

Guidelines for Use of Zebrafish in the NIH Intramural Research Program

Background:

The PHS Policy on Humane Care and Use of Laboratory Animals defines animal as, “any live, vertebrate animal used or intended for use in research, research training, experimentation or biological testing or for related purposes.”¹ Furthermore, OLAW interpretation of PHS policy considers aquatic species as “live, vertebrate animals” at hatching.² Although this is an imprecise stage for zebrafish it can be approximated at 72 hours post fertilization.

General Guidelines for Animal Study Proposals (ASPs):

- For purposes of animal number accountability, all stages of development greater than three days post fertilization(dpf) should be counted in an approved ASP. Due to the high throughput nature of the zebrafish model, large numbers of animals may be listed on ASPs (Section B and Section H in the NIH ASP Template). However, the majority of studies are completed on larval zebrafish between 4-7dpf. Therefore, to understand animal use, an Animal Care and Use Committee (ACUC) may request that investigators report their annual animal numbers separately as larval stages (4-7 dpf) and those animals reared past ≥ 8 dpf.
- Brain structures required for the affective experience of noxious stimuli are immature at early larval stages (up to 7 dpf).³⁻⁹ The number of larvae used may be provided as an estimate considering their size and normal housing conditions.
- When evaluating “Sex as a biological variable...”, the Principal Investigator (PI) and ACUC must consider age since sex is undetermined until at least 25 dpf.^{10,11}

Common Zebrafish Procedures:

Tissue Collection for Genotyping

- Fin clipping for DNA analysis and/or genotyping must be described in the ASP and approved by the IC ACUC.
- Individuals performing the procedure must be trained and show proficiency in the technique.
- Zebrafish usually undergo tissue collection ≥ 2 month old. The caudal (tail) fin is the preferred tissue collection site. When done correctly, the fin will not bleed and should regenerate in 7-10 days.
- The fin biopsy length should be limited to the smallest amount possible. In general, approximately 2-3mm of the caudal fin is sufficient to generate DNA for multiple PCR reactions and to prevent damage to the fin. For other fins, smaller amounts of tissue collected is recommended.
- Anesthesia is frequently used to assist with fish handling.¹²
- Other alternative methods for genotyping are available.^{13,14}

Euthanasia Guidelines

- Euthanasia of animals is an important consideration in all ASPs and must be described. The PI and all investigators on the protocol must be trained in the proper technique, equipment, and agents for euthanasia and will be held responsible for the correct implementation of these guidelines.

- All methods of euthanasia of zebrafish described below are the most commonly used methods in the Division of Intramural Research and are in accordance with the [AVMA Guidelines on Euthanasia: 2020 Edition](#)¹⁵. IC ACUC may require scientific justification for other euthanasia methods.
- The effectiveness of euthanasia methods may vary by life stage. Using adjunctive methods to guarantee death is recommended.
- Zebrafish carcasses should be disposed of as Medical Pathological Waste according to NIH policies.

Euthanasia Procedures

1. For zebrafish embryos/ larvae ≤ 7 dpf, euthanasia can be completed using one of the following methods:

- a. Sodium Hypochlorite (bleach):
 - i. They should remain in this solution at least five minutes prior to disposal to ensure death. *Extreme caution must be used with this method to avoid any possibility of bleach entering the aquatic housing system water.
 - ii. For bleach solutions, verify the sodium hypochlorite concentrations:
 1. 6.15% sodium hypochlorite, add to the culture system water at ≥ 1 part bleach to 5 parts system water.
 2. 8.25% sodium hypochlorite, add to the culture system water at ≥ 1 part bleach to 7 parts system water.
- b. Rapid chilling (hypothermic shock) followed by an adjunctive method of immersion in dilute sodium hypochlorite or calcium hypochlorite solution (2-step method).

2. For zebrafish larvae up to 8-15 dpf euthanasia requires a secondary method in order to ensure death. This age group can survive anesthetic overdose and rapid chilling even after prolonged absence of heartbeat. They can revive if returned to water that is within their normal environmental parameters.

- a. Euthanasia by rapid chilling with adjunctive method:¹⁶
 - i. Submersion into ice water bath (5 parts ice/1 part water, 2-4^o C) for at least 20 minutes to ensure death.
 1. The fish must not come in direct contact with the ice chips; for example, a mesh-bottom inner cage of a breeder tank can be pushed into the ice slurry as a barrier.
 2. Since some zebrafish up to 15 dpf can survive rapid chilling even after prolonged absence of a heartbeat, a secondary method should be used to ensure death in embryos <15 dpf.
 - ii. Adjunctive methods after fish have been rendered unconscious prior to their application are freezing or other physical or chemical methods for destroying the brain function.
- b. General anesthesia with pharmaceutical grade buffered MS-222 followed by adjunctive methods such as freezing or other physical or chemical methods for destroying the brain function.

3. For zebrafish ≥ 15 dpf, euthanasia can be done using the following methods:

- a. Euthanasia by rapid chilling^{17,18}:
 - a. Submersion in ice water bath (5 parts ice/1part water, 2-4° C) for at least 10 minutes after cessation of opercular (i.e., gill) movement.
 - i. The fish must not come in direct contact with the ice chips; for example, a mesh-bottom inner cage of a breeder tank can be pushed into the ice slurry as a barrier.
 - ii. In any fish where it is difficult to visualize opercular movement, fish should be left in the ice water for at least 30 minutes after cessation of all movement to ensure death by hypoxia.
 - b. Water temperature must be monitored with a thermometer and maintained between 1-4° C to ensure proper euthanasia
- b. Overdose by prolonged immersion in buffered pharmaceutical-grade MS-222 in 250-500 mg/L solution.
 - a. Buffering with sodium bicarbonate should result in a pH between 7.0 and 7.5. Non-buffered MS-222 is acidic and causes an aversive reaction in unanesthetized fish.
 - b. Fish should be left in the solution for at least 30 minutes following cessation of opercular movement.
 - c. Fish should not be left in the euthanasia solution unattended, nor should they be left in the solution for more than one hour.
- c. Anesthesia with pharmaceutical grade buffered MS-222 (100-200 mg/L) followed by an adjunctive method after fish have been rendered unconscious prior to their application are decapitation, exsanguination, freezing or other physical or chemical methods for destroying the brain function.

References:

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