.

This room contains a state of the art aquatic facility for raising and maintaining zebrafish. There is no other housing for zebrafish on campus.

6) Maximum period that animals will be housed here.

They will be housed in this facility their whole lived, typically 2-3 years.

Please attach a copy of your Standard Operating Procedures for extramural housing.

IACUC Use Only:

Initial Inspection Date:		**Expiration Date:**	
Inspected by:		**Inspected by:**	

-- end of Attachment L --

Attachment M: Manipulation of Diet or Environment

table of contents (attachments are to be completed only as required by the checklist)

Describe dietary restrictions or manipulations, and environmental manipulations, using a separate attachment for each species and procedure. *General guidelines to dietary restriction are available at:* http://iacuc.cwru.edu/guide/guide2.html#mcua.

Species and Procedure: **Danio rerio, raising embryos at different temperatures**

1) Describe the dietary manipulations or manipulation of the environment. Describe relevant parameters including duration, nutrients removed or supplemented, weight criteria, endpoints, etc.) Describe alterations to lighting, temperature or other environmental variables or conditions and their duration.

Embryos will be raised at slightly higher or slightly lower temperatures during the first seven days of development (0 days post fertilization to 7 days post fertilization). For instance, zebrafish are typically raised at 28.5 degrees Celsius. We will raise fish at 5 degrees above and below this to determine if there is an effect on the sex of the fish.

2) What physical or physiological effect(s) will result from this treatment and what is their expected duration?

It may influence whether the fish develop as males or females, but there will be no other effects.

3) If pain, distress or significant symptomatology are possible, list the criteria to be used to determine when euthanasia is to be performed. *(For example, signs of extreme distress in rodents include hunched posture, disheveled coat, reduced food consumption, emaciation, inactivity, difficulty in ambulation, respiratory problems, and solid tumor growth.)*

N/A

-- end of Attachment M --

Attachment N: Behavioral Tests

table of contents (attachments are to be completed only as required by the checklist)

NIH guidelines for manipulation of diet in behavioral studies
http://iacuc.cwru.edu/policy/nihpolicies/dietctrl.htm.

Describe Behavioral Tests, using a separate attachment for each species or procedure.

Species and Procedure:

Complete the appropriate sections below.

Conditioning

1) What is the purpose of the conditioning?

2) Describe the reinforcement techniques which will be used.

- No reinforcement.
- Food reward. Describe:
- Electrical shock. Give strength and duration:
- Food deprivation. State duration:
- Water deprivation. State duration:
- Other. Give strength and duration:

3) What criteria will be used to monitor the health and welfare of the animals?

Other Behavioral Training And Testing

1) What is the purpose of the training and testing?

2) Describe the procedure, detailing any aversive stimuli or conditions, including their strength and duration. If aversive stimuli or conditions will be used, describe how the health and welfare of the animals will be monitored.

-- end of Attachment N --

Attachment O: Other Procedures

table of contents (attachments are to be completed only as required by the checklist)

For procedures not fitting the preceding categories, give the following information.

Complete a separate Attachment for each species or procedure.

1) Species and Procedure:

2) Describe the procedure in detail.

3) How long will the animals be maintained after the procedure?

4) Describe the plan for monitoring the animals after the procedure, including frequency and length of observation, and clinical signs which will be monitored.

5) Describe the experimental endpoints.

6) What are the expected physiological or clinical effects of these procedures?

7) Describe the criteria for interventional euthanasia of animals too sick to continue in the study.

-- end of Attachment O --

Attachment P: Field Studies

table of contents (attachments are to be completed only as required by the checklist)

If animals in the wild will be used, identify the species; describe how they will be observed, any interactions with the animals, whether the animals will be disturbed or affected, and any special procedures anticipated. Indicate if Federal permits are required and whether they have been obtained. Information on regulations governing field studies: http://iacuc.cwru.edu/guide/guide1.html#finv.

-- end of Attachment P --

Attachment Q: Rabies Pre-Exposure Immunization Consent Form

Case Western Reserve University ** University Health Service
2145 Adelbert Road ** Cleveland, Ohio 44106 ** (216) 368-2450

It is recommended that persons handling certain species of animals on a regular basis be vaccinated against rabies because of a small risk of contracting this potentially fatal disease. The vaccine administered is the Human Diploid Vaccine and involves three injections in the upper arm, one injection on day, one on day 7 and one on day 28. A local reaction can be expected in the area consisting of tenderness, redness, itching or hardness. This will last for a few days.

Occasionally, other reactions occur – headache, malaise, fewer, muscle or joint aching, general itching or rash and occasional nausea and vomiting. Please contact the UHS at 368-2450 if any such reaction occurs.

The duration of protection from the rabies vaccine varies from person to person. Consequently, we recommend that persons receiving the vaccine have antibody to the rabies virus measured every 2 years. If the titers of the antibody have fallen below a certain level, a booster dose of the vaccine should be administered to provide adequate protection. Blood should not be donated for a 6-month period after immunizations.

I have read the above information and am aware of the indications for taking the rabies vaccination and the side effects of the vaccination.

I consent to taking this immunization.

I do not wish to avail myself of this immunization

Sign Name:

Print Name: Date:

-- end of Attachment Q --