# Investigating the Immune Response of *D. rerio* Using Keyhole Limpet Hemocyanin

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## Table of Contents

## I. Abstract:

In this project, immunization of a model aquatic organism is used in the classroom to facilitate understanding of molecular biology, evolution and the vertebrate adaptive immune system. Molecular immunoglobulin and T cell receptor cloning, sequencing and repertoire analysis techniques that I learned in the Criscitiello Comparative Immunogenetics Lab at Texas A&M this summer were applied to analysis of the lymphocyte antigen receptor repertoire in zebrafish. Students at A&M Consolidated High School performed experiments to quantify and qualify various aspects of the immune response of the model organism, *D. rerio*. Hemocytometry was used to measure leukocyte counts before and after immunization with keyhole limpet hemocyanin. Serum IgM was analyzed using ELISA. Repertoire analysis was performed using PCR and DNA sequencing. The data from these experiments was used in the classroom to discuss the evolution of vertebrate adaptive immunity, immunization techniques, and vaccination design. Furthermore, the repertoire sequence data contributed to an important phylogenetic perspective for ongoing NSF funded studies at A&M on the plasticity of B and T cell receptor immunogenetics. One goal is for high school students to earn co-authorship on journal publications from the Criscitiello lab. This was the first stage in an iterative system of teaching and research that will continue to include reciprocal pedagogical exchange and hands-on experience in comparative molecular immunology.

## II. Science Background:

This unit focused on teaching and demonstrating the basic concepts of the vertebrate immune system. The history of immunology is discussed to provide conversational context, and to point out how much we have learned and how much is left to discover. The remainder of the lecture content includes a focus on vocabulary needed to understand the above-mentioned concepts.

Immunology, stemming from the Latin root *immunis* meaning “exempt,” is the study of the interplay between an organism’s specific (adaptive) and non-specific (innate) immune responses. The non-specific, or innate immune system, is the subsystem that comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. Cells of the innate immune system include:

**Mast Cells** – These cells reside in the connective tissue and mucus membranes of an organism. They are associated with wound healing and defense against pathogens.
**Macrophages** – Greek: “larger eater.” These cells can move outside the vascular system in pursuit of invading pathogens. Macrophages are the most efficient phagocytes, and can individually destroy a substantial number of foreign cells.
**Neutrophils** – These cells are considered granulocytes, and they contain a variety of toxic substances that can kill or inhibit the growth of bacteria and fungus.
**Dendritic Cells** – These cells are present in tissues that are in contact with the external environment (skin, nose, lungs, stomach, and intestines). One of their key roles is in antigen presentation, and they serve as a link between the innate and adaptive immune system.

**Basophils/Eosinophils** – These cells are mostly responsible for a body's allergies. Basophils release histamine, and eosinophils release a variety of toxic proteins that can kill bacteria and parasites as well as damage a host's tissue.
**Natural Killer (NK) Cells** – These cells are responsible for destroying compromised host cells such as cancer cells or cells infected with virus.

The adaptive immune system is a highly complicated subsystem that is comprised of highly specialized cells and processes that eliminate or prevent pathogen growth. The adaptive immune system can only be found in vertebrates. Humoral immunity and cell-mediated immunity are subsets of the adaptive immune system.

The humoral immune system is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides. The system is named as such after the classic "humors," or body fluids. Generally, the humoral system refers to antibody production and the accessory processes that accompany it.

The cell-mediated immune system does not involve antibodies *per se*, but also involves the activation of phagocytes, antigen-specific cells, and cytokines. Cell-mediated immunity plays an important role in protection generated due to immunizations.

Throughout a vertebrate's lifetime, it can generate an almost infinite variety of cells and substances in an attempt to keep the host healthy. The response to a foreign body in the host can manifest in one of two ways depending on that host's prior exposure to the foreign body. If this is the initial exposure to the foreign body, the host will have an effector response that has the goal of eliminating or neutralizing the foreign body. If this is not the initial exposure to a foreign body, the host's "memory" will assist in a more rapid and efficient response to eliminating or neutralizing the foreign body. [1]

A Brief History of Immunology:

Documents have shown that humans have made note of immunological concepts dating back to around 400 B.C.E. These documents surround the resistance of plague survivors to additional exposure to plague events. Also, a standard disinfecting treatment for a plague victim's household was to fumigate the area with sulfur vapors. Around 50 B.C.E. the beginnings of germ theory surfaced with Lucretius' suggestion that disease was caused by invisible living creatures. During the 10$^{th}$ century it has been documented that the Turks inoculated their children against smallpox by exposing them to particles from smallpox blisters.

Prior to a modern understanding of human anatomy and physiology, the classic causes of disease were attributed to the body's humors: Blood, yellow bile, black bile, and phlegm. Each one of these humors was connected to a season, a basic element (air, fire, earth, water), an organ, and human qualities/symptoms (warm & moist, warm & dry, cold & dry, cold & moist). Medical professionals of the time specialized in analyzing the

humors of an ill person and treating the illness with ancient remedies such as sweating, blood-letting, herbal medications, etc. [2]

As immunology made its way into the modern era, the focus started in the 1700's in the war on smallpox. When Lady Montague became aware of the practice of inoculating against smallpox, she set a political and stature-based precedent by her children treated. In the late 1700's Edward Jenner made note that milkmaids, who were exposed to cowpox in the course of their work, were immune to smallpox. In experiments that would be considered unethical and illegal under today's standards, Jenner began injecting fluids from cowpox blisters into orphans and prisoners to see if the immunity could be transferred via injection. From those beginnings, the war on smallpox continued for hundreds of years until the WHO (World Health Organization) declared that smallpox had been eradicated from the planet in 1989. [3]

The following are other noteworthy immunological discoveries that can be expanded upon by the instructor, or assigned as a research project/presentation for the students:

1. Louis Pasteur – In 1879, Pasteur discovered that some aged bacterial cultures lost virulence after time. These weakened bacteria could be used as a vaccine to prevent damage to a host if exposed to a stronger version of the same bacteria type. He applied this technique to anthrax, and eventually to rabies.
2. Elie Metchnikoff – He discovered that phagocytic cells ingest microbes and their particles, therefore that cells conferred immunity.
3. Emil von Behring – He discovered that blood serum could transfer immunity, therefore blood conferred immunity.
4. Karl Landsteiner – He discovered that antibodies could be produced to address almost any organic compound. In his later career, he discovered that antibody specificity reveals an almost unlimited range of reactivity.

The following are basic vocabulary that the instructor should be familiar with and that the students should master throughout the unit:

1. **Immunity** is defined as resistance to infectious disease and the collection of cells and tissues that protects the body from infection is known as the **immune system**. The coordinated reaction of the cells of the immune system to a pathogen is known as the **immune response**.
2. **Antigen** is a general term that applies to molecules that bind to antibodies or T cell receptors with high affinity. Antigens come in many forms: for example, small molecules in the environment and a huge array of bacterial and viral surface proteins might all act as antigens. Many times, you will see the terms antigen and microbe used interchangeably, since most antigens are derived from larger pieces of a microbe.
3. **Antibody (Ab)** Protein molecule that is synthesized on exposure to antigen and that combines specifically with that antigen.
4. **Antigen presenting cell (APC)** A cell which carries antigen in a form that can stimulate lymphocytes. Macrophages are the most common APCs.

5. **Immunoglobulin (Ig)** A glycoprotein composed of H and L chains that function as antibody. Immunoglobulin function as antibodies.
6. **Leukocyte** A white blood cell. This general term covers all the nucleated cells of mammalian blood.
7. **Thymus** The central lymphoid organ that is located in the thorax which controls the ontogeny of T cells.
8. **Lymphocytes** are cells found in the blood, lymphoid tissues and most organs of the body that express receptors for specific antigens and mediate immune responses. The lymphocytes that we will talk the most about are **B cells** and **T cells** (B cell = B lymphocyte; T cell = T lymphocyte).
9. When B and T cells become activated, they divide and mature into **effector cells** that actually do the job of fighting the microbe. Mature B cells are called **plasma cells**; plasma cells secrete **antibodies**, which are glycoprotein molecules that bind antigens with high affinity and help to eliminate those antigens. Mature T cells are called **effector T cells**. Effector T cells either assist ("help") leukocytes to kill ingested microbes or directly kill infected cells.
10. **Humoral immunity** is the type of adaptive immunity that is mediated by antibodies produced by plasma cells (B-cells [white blood cells]). Humoral immunity is the main mechanism for defending against extracellular microbes and their toxins.
11. **Cell-mediated immunity** is the type of adaptive immunity mediated by T lymphocytes; cell-mediated immunity is the main defense mechanism against microbes that survive within phagocytes (i.e. the bacteria that causes Tuberculosis) or that infect the cytosol of non-phagocytic cells (i.e. many viruses).
12. **Cytokines** are secreted proteins that work as mediators of immune and inflammatory reactions. Cytokines provide a mechanism for cells of the immune system to "talk" to one another to coordinate a response. **Interleukin** is another term for a cytokine that acts on other leukocytes. [4]

## III. Student Outcomes:

The students will master lab techniques such as live injections, dissections, creating blood smears, staining cells, and cell counting using an oil-immersion light microscope.
The students will learn about experimental design and laboratory collaboration.
The students will design and author a scientific poster & oral presentation via power point.

## IV. Learning Objectives:

The students will learn the key terms and processes of the basics of the immune system.

**A. Next Generation Science Standards (NGSS)**

1. HS-LS4-1
2. HS-LS4-2
3. HS-LS4-3
4. HS-LS4-4
5. HS-ETS1-2
6. HS-LS1-1
7. HS-LS1-3

## V. Time Requirements:

The unit is designed for 50-minute class periods over 10 days. The unit includes one full week of lecture, review, and practice and one day a week for the following four weeks to boost and analyze the fish. Finally, at least one full day will be dedicated to analyzing data.

## VI. Advanced Preparation:

Three stable and separate populations of zebrafish, numbering at least ten per group, should be established in the week prior to the experiment. The same should be done with a larger example set of fish if a practice group is desired.

## VII. Materials and Equipment: Whole Class Check List

Zebrafish – 3 groups of 20 Zebrafish
DNP-KLH – 5 mg DNP-KLH
Finquel/MS-222 – 100 grams
10 scalpels and blades
25 dissection pins
5 dissection scissors
Light Microscope
Capillary Tubes – Pack of 100
Wright's Stain Kit
Glass Slides – Pack of 50
Glass Coverslips – Pack of 50
1 mL syringes needle – Pack of 25
Sterile Saline – 10 mL
Air Stone & Aquarium Bubbler x 3
Larger Example Fish, such as a cichlid (optional) – 3 fish in total

## VIII. Students Prior Knowledge:

Students should have a basic understanding of the immune system. In addition, they should be familiar with bare basics of an immune response and how organisms respond to various treatments, such as vaccines.

## IX. Daily Unit Plans:

**Day One:**

Objectives:

1. The students will establish a knowledge base of immunology via pre-quiz/test.
2. The teacher will lecture over a few of the basics of immunology.
3. The teacher will explain the overall project & project goals, and the students will chose which lab group they would like to work in (bench science, analysis, writing/presenting).

Anticipatory Set:

1. Sign-in to m.socrative.com to take our immunology pre-quiz

Activities:

1. The students will complete their pre-quiz/test within 15 minutes at the start of class.
2. Lecture: The teacher will briefly review the pre-quiz questions so that the class can see who already has a background in immunology. From there, the teacher will give an introductory lecture over the specific versus non-specific immune systems.
3. The teacher will suggest some general working groups for our immunology project: Bench Science Team, Data Analysis Team, and Writing/Presentation Team. Students will select which groups they would like to prioritize, and students will be able to freely move from group to group based on their skill set and interest.

Evaluation:

The teacher will gauge the general knowledge that my students have over immunology based on their responses from the socrative pre-test.

Vocabulary:

Specific Immunity, Non-Specific Immunity, Immunology, *immunis,* Adaptive Immunity

**Day Two:**

Objectives:

1. The teacher will continue to lecture over the basics of the immune system.
2. The students will practice anaesthetizing and injecting fish, and observing their recovery in well oxygenated water.

Anticipatory Set:

1. Give a general example of a specific and non-specific reaction that your body would have to an exposure to an antigen.
2. Gather into your group(s) that were established yesterday and work on deciding on leadership roles within the group.

Activity:

1. The class will discuss Humoral versus Cell-mediated immunity, and foreign recognition (effector response and memory).
2. The teacher will demonstrate how to dose a fish with MS-222, what to look for as the fish becomes anaesthetized, and how to inject fish (using sterile blue saline).
3. The students will then split into groups and do the above themselves. They will be encouraged to help each other prior to asking the teacher for help.

Evaluation:
The teacher will observe and check to make sure that students are working and that everyone has a good grasp of the process and the goals. The teacher will take note of student leaders within each group.

Vocabulary:
Foreign recognition, effector response, memory, historic humors (yellow bile, black bile, blood, phlegm).

**Day Three:**
Objectives**:**

1. Students will practice anaesthetizing the "practice" fish for mucosal exposure, timing the rotation of the fish through the "antigen" solution, and placing the fish in a recovery tank.

Anticipatory Set:

1. What went well yesterday? What do we need to improve upon prior to beginning our actual experiment?

Activities:

1. The teacher will lecture for approximately 30 minutes; with the students assigned to write down any questions they may have as well as begin documenting a vocabulary list for our project.
2. The teacher will walk the students through anaesthetizing the fish for a mucosal exposure, and then help monitor the students as they do this procedure themselves.

Evaluation:
Homework – The students are expected to write at least 5 questions that they had during the lecture.

Vocabulary:
B cells, T cells, lymphocytes, plasma cells, antigens, antibodies, effector cells, cytokines, interleukin, leukocytes

**Day Four:**

Objectives:

1. The class will research and answer the questions the students wrote as homework from yesterday.
2. The students will practice euthanizing some sample fish, dissecting out the spleen, and drawing blood to make a blood smear.

Anticipatory Set:
Compare the questions you wrote from yesterday's lecture with those around you. As a group, come up with the top 5 questions you have.

Activity:
1. The students will ask their top questions. The students will be responsible for this material at the end of the unit.
2. The teacher will demonstrate the appropriate steps to take in euthanizing a fish, identifying and dissecting out the spleen, drawing blood using a capillary tube, and making a blood smear.

Evaluation:
1. Full participation is expected from each student.
2. For those students that do not want to participate in euthanizing and/or dissecting a fish, they will be expected to write the procedure that each small group uses to complete this step of the experiment.
3. The students will be quizzed on the answered questions from the end of class. This quiz will occur several days from now.

Vocabulary:
Louis Pasteur, *Pasteurella*, small pox, World Health Organization, specificity, APC, Ig, leukocyte, thymus

**Day Five:**

Objective:
The students will begin their zebrafish experiment.

Anticipatory Set:
Break into your working groups, put on the appropriate personal protection equipment (gloves, goggles, lab coats), and wait for further instructions.

Activity:
1. The students will split into the following groups:
    a. Control Group
    b. Injection Experimental Group
    c. Mucosal Exposure Experimental Group
    d. Scribes
    e. Data Analysis
    f. Small Dissection Team

2. Control Group:
    a. In rotations of 5 fish at a time, complete the following steps until all fish have been treated:
    b. Move 5 fish from their holding tank into a 250 mL beaker containing sufficient MS-222 at a concentration of 50 µg/L.
    c. Leave the fish in the MS-222 until fully anaesthetized.
    d. Remove each fish and hold it in a paper towel for stability and grip.
    e. Inject 50 µL of sterile saline into the abdomen of each fish.
    f. Move the fish into a recovery tank/beaker that is well oxygenated with a bubbler and air stone.
3. Injection Experimental Group:
    a. In rotations of 5 fish at a time, complete the following steps until all fish have been treated:
        i. Move 5 fish from their holding tank into a 250 mL beaker containing sufficient MS-222 at a concentration of 50 µg/L.
        ii. Leave the fish in the MS-222 until fully anaesthetized.
        iii. Remove each fish and hold it in a paper towel for stability and grip.
        iv. Inject 50 µL of 1 mg/mL DNP-KLH into the abdomen of each fish.
        v. Move the fish into a recovery tank/beaker that is well oxygenated with a bubbler and air stone.
4. Mucosal Exposure Group:
    a. In rotations of 5 fish at a time, complete the following steps until all fish have been treated:
        i. Move 5 fish from their holding tank into a 250 mL beaker containing sufficient MS-222 at a concentration of 50 µg/L.
        ii. Leave the fish in the MS-222 until they begin to lose the ability to regulate their buoyancy, but not so anaesthetized that their gills quit fluttering.
        iii. Place each fish into a solution of 35 mg/L of DNP-KLH for 15 minutes.
        iv. Move the fish into a recovery tank/beaker that is well oxygenated with a bubbler and air stone.

5. Scribes:
    a. Each group will be assigned a scribe who will write down each step and occurrence as it is done.
6. Data Analysis:
    a. The data analysis group will stain the blood slides created that week, and will work on cell counting from stained slides from the previous week.
    b. For the first week, this group will practice counting cells from the slides made during a practice round of fish (cichlids).
7. Small Dissection Team:
    a. One fish from each group will be euthanized using a solution of 500 mg/L. If the fish does not die within a few seconds, increase the dosage to shorten the time until death.

b. Cut off the tail of each fish and using a capillary tube, draw out a few drops of blood from the caudal vein.
c. Place the drops of blood onto a slide to be smeared & stained.
d. Once the blood has been removed, dissect out the spleen and place it into a 1.7 mL tube containing RNALater. Label this tube for transport and storage prior to analyzing the sample via qPCR.

Evaluation:
Full participation is required. Full clean up from each group is required.

Vocabulary:
All unit vocabulary

**Day Six:**
**(Week #1):** The steps of Day 5 should be repeated one week following Day 5 (Week 0).

**Day Seven**:
**(Week #2):** The steps of Day 5 should be repeated one week following Day 6 (Week #1)

**Day Eight**:
**(Week #3):** The steps of Day 5 should be repeated one week following Day 7 (Week #2)

**Day Nine:**
Objectives:
All experimental organisms will be euthanized for analysis.

Anticipatory Set:
Break into your working groups, put on the appropriate personal protection equipment (gloves, goggles, lab coats), and wait for further instructions.

Activity:
1. Each group will euthanize their fish one-by-one.
2. A member from the small dissection team will help members of each working group to draw blood, create a smear, and dissect out each specimen's spleen.
3. All equipment will be sterilized, and all remains of experimental organisms will be autoclaved prior to disposal.

Evaluation:
Full participation is required. Full clean up from each group is required.

Vocabulary: All Unit Vocabulary

**Day Ten:**
Objectives:

Each student will work on cell counting. They will determine a lymphocyte percentage per slide prior to each slide being reviewed by a peer.

Anticipatory Set:
Setup a light microscope at your seat, and jot down the steps for using an oil-immersion microscope for review prior to receiving your slide.

Activity:
1. Each student will setup a light microscope, and identify where the bottles of oil are located around the classroom.
2. Each student will receive a blood smear and will take note of the week and group it is from.
3. They will view the slides under oil immersion and count how many lymphocytes they find for every 100 leukocytes.
4. They will trade slides with their peers to have their data reviewed.
5. This may take more than one day.

Evaluation:
Each student will turn in a document with their data, and the name and data of the student that reviewed their slide(s).

Vocabulary:
All unit vocabulary

## X. Summative Assessment:

1. The students will present their poster and oral presentation to the science department.
2. The students will retake the pre-quiz to evaluate how much they learned from the start of the project to its end.

## XI. Additional Notes for Curriculum Developers:

Materials for this lab activity can be found through the following vendors:

- Carolina
- Flinn Scientific
- Sigma-Aldrich
- Zebrafish – Any standard retailer of fish such as PetCo, PetsMart, LiveAquaria.com, etc.

Zebrafish are used in the lab activity because they are a model organism. The fish are cheap, small, and easy to care for. As long as they are provided with enough clean and dechlorinated water the fish do not require any special foods or treatments. They can live at relatively high populations in relatively small containers.

The fish should be kept in at least three different containers/tanks prior to the lab to limit exposure to one another. It is recommended that the fish be purchased several weeks prior to the start of the experiment to adjust for any fish that may be sick when

purchased or may become sick from the stress of purchase and movement to their new tanks.

**Student Section:**

I. Rationale:

Students should be able to comprehend immunology and immune response in regards to lymphocytes. Students will understand key reactions of cells in the immune system during immune response.

Students will be able to differentiate an immune response to an antigen injected directly into the abdomen of a model organism, versus the immune response of that same model organism exposed to the same antigen via mucosal introduction.

II. Materials:

Listed Above

III. Procedure:

Specific daily experimental procedures are listed above on days 5, 9, and 10

IV. Data Collection:

Data was collected via cell counting, observing the total number of lymphocytes per 100 leukocytes visible on their slides.

qPCR data was gathered by students who were working in the Criscitiello Lab

V. Discussion/Analysis

The lymphocyte count for both the systemic exposure and the mucosal exposure to keyhole limpet hemocyanin was very similar, though significantly different than the control.

When considering the qPCR data, the IgM and IgZ response was thirty times higher in a systemic exposure test group in comparison to a mucosal exposure test group.

**Pre/Post Quiz:**

1) What is the difference between a specific and a non-specific immune response?
2) What is the difference in humoral versus cell-mediated immunity?
3) What is an effector response and how is it connected to immunological memory?
4) Describe two different types of lymphocytes.
5) How are antigens and antibodies connected?
6) Describe antibody-antigen specificity.

## Student Protocols:

### Model Organism Anaesthetizing and Practice Saline Injection:

1. Make 250 mL of 50 mg/L MS-222 solution.
2. One at a time, scoop the fish out of their holding tank using a net and place them into the MS-222 solution.
3. After several minutes the fish should lose its ability to regulate its buoyancy, and will roll over in the water.
4. Once the fish has lost its ability to regulate buoyancy, scoop the fish out of the MS-222 and place in a paper towel.
5. Gently hold the fish in the paper towel with the ventral side of the fish facing you.
6. Using the syringe filled with 1 mL of dyed blue sterile saline, gently push the needle through the scales of the fish and into its abdominal cavity. This should be done above the cloaca, but below the ribs.
7. Slowly inject 50 µL of the sterile saline into the fish. You should be able to see a color change in the abdomen of the fish if you have successfully injected the solution.
8. Gently pull the needle straight out of the fish.
9. Place the fish in a well-oxygenated tank and observe it as it recovers. The fish should be back to swimming normally within five to ten minutes.

### Control Group Weekly Protocol:

In rotations of 5 fish at a time, complete the following steps until all fish have been treated:

1. Move 5 fish from their holding tank into a 250 mL beaker containing MS-222 at a concentration of 50 mg/L.
2. After several minutes the fish should lose its ability to regulate its buoyancy, and will roll over in the water.
3. Once the fish has lost its ability to regulate buoyancy, scoop the fish out of the MS-222 and place in a paper towel.
4. Using the syringe filled with 1 mL of dyed blue sterile saline, gently push the needle through the scales of the fish and into its abdominal cavity. This should be done above the cloaca, but below the ribs.
5. Slowly inject 50 µL of the sterile saline into the fish. You should be able to see a color change in the abdomen of the fish if you have successfully injected the solution.
6. Gently pull the needle straight out of the fish.
7. Place the fish in a well-oxygenated tank and observe it as it recovers. The fish should be back to swimming normally within five to ten minutes.
8. Select at least one fish to be killed and dissected.

**Experimental Injection Group Weekly Protocol:**

In rotations of 5 fish at a time, complete the following steps until all fish have been treated:

1. Move 5 fish from their holding tank into a 250 mL beaker containing MS-222 at a concentration of 50 mg/L.
2. After several minutes the fish should lose its ability to regulate its buoyancy, and will roll over in the water.
3. Once the fish has lost its ability to regulate buoyancy, scoop the fish out of the MS-222 and place in a paper towel.
4. Using the syringe filled with 1 mL of 1 mg/mL DNP-Hemocyanin Conjugate, Keyhole Limpet, gently push the needle through the scales of the fish and into its abdominal cavity. This should be done above the cloaca, but below the ribs.
5. Slowly inject 50 µL of the 1 mg/mL DNP-Hemocyanin Conjugate, Keyhole Limpet into the fish. You should be able to see a color change in the abdomen of the fish if you have successfully injected the solution.
6. Gently pull the needle straight out of the fish.
7. Place the fish in a well-oxygenated tank and observe it as it recovers. The fish should be back to swimming normally within five to ten minutes.
8. Select at least one fish to be killed and dissected.

**Experimental Mucosal Exposure Group Weekly Protocol:**

In rotations of 5 fish at a time, complete the following steps until all fish have been treated:

1. Move 5 fish from their holding tank into a 250 mL beaker containing MS-222 at a concentration of 50 mg/L.
2. Place the fish in 250 mL of 35 mg/L DNP-Hemocyanin Conjugate, Keyhole Limpet for 15 minutes
3. Place the fish in a well-oxygenated tank and observe it as it recovers. The fish should be back to swimming normally within five to ten minutes.
4. Select at least one fish to be killed and dissected.

**Dissection Protocol:**

1. One at a time, please place the fish to be dissected into 250 mL of 500 mg/L MS-222.
2. The fish should die within seconds, and if this does not occur, increase the concentration of MS-222.
3. Once the fish has died, remove it from the solution and cut off the tail (caudal fin) of the fish.
4. Using a capillary tube, draw up a few drops of blood from the caudal vein. If only a limited amount of blood has been removed, gently squeeze the fish to push out more blood.
5. Place the drops of blood onto a glass slide, and make a blood smear by dragging a new slide across the blood.
6. Stain the slide using a Wright's Stain protocol.
7. If the lab has access to a qPCR, remove the spleen from the fish and place it in a 1.7 mL tube containing 0.5 mL of RNALater.

**Cell Counting Protocol:**

1. Setup of a light microscope with oil immersion capabilities.
2. Each student will receive a blood smear and will take note of the week and group it is from.
3. The students will view the slides under oil immersion and count how many *lymphocytes* they find for every 100 *leukocytes*.
4. They will trade slides with their peers to have their data reviewed.
5. The class can aggregate their data to find an overall lymphocyte percentage for each group for each week.

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