IACUC Appendix: [redacted] September 2017 – August 2020

TABLE OF CONTENTS

I. KEY WORDS, ABREVIATIONS, DEFINITIONS and DESCRIPTIONS:

Embryo – 1 cell zygote or <3dpf. The choice of time post fertilization as opposed to using "hatching" (exiting the chorion) is appropriate because many experimental procedures benefit from manually hatching "dechorionation" which can be done as early as egg laying and is often done at 1-2dpf for a number of reasons. Embryonic development including formation of organ systems is incomplete until approximately 3dpf.
Larvae – 3 to 7±1dpf, yolk may still be available for nutrition but the animals can feed. Most organ systems are developed and the animals are motile although those nearest 3dpf may predominately lay on the bottom of their housing container laregely due to the lack of a functional swim bladder. By 7dpf a functional swim bladder will have formed enabling larvae to swim throughout the water column.
Fry/Juveniles – 7±1dpf to 90±30dpf, are motile can forage for food and feed, the yolk is depleted at this point, and the animals are not reproductively mature.
Adult – 90±30dpf the key feature of the adult zebrafish is reproductive maturity.
OAL – Overall length measured from the lip of lower jaw to fork in tail fin.
Chorion – Outer egg membrane for zebrafish eggs. The chorion is a slightly opaque "bag" that surrounds the egg or developing embryo.
Clutch – Progeny from spawning a single mating pair.
Cohort – Group of animals created/born at the same time under the same conditions.
Siblings – Individuals from a single clutch.
Housing – Aquatic habitat on or off main system; i.e. tank.
Static tank – Aquatic habitat not connected to a water supply; relies on manual water change.
Recirculating system – Aquatic habitat with automatic filtration, and water exchange.
Rotifers – Rotifers are saltwater zooplankton that can be cultured in the lab for live feeding zebrafish larvae
Artemia – Brine shrimp; primary live food for juvenile and adult zebrafish.

Dry feed – Desiccated pellet feed for zebrafish. Comes in several sized pellets to accommodate various stages of development.
Noldus® – Commercial behavioral analysis system conducive for studies from embryo to adult. The device controls temperature and lighting. And can track the movement of zebrafish larvae with via video recording and tracking software. Video capture can occur with visible or infrared lighting so light v dark behavior can be directly assessed.
Feeding cone – Culturing device Rotifers or Artemia.
ZIRC – Zebrafish International Resource Center is the primary source for wildtype and genetically modified zebrafish. www.zebrafish.org
ZFIN – Zebrafish Information Network, model system data repository. ZFIN provides a free accessible portal for data, protocols and community discussion relevant to zebrafish research and maintenance. www.zfin.org
Transgenesis – Addition of foreign DNA to host genome.
Gene editing – Editing of the host genome, which may or may not include addition of DNA.
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats; RNA guided nuclease used for *in vivo* gene editing.
Morpholino® – Modified antisense oligonucleotide used for transient silencing of gene expression *in vivo.* www.gene-tools.com
Microinjection – Injection system for delivery of soluble or liquid suspended substances into embryos or larvae. May include but not limited to, drugs, toxins, mRNA, DNA, protein, or cells. Typically, a gas operated microinjection pump and glass capillary tubes pulled to a sharp point are used for injection.
Intraperitoneal injection or IP – Injection of soluble or liquid suspended substances into the intraperitoneal space of juvenile or adult animals. Typically, a Hamilton syringe for volumes ranging from 1-10ul are used.
Graft – Surgical implantation, or injection of solid substances, i.e. bacterium, tissue, beads.
NAU ZebTEC® – Main zebrafish housing units located in the [redacted]. There are two ZebTEC designated "**Nursery**" and "**Main**".
Nursery – Zebrafish housing for larvae and fry, situated on the bottom row of the Nursery NAU ZebTEC. Specialized tanks allow for shallow water depth, while maintaining circulation, both optimal parameters for raising zebrafish.
External fish – Zebrafish ≥72hpf born outside of, or removed from, [redacted], and that have not been disinfected using standard embryo bleaching protocol. *Examples:* (1) Embryos ordered from ZIRC are disinfected prior to distribution and therefor may enter NAU ZebTEC. (2) Adult fish ordered from ZIRC cannot enter the NAU ZebTECs because they cannot be disinfected. (3) Fish removed from [redacted] for any reason cannot re-enter the NAU ZebTECs.
SOP – Standard operating procedure/protocol. Protocols for zebrafish care and research pursuits.
Density – Number of individual animals per liter of water.
MS-222 or Tricaine – Ethyl 3-aminobenzoate methanesulfonate, CAS# 886-86-2; used as anesthetic for aquatic vertebrates including zebrafish.
Danieau's solution – Buffered water for rearing zebrafish embryos; suitable for <7dpf larvae.
RO water – Reverse osmosis water.
DI water – Deionized water.

System water – Water that has been conditioned for use in ZebTEC housing or taken directly from ZebTEC system.
Media – Aqueous solution for maintaining zebrafish; includes system water and Danieau's solution
Alkaline hydrolysis – Method for dissolving animal tissue using concentrated (1-2M) sodium hydroxide (NaOH) in an aqueous solution.
BSA – Biological Sciences Annex; NAU vivarium located in Bldg 21 and the location of the NAU ZebTEC.
DMSO – Dimethyl sulfoxide is a solvent routinely used to prepare stock solutions for exposure studies, or as the vehicle/solvent control.

II. ZEBRAFISH HOUSING

a. Main Housing Systems

Zebrafish are housed in two state-of-the-art recirculating aquatic habitats (Tecniplast ZebTEC Active Blue) holding polycarbonate 3.5L and/or 8L tanks and partitioned 1L (for nursery, breeding and isolation) polycarbonate tanks that are located in [redacted]. The ZebTEC systems use mechanical and biological filtration to maintain optimal water quality while reducing system consumable waste and effluent. The systems are fed with RO water, that is automatically conditioned with sea salt and buffer to achieve optimal water. The ZebTEC systems are connected to the web allowing for remote real time monitoring and automatic alarm reporting. Additional details quoted from ZebTEC Active Blue product brochure:

> **Active Blue Technology** Maintenance Free System, no more consumables, saving you time and money achieving optimal water quality! Tecniplast introduces a self-cleaning mechanical filtration at stand-alone rack level, a real unique innovation. Entirely thought, designed and manufactured at Tecniplast, our technology guarantees the finest filtration available on the market for this product. A unique self-cleaning drum filter is used to obtain a 40 micron or even 26 micron fine mechanical filtration, eliminating the need to have pre-filters and fine mechanical filters. When the drum filter nets get dirty, the system automatically back-flushes it, removing all the suspended solids and guaranteeing the best water quality for your animals without manual maintenance. The back flushing water is counted as replacement water volume, thus saving in extra water and consequently extra costs.
>
> • No more need for stocking consumables.
>
> • Greatly reduced running costs.
>
> • Labor saving and less stress for the animals.
>
> • Better fish welfare and thus increased research level!
>
> **The New ZebTEC Stand Alone** Reaching excellence not only in self-cleaning filtration! On top of featuring the Active Blue technology, Tecniplast New Stand Alone system brings a series of great advantages and innovative solutions at different levels:
>
> **Fine mechanical filtration:** enhanced consumables free fine mechanical filtration, down to 40 and even 26 micron using the Active Blue technology.
>
> **Water disinfection:** Highest UV disinfection killing rate (up to 180.000 $\mu W/cm^2/sec$).
>
> **Biofiltration:** Tecniplast is now using the most innovative biofilter material, called Biochip. It is a unique biofiltering support able to provide the best and highest ratio between space utilized and nitrifying surface available. Thanks to the unique shape, this material is able to clean itself and every biofilm accumulation is avoided thanks to the continuous movement of the disks. Being entirely injection molded, its durability is basically everlasting.
>
> **Chemical filtration:** Available as an optional item, our activated and acid washed carbon bag can be added whenever needed.
>
> **Advanced monitoring System:** Better graphic and remote control. Great visibility and ease of use are the basic features of our new monitoring system. All the main important information are reported on the first page. Thanks to the USB port, data and graphics are easily downloadable using a pen drive. Moreover, you can manage remotely the system through the web and be notified of alarms via text message or e-mail.

Robustness: All the material we used and the design we have implemented are focused to achieve an almost everlasting product. Molded sump, painted 316L stainless steel racks are just some of the important features which show our robust approach in product design.

Flexibility: The new ZebTEC SA comes in different configurations: 4, 5 or 6 levels and can feature 3,5 l 8,0 l or 1,1 l tanks on the same rack without any need of replacing the manifolds. Divider options available.

Easy tank handling: All our tanks are positioned on dedicated black runners, which makes it extremely easy to slide them in and out from the rack. Moreover, the dark color provides environmental enrichment and keeps fish calm: they know where they are swimming.

Unique technical features

- "push and pull" valve with fine tune flow control prevents accidental water loss and allows for the end user to adjust flow to each tank. When water is delivered the valve locks the tank in the proper position. By pulling the valve up the water flow is stopped and the tank is easily removed. Each valve has a visual indicator which allows the operator to see at a glance if a tank has the proper water flow. Many of our systems come standard with pressure regulation to guarantee consistent flow from 5 WCH to a steady drip and everything in between.
- Clean tank technology, reducing maintenance and saving you money! You will find that by utilizing our patented unique active siphon system along with the special tank shape and color, the maintenance required is significantly reduced, unlike other manual systems that use manual tank siphoning and algae removal, saving you time and money.

b. Quarantine housing

Zebrafish born outside the NAU zebrafish rooms or removed from the Main Housing (Animal Annex) **may not** enter or re-enter the ZebTEC housing systems. External fish will be housed in standard glass or acrylic aquaria until sexually mature (or a minimum of 2 weeks for observation). Sexually mature external fish will be breed in [redacted] and derived embryos will be disinfected, and moved to the NAU zebrafish room. These procedures are designed to prevent the accidental spread of diseases.

c. Locations for experimental procedures

Table: Locations for Experimental Procedures		
Experiment type	**Location(s) Bldg:Rm**	**Rationale and clarification**
Chemical exposure in embryos, larvae or adult zebrafish (does not include microinjection of nucleic acids or protein).	[redacted]	**No chemicals may enter [redacted] with two exceptions: 1. chemicals required for facility operation; 2. nucleic acids or protein for microinjection into embryos.**
Microinjection into embryos for genetic manipulation.	[redacted]	**Injection of mRNA, DNA, protein, and modified nucleic acids are permitted in [redacted] to establish new genetic lines. Embryos microinjected in [redacted] are used for immediate gain-of-function and/or loss-of-function analysis and will not be raised AND CANNOT reenter [redacted].**
Behavioral assessments (e.g. novel tank assay; optomotor	[redacted]	**Behavioral assessments using individuals that have NOT been exposed to chemicals and have NEVER left**

response; thigmotaxis; visual motor response).		**[redacted] can be performed in [redacted]. Behavioral assessments on exposed embryos, larvae or adults will be performed in [redacted] and those animals CANNOT reenter [redacted].**
Gene expression analysis	[redacted]	**Embryos, larvae or adults used for gene expression analysis will be moved to [redacted] for nucleic acid or protein isolation and expression analysis.**
Tissue isolation for genotyping (i.e finclip)	[redacted]	**Tissue isolation for genotyping with the intent of line maintenance and/or mutant generation will be performed in [redacted]. Tissue isolation and genotyping on individuals NOT for line generation or maintenance will be carried out in [redacted]. Animals brought into [redacted] CANNOT reenter [redacted].**

III. Feeding (*for general references see*: (Harper and Lawrence, 2011; Lawrence et al., 2016; Nüsslein-Volhard and Dham, 2002; Westerfield, 2007)

a. Schedule ***(see Section IX "Feeding schedule")***

i. Free swimming zebrafish (>5dpf) are fed between 1 and 3 times daily which may include live and/or dry food.

b. Live feed

i. *Artemia* nauplii (first larval stage of brine shrimp) can be reared in the laboratory using a feeding cone culturing device. Freshly hatched *Artemia* (i.e. nauplii) are free swimming subsist on their yolk reserve for nutrition. Because nauplii are consuming their yok supply it is best to collect them as close to the time of hatching as possibly to maximize the nutritional capacity as a food source for zebrafish. Due to the size of *Artemia* nauplii (~475-500um) it may be difficult for early zebrafish larvae to fit the nauplii into their mouth.

ii. *Branchionus* (rotifer) are considerably smaller, 100-250µm, then *Artemia* nauplii, and are slow swimming making them an ideal food source for larval zebrafish (5-21dpf)

iii. Live food provides environmental enrichment. Aside from the nutritional benefits of using life food sources, the live food stimulates normal zebrafish foraging and predatory behaviors (Harper and Lawrence, 2011; Lawrence et al., 2016; Westerfield, 2007).

c. Dry feed

i. Dry feed or pellet feed can be obtained from commercial vendors (www.zebrafish.skrettingusa.com) and offers well balanced nutritional content suitable for all stages of zebrafish growth. However, uneaten dry food decays rapidly in water which can negatively affect water quality. Therefore, it is important to offer only enough dry food per tank as the occupants a can consume in a 10-15 minute period. Nursery tanks receiving low water circulation should not be fed dry food, as larval stock can easily succumb to poor water quality.

IV. Standard Operating Procedures/Protocols

a. Quarantine zone: *Adapted from -* (Snider and Clegg, 1975)

☐Fish brought in from the outside (i.e. not born in NAU ZebTEC) or removed from NAU ZebTEC must be isolated in quarantine zones to reduce the risk of contaminating the existing stocks with infectious diseases.

☐Quarantine zones include a designated incubator located in [redacted] and a small bench top space for static aquaria.

☐Secondary containment, such as Tupperware trays to prevent spillage from tanks or petri dishes, must also be used in quarantine zones.

☐Quarantine incubator and zones must be disinfected with bleach between uses.

☐Only pre-authorized people can access quarantine zones.

☐Jewelry must be removed and sleeves rolled up prior to working in quarantine zones. *One exception to this rule are wedding bands which may be worn in the quarantine zones only after they gone through vigorous hand washing.*

☐Always wash hands (and arms) carefully in the local sink using antiseptic soap before and after working in the quarantine room.

☐All incoming fish are examined and, if necessary, treated for curable diseases.

☐All new fish are to be checked daily for two weeks for signs of illness. Remove any potentially sick fish.

☐Dead fish are collected in a plastic bag and disposed of according to BSA procedures.

☐No live adult fish are to be moved from the quarantine zones unless they leave the building entirely.

☐Embryos obtained in the quarantine room (after the initial 2 week observation period) must be treated in bleach for surface disinfection before leaving the room ***(See Section IV b. "Bleaching embryos")***

☐No equipment can leave the quarantine zone without being disinfected (bleached or autoclaved).

☐No equipment is to be moved in or out of the quarantine zone without prior approval.

☐When working in the quarantine area, assume that everything (including yourself) is contaminated. Think about what you are doing!

b. Bleaching embryos: *Adapted from -* (Snider and Clegg, 1975)

☐Prepare three beakers of bleach solution, containing 0.1 ml of 5% sodium hypochlorite (bleach) in 170 ml of system water ***(See Section VI c. "System water")***

☐Mix thoroughly.

☐Place the embryos in the first beaker, and allow them to stand for 5 minutes.

☐Pour off the bleach solution, and rinse the embryos with system water.

☐Allow the embryos to stand in system water for 5 minutes.

☐Place the embryos in the second beaker of bleach solution for 5 minutes.

☐Pour off the bleach solution, and rinse the embryos with system water.

☐Allow the embryos to stand in system water for 5 minutes.

☐Place the embryos in the third beaker of bleach solution for 5 minutes.

☐Pour off the bleach solution, and rinse the embryos with copious amounts of system water.

☐Transfer embryos to sterile or disposable petri dishes. Embryos can now be transferred safely from the quarantine zone to [redacted].

c. Feeding fish and larvae

i. Refer to feeding schedule to determine appropriate food type ***(See Section IV c. "Feeding fish and larvae" & Section IX a. "Feeding schedule")***.

☐Rotifers
☐Brine shrimp
☐Dry food

ii. In NAU ZebTEC

☐Turn water flow OFF by pressing "PAUSE TO FEED" button.

☐Add enough food (rotifers, brine shrimp or dry feed) so that all fish are able to feed, but not more than the animals can consume in a 10-15 minute window.

☐Turn water flow ON by pressing "RESUME" buttom.

iii. In static tanks - ***animals in static tanks get live food only***

☐Add enough food so that all fish are able to feed, but not more than the animals can consume in a 10-15 minute window (N.B. in static systems *less* is often better, to prevent water fouling between water replacement)

☐Siphon out uneaten food.

d. Breeding fish

☐On the afternoon prior to the desired breeding morning, identify individuals be breed.

☐Note sex and genotype for each individual that will be used ***(Critically important so you know where to put them back!)***

☐Using clean nets (per parent tank), move an equal number of male and female adults to a breeding tank containing breeding insert with perforated bottom, lid, divider, plastic foliage, and fresh system water.

☐Separate males from females with divider.

☐Leave animals on bench overnight.

☐At "dawn" (time when lights begin coming on) pull dividers. Within minutes fish should start displaying mating behaviors, including chasing and hovering near foliage

☐Check bottom of breeding tank for presence of eggs/embryos.

☐If no eggs/embryos are present leave tank alone (come back later).

☐If eggs/embryos are present fill a second breeding tank (without insert) with system water.

☐Transfer insert (and that is contained therein – fish, foliage) from original breeding tank to fresh tank, and check again later for more eggs/embryos.

☐At the sink take tank with newly laid eggs/embryos and poor through tea strainer.

☐Rinse eggs/embryos with system water. Eggs/embryos should remain in strainer; debris should pass through.

☐Invert strainer with eggs/embryos over clean and labeled petri dish and squirt system water over the back of the strainer to dislodge eggs/embryos so that they fall into the petri dish.

☐Move petri dish to 28.5C environment (fish room or incubator).

☐Record which tanks/individuals breed, approximate number of progeny, and approximate time eggs were laid (this is useful information for planning future breeding).

☐Having given fish several hours to breed move fish back to their original holding tanks using clean nets.

☐Feed small amount of brine or dry food (note these fish will have missed morning feeding).

☐In the afternoon (~6hpf) examine plates with embryos and aspirate out any unfertilized, developmental defective or otherwise non-viable eggs/embryos from dish.

☐If collected embryos are to be raised as new broodstock, replace system water with 0.3X Danieau's solution ***(See Section VI a. "Danieau's solution")*** supplemented with methylene blue ***(See Section VI. d. "Methylene blue").***

e. Euthanasia – Ice water immersion (Leary et al., 2013)
The ethical culling zebrafish embryos, larvae, juveniles or adults are carried out in a manner that minimizes stress and pain for the animal subject.

i. **Adults and large juveniles – euthanasia**

☐Identify individuals to be euthanized

☐Move individuals to breeding tank with perforated insert
- not to exceed 10 fish/liter for full sized adults 1-2.5" OAL
- not to exceed 20 fish/liter for juveniles <1 OAL
- individuals must not be able to pass through perforations in breeding insert.

☐In sink or near floor drain, fill rectangular Tupperware with ice
- Tupperware is marked "EUTHANASIA BIN".

☐Add system water to top of ice
- Allow water temperature to equilibrate with ice >5 min

☐Rapidly plunge perforated inserts with lids and fish into ice water.
- Fish will immediately begin to seize and become non-responsive in seconds.
- The purpose of the perforated insert it to prevent the direct contact of ice to the fish because it may be painful to the fish.

☐Leave fish on ice for >10 minutes
- Confirm operculum (gill covers) are not moving.

☐Dispose of unexposed carcasses by wrapping in paper towel and placing in sealed plastic bag.

- Fish exposed to chemical and/or biological agents must be disposed of in accordance with EHS procedures ***(See Section IV h. "Alkaline hydrolysis")***

ii. **Larvae and juveniles (>3dpf)**

☐Identify individuals to be euthanized

☐Move individuals to breeding tank with fine mesh liner/bag

- Gallon-sized paint strainer works well
- Animals must not be able to pass through mesh.

☐In sink or near floor drain, fill rectangular Tupperware with ice.

- Tupperware is marked "EUTHANASIA BIN".

☐Add system water to top of ice

- Allow water temperature to equilibrate with ice >5 min

☐Rapidly plunge perforated inserts with lids and fish into ice water.

- Fish will immediately begin to seize and become non-responsive in seconds.
- The purpose of the perforated insert it to prevent the direct contact of ice to the fish because it may be painful to the fish.

☐Leave fish on ice for >10 minutes

☐Dispose of unexposed carcasses by wrapping in paper towel and placing in sealed plastic bag, frozen for >12hrs and disposed in the Biological Sciences Animal Annex.

- Fish exposed to chemical and/or biological agents must be disposed of in accordance with EHS procedures ***(See Section IV h. "Alkaline hydrolysis")***

iii. **Embryos and larvae <7dpf**

☐Embryos and larvae a week or less old can moved to petri dishes containing Danieau's solution or system water.

- 100-200 embryos per 10cm plate

☐Plates are place directly into 4°C refrigerator overnight.

☐Dispose of unexposed carcasses by flushing down sink or floor drain with several gallons of water.

- Fish exposed to chemical and/or biological agents must be disposed of in accordance with EHS procedures ***(See Section IV h. "Alkaline hydrolysis")***

f. Anesthetization with tricaine (MS-222)

Often it is necessary to anesthetize zebrafish larvae, juveniles or adults for a number of procedures including fin clipping, microinjection, IP injection, *in vitro* fertilization and terminal sampling. ***(See Section VI b. "Tricaine solutions")***

☐Prepare 1X working strength tricaine [0.016%w/v] in clean beaker or falcon tube

- Volume of tricaine will depend on size and number of animals set for anesthesia (e.g. adult fish can be anesthetized one by one in a volume of 50-100mls, or in groups by increasing the overall volume at approximately 1 fish for every 50mls.

☐Anesthetize by dropping animals into container containing 1X tricaine solution.

- **Do not put fish from different tanks into the same tricaine bath.** For example, if animals are to be fin clipped from two different system tanks, then two separate tricaine baths must be prepared - one for each tank.

☐Observe animals for decreased swimming and slowing of the operculum

- Approximately 1-2 minutes for adult fish.

☐Keep animals moist with 1x tricaine solution using a tricaine soaked paper towel during procedure (e.g. biopsy, injection, etc...)

☐Recover anesthetized animals by moving to fresh system water (≥7dpf) or Danieau's buffer (≤6dpf).

☐Discard tricaine solution after 1-2hours of use.

g. Terminal sampling

Many experiments seek to investigate and ultimately understand the consequences of a perturbation on molecular, physiological, biochemical processes in zebrafish cells, embryos, larvae, juveniles or adults. In pursuit of this understanding many experimental endpoints involve euthanizing control and experimental groups and harvesting tissues, biofluids, or cells so that they can be processed for extraction and isolation of organic and inorganic compounds, proteins, lipids, and nucleic acids for laboratory analysis.

***i.* Adults and large juveniles – euthanasia and sampling**

☐Identify experimental animals to be sampled.

☐Can the animals be euthanized prior to sampling using standard ice immersion?

- Yes – go to "Ice Immersion"
- No – continue in this section.
- ***(See Section IV f. "Anesthetization with tricaine")***

☐Prepare tricaine solution, 200mL - 1L in system water at 25-28°C.
- Volume will depend on number of individuals to be anesthetized.
- ***(See Section VI b. "Tricaine solutions")***

☐Capture individuals with net and drop into tricaine solution

☐Wait until individuals stop swimming and opercula have slowed or ceased.

☐Remove individual from tricaine solution and place on paper towel moistened with tricaine solution.

☐Dispatch individual with cervical, brain or heart transection.
- Method for dispatching may change depending on the desired material to harvest.
- Example (1) if the spinal cord is to be harvested heart transection would be appropriate.
- Example (2) if whole brain is to be harvested a heart transection would be appropriate.

☐Isolate and collect desired material.

☐Dispose of unexposed carcasses by wrapping in paper towel and placing in sealed plastic bag for disposal according to BSA policy.
- Fish exposed to chemical and/or biological agents must be disposed of in accordance with EHS procedures ***(See Section IV h. "Alkaline hydrolysis")***

ii. **Larvae and embryos (<7dpf) euthanasia and sampling**

☐Identify experimental animals to be sampled.

☐Can the animals be euthanized prior to sampling using ice immersion?
- Yes – go to "Ice Immersion"
- No – continue in this section.

☐Prepare 50ml of 2X concentration working strength tricaine solution, in system water or Danieau's at 25-28°C. ***(See Section VI b. "Tricaine solutions")***

☐Add equal volume of 2X tricaine to culturing plate/well holding larvae
- Example: zebrafish larvae are contained in 6 well plate with 5ml Danieau's per well, therefor add 5ml of 2x tricaine to each well to reach 1X working strength.

☐Wait ~5min until larvae do not move when the dish is tapped.

☐Collect desired material.

- Perform dissections on a clean inverted plastic petri dish lid with a drop of tricaine solution sufficient to cover the larva.
- For sampling whole embryos simply move individuals directly to collection tube.

☐Dispose of unexposed remains by flushing down sink drain with > 1 gallon tap water.

- Fish exposed to chemical and/or biological agents must be disposed of in accordance with EHS procedures ***(See Section IV h. "Alkaline hydrolysis")***

h. Alkaline hydrolysis

Provides a method for dissolving animal tissue in a concentrated solution of sodium hydroxide (NaOH, also known as caustic soda), that destroys biological contaminants and produces liquid waste that can be discarded following EHS hazardous waste disposal protocols.

☐Prepare 1M Na0H in glass beaker with stir bar

- 10ml for ~1ml carcass volume or 1g carcass weight

☐Add tissue/carcasses to NaOH solution

- stir until dissolved

☐Pour liquid into compatible chemical waste collection container

- follow standard EH&S protocol for hazardous waste pickup and disposal.

V. General Experimental approaches

a. Exposure studies

Exposure studies are an important method for determining the effects a chemical may have on molecular, physiological, and biochemical processes in zebrafish cells, embryos, larvae, juveniles or adults.

There are several considerations that must be addressed prior to conducting an exposure study, such as: dosage, duration, method of administration, and solubility of the chemical. ***This section describes general protocols related to exposure administration***. Dosage and duration must be established empirically once an appropriate administration regime is established.

i. Preparation of chemical stocks

Chemicals are prepared as concentrated stocks 10X – 1000X using an appropriate solvent based on the properties of the chemical to be dissolved. Examples of solvents routinely used in zebrafish exposure studies are: water, dimethyl sulfoxide (DMSO), acetone, ethanol (EtOH), and mineral or corn oil. Chemical stocks in their appropriate solvent are diluted using PBS or water to the final working concentration.

ii. Water borne exposures in embryos or larvae ≤7dpf

Water borne exposures for embryos and larvae are done using Danieau's buffer supplemented with working strength chemical or solvent control. The density of animals should not exceed 1 animal per milliliter final volume. For example, a group of 5 embryos would be exposed in a minimum volume of 5 milliliters. Exposures are carried out using glass or polycarbonate plastic plates or vials depending on the type of chemical and necessary treatment volume. Common containers used are 6 to 48well culture plates, and 20 milliliter glass sample vials with loose fitting caps (for gas exchange). Some compounds especially those that are lipophilic may preferentially bind to plastic and exposures for that class of chemicals should be carried out in glass.

iii. Microinjection in embryos or larvae <3dpf

Direct delivery of chemical to embryos or larvae can be carried out by microinjection. Advantages of microinjection over waterborne exposure include direct and acute exposure to a precisely measured amount of chemical, reduction in the amount of chemical necessary to carry out the exposure, reduced chemical waste, and the ability to target the region of the embryo where exposure is desired (i.e. circulation, yolk). A disadvantage of microinjection mediated exposures are that sample sizes tend to be reduced because injections (micro or IP) are more specialized procedures that take skilled hands and more time to carryout then simple waterborne exposures.

iv. Water borne exposures in juvenile or adults

The ease and relatively low stress (for the animals) of waterborne exposures in juveniles or adult make this method attractive, however several limitations are present. For example, in a waterborne exposure it is extremely difficult to know how much compound is actually entering the animal. Along those lines uncertainty regarding the persistence of the compound in the aqueous environment presents another challenge for interpreting outcomes.

v. Intraperitoneal injection in adults

Direct delivery of chemical to juvenile or adult zebrafish can be carried out by intraperitoneal injection. IP injections utilize fine metal hypodermic needles 28 – 32gauge, and glass Hamilton syringes typically in the 1-10microlitter range. Advantages of IP injection over waterborne exposure mirror those described for microinjection in embryos or larvae. Disadvantages for IP injection, can include stress to the animals because they will have to be anesthetized and the inherent risk of puncturing the cavity of the fish. [redacted] is experienced in this procedure and so far, has not lost any fish due to apparent injection trauma. Typically, IP injection mediated exposures encompass a 12 – 72hour exposure duration followed by terminal sampling. Importantly the fish are only ever injected once. IP injected animals are maintained in glass aquaria following normal static tank procedures that include feeding and manual water changes.

vi. Exposure through feeding.

Exposure to chemicals through natural feeding is a newer approach that necessitates more studies to know if it is a valid and/or useful method for understanding the consequences of exposure to various chemicals (Banote et al., 2013; Kulkarni et al., 2014; Zang et al., 2011). In essence it requires the compound to be dissolved in solvent combined with powdered dried feed to make a homogenous "paste". The paste is then dried and pelleted to the appropriate size for the fish that will be fed. This method does not allow the investigator to know how much chemical is entering the animal, however, it may be an appropriate method to recapitulate ecological scenarios in which fish are exposed to chemicals presumably through their food supply. Additionally, chemicals that are consumed and pass through digestive mechanisms may provide new insights on the chemical disposition *in vivo*, that may have been missed by waterborne or IP injection into non-digestive spaces. It's possible that weighing the excrement collected at the bottom of the housing container over the course of the exposure could be used to estimate the amount of chemical consumed. As this is a newer not thoroughly tested exposure strategy, we will work to assess the efficacy of this method in comparison to the other methods described above.

b. Gene expression analysis ***(Note Section IV e – h. are relevant to this section)***

Gene expression analysis is a fundamental method for determining the transcriptional effects (*e.g. turning genes on or off*) of a perturbation, whether it be from chemical exposure, physical or genetic manipulation. Gene expression analysis may include probing for the presence, absence or change in *mRNA* or protein levels. Two general methods are applied to gene expression experiments in [redacted] laboratory, they include direct *in situ* analysis and indirect molecular analysis. In simple terms in situ analysis is used to evaluate the temporal and spatial expression characteristics for a gene. Methods for *in situ* include fixing then staining whole, sectioned or dissected embryos with dye labelled antisense RNA (binds to a specific RNA sequence that can be customized to match nearly any expressed sequence) or a specific antibody (immune-protein that specifically and tightly attaches to another molecule, usually another protein). The result is tissue staining that represents the physical location that the gene of interest is expressed.

i. Whole mount RNA *in situ* hybridization or antibody labelling.
ii. Histological section RNA *in situ* hybridization or antibody labelling.
iii. Reverse transcriptase - PCR (RT-PCR)
iv. Protein isolation and immunoblotting

VI. Recipes

a. **Danieau's solution** (*from CSHL press -* http://cshprotocols.cshlp.org/content/2011/7/pdb.rec12467.full)(CSHL, 2011)

Reagent	Amount per 1 L (g)	Final concentration [mM]
NaCl	**101.7**	**1740**
KCl	**1.56**	**21**
$MgSO_4 \bullet 7H_2O$	**2.96**	**12**
$Ca(NO_3)_2$	**4.25**	**18**
HEPES buffer	**35.75**	**150**
Adjust pH to 7.6 with NaOH or HCl; Autoclave; store at 4°C; dilute with RO/DI water 1:100 for 0.3X working concentration.		

b. **Tricaine solutions** (CAS# 886-86-2) (*Adapted from -* Westerfield, M. (2007) THE ZEBRAFISH BOOK, 5th Edition)

- Stock solution [0.4% $^{w/v}$]
  - Dissolve 400mg tricaine powder in 90ml RO/DI water
  - Add 2.1ml 1M Tris (pH 9)
  - pH to 7.4 – 7.6 with NaOH
  - Add RO/DI water to 100ml final volume
  - Aliquot solution (1 – 5ml)
  - Store at -20°C out of light (can use amber tubes).
- Working strength [0.016%$^{w/v}$]
  - Thaw aliquots and add directly to Danieau's or System water at 40ul per 1ml media.

c. **System water**: ZebTEC aquatic housing units automatically doses RO water to generate system water appropriate for maintaining zebrafish. To obtain system water take water directly from a ZebTEC or prepare water according to the following parameters.

Parameter	**Range**
pH	7.2-7.6
Temperature	28C
Conductivity	500 µS
Alkalinity	Stable within 50-150 ppm (mg/L)
Hardness	Stable within 75-200 ppm (mg/L)

Salinity	Stable within 0.5-2.0 ppt (g/L)
Dissolved oxygen	No less than 4 ppm (mg/L)
Carbon dioxide	No more than 20 ppm (mg/L)

d. Methylene blue (CAS# 122965-43-9)

i. Stock solution: 0.1%$^{v/v}$ methylene blue in DI H_2O

ii. Working strength: 0.0002%$^{v/v}$ system water or Danieau's solution.

VII. Husbandry sheets

Excel sheets that provide breeding schedules and overall animal numbers.

These values represent post-hatched (>3dpf) larvae through adults maintained on ZebTECs

Strain/Line	Into System	Out of System	On system
AB	1400	900	500
TUB	1400	1140	260
GOLDEN	1280	980	300
MITFA	1280	980	300
PXRe2$^{(MS00\ )}$	1280	980	300
CYP20A1e2$^{(MS060)}$	1280	980	300
CYP20A1e2e3$^{(MS06\ )}$	1280	980	300
PXRe7e8$^{(MS087)}$	1280	980	300
PXRe7e8$^{(MS09\ )}$	1280	980	300
PXRe7e8$^{(MS098)}$	1280	980	300
expected new line$^{(MS\ \ )}$	1340	1080	260
expected new line$^{(MS\ \ )}$	1340	1080	260
expected new line$^{(MS\ \ )}$	1340	1080	260
expected new line$^{(MS\ \ )}$	1340	1080	260
expected new line$^{(MS\ \ )}$	900	795	105
expected new line$^{(MS\ \ )}$	900	795	105
expected new line$^{(MS\ \ )}$	900	795	105
expected new line$^{(MS\ \ )}$	900	795	105
TOTALS	**22000**	**17380**	**4620**

WILDTYPE "AB" STRAIN

Typical husbandry schedule over a 3 year period for colony maintenance

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	# progeny from spawning	Cross	Cull (**)	Take for Experiment (**)	Genotype / Background	Notes
YR	Sep-20 7		0	Z RC	200	*200*	TBD	*0*	N	0	0	Z RC	u c ase c u c of AB W LDTY E f om Z RC
	Dec-20 7	90	0	Z RC	0	*100*	50 50	*0*	N	00	0	Z RC	Sex a ma s a d cu dow o 00 f s w 50 50 sex a o
	Ma -20 8	80	0	Z RC	0	*100*	50 50	*200*	N	0	0	Z RC	Spaw (0 c oss) a 6 mo s o keep s ocks "f es "
	Ma -20 8			NAU	200	*200*	TBD	*0*	N	0	0	NAU	Spaw ed f om 0 c oss
	Ju -20 8	90		NAU	0	*200*	50 50	*0*	N	00	0	NAU	Sex a ma s a d cu dow o 00 f s w 50 50 sex a o
YR2	Sep-20 8	80		NAU	0	*100*	50 50	*200*	N	0	0	NAU	Spaw a 6 mo s o keep s ocks "f es "
	Sep-20 8	360	0	Z RC	0	*100*	50 50	*0*	N	0	00	Z RC	A ma s a e used fo expe me a d sac f ced
	Sep-20 8		2	NAU	200	*200*	TBD	*0*	N	0	0	NAU	Spaw ed f om c oss
	Sep-20 8		0	Z RC	200	*200*	TBD	*0*	N	0	0	Z RC	u c ase ew c u c of AB W LDTY E f om Z RC
	Dec-20 8	90	2	NAU	0	*100*	50 50	*0*	N	0	0	NAU	Sex a ma s a d cu dow o 00 f s w 50 50 sex a o
	Dec-20 8	90	0	Z RC	0	*100*	50 50	*0*	N	00	0	Z RC	Sex a ma s a d cu dow o 00 f s w 50 50 sex a o
	Ma -20 9	80	2	NAU	0	*100*	50 50	*200*	**Y**	0	0	NAU/Z RC	C oss NAU 2 w Z RC20 8 0
	Ma -20 9	80	0	Z RC	0	*100*	50 50	*200*	**Y**	0	0	NAU/Z RC	C oss NAU 2 w Z RC20 8 0
	Ma -20 9	360		NAU	0	*100*	50 50	*0*	N	0	00	NAU	A ma s a e used fo expe me a d sac f ced
	Ju -20 9	90	3' [2]	NAU	200	*200*	TBD	*0*	N	0	0	NAU/Z RC	Spaw (u e a ed f s) a 6 mo s o keep s ocks "f es "
YR3	Sep-20 9	80	3'	NAU	0	*00*	50 50	*0*	N	00	0	NAU/Z RC	Sex a ma s a d cu dow o 00 f s w 50 50 sex a o
	Sep-20 9	360	2	NAU	0	*100*	50 50	*200*	N	0	0	NAU/Z RC	Spaw a 6 mo s 3' c oss
	Sep-20 9	360	0	Z RC	0	*100*	50 50	*0*	N	0	00	NAU	A ma s a e used fo expe me a d sac f ced
	Sep-20 9		4	NAU	200	200	TBD	*0*	N	0	0	NAU	Spaw ed f om 3' c oss
	Ma -2020	90	4	NAU	0	*00*	50 50	*0*	N	00	0	NAU	Sex a ma s a d cu dow o 00 f s w 50 50 sex a o
	Ma -2020	360	3'	NAU	0	*00*	50 50	*0*	N	0	00	NAU/Z RC	A ma s a e used fo expe me a d sac f ced
	Ju -2020	80	4	NAU	0	*00*	50 50	*200*	N	0	0	NAU	Spaw a 6 mo s o keep s ocks "f es "
	Ju -2020		5	NAU	200	*200*	TBD	*0*	N	0	0	NAU	Spaw ed f om 4 c oss
	Colony totals				*** Into system: 1400**				**** Out of system: 900**		**On system total: 500**		

# of embryos produced for stock maintenance: 1200

Footnotes: T ese va ues ep ese e umbe of d v dua s pe a spec f c ge e a o a a spec f c da e (o a ua o a s)

2 #' d ca es a s ge e a o was ge e a ed by a c oss

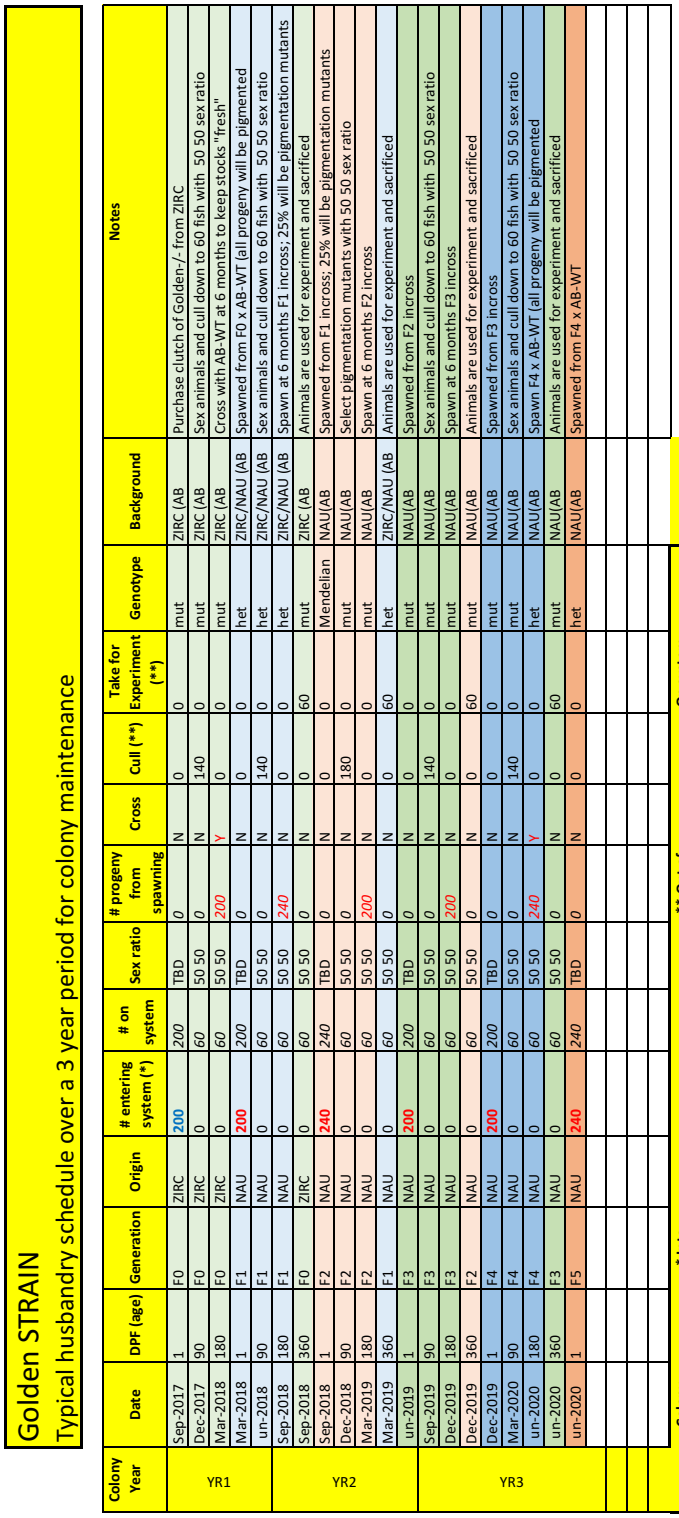
WILDTYPE "Tubingen" STRAIN

Typical husbandry schedule over a 3 year period for colony maintenance

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	# progeny from spawning	Cross	Cull (**)	Take for Experiment (**)	Genotype / Background	Notes
YR	Sep-20 7		0	Z RC	200	*200*	TBD	*0*	N	0	0	Z RC	u c ase c u c of TUB W LDTY E f om Z RC
	Dec-20 7	90	0	Z RC	0	*60*	50 50	*0*	N	40	0	Z RC	Sex a ma s a d cu dow o 60 f s w 50 50 sex a o
	Ma -20 8	80	0	Z RC	0	*60*	50 50	*200*	N	0	0	Z RC	Spaw (0 c oss) a 6 mo s o keep s ocks "f es "
	Ma -20 8			NAU	200	*200*	TBD	*0*	N	0	0	NAU	Spaw ed f om 0 c oss
	Ju -20 8	90		NAU	0	*60*	50 50	*0*	N	40	0	NAU	Sex a ma s a d cu dow o 60 f s w 50 50 sex a o
YR2	Sep-20 8	80		NAU	0	*60*	50 50	*200*	N	0	0	NAU	Spaw a 6 mo s o keep s ocks "f es "
	Sep-20 8	360	0	Z RC	0	*60*	50 50	*0*	N	0	60	Z RC	A ma s a e used fo expe me a d sac f ced
	Sep-20 8		2	NAU	200	*200*	TBD	*0*	N	0	0	NAU	Spaw ed f om c oss
	Dec-20 8	90	0	Z RC	200	*200*	TBD	*0*	N	0	0	Z RC	u c ase ew c u c of TUB W LDTY E f om Z RC
	Dec-20 8	90	2	NAU	0	*60*	50 50	*0*	N	40	0	NAU	Sex a ma s a d cu dow o 60 f s w 50 50 sex a o
	Dec-20 8	90	0	Z RC	0	*60*	50 50	*0*	N	40	0	Z RC	Sex a ma s a d cu dow o 60 f s w 50 50 sex a o
	Ma -20 9	80	2	NAU	0	*60*	50 50	*200*	Y	0	0	NAU/Z RC	C oss NAU 2 w Z RC20 8 0
	Ma -20 9	80	0	Z RC	0	*60*	50 50	*200*	Y	0	0	NAU/Z RC	C oss NAU 2 w Z RC20 8 0
	Ma -20 9	360		NAU	0	*60*	50 50	*0*	N	0	60	NAU	A ma s a e used fo expe me a d sac f ced
	Ju -20 9	90	3' [2]	NAU	200	*200*	TBD	*0*	N	0	0	NAU/Z RC	Spaw (u e a ed f s) a 6 mo s o keep s ocks "f es "
YR3	Sep-20 9	80	3'	NAU	0	*60*	50 50	*0*	N	40	0	NAU/Z RC	Sex a ma s a d cu dow o 60 f s w 50 50 sex a o
	Sep-20 9	360	2	NAU	0	*60*	50 50	*200*	N	0	0	NAU/Z RC	Spaw a 6 mo s 3' c oss
	Sep-20 9	360	0	Z RC	0	*60*	50 50	*0*	N	0	60	NAU	A ma s a e used fo expe me a d sac f ced
	Sep-20 9		4	NAU	200	200	TBD	*0*	N	0	0	NAU	Spaw ed f om 3' c oss
	Ma -2020	90	4	NAU	0	*60*	50 50	*0*	N	40	0	NAU	Sex a ma s a d cu dow o 60 f s w 50 50 sex a o
	Ma -2020	360	3'	NAU	0	*60*	50 50	*0*	N	0	60	NAU/Z RC	A ma s a e used fo expe me a d sac f ced
	Ju -2020	80	4	NAU	0	*60*	50 50	*200*	N	0	0	NAU	Spaw a 6 mo s o keep s ocks "f es "
	Ju -2020		5	NAU	200	*200*	TBD	*0*	N	0	0	NAU	Spaw ed f om 4 c oss
	Colony totals		*** Into system:**		**1400**			**** Out of system:**		**1140**	**On system total:**		**260**
			# of embryos produced:		**1200**								

Footnotes: T ese va ues ep ese e umbe of d v dua s pe a spec f c ge e a o a a spec f c da e (o a ua o a s)

2 #' d ca es a s ge e a o was ge e a ed by a c oss



Golden STRAIN

Typical husbandry schedule over a 3 year period for colony maintenance

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	# progeny from spawning	Cross	Cull (**)	Take for Experiment (**)	Genotype	Background	Notes
YR1	Sep-2017	1	F0	ZIRC	200	200	TBD	0	N	0	0	mut	ZIRC (AB	Purchase clutch of Golden-/- from ZIRC
	Dec-2017	90	F0	ZIRC	0	60	50 50	0	N	140	0	mut	ZIRC (AB	Sex animals and cull down to 60 fish with 50 50 sex ratio
	Mar-2018	180	F0	ZIRC	0	60	50 50	200	Y	0	0	mut	ZIRC (AB	Cross with AB-WT at 6 months to keep stocks "fresh"
	Mar-2018	1	F1	NAU	200	200	TBD	0	N	0	0	het	ZIRC/NAU (AB	Spawned from F0 x AB-WT (all progeny will be pigmented
	un-2018	90	F1	NAU	0	60	50 50	0	N	140	0	het	ZIRC/NAU (AB	Sex animals and cull down to 60 fish with 50 50 sex ratio
YR2	Sep-2018	180	F1	NAU	0	60	50 50	240	N	0	0	het	ZIRC/NAU (AB	Spawn at 6 months F1 incross; 25% will be pigmentation mutants
	Sep-2018	360	F0	ZIRC	0	60	50 50	0	N	0	60	mut	ZIRC (AB	Animals are used for experiment and sacrificed
	Sep-2018	1	F2	NAU	240	240	TBD	0	N	0	0	Mendelian	NAU(AB	Spawned from F1 incross; 25% will be pigmentation mutants
	Dec-2018	90	F2	NAU	0	60	50 50	0	N	180	0	mut	NAU(AB	Select pigmentation mutants with 50 50 sex ratio
	Mar-2019	180	F2	NAU	0	60	50 50	200	N	0	0	mut	NAU(AB	Spawn at 6 months F2 incross
	Mar-2019	360	F1	NAU	0	60	50 50	0	N	0	60	het	ZIRC/NAU (AB	Animals are used for experiment and sacrificed
YR3	un-2019	1	F3	NAU	200	200	TBD	0	N	0	0	mut	NAU(AB	Spawned from F2 incross
	Sep-2019	90	F3	NAU	0	60	50 50	0	N	140	0	mut	NAU(AB	Sex animals and cull down to 60 fish with 50 50 sex ratio
	Dec-2019	180	F3	NAU	0	60	50 50	200	N	0	0	mut	NAU(AB	Spawn at 6 months F3 incross
	Dec-2019	360	F2	NAU	0	60	50 50	0	N	0	60	mut	NAU(AB	Animals are used for experiment and sacrificed
	Dec-2019	1	F4	NAU	200	200	TBD	0	N	0	0	mut	NAU(AB	Spawned from F3 incross
	Mar-2020	90	F4	NAU	0	60	50 50	0	N	140	0	mut	NAU(AB	Sex animals and cull down to 60 fish with 50 50 sex ratio
	un-2020	180	F4	NAU	0	60	50 50	240	Y	0	0	het	NAU(AB	Spawn F4 x AB-WT (all progeny will be pigmented
	un-2020	360	F3	NAU	0	60	50 50	0	N	0	60	mut	NAU(AB	Animals are used for experiment and sacrificed
	un-2020	1	F5	NAU	240	240	TBD	0	N	0	0	het	NAU(AB	Spawned from F4 x AB-WT
	Colony totals				* Into system:	1280			** Out of system:	980	On system total:	300		
					# of embryos produced:	1080								

Footnotes: 1. These values represent the number of individuals per a specific gneration at a specific date (not annual totals .

MITFA STRAIN

Typical husbandry schedule over a 3 year period for colony maintenance

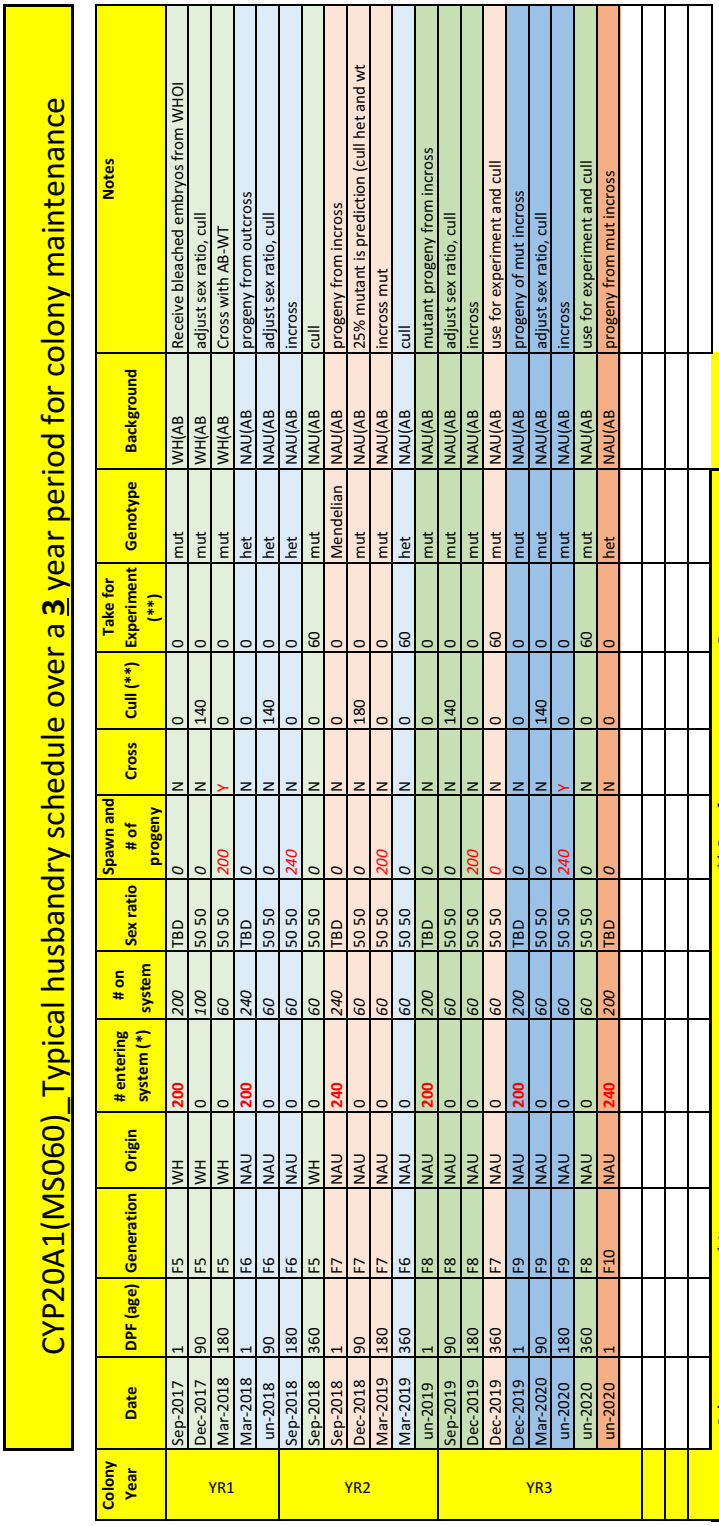
Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	# progeny from spawning	Cross	Cull (**)	Take for Experiment (**)	Genotype	Background	Notes
YR1	Sep-2017	1	F0	ZIRC	200	200	TBD	0	N	0	0	mut	ZIRC (AB	Purchase clutch of MITFA -/- from ZIRC
	Dec-2017	90	F0	ZIRC	0	60	50 50	0	N	140	0	mut	ZIRC (AB	Sex animals and cull down to 60 fish with 50 50 sex ratio
	Mar-2018	180	F0	ZIRC	0	60	50 50	200	Y	0	0	mut	ZIRC (AB	Cross with AB-WT at 6 months to keep stocks "fresh"
	Mar-2018	1	F1	NAU	200	200	TBD	0	N	0	0	het	ZIRC/NAU (AB	Spawned from F0 x AB-WT (all progeny will be pigmented
	un-2018	90	F1	NAU	0	60	50 50	0	N	140	0	het	ZIRC/NAU (AB	Sex animals and cull down to 60 fish with 50 50 sex ratio
YR2	Sep-2018	180	F1	NAU	0	60	50 50	240	N	0	0	het	ZIRC/NAU (AB	Spawn at 6 months F1 incross; 25% will be pigmentation mutants
	Sep-2018	360	F0	ZIRC	0	60	50 50	0	N	0	60	mut	ZIRC (AB	Animals are used for experiment and sacrificed
	Sep-2018	1	F2	NAU	240	240	TBD	0	N	0	0	Mendelian	NAU/AB	Spawned from F1 incross; 25% will be pigmentation mutants
	Dec-2018	90	F2	NAU	0	60	50 50	0	N	180	0	mut	NAU/AB	Select pigmentation mutants with 50 50 sex ratio
	Mar-2019	180	F2	NAU	0	60	50 50	200	N	0	0	mut	NAU/AB	Spawn at 6 months F2 incross
	Mar-2019	360	F1	NAU	0	60	50 50	0	N	0	60	het	ZIRC/NAU (AB	Animals are used for experiment and sacrificed
	un-2019	1	F3	NAU	200	200	TBD	0	N	0	0	mut	NAU/AB	Spawned from F2 incross
YR3	Sep-2019	90	F3	NAU	0	60	50 50	0	N	140	0	mut	NAU/AB	Sex animals and cull down to 60 fish with 50 50 sex ratio
	Dec-2019	180	F3	NAU	0	60	50 50	200	N	0	0	mut	NAU/AB	Spawn at 6 months F3 incross
	Dec-2019	360	F2	NAU	0	60	50 50	0	N	0	60	mut	NAU/AB	Animals are used for experiment and sacrificed
	Dec-2019	1	F4	NAU	200	200	TBD	0	N	0	0	mut	NAU/AB	Spawned from F3 incross
	Mar-2020	90	F4	NAU	0	60	50 50	0	N	140	0	mut	NAU/AB	Sex animals and cull down to 60 fish with 50 50 sex ratio
	un-2020	180	F4	NAU	0	60	50 50	240	Y	0	0	het	NAU/AB	Spawn F4 x AB-WT (all progeny will be pigmented
	un-2020	360	F3	NAU	0	60	50 50	0	N	0	60	mut	NAU/AB	Animals are used for experiment and sacrificed
	un-2020	1	F5	NAU	240	240	TBD	0	N	0	0	het	NAU/AB	Spawned from F4 x AB-WT
	Colony totals				* Into system: 1280			** Out of system: 980			On system total: 300			
					# of embryos produced: 1080									

Footnotes: 1. These values represent the number of individuals per a specific generation at a specific date (not annual totals .

PXRe2(MS017)_Typical husbandry schedule over a **3** year period for colony maintenance

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	Spawn and # of progeny	Cross	Cull (**)	Take for Experiment (**)	Genotype	Background	Notes
YR	Sep-20 7		5	WH	200	*200*	TBD	*0*	N	0	0	mu	WH(AB)	Rece ve b eac ed emb yos f om WHO
	Dec-20 7	90	5	WH	0	*100*	50 50	*0*	N	40	0	mu	WH(AB)	ad us sex a o, cu
	Ma -20 8	80	5	WH	0	*60*	50 50	*200*	Y	0	0	mu	WH(AB)	C oss w AB-WT
	Ma -20 8		6	NAU	200	*240*	TBD	*0*	N	0	0	e	NAU(AB)	p oge y f om ou c oss
	Ju -20 8	90	6	NAU	0	*60*	50 50	*0*	N	40	0	e	NAU(AB)	ad us sex a o, cu
YR2	Sep-20 8	80	6	NAU	0	*60*	50 50	*240*	N	0	0	e	NAU(AB)	c oss
	Sep-20 8	360	5	WH	0	*60*	50 50	*0*	N	0	60	mu	NAU(AB)	cu
	Sep-20 8		7	NAU	240	*240*	TBD	*0*	N	0	0	me de a	NAU(AB)	p oge y f om c oss
	Dec-20 8	90	7	NAU	0	*60*	50 50	*0*	N	80	0	mu	NAU(AB)	25% mu a sp ed c o (cu e a d w)
	Ma -20 9	80	7	NAU	0	*60*	50 50	*200*	N	0	0	mu	NAU(AB)	c oss mu
	Ma -20 9	360	6	NAU	0	*60*	50 50	*0*	N	0	60	e	NAU(AB)	cu
	Ju -20 9		8	NAU	200	*200*	TBD	*0*	N	0	0	mu	NAU(AB)	mu a p oge y f om c oss
YR3	Sep-20 9	90	8	NAU	0	*60*	50 50	*0*	N	40	0	mu	NAU(AB)	ad us sex a o, cu
	Dec-20 9	80	8	NAU	0	*60*	50 50	*200*	N	0	0	mu	NAU(AB)	c oss
	Dec-20 9	360	7	NAU	0	*60*	50 50	*0*	N	0	60	mu	NAU(AB)	use fo expe me a d cu
	Dec-20 9		9	NAU	200	*200*	TBD	*0*	N	0	0	mu	NAU(AB)	p oge y of mu c oss
	Ma -2020	90	9	NAU	0	*60*	50 50	*0*	N	40	0	mu	NAU(AB)	ad us sex a o, cu
	Ju -2020	80	9	NAU	0	*60*	50 50	*240*	Y	0	0	mu	NAU(AB)	c oss
	Ju -2020	360	8	NAU	0	*60*	50 50	*0*	N	0	60	mu	NAU(AB)	use fo expe me a d cu
	Ju -2020		0	NAU	240	*200*	TBD	*0*	N	0	0	e	NAU(AB)	p oge y f om mu c oss
	Colony totals				* Into system: **1280**			** Out of system: **980**			On system total: **300**			
					# of embryos produced: **1080**									

Footnotes: T ese va ues ep ese e umbe of d v dua s pe a spec f c ge e a o a a spec f c da e (o a ua o a s)



CYP20A1(MS060)_Typical husbandry schedule over a **3** year period for colony maintenance

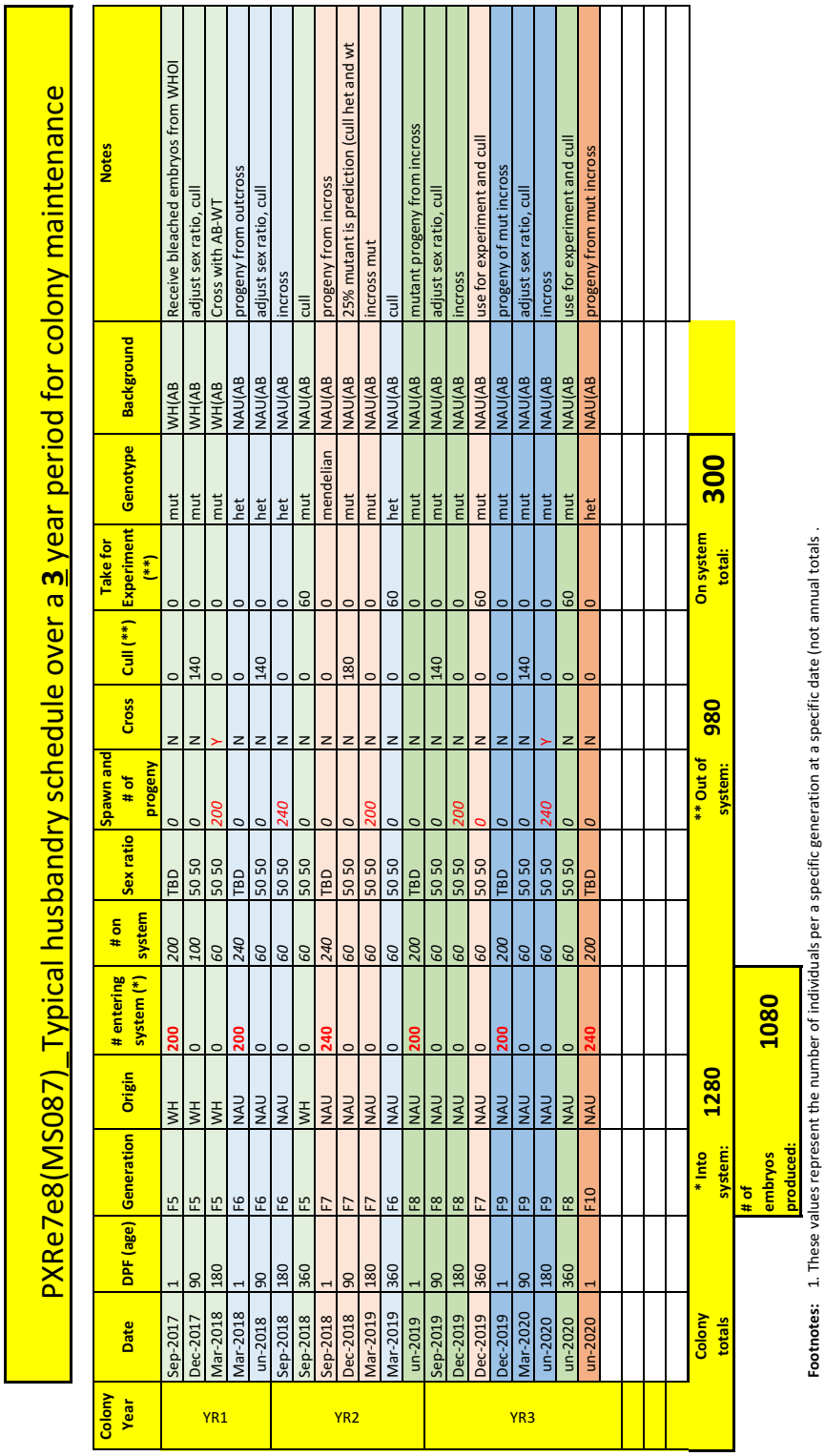
Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	Spawn and # of progeny	Cross	Cull (**)	Take for Experiment (**)	Genotype	Background	Notes
YR1	Sep-2017	1	F5	WH	200	*200*	TBD	*0*	N	0	0	mut	WH(AB	Receive bleached embryos from WHOI
	Dec-2017	90	F5	WH	0	*100*	50 50	*0*	N	140	0	mut	WH(AB	adjust sex ratio, cull
	Mar-2018	180	F5	WH	0	*60*	50 50	*200*	Y	0	0	mut	WH(AB	Cross with AB-WT
	Mar-2018	1	F6	NAU	200	*240*	TBD	*0*	N	0	0	het	NAU(AB	progeny from outcross
	un-2018	90	F6	NAU	0	*60*	50 50	*0*	N	140	0	het	NAU(AB	adjust sex ratio, cull
	Sep-2018	180	F6	NAU	0	*60*	50 50	*240*	N	0	0	het	NAU(AB	incross
	Sep-2018	360	F5	WH	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	cull
YR2	Sep-2018	1	F7	NAU	240	*240*	TBD	*0*	N	0	0	Mendelian	NAU(AB	progeny from incross
	Dec-2018	90	F7	NAU	0	*60*	50 50	*0*	N	180	0	mut	NAU(AB	25% mutant is prediction (cull het and wt
	Mar-2019	180	F7	NAU	0	*60*	50 50	*200*	N	0	0	mut	NAU(AB	incross mut
	Mar-2019	360	F6	NAU	0	*60*	50 50	*0*	N	0	60	het	NAU(AB	cull
	un-2019	1	F8	NAU	200	*200*	TBD	*0*	N	0	0	mut	NAU(AB	mutant progeny from incross
YR3	Sep-2019	90	F8	NAU	0	*60*	50 50	*0*	N	140	0	mut	NAU(AB	adjust sex ratio, cull
	Dec-2019	180	F8	NAU	0	*60*	50 50	*200*	N	0	0	mut	NAU(AB	incross
	Dec-2019	360	F7	NAU	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	use for experiment and cull
	Dec-2019	1	F9	NAU	200	*200*	TBD	*0*	N	0	0	mut	NAU(AB	progeny of mut incross
	Mar-2020	90	F9	NAU	0	*60*	50 50	*0*	N	140	0	mut	NAU(AB	adjust sex ratio, cull
	un-2020	180	F9	NAU	0	*60*	50 50	*240*	Y	0	0	mut	NAU(AB	incross
	un-2020	360	F8	NAU	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	use for experiment and cull
	un-2020	1	F10	NAU	240	*200*	TBD	*0*	N	0	0	het	NAU(AB	progeny from mut incross
	Colony totals				*** Into system: 1280**				**** Out of system: 980**		**On system total: 300**			
					# of embryos produced: 1080									

Footnotes: 1. These values represent the number of individuals per a specific generation at a specific date (not annual totals .

CYP20A1(MS061)_Typical husbandry schedule over a **3** year period for colony maintenance

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	Spawn and # of progeny	Cross	Cull (**)	Take for Experiment (**)	Genotype	Background	Notes
YR1	Sep-2017	1	F5	WH	200	*200*	TBD	*0*	N	0	0	mut	WH(AB	Receive bleached embryos from WHOI
	Dec-2017	90	F5	WH	0	*100*	50 50	*0*	N	140	0	mut	WH(AB	adjust sex ratio, cull
	Mar-2018	180	F5	WH	0	*60*	50 50	*200*	Y	0	0	mut	WH(AB	Cross with AB-WT
	Mar-2018	1	F6	NAU	200	*240*	TBD	*0*	N	0	0	het	NAU(AB	progeny from outcross
	un-2018	90	F6	NAU	0	*60*	50 50	*0*	N	140	0	het	NAU(AB	adjust sex ratio, cull
	Sep-2018	180	F6	NAU	0	*60*	50 50	*240*	N	0	0	het	NAU(AB	incross
	Sep-2018	360	F5	WH	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	cull
YR2	Sep-2018	1	F7	NAU	240	*240*	TBD	*0*	N	0	0	mendelian	NAU(AB	progeny from incross
	Dec-2018	90	F7	NAU	0	*60*	50 50	*0*	N	180	0	mut	NAU(AB	25% mutant is prediction (cull het and wt
	Mar-2019	180	F7	NAU	0	*60*	50 50	*200*	N	0	0	mut	NAU(AB	incross mut
	Mar-2019	360	F6	NAU	0	*60*	50 50	*0*	N	0	60	het	NAU(AB	cull
	un-2019	1	F8	NAU	200	*200*	TBD	*0*	N	0	0	mut	NAU(AB	mutant progeny from incross
	Sep-2019	90	F8	NAU	0	*60*	50 50	*0*	N	140	0	mut	NAU(AB	adjust sex ratio, cull
	Dec-2019	180	F8	NAU	0	*60*	50 50	*200*	N	0	0	mut	NAU(AB	incross
	Dec-2019	360	F7	NAU	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	use for experiment and cull
YR3	Dec-2019	1	F9	NAU	200	*200*	TBD	*0*	N	0	0	mut	NAU(AB	progeny of mut incross
	Mar-2020	90	F9	NAU	0	*60*	50 50	*0*	N	140	0	mut	NAU(AB	adjust sex ratio, cull
	un-2020	180	F9	NAU	0	*60*	50 50	*240*	Y	0	0	mut	NAU(AB	incross
	un-2020	360	F8	NAU	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	use for experiment and cull
	un-2020	1	F10	NAU	240	*200*	TBD	*0*	N	0	0	het	NAU(AB	progeny from mut incross
	Colony totals		* Into system:		1280			** Out of system:		980		On system total:		300
			# of embryos produced:		1080									

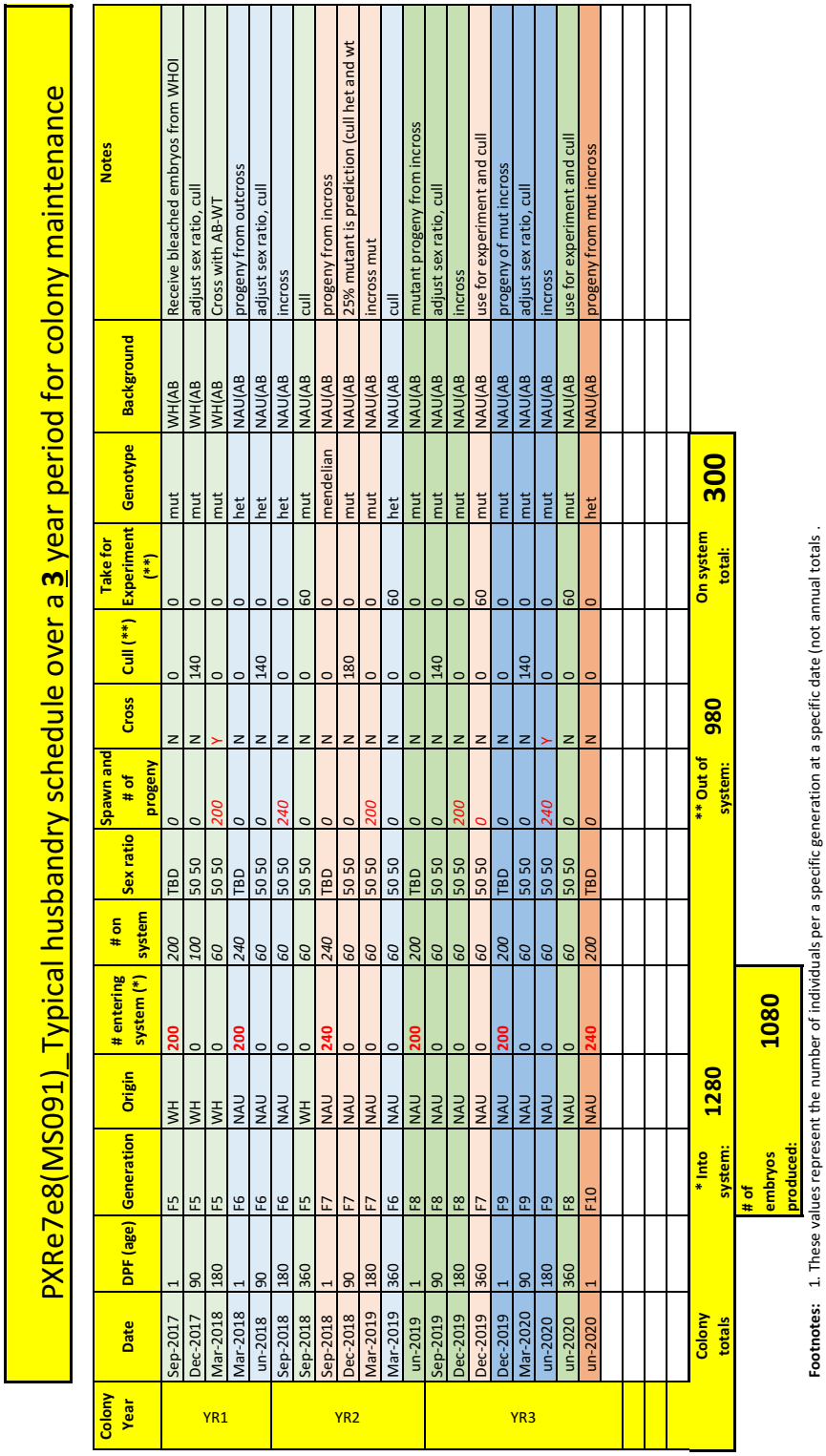
Footnotes: 1. These values represent the number of individuals per a specific generation at a specific date (not annual totals .



PXRe7e8(MS087)_Typical husbandry schedule over a **3** year period for colony maintenance

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	Spawn and # of progeny	Cross	Cull (**)	Take for Experiment (**)	Genotype	Background	Notes
YR1	Sep-2017	1	F5	WH	200	*200*	TBD	*0*	N	0	0	mut	WH(AB	Receive bleached embryos from WHOI
	Dec-2017	90	F5	WH	0	*100*	50 50	*0*	N	140	0	mut	WH(AB	adjust sex ratio, cull
	Mar-2018	180	F5	WH	0	*60*	50 50	*200*	Y	0	0	mut	WH(AB	Cross with AB-WT
	Mar-2018	1	F6	NAU	200	*240*	TBD	*0*	N	0	0	het	NAU(AB	progeny from outcross
	un-2018	90	F6	NAU	0	*60*	50 50	*0*	N	140	0	het	NAU(AB	adjust sex ratio, cull
YR2	Sep-2018	180	F6	NAU	0	*60*	50 50	*240*	N	0	0	het	NAU(AB	incross
	Sep-2018	360	F5	WH	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	cull
	Sep-2018	1	F7	NAU	240	*240*	TBD	*0*	N	0	0	mendelian	NAU(AB	progeny from incross
	Dec-2018	90	F7	NAU	0	*60*	50 50	*0*	N	180	0	mut	NAU(AB	25% mutant is prediction (cull het and wt
	Mar-2019	180	F7	NAU	0	*60*	50 50	*200*	N	0	0	mut	NAU(AB	incross mut
	Mar-2019	360	F6	NAU	0	*60*	50 50	*0*	N	0	60	het	NAU(AB	cull
	un-2019	1	F8	NAU	200	*200*	TBD	*0*	N	0	0	mut	NAU(AB	mutant progeny from incross
	Sep-2019	90	F8	NAU	0	*60*	50 50	*0*	N	140	0	mut	NAU(AB	adjust sex ratio, cull
	Dec-2019	180	F8	NAU	0	*60*	50 50	*200*	N	0	0	mut	NAU(AB	incross
	Dec-2019	360	F7	NAU	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	use for experiment and cull
YR3	Dec-2019	1	F9	NAU	200	*200*	TBD	*0*	N	0	0	mut	NAU(AB	progeny of mut incross
	Mar-2020	90	F9	NAU	0	*60*	50 50	*0*	N	140	0	mut	NAU(AB	adjust sex ratio, cull
	un-2020	180	F9	NAU	0	*60*	50 50	*240*	Y	0	0	mut	NAU(AB	incross
	un-2020	360	F8	NAU	0	*60*	50 50	*0*	N	0	60	mut	NAU(AB	use for experiment and cull
	un-2020	1	F10	NAU	240	*200*	TBD	*0*	N	0	0	het	NAU(AB	progeny from mut incross
	Colony totals		* Into system:		1280			** Out of system:		980	On system total:		300	
			# of embryos produced:		1080									

Footnotes: 1. These values represent the number of individuals per a specific generation at a specific date (not annual totals .



PXRe7e8(MS091)_Typical husbandry schedule over a **3** year period for colony maintenance

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	Spawn and # of progeny	Cross	Cull (**)	Take for Experiment (**)	Genotype	Background	Notes
YR1	Sep-2017	1	F5	WH	200	200	TBD	0	N	0	0	mut	WH(AB	Receive bleached embryos from WHOI
	Dec-2017	90	F5	WH	0	100	50 50	0	N	140	0	mut	WH(AB	adjust sex ratio, cull
	Mar-2018	180	F5	WH	0	60	50 50	200	Y	0	0	mut	WH(AB	Cross with AB-WT
	Mar-2018	1	F6	NAU	200	240	TBD	0	N	0	0	het	NAU(AB	progeny from outcross
	un-2018	90	F6	NAU	0	60	50 50	0	N	140	0	het	NAU(AB	adjust sex ratio, cull
YR2	Sep-2018	180	F6	NAU	0	60	50 50	240	N	0	0	het	NAU(AB	incross
	Sep-2018	360	F5	WH	0	60	50 50	0	N	0	60	mut	NAU(AB	cull
	Sep-2018	1	F7	NAU	240	240	TBD	0	N	0	0	mendelian	NAU(AB	progeny from incross
	Dec-2018	90	F7	NAU	0	60	50 50	0	N	180	0	mut	NAU(AB	25% mutant is prediction (cull het and wt
	Mar-2019	180	F7	NAU	0	60	50 50	200	N	0	0	mut	NAU(AB	incross mut
	Mar-2019	360	F6	NAU	0	60	50 50	0	N	0	60	het	NAU(AB	cull
	un-2019	1	F8	NAU	200	200	TBD	0	N	0	0	mut	NAU(AB	mutant progeny from incross
	Sep-2019	90	F8	NAU	0	60	50 50	0	N	140	0	mut	NAU(AB	adjust sex ratio, cull
	Dec-2019	180	F8	NAU	0	60	50 50	200	N	0	0	mut	NAU(AB	incross
	Dec-2019	360	F7	NAU	0	60	50 50	0	N	0	60	mut	NAU(AB	use for experiment and cull
YR3	Dec-2019	1	F9	NAU	200	200	TBD	0	N	0	0	mut	NAU(AB	progeny of mut incross
	Mar-2020	90	F9	NAU	0	60	50 50	0	N	140	0	mut	NAU(AB	adjust sex ratio, cull
	un-2020	180	F9	NAU	0	60	50 50	240	Y	0	0	mut	NAU(AB	incross
	un-2020	360	F8	NAU	0	60	50 50	0	N	0	60	mut	NAU(AB	use for experiment and cull
	un-2020	1	F10	NAU	240	200	TBD	0	N	0	0	het	NAU(AB	progeny from mut incross
	Colony totals		* Into system:		1280			** Out of system:		980		On system total:		300
			# of embryos produced:		1080									

Footnotes: 1. These values represent the number of individuals per a specific generation at a specific date (not annual totals .

PXRe7e8(MS097)_Typical husbandry schedule over a **3** year period for colony maintenance

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	Spawn and # of progeny	Cross	Cull (**)	Take for Experiment (**)	Genotype	Background	Notes
YR	Sep-20 7		5	WH	200	*200*	TBD	*0*	N	0	0	mu	WH(AB)	Rece ve b eac ed emb yos f om WHO
	Dec-20 7	90	5	WH	0	*100*	50 50	*0*	N	40	0	mu	WH(AB)	ad us sex a o, cu
	Ma -20 8	80	5	WH	0	*60*	50 50	*200*	Y	0	0	mu	WH(AB)	C oss w AB-WT
	Ma -20 8		6	NAU	200	*240*	TBD	*0*	N	0	0	e	NAU(AB)	p oge y f om ou c oss
	Ju -20 8	90	6	NAU	0	*60*	50 50	*0*	N	40	0	e	NAU(AB)	ad us sex a o, cu
YR2	Sep-20 8	80	6	NAU	0	*60*	50 50	*240*	N	0	0	e	NAU(AB)	c oss
	Sep-20 8	360	5	WH	0	*60*	50 50	*0*	N	0	60	mu	NAU(AB)	cu
	Sep-20 8		7	NAU	240	*240*	TBD	*0*	N	0	0	me de a	NAU(AB)	p oge y f om c oss
	Dec-20 8	90	7	NAU	0	*60*	50 50	*0*	N	80	0	mu	NAU(AB)	25% mu a sp ed c o (cu e a d w)
	Ma -20 9	80	7	NAU	0	*60*	50 50	*200*	N	0	0	mu	NAU(AB)	c oss mu
	Ma -20 9	360	6	NAU	0	*60*	50 50	*0*	N	0	60	e	NAU(AB)	cu
	Ju -20 9		8	NAU	200	*200*	TBD	*0*	N	0	0	mu	NAU(AB)	mu a p oge y f om c oss
YR3	Sep-20 9	90	8	NAU	0	*60*	50 50	*0*	N	40	0	mu	NAU(AB)	ad us sex a o, cu
	Dec-20 9	80	8	NAU	0	*60*	50 50	*200*	N	0	0	mu	NAU(AB)	c oss
	Dec-20 9	360	7	NAU	0	*60*	50 50	*0*	N	0	60	mu	NAU(AB)	use fo expe me a d cu
	Dec-20 9		9	NAU	200	*200*	TBD	*0*	N	0	0	mu	NAU(AB)	p oge y of mu c oss
	Ma -2020	90	9	NAU	0	*60*	50 50	*0*	N	40	0	mu	NAU(AB)	ad us sex a o, cu
	Ju -2020	80	9	NAU	0	*60*	50 50	*240*	Y	0	0	mu	NAU(AB)	c oss
	Ju -2020	360	8	NAU	0	*60*	50 50	*0*	N	0	60	mu	NAU(AB)	use fo expe me a d cu
	Ju -2020		0	NAU	240	*200*	TBD	*0*	N	0	0	e	NAU(AB)	p oge y f om mu c oss
	Colony totals		# of embryos produced:		* Into system: 1280			** Out of system: 980			On system total: 300			

Footnotes: T ese va ues ep ese e umbe of d v dua s pe a spec f c ge e a o a a spec f c da e (o a ua o a s)

New line for Year 2 - Typical husbandry schedule over a **3** year period for colony maintenance for a single line

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system	Sex ratio	# progeny from spawning	Cross	Cull or failure to thrive (**)	Take for Experiment (**)	Genotype	Background	Notes
YR1														
YR2	Sep-2017	1		NAU	400	400	TBD	0	N	0	0	mosiac	AB(NAU	CRISPR-HDR mutagenesis with selcetion marker (cry gfp
	Dec-2017	90		NAU	0	80	TBD	0	N	320	0	mosiac / cry gfp	AB(NAU	Select cry gfp+ males and females. Estimated < 20% penetrance
	Mar-2018	180		NAU	0	80	TBD	500	Y	0	0	mosiac / cry gfp	AB(NAU	Select cry gfp+ larvae Estimated 1-10% penetrance (showing 5% ; outX to WT
	Mar-2018	1		NAU	500	25	TBD	0	N	475	0	het / cry gfp	AB(NAU	Select cry gfp+ larvae Estimated 1-10% penetrance (showing 5%
	un-2018	90		NAU	0	25	TBD	0	N	0	0	het / cry gfp	AB(NAU	Confirm mutant sequence
	Sep-2018	180		NAU	0	25	TBD	240	N	0	0	het / cry gfp	AB(NAU	Incross F1 to produce 25% mut / cry gfp
	Sep-2018	360		NAU	0	80	TBD	0	N	80	0	mosiac / cry gfp	AB(NAU	Cull founders
YR3	Sep-2018	1		NAU	240	240	TBD	0	N	60	0	cry gfp	AB(NAU	Cull cry gfp negative ~25%
	Dec-2018	90		NAU	0	180	TBD	0	N	120	0	mixed / cry gfp	AB(NAU	PCR to confirm mut or het; predict 25% mut; cull het
	Mar-2019	180		NAU	0	60	TBD	200	N	0	0	mut / cry gfp	AB(NAU	Pure population of mut
	Mar-2019	360		NAU	0	25	TBD	0	N	25	0	het / cry gfp	AB(NAU	cull F1 hets
	un-2019	1		NAU	200	200	TBD	0	N	0	0	mut / cry gfp	AB(NAU	

Colony totals	* Into system:	1340	** Out of system:	1080	On system total:	260
	# of embryos produced:	940				

Footnotes: 1. These values represent the number of individuals per a specific generation at a specific date (not annual totals .

New line for Year 3 -Typical husbandry schedule over a **3** year period for colony maintenance for a single line

Colony Year	Date	DPF (age)	Generation	Origin	# entering system (*)	# on system[1]	Sex ratio	# progeny from spawning	Cross	Cull or failure to thrive (**)	Take for Experiment (**)	Genotype	Background	Notes
YR1														
YR2														
YR3	Sep 2018	1	F0	NAU	400	400	BD	0	N	0	0	mosiac	AB(NAU	CRISPR HDR mutagenesis with selcetion marker (cry gfp
	Dec 2018	90	F0	NAU	0	80	BD	0	N	320	0	mosiac / cry gfp	AB(NAU)	Select cry gfp+ males and females. Estimated < 20% penetrance
	Mar 2019	180	F0	NAU	0	80	BD	500	Y	0	0	mosiac / cry gfp	AB(NAU)	Select cry gfp+ larvae Estimated 1 10% penetrance (showing 5%); outX to W
	Mar 2019	1	F1	NAU	500	25	BD	0	N	475	0	het / cry gfp	AB(NAU)	Select cry gfp+ larvae Estimated 1 10% penetrance (showing 5%)
	un 2019	90	F1	NAU	0	25	BD	0	N	0	0	het / cry gfp	AB(NAU)	Confirm mutant sequence
Colony totals		* Into system:		900			** Out of system:		795		On system total:	105		
			# of embryos produced:		500									

Footnotes: 1. hese values represent the number of individuals per a specific generation at a specific date (not annual totals).

VIII. Embryo production numbers

Maximum embryo (0-3dpf) production

It is difficult to accurately predict the number of embryos that will be produced under normal breeding regimes for routine experimental purposes with endpoints no later then 3dpf. Here, we describe the theoretical maximum number of embryos produced over three years, and we emphasize that it is unlikely that realized embryo production will reach the theoretical maximum. Note these animals are NOT for line maintenance or new line production, numbers of larva obtained for those purposes are calculated separately.

Breeding for embryo production may be carried out up to 5 times per week. A typical breeding setup will include 6-10 mating pairs. If we predict an average embryo yield of 200 embryos per female and 10 females, then 1000-2000 embryos are produced in a given breeding day. Five breeding days per week, would produce up to 10,000 embryos in a week. Breeding for embryo production for routine experiments will be heavy during the academic year (Fall and Spring) with reduced workflow in the Summer and Winter recesses. To adjust for this we subtract 8 weeks of breeding leaving 44 weeks per year for embryo production. then the theoretical maximum production is 10,000 embryos x 44 weeks = 440,000 embryos per year and 1,320,000 embryos over the course of three years. We reiterate that 1,320,000 represents a theoretical maximum and we expect realized embryo production to be considerably less.

To put these numbers in perspective the cohort size (# of embryos per treatment) for a typical microinjection experiment is 200 - 500 embryos, and at minimum, two treatment groups (control v experimental). Because, an individual breeding pair often does not produce embryos of sufficient quantity or quality it is necessary to initiate spawning across multiple pairs in excess of what would be required if every animal breed and produced embryos at maximum potential**.**

IX. Database searches

Database: Pubmed
When: June 26, 2017
Query criteria: search in title and abstract

Search terms zebrafish + exposure + development	**Number of references found** 1062	**Range of publication dates** 1992 -2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. The zebrafish has matured into an indispensable model system for studying exposure and development. Although these search results do not offer RRR for zebrafish use, it is reasonable to think that the use of zebrafish may **reduce** the number of rodents used in exposure studies by **replacing** with the less sentient zebrafish.		

Database: Pubmed
When: June 26, 2017
Query criteria: search in title and abstract

Search terms mice or rat + exposure + development	Number of references 16,006	Range of publication dates 1933 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in Dr. [redacted] IACUC protocol. Similar search using “mice or rat” instead of “zebrafish” for comparison.		

Database: Pubmed
When: June 26, 2017
Query criteria: search in title and abstract

Search terms zebrafish + fin clip	Number of references 3	Range of publication dates 2013 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. Two, alternative methods to fin clipping adult zebrafish for DNA collection and genotyping are included in these literature results. (**Breacker et al., 2017; Wilkinson et al., 2013**) the third reference evaluates cooling versus MS222 for euthanasia. **Breacker et al.** evaluates the usefulness of skin swabbing for collection of gDNA. **Wilkinson et al.** describe a method for genotyping by fin clipping of 72hpf larvae. **The DNA collection methods described in these publications represent refinements to the standard fin clip methods routine for zebrafish research. Notably the skin swab method does not require anesthesia and eliminates trauma associated with a biopsy. Although it is reasonable to assume that the swab method would be less stressful to the animal, direct assessments of stress following DNA collection by swab or fin biopsy are lacking.** We will adopt the “Breacker” method for genotyping fish (>2cm OAL) and the “Wilkinson” method for larval genotyping (except we plan to perform the fin clipping in 1-2dpf embryos similar to Samuel et al 2015 – *derived from alternative database search, see below*). Because these approaches are relatively new, in many cases we will still need to validate genotype by traditional fin clip methods.		

IACUC Appendix: [redacted] September 2017 – August 2020

Database: Pubmed
When: June 26, 2017
Query criteria: search in title and abstract

Search terms	Number of references	Range of publication dates
zebrafish + intraperitoneal	104	1997 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. Intraperitoneal injection in zebrafish is an established method for chemical exposure in juvenile or adult zebrafish. References located in this search did not present methods for **refinement** of the approach. As stated earlier however, the use of zebrafish instead of mammalian models in exposure experiments supports **reduction** of mammalian research subjects.		

Database: Pubmed
When: June 26, 2017
Query criteria: search in title and abstract

Search terms	Number of references	Range of publication dates
zebrafish OR Danio + intraperitoneal + waterborne + exposure	0	n/a
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. No results were found. The lack of search results may indicate the absence of any direct comparison of IP injection to waterborne exposures.		

Database: Pubmed
When: June 26, 2017
Query criteria: search in title and abstract

Search terms	Number of references	Range of publication dates
zebrafish + xenograft	135	2007 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. The xenograft experiment using zebrafish as a host have recently emerged and show great promise. Implementing zebrafish for these experiments will likely **reduce** the number of rodents (typical host animal) used in the field by **replacing** with the less sentient zebrafish.		

Database: Pubmed
When: June 26, 2017
Query criteria: search in title and abstract

Search terms	Number of references	Range of publication dates
mice or rat + xenograft	13,303	1969 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. Similar search using “mice or rat” instead of “zebrafish” for comparison.		

IACUC Appendix: [redacted] September 2017 – August 2020

Database: Pubmed
When: June 26, 2017
Query criteria: search in title and abstract

Search terms	Number of references	Range of publication dates
zebrafish + euthanasia	11	2009 - 2017

Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol.

11 articles were found in the search with 10 that directly investigate euthanasia in zebrafish. Articles discuss the effectiveness of, lidocaine hydrochloride, clove oil (eugenol), MS222, hypothermic shock (rapid chilling). At this point there does not appear to be a consensus on the most humane method of euthanasia for zebrafish. Furthermore, the 'best' method is likely to be developmental-stage dependent. Additionally, endpoint assessment must also be considered as some methods may adversely influence experimental outcomes irrespective to the purpose of the experiment.

[redacted] proposed methods include rapid chilling and MS222 followed by secondary measures to ensure death (freezing @ -20C, aldehyde fixation, decapitation, or maceration). Strykowski and Schech recently explored MS222, eugenol and rapid chilling in 14dpf larvae using *time to no heartbeat cessation and rate of recovery* as their metrics for efficient death. The study found that rapid chilling was the most effective method for euthanizing zebrafish, with a recovery rate near 0% in comparison to MS222 which showed a near 100% recovery (Strykowski and Schech, 2015). A study by Wilson et al. 2009 also indicated that rapid chilling was a more efficient method for euthanasia. Additioanly the study also that rapid chilling appeared to elicit less aversive behavior compared to MS222 (Wilson et al., 2009). In light of these studies, and professional experience with both methods, [redacted] proposes to use rapid chilling as the primary method for euthanasia and MS222 mediated euthanasia as an alternative method if rapid chilling is not compatible with the experimental approach. In either case secondary measures will be carried out to ensure death, and the method for euthanasia will be carefully considered with respect to animal welfare and experimental endpoints.

Database: Web of Science (WOS)
When: June 27, 2017
Query criteria: Topic

Search terms	**Number of references found**	**Range of publication dates**
zebrafish + exposure + development	1787	1992 - 2017

Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol.

Increased publication number in search results is likely due to less restrictive search criteria ("Topic" versus "Title/Abstract").

Despite the differences in WOS query numbers the content of the search is largely similar to Pubmed showing a diverse range of manuscripts covering exposure testing and developmental biology in zebrafish. As stated earlier in this document it is reasonable to think that the use of zebrafish may **reduce** the number of rodents used in exposure studies by **replacing** with the less sentient zebrafish.

Database: Web of Science
When: June 27, 2017
Query criteria: Topic

Search terms	Number of references	Range of publication dates
	1,425,674	1900 - 2017

mice or rat + exposure + development		
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. Similar search using “mice or rat” instead of “zebrafish” for comparison.		

Database: Web of Science
When: June 27, 2017
Query criteria: Topic

Search terms	Number of references	Range of publication dates
zebrafish + fin clip	17	2000 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. 17 articles were found of which 12 discussed or used fin clipping in zebrafish work. One publication that did not appear in the Pubmed search is R. Samuel et al. in *Biomed Microdevices* (Samuel et al., 2015). The approach described in that manuscript is similar to Wilkinson et al. 2013 in that it is useful for embryonic/larval fish, however there a few key differences, which may offer **refinement** to traditional methods of genotyping. One major differneces is that the authors were able to show that DNA isolated from the chorionic fluid of 1-2dpf embryos was sufficient for accurate PCR genotyping in approximately 78% of embryos (as opposed to 100% from fin clip). The limitation to the approach is the requirement for custom microfluidic equipment, a failure rate of 22%, and PCR amplification that required cycling numbers far above standard protocol (60 cycles; as cycle number increases, so does the potential for erroneous amplification. A typical PCR cycle number is between 30 and 40). Although the chorionic microfluidic approach holds promise the limitation for necessary equipment and high failure rate seem to outweigh the positive aspects. The authors also compared fin clip DNA in the same microfluidic chips from 1-2dpf embryos, which suggests a 48hpf embryo can be fin clipped. This is 24hrs earlier then the Wilkinson et al. 2013 reported and is certainly a **refinement** of the embryonic fin clip method. A 1-2dpf embryo would not need to be anesthetized prior to fin clipping, and as the authors show regeneration of the fin is complete within 5dpf.		

Database: Web of Science
When: June 27, 2017
Query criteria: Topic

Search terms	Number of references	Range of publication dates
zebrafish + intraperitoneal	133	1996 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. The alternative data base search (WOS) revealed similar content to Pubmed. One publication, Stewart et al. 2011 in *Zebrafish Neurobehavioral Protocols* discusses the advantages of IP injection as a method for delivering drugs to adult zebrafish (Stewart et al., 2011) . Publications from Zang et al. 2011in *Zebrafish* and Kulkarni et al. 2014 in *Pharmacological Reports*, Describe methods for oral administration of chemicals by oral gavage or by feeding food-chemical mixtures. These approaches are promising and may **refine** exposures typically administered by IP injections by enabling a *less* invasive method for chemical delivery. Additionally, oral exposures have the advantage (or disadvantage) of addressing gut metabolism as an influence on chemical disposition *in vivo*. Looking forward [redacted] is interested in implementing oral gavage for exposure studies however, elimination of IP injection is unlikely as it is a highly controllable method of delivery that is only marginally more invasive than oral gavage (animals will still need to be anesthetized and handled). Food based methods for chemical delivery will also be pursued, however, this approach is complicated		

by the uncertainty of how a particular chemical may (or may not react) with the food. Although promising more studies to evaluate the food based exposure methods must be carried to better address uncertainties.

Database: Web of Science
When: June 27, 2017
Query criteria: Topic

Search terms	Number of references	Range of publication dates
zebrafish + Danio + intraperitoneal + waterborne + exposure	2	2005 - 2015
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. WOS search reproduced items found with “zebrafish + intraperitoneal”.		

Database: Web of Science
When: June 27, 2017
Query criteria: Topic

Search terms	Number of references	Range of publication dates
zebrafish + xenograft	152	2001 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. WOS search largely reproduced content from Pubmed search. Increase in items located likely due to less restrictive search criterion “Topic” vs “Title/Abstract”.		

Database: Web of Science
When: June 27, 2017
Query criteria: Topic

Search terms	Number of references	Range of publication dates
mice + xenograft	18,598	1969 - 2017
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol. Replacement of “zebrafish” with “mice” for comparison		

Database: Web of Science
When: June 27, 2017
Query criteria: Topic

Search terms	Number of references	Range of publication dates
zebrafish + euthanasia	15	2008 - 2017

IACUC Appendix: [redacted] September 2017 – August 2020

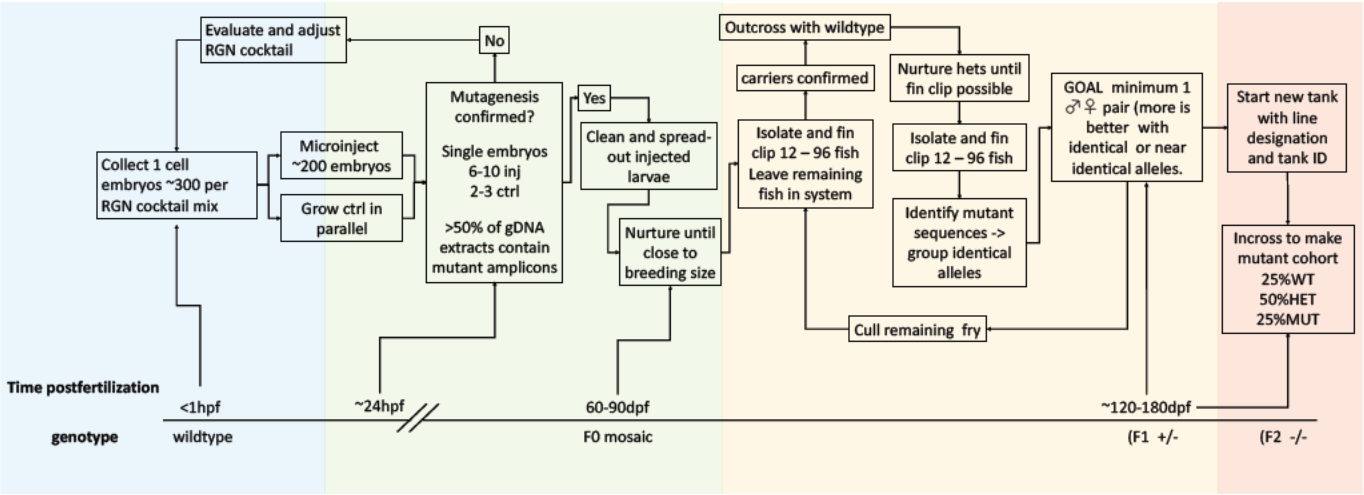
Findings related to Replacement, Reduction, and Refinement (RRR). And statement on inclusion (or not) in [redacted] IACUC protocol.
WOS search largely reproduced items found with Pubmed. One paper that was not revealed in the Pubmed search was, Collymore et al. 2014 in *Journal of the American Association for Laboratory Animal Science* (Collymore et al., 2014). Although the article does not specifically look at euthanasia it does evaluate common anesthetics used for zebrafish and concluded that MS222 is the best drug for maintaining a “surgical plane” of anesthesia.

X. Figures

a. Feeding schedule

Feeding begins at 5dpf with rotifers
Brine shrimp is added to diet at ~12dpf, with proportional reduction in rotifer
Dry feed is added to diet at approximately 20dpf

b. Nuclease mediated mutagenesis work flow (CRISPR-Cas/TALEN)



Note: "RGN" symbolizes CRISPR or TALEN

XI. Training records

CITI PROGRAM

Completion Date 15-Jun-2017
Expiration Date 14-Jun-2020
Record ID [redacted]

This is to certify that:

[redacted]

Has completed the following CITI Program course:

Working with the IACUC (Curriculum Group)
Investigators, Staff and Students (Course Learner Group)
1 - Basic Course (Stage)

Under requirements set by:

Northern Arizona University

CITI

Verify at www.citiprogram.org/verify/?wf7702fc1-a055-4abc-aca8-dc8c118568d7-23575738

[redacted]

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COMPLETION REPORT - PART 1 OF 2
COURSEWORK REQUIREMENTS*

* NOTE: Scores on this Requirements Report reflect quiz completions at the time all requirements for the course were met. See list below for details. See separate Transcript Report for more recent quiz scores, including those on optional (supplemental) course elements.

- **Name:** [redacted]
- **Institution Affiliation:** Northern Arizona University (ID: 1192)
- **Institution Email:** [redacted]
- **Institution Unit:** Biological Sciences
- **Phone:** [redacted]

- **Curriculum Group:** Working with Fish in Research Settings
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - Lab Animal Research

- **Record ID:** [redacted]
- **Completion Date:** 15-Jun-2017
- **Expiration Date:** 14-Jun-2020
- **Minimum Passing:** 80
- **Reported Score*:** 97

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED	SCORE
Introduction, Taxonomy, Research Mandates, and Occupational Health (ID: 15315)	15-Jun-2017	4/5 (80%)
Alternatives, Humane Standards, Housing, Source, and Acclimation and Quarantine (ID: 15316)	15-Jun-2017	5/5 (100%)
Biological Features (ID: 15317)	15-Jun-2017	5/5 (100%)
Recognizing Pain and Distress, Blood Collection, Identification and Routes of Administration (ID: 15318)	15-Jun-2017	5/5 (100%)
Anesthesia, Analgesia, Surgery, and Postoperative Care (ID: 15319)	15-Jun-2017	5/5 (100%)
Euthanasia (ID: 15320)	15-Jun-2017	5/5 (100%)

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

Verify at: www.citiprogram.org/verify/?k6e46dd9a-16e5-49c0-a935-6b1ad607e492-23575741

Collaborative Institutional Training Initiative (CITI Program)
Email: support@citiprogram.org
Phone: 888-529-5929
Web: https://www.citiprogram.org

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COMPLETION REPORT - PART 1 OF 2
COURSEWORK REQUIREMENTS*

* NOTE: Scores on this Requirements Report reflect quiz completions at the time all requirements for the course were met. See list below for details. See separate Transcript Report for more recent quiz scores, including those on optional (supplemental) course elements.

- **Name:** [redacted]
- **Institution Affiliation:** Northern Arizona University (ID: 1192)
- **Institution Email:** [redacted]
- **Institution Unit:** Biological Sciences
- **Phone:** [redacted]

- **Curriculum Group:** Responsible Conduct of Research (RCR)
- **Course Learner Group:** Researchers Responsible Conduct of Research
- **Stage:** Stage 1 - Basic Course

- **Record ID:** [redacted]
- **Completion Date:** 15-Jun-2017
- **Expiration Date:** N/A
- **Minimum Passing:** 80
- **Reported Score*:** 100

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED	SCORE
Authorship (RCR-Basic) (ID: 16597)	15-Jun-2017	5/5 (100%)
Collaborative Research (RCR-Basic) (ID: 16598)	15-Jun-2017	5/5 (100%)
Conflicts of Interest (RCR-Basic) (ID: 16599)	15-Jun-2017	5/5 (100%)
Data Management (RCR-Basic) (ID: 16600)	15-Jun-2017	5/5 (100%)
Mentoring (RCR-Basic) (ID: 16602)	15-Jun-2017	5/5 (100%)
Peer Review (RCR-Basic) (ID: 16603)	15-Jun-2017	5/5 (100%)
Research Misconduct (RCR-Basic) (ID: 16604)	15-Jun-2017	5/5 (100%)
Plagiarism (RCR-Basic) (ID: 15156)	15-Jun-2017	5/5 (100%)
Research Involving Human Subjects (RCR-Basic) (ID: 13566)	15-Jun-2017	5/5 (100%)

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

Verify at: www.citiprogram.org/verify/?ka02133f9-84d6-4711-96ac-a5a00bd14270-23575742

Collaborative Institutional Training Initiative (CITI Program)
Email: support@citiprogram.org
Phone: 888-529-5929
Web: https://www.citiprogram.org

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COMPLETION REPORT - PART 2 OF 2
COURSEWORK TRANSCRIPT**

** NOTE: Scores on this Transcript Report reflect the most current quiz completions, including quizzes on optional (supplemental) elements of the course. See list below for details. See separate Requirements Report for the reported scores at the time all requirements for the course were met.

- **Name:** [redacted]
- **Institution Affiliation:** Northern Arizona University (ID: 1192)
- **Institution Email:** [redacted]
- **Institution Unit:** Biological Sciences
- **Phone:** [redacted]

- **Curriculum Group:** Responsible Conduct of Research (RCR)
- **Course Learner Group:** Researchers Responsible Conduct of Research
- **Stage:** Stage 1 - Basic Course

- **Record ID:** [redacted]
- **Report Date:** 15-Jun-2017
- **Current Score**:** 100

REQUIRED, ELECTIVE, AND SUPPLEMENTAL MODULES	MOST RECENT	SCORE
Using Animal Subjects in Research (RCR-Basic) (ID: 13301)	15-Jun-2017	5/5 (100%)
Research Involving Human Subjects (RCR-Basic) (ID: 13566)	15-Jun-2017	5/5 (100%)
Plagiarism (RCR-Basic) (ID: 15156)	15-Jun-2017	5/5 (100%)
Authorship (RCR-Basic) (ID: 16597)	15-Jun-2017	5/5 (100%)
Collaborative Research (RCR-Basic) (ID: 16598)	15-Jun-2017	5/5 (100%)
Conflicts of Interest (RCR-Basic) (ID: 16599)	15-Jun-2017	5/5 (100%)
Data Management (RCR-Basic) (ID: 16600)	15-Jun-2017	5/5 (100%)
Mentoring (RCR-Basic) (ID: 16602)	15-Jun-2017	5/5 (100%)
Peer Review (RCR-Basic) (ID: 16603)	15-Jun-2017	5/5 (100%)
Research Misconduct (RCR-Basic) (ID: 16604)	15-Jun-2017	5/5 (100%)

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

Verify at: www.citiprogram.org/verify/?ka02133f9-84d6-4711-96ac-a5a00bd14270-23575742

Collaborative Institutional Training Initiative (CITI Program)
Email: support@citiprogram.org
Phone: 888-529-5929
Web: https://www.citiprogram.org

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COMPLETION REPORT - PART 2 OF 2
COURSEWORK TRANSCRIPT**

** NOTE: Scores on this Transcript Report reflect the most current quiz completions, including quizzes on optional (supplemental) elements of the course. See list below for details. See separate Requirements Report for the reported scores at the time all requirements for the course were met.

- **Name:** [redacted]
- **Institution Affiliation:** Northern Arizona University (ID: 1192)
- **Institution Email:** [redacted]
- **Institution Unit:** Biological Sciences
- **Phone:** [redacted]

- **Curriculum Group:** Working with Fish in Research Settings
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - Lab Animal Research

- **Record ID:** [redacted]
- **Report Date:** 15-Jun-2017
- **Current Score**:** 97

REQUIRED, ELECTIVE, AND SUPPLEMENTAL MODULES	MOST RECENT	SCORE
Introduction, Taxonomy, Research Mandates, and Occupational Health (ID: 15315)	15-Jun-2017	4/5 (80%)
Alternatives, Humane Standards, Housing, Source, and Acclimation and Quarantine (ID: 15316)	15-Jun-2017	5/5 (100%)
Biological Features (ID: 15317)	15-Jun-2017	5/5 (100%)
Recognizing Pain and Distress, Blood Collection, Identification and Routes of Administration (ID: 15318)	15-Jun-2017	5/5 (100%)
Anesthesia, Analgesia, Surgery, and Postoperative Care (ID: 15319)	15-Jun-2017	5/5 (100%)
Euthanasia (ID: 15320)	15-Jun-2017	5/5 (100%)

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

Verify at: www.citiprogram.org/verify/?k6e46dd9a-16e5-49c0-a935-6b1ad607e492-23575741

Collaborative Institutional Training Initiative (CITI Program)
Email: support@citiprogram.org
Phone: 888-529-5929
Web: https://www.citiprogram.org

XII. Bibliography

Banote, R.K., Koutarapu, S., Chennubhotla, K.S., Chatti, K., and Kulkarni, P. (2013). Oral gabapentin suppresses pentylenetetrazole-induced seizure-like behavior and cephalic field potential in adult zebrafish. Epilepsy Behav. *27*, 212–219.

Breacker, C., Barber, I., Norton, W.H.J., McDearmid, J.R., and Tilley, C.A. (2017). A Low-Cost Method of Skin Swabbing for the Collection of DNA Samples from Small Laboratory Fish. Zebrafish *14*, 35–41.

Collymore, C., Tolwani, A., Lieggi, C., and Rasmussen, S. (2014). Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). J. Am. Assoc. Lab. Anim. Sci. *53*, 198–203.

CSHL (2011). Cold Spring Harbor Protocols.

Harper, C., and Lawrence, C. (2011). The laboratory zebrafish.

Kulkarni, P., Chaudhari, G.H., Sripuram, V., Banote, R.K., Kirla, K.T., Sultana, R., Rao, P., Oruganti, S., and Chatti, K. (2014). Oral dosing in adult zebrafish: Proof-of-concept using pharmacokinetics and pharmacological evaluation of carbamazepine. Pharmacol. Reports *66*, 179–183.

Lawrence, C., Best, J., Cockington, J., Henry, E.C., Hurley, S., James, A., Lapointe, C., Maloney, K., and Sanders, E. (2016). The Complete and Updated "Rotifer Polyculture Method" for Rearing First Feeding Zebrafish. J. Vis. Exp. e53629.

Leary, S., Underwood, W., Anthony, R., Cartner, S., Corey, D., Grandin, T., Greenacre, C., Gwaltney-Brant, S., McCrackin, M.A., Meyer, R., et al. (2013). The AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. In The AVMA Guidelines for the Euthanasia of Animals, (Elsevier), pp. 69–74.

Nüsslein-Volhard, C., and Dham, R. (2002). Zebrafish: A practical approach. New York Oxford Univ. Press 2002.

Samuel, R., Stephenson, R., Roy, P., Pryor, R., Zhou, L., Bonkowsky, J.L., and Gale, B.K. (2015). Microfluidic-aided genotyping of zebrafish in the first 48 h with 100% viability. Biomed. Microdevices *17*.

Snider, D.R., and Clegg, E.D. (1975). Alteration of phospholipids in porcine spermatozoa during in vivo uterus and oviduct incubation. J. Anim. Sci. *40*, 269–274.

Stewart, A., Cachat, J.M., Suciu, C., Hart, P.C., Gaikwad, S., Utterback, E., DiLeo, J., and Kalueff, A. V (2011). Intraperitoneal Injection as a Method of Psychotropic Drug Delivery in Adult Zebrafish. In Zebrafish Neurobehavioral Protocols, A. V Kalueff, and J.M. Cachat, eds. (Totowa, NJ: Humana Press), pp. 169–179.

Strykowski, J.L., and Schech, J.M. (2015). Effectiveness of recommended euthanasia methods in larval zebrafish (Danio rerio). J. Am. Assoc. Lab. Anim. Sci. *54*, 81–84.

Westerfield, M. (2007). The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition. Univ. Oregon Press. Eugene.

Wilkinson, R.N., Elworthy, S., Ingham, P.W., and van Eeden, F.J.M. (2013). A method for high-throughput PCR-based genotyping of larval zebrafish tail biopsies. Biotechniques *55*, 314–316.

Wilson, J.M., Bunte, R.M., and Carty, A.J. (2009). Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (Danio rerio). J. Am. Assoc. Lab. Anim. Sci. *48*, 785–789.

Zang, L., Morikane, D., Shimada, Y., Tanaka, T., and Nishimura, N. (2011). A Novel Protocol for the Oral Administration of Test Chemicals to Adult Zebrafish. Zebrafish *8*, 203–210.