J. Chosun Natural Sci.
Vol. 14, No. 4 (2021) pp. 155 – 158
https://doi.org/10.13160/ricns.2021.14.4.155

# A Practical Protocol of Zebrafish Heart Rate Measurement for High School Students

**Jeong Hoon Cho**†
*Department of Biology Education, Chosun University, Gwangju, Korea*

## Abstract

To study the effects of hormones and neurotransmitters, zebrafish (*Danio rerio*) are a great substitute for water fleas (*Daphnia)*. The zebrafish is an ideal vertebrate model because it has a transparent embryonic stage. It is easy to get consistent heart rate measurements in embryonic zebrafish when treating them with hormones and neurotransmitters. To observe the heart rate, two to three embryonic zebrafish are anesthetized with MS-222 and then transferred to a glass slide specifically designed for heart observation and easy application of various chemicals. After the heartbeats are counted for 2 minutes, apply either 100 μM epinephrine or 100 μM acetylcholine to the zebrafish. Wait 5, 10, and 20 minutes and count the heartbeats at each time point. All procedures are repeated three times. The final results are averaged and analyzed by using statistical methods. The above method which we have developed is practical enough for high school students to measure the heart rate in zebrafish under various conditions and to analyze the data set.

**Keywords:** Zebrafish, Circulatory system, Heart rate, Epinephrine, Acetylcholine

(Recceived November 9, 2021 Revised December 12, 2021 Accepted December 29, 2021)

## 1. Introduction

In schools and science camps, taking heart rate (HR) measurements of *Daphnia* is a readily available and common experiment because *Daphnia* are transparent, inexpensive, and are easy to manipulate[1]. *Daphnia*'s transparency allows students to observe the heart and count HR with almost no training. In addition, due to *Daphnia* being easy to manipulate, students are able to apply various chemicals and investigate the effects on heart rate. However, *Daphnia* are crustaceans, invertebrates with an open heart system. One of the popular experiments is to investigate the hormonal and neurotransmitter effects on heart rate such as those caused by epinephrine and acetylcholine. Even though the invertebrate *Daphnia* respond the hormones, the reaction mechanism is not the same as the one in vertebrates. Moreover, there are inconsistent results between studies using *Daphnia*. Depending on the study, epinephrine and acetylcholine may increase or decrease HR in *Daphnia*[1,2]. In contrast, heart rate studies using zebrafish (*Danio rerio*) treated with epinephrine and acetylcholine are consistent. Zebrafish HR increases in response to epinephrine after 4 days post fertilization (dpf) and the HR decreases in response to acetylcholine after 5 dpf [3]. Thus, a model vertebrate animal, such as the zebrafish, is superior to *Daphnia* for investigating heart rate in response to hormones and neurotransmitters.

The zebrafish (*Danio rerio*) is an important vertebrate model organism used in many scientific studies because of its transparency, fast development, and easy care[4-6].

†Corresponding author : renocho@chosun.ac.kr

Consistent results can be obtained when treating zebrafish with hormones and neurotransmitters. Taken together, zebrafish are an ideal model for high school students to learn the heart system and understand hormonal or neurotransmitter action. Here, we have developed an easy protocol for high school students to take HR measurements in zebrafish. Our zebrafish system enables untrained students 1) to observe the heart and the circulatory system of the embryonic zebrafish, 2) to measure changes in heart rate in response to hormones and neurotransmitters, such as adrenaline and acetylcholine, 3) to interpret the collected data set and understand the effects of hormones and neurotransmitters on heart rate, and 4) to draw a conclusion from the experimental results.

## 2. Methodology

### 2.1. Experimental design

To understand hormonal and other chemical effects on vertebrate heart rate, embryonic zebrafish (a vertebrate) are required. The clear embryonic fish can be obtained by using our zebrafish egg collecting method and equipment[7]. To observe the heart system and measure the heart rate easily, our specific glass slide with a silicon pad is required (Fig. 1).

#### Materials

Embryonic zebrafish: 5 and older days post fertilization (dpf)

Viewing slide (a specifically designed shape for this observation)

Cover slide

Forceps

Cut filter papers

1.5 mL tubes

Disposable dropping Pipettes

Micropipette (100, 200, 1000 μL)

#### Equipment

A stero/dissecting/light Microscope

Hand-held Counter

Timer

#### Reagents

1. Ringer's solution: Ringers solution 1/4 strength tablets (Sigma, St. Louis, USA)

Dissolve 1 tablet in 500 mL of deionized water and sterilized by autoclaving at 121°C for 15 minutes.

2. Ethyl 3-aminobenzoate methanesulfonate (MS-222, Tricaine, Sigma-Aldrich, St. Louis, USA)

Make a 10X MS-222 solution. Dissolve 1 g of MS-222 powder in 1 L of Ringer's solution. Adjust the pH to 7.2 by adding 0.1 g of sodium bicarbonate in 200 mL of MS-222 solution (final working concentration of MS-222 will be 0.1 g/L). The stock is stored at 4°C.

*A high concentration of MS-222 slows the heartbeat of zebrafish larvae[8]. Therefore, start with a half concentration of MS-222 solution (0.05 g/L).

3. Adrenaline ((−)-Epinephrine (+)-bitartrate salt, Sigma-Aldrich, St. Louis, USA)

Prepare a 1 M stock solution (1,000X) in distilled water and separate into 1 mL aliquots.

All stock solutions should be stored at -80°C (if not available, −20°C).

The final working solution is prepared by making a 1 to 1000 dilution of the stock solution in 0.5X MS-222 solution (final concentration=100 μM, 1X).

*It is easily oxidized with oxygen, which is indicated by a pink or dark red color. Check the color before using it. If the color is pink, it shouldn't be used because the concentration is not correct[9-11].

4. Acetylcholine (Sigma-Aldrich, St. Louis, USA)

Prepare a 1 M stock solution (1,000X stock) in distilled water and store it at −20°C or 4°C.

The final working solution is prepared by making a 1 to 1,000 dilution of the stock solution in 0.5X MS-222 solution (final concentration=100 μM, 1X).

### 2.2. Experimental Methods

Procedure 1: Prepare the Slide of Anaesthetized Zebrafish

1. Collect three zebrafish larvae (6 dpf) from the zebrafish larvae swimming tube/dish by using a disposable dropping pipet and put them into a 1.5 mL tube. Take note of the volume of water the zebrafish are in. You will need this for the next step.

2. Add the same volume of 1X MS-222 solution (final working concentration will be 0.5X MS-222 solution) as you used in step 1 for the zebrafish in water. Wait 2 minutes.

3. Transfer the anaesthetized animals to the special glass slide. Make sure they are in liquid; the center hole of the slide should have liquid in it, but not overflow. Position the fish in the center of the hole (see Fig. 1).

4. Carefully put a cover slide on top of the center. Remove excess water with the cut filter paper.

5. Put the slide on top of the stage of a microscope.

Procedure 2: Count the Heartbeats

1. Examine the heart of the embryonic zebrafish under low magnification (Fig. 2).

2. Focus on the heart under high magnification.

3. Count the heartbeats for a minute* by using a hand-held counter.

4. Count at least 3 times for replication per each embryonic fish and record the numbers in a data sheet.

*Counting for two minutes is more accurate and is preferable.

Caution: Do not exceed 30 minutes from the initial anesthetizing.

Procedure 3: Chemical Treatment

1. Remove the above slide from the microscope stage.

2. Carefully add 200 μL of 100 μM (1X) epinephrine** solution to one end of the slide by using a micropipette. Use enough solution to completely fill the slide and prevent air bubbles. Liquid will be pushed out as you do this, so at the same time use filter paper at the other end of the slide to absorb the liquid that overflows (Fig. 1).

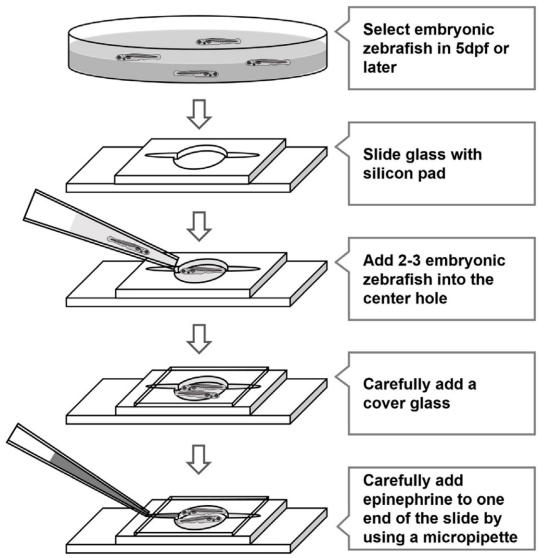


**Fig. 1.** Processes of the embryonic zebrafish slide preparation.

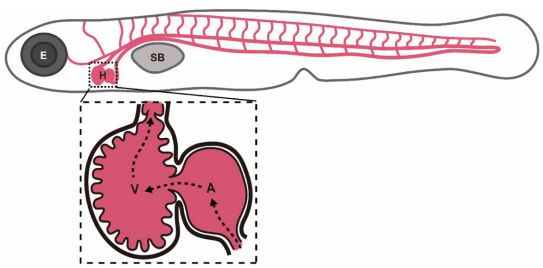


**Fig. 2.** Structure of zebrafish Heart. E, eye; SB, swim bladder; H, heart; V, ventricle; A, atrium

3. Repeat step 2 to ensure the solution is completely inside the hole (Fig. 1).

4. For each 5-, 10-, and 20-minute treatment, measure the heart rate per minute (by counting heartbeats) and repeat this three times per treatment (i.e. time point).

** For the control treatment, add 200 μL of 0.5X MS-222 solution (0.5 g/L).

For the acetylcholine treatment, add 200 μL of 100 μM (1X) acetylcholine solution.

**Table 1.** Heart rates in 5 dpf embryonic zebrafish

| 5dpf embryonic zebrafish | control | +100 μM Epinephrine | +100 μM Acetylcholine |
|---|---|---|---|
| Heart rate/ min | 160±10 n=30 | 176±11* n=30 | 148.8±7* n=30 |

A mean of heart beats per minute (±S.E.) is given for the control, the 100 μM epinephrine-treated, and the 100 μM acetylcholine-treated embryonic zebrafish. (Unpaired *t*-tests between the control and either the epinephrine or acetylcholine treatments were used with *p<0.05.)

## 3. Results

On average, the heart rate is 160±10/min at 25°C for 5 dpf embryonic zebrafish (Table 1), which is consistent with the results of Schwerte, 2006. The 100 μM epinephrine treatment increased the heart rate an average of 110% (range, 105~122%, n=30). Treatment of 100 μM acetylcholine decreased the heart rate by an average of 93% (range, 91-103%, n=30). Depending on the age of the embryonic zebrafish, the concentration of the chemicals, and the temperature, the results can vary.

## 4. Data Management and Analysis

The final data sets should be analyzed using a statistical method such as a student t-test. This helps students understand the concept of statistical data analysis, which is critical to any science education.

## Acknowledgements

The study was supported by a research fund from Chosun University, 2020. We would also like to thank Dr. Eleanor Thorne for her careful reading of the manuscript and for great comments to improve and clarify the manuscript.

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