

# Guidelines for Use of Zebrafish in the NIH Intramural Research Program

## Euthanasia Guidelines

Euthanasia of zebrafish must be carried out by one of the following methods.

1. For zebrafish  $\geq 8$  dpf the following methods are acceptable for euthanasia:
  - Immobilization by submersion in ice water (5 parts ice/1 part water, 0-4° C) for at least 10 minutes following cessation of opercular (i.e., gill) movement. In any fish where it is difficult to visualize opercular movement, fish should be left in the ice water for at least 20 minutes after cessation of all movement to ensure death by hypoxia.
  - Overdose of tricaine methane sulfonate (MS222, 200-300 mg/l) by prolonged immersion. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement.
  - Anesthesia with tricaine methane sulfonate (MS222, 168 mg/l) followed by rapid freezing in liquid nitrogen.
2. For zebrafish 4-7 days post-fertilization (dpf) the following methods are acceptable for euthanasia:
  - Immobilization by submersion in ice water (5 parts ice/1 part water, 0-4° C) for at least 20 minutes to ensure death by hypoxia.
  - Addition of bleach solution (sodium hypochlorite 6.15%) to the culture system water at 1 part bleach to 5 parts water. They should remain in this solution at least five minutes prior to disposal to ensure death. As detailed above in the scientific background section, pain perception has not developed at these earlier stages so this is not considered a painful procedure.
3. Recent observations have indicated that zebrafish larvae up to at least 15 dpf can survive anesthetic overdose and rapid chilling. Despite prolonged absence of heartbeat they can revive once returned to water that is within their normal environmental parameters. When these methods are used on larvae up to 15 dpf, an adjunct method such as adding sodium hypochlorite as described in B2 above should be used after the heart beat has stopped in order to ensure death.
4. For embryos  $\leq 3$  dpf, development should be terminated using bleach as described in section B2 above.
5. Additional methods can be used if required by experimental design and approved by the IC Institutional ACUC committee:
  - Decapitation with a sharp blade by a trained individual.
  - Anesthetic overdose or rapid chilling followed by fixation in paraformaldehyde or other fixative
  - For embryos  $< 8$  dpf: rapid freezing in -70 freezer. Embryos should be contained in a minimum amount of water to insure rapid freezing and death.

- Maceration using a well maintained macerator designed for the size of the fish being euthanized.

Zebrafish carcasses from any of these methods should be disposed of as Medical Pathological Waste according to NIH policies.

These methods ensure death provided the timeframes above are followed. The ice water method should not be extrapolated to other aquatic species without first confirming the effectiveness for that species. Aquatic species, native to a colder environment than zebrafish, may be more resistant to hypothermic shock and may recover subsequently.

### **General Guidelines**

Current OLAW interpretation of PHS policy considers aquatic species as "live, vertebrate animals" at hatching. Although this is an imprecise stage for zebrafish it can be approximated at 72 hours post fertilization. For purposes of accountability all stages of development greater than three days of age should be described in an approved Animal Study Proposal. Thus an estimate of the number of larval zebrafish from day 4 - 7dpf should be included in Animal Requirements (Section B in the NIH ASP form).

Since these early stages (4-7dpf) do not to feel pain or distress, it is preferable that their numbers be separated from zebrafish  $\geq 8$ dpf. This number can be listed as Column C in the Pain and Distress Category (Section H) of zebrafish ASPs as a separate number from zebrafish  $\geq 8$ dpf.

The pain and distress categorization of the  $\geq 8$ dpf fish should be determined by the investigator based on the specific procedures described in the protocol. The number of animals used may need to be provided as an estimate, particularly with these young larvae, considering their size and normal housing conditions. Estimated numbers may still be used after they have matured to adults if they are group housed.

### **References**

1. University of Oregon (2008) Final Report to OLAW on Euthanasia of Zebrafish.
2. National Institutes of Health (2009) Final Report to OLAW on Euthanasia of Zebrafish.
3. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition.
4. Matthews and Varga. (2012) Anesthesia and Euthanasia in Zebrafish *ILAR Journal* 53(2):192-204.

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