Objectives:
1. To explore the nature of cellular membranes by investigating environmental conditions which stress them.
2. To learn methods for measuring the extent of stress on the membranes using spectrophotometry.
3. To explore conditions that impact homeostasis in living cells.

I. BACKGROUND MATERIAL

If you have ever cooked fresh beets, you know how much beet pigment pours out of cut beets into the cooking water. The color is due to betacyanin in the cells. Similarly, the color of red onions is due to pigments (including anthocyanin) in a thin layer of cells. In both cases, the pigments are enclosed in membrane-bound vacuoles inside the cells, and we can observe membrane function by observing pigment movement. In this laboratory, we will explore the nature of cellular membranes and how environmental conditions affect their function using beets and red onions as experimental organisms.

A. MEMBRANES

Membrane: a lipid and protein bilayer, which covers all living cells, and most of their internal organelles.

Since lipids are hydrophobic, when arranged in a bilayer as in a cellular membrane they act as a barrier to prevent the mixing of neighboring water-based solutions. For instance, the cytoplasm of a cell and the fluids outside the cell are water-based solutions, so the cell membrane or plasma membrane forms a barrier between them. In other words, biological membranes maintain the interior conditions required for life inside the cell, despite the external conditions. The property of maintaining conditions agreeable to an organism is described as homeostasis. The lipid bilayer is quite thin (6-10 nm, or billionths of a meter), and is spanned by protein molecules. These proteins allow membranes to control transport of molecules into and out of cells. Inside cells, membrane-bound organelles likewise maintain distinct conditions from the remainder of the cytoplasm. Other membrane-bound proteins function as receptor molecules that detect other molecules, or cells, allowing communication with the environment or other cells.

The structure of biological membranes is the basis for their many functions. The physical and chemical integrity of a membrane is crucial for the proper functioning of the cell or organelle. Cellular membranes can only withstand a certain range of external conditions. When cells are exposed to conditions outside of that range, membrane function will breakdown and cellular function is impaired, perhaps even leading to cell death.
Environmental conditions that can stress membranes include:
1) High temperatures causing violent molecular collisions that can physically destroy a membrane.
2) Low temperatures causing water to crystallize as ice, expanding and pushing membrane components apart, often rupturing membranes.
3) High or low salinity environments altering the movement of water in and out of cells
4) High or low pH environments, disrupting the protein components of the membrane
5) Other chemicals in the environment
6) Application of physical stress, such as cutting, can also rupture membranes.
7) Organic solvents may dissolve a membrane’s lipids, in effect reducing the membrane to tatters.

**B. BEET CELLS AND BETACYANIN**

Vacuoles are fluid-filled organelles found in plant cells that generally contain solutions distinct from the remainder of the cell. We will use the vacuoles of beet cells to investigate conditions affecting membrane function. Roots of beet (*Beta vulgaris*) are red due to the water-soluble reddish pigment called betacyanin, localized almost entirely in the large central vacuoles of cells. In undamaged cells, betacyanin remains inside the vacuole, not being able to pass through the vacuolar membrane. What would happen if environmental stress impaired the function of this membrane? As environmental conditions became more severe, what would you expect to happen?

If you suspect that betacyanin might leak out of the vacuole and out of the cells you are right. Since betacyanin is a molecule that has a visible color, we can measure (quantify) how much of it is in a solution using an instrument called a spectrophotometer.

**C. SPECTROPHOTOMETRY**

*Spectrophotometer*: An instrument used to measure the relative intensities of light wavelengths in a spectrum.

Visible light is composed of colors with distinct wavelengths. Spectrophotometry is based on the principle that substances absorb light in particular wavelengths and reflect light in other wavelengths. Each substance has an “absorption signature,” measured as the amount of absorption for each wavelength. The *amount* of light absorbed is dependent upon the *concentration* (amount per unit volume) of the substance in solution, such as the pigment in the beet juice.
A spectrophotometer projects a beam of light of a particular wavelength through a solution contained in a cuvette, a test tube made of optically clear glass or plastic. The solution will absorb some of the light, and the amount absorbed is proportional to the quantity of absorbing substance present in the cuvette. This proportionality is called the Beer-Lambert Law:

$$A = Elc$$

This relationship states that the absorption (A) of light of a particular wavelength is proportional to the length of the light path through the solution (l) times the concentration (c) of the substance. The E (epsilon) in the equation is the molar extinction coefficient that is a constant for a particular wavelength and particular substance. Different substances absorb different amounts of a particular wavelength of light and therefore have different E values. Note that there is a linear relationship between absorbance and concentration. As the concentration of the absorbing substance increases, the amount of light it absorbs increases.

Figure 2: The path of light through the spectrophotometer. Note that the prism can be rotated, allowing light of different wavelength to shine through the test substance.
The “spec” is a very delicate instrument. When using a spec, please be careful and ask your TA if you encounter any difficulties! As with any delicate machine, using it well takes practice.

**Spectrophotometer instruction summary:**

*Allow spec to warm up for 30 minutes before use.*

1. Press the “A/T/C” button to change the spec between modes; absorbance, transmittance, and concentration. You want to measure absorbance, so make sure the display indicates absorbance mode.

2. Press the “nm Δ” or “nm ∇” button to select the wavelength you want to measure absorbance of.

3. Insert a cuvette filled with the “blank” solution into cell holder, and close sample door.

4. Press the “0 ABS/100% T” to zero the absorbance for the “blank” solution.

5. Remove blank and insert a cuvette filled your sample into cell holder, and close the door. Read the absorbance value on the display.

**II. A CONTEXT FOR THE EXERCISE**

Recall the steps in Scientific Process:

1. Make observations about the natural world.
2. Ask questions about, or formulate a reasonable testable hypothesis to explain observations.
3. Design and execute experiments to generate results, which could answer the question or test the hypothesis.
4. Analyze results and draw inferences regarding the veracity of the hypothesis and, if true, how it might fit with “established” knowledge.

Keep these steps in mind as you approach the analysis of any laboratory exercise.

To guide your experiments for today’s laboratory, we will provide focused observations, questions, and hypotheses for you.
In the present case we might consider:

**Observation:** Cell vacuoles are surrounded by membranes that function to control the internal environment of the cell, or help maintain homeostasis. Environmental conditions (external to the cell) may affect membrane function, and thus a cell's ability to maintain homeostasis.

**Questions:** What environmental conditions damage membrane function? Are high or low temperatures more stressful? Do chemicals in the environment affect membrane function?

**Hypotheses:** Potential answers to our questions form our testable hypotheses. We might hypothesize:
1. Temperatures approaching boiling are more damaging to cell membranes than low temperatures since plants can survive low temperatures in nature.
2. Increasing concentrations of solvents in the environment will cause increased damage to cellular membranes.

**Experiment:**
You know that you will use *spectrophotometry* and beet tissues exposed to different conditions to test your hypotheses. Betacyanin concentration in extracellular fluid will be used as the measure of membrane damage. The exact procedures you will follow to test these hypotheses are in section III.

**Predictions:**
Once you have an experimental protocol, you should make predictions of what you expect to find if your hypothesis is correct. For instance, if hypothesis 1 is correct, you might predict that more betacyanin will leak out of the cells that are exposed to high temperatures, and the spectrophotometer will record an increased absorption of non-red light (why not red light?). What would you expect for results if hypothesis 2 is correct?

**Results:** You should record your results in self-explanatory tables and graphs and then analyze them to determine if your predictions were correct, and your data support or reject your hypotheses (match or are different from your predictions). What inferences can you make from your own results and from compiled results among your fellow students?

**III. METHODS**

*Work in groups of four or as directed by your TA.*

These procedures will guide you through an experiment to test the hypotheses given, and also teach you methods that are used to conduct experiments like this by scientists. Pay attention to what you are doing, and think about what and why you are doing it. Otherwise, it’s just a waste of time!
A. Preparation

1. Prepare nine uniform beet cores using a cork borer with an 8 mm inside diameter, and trim each core to 15 mm in length. Four of these cores will be used for the temperature-related treatments, and five will be used for the solvent treatments.

2. Place cores in a beaker and rinse with room temperature tap water for 2 minutes to remove betacyanin that has leaked from damaged (cut) cells.

3. Place one core in each of nine capped vials labeled appropriately.

B. Temperature-related treatments

1. Place a test tube in the ice bath (0° C) several minutes before you add the beet core so it is at the correct temperature. Once you add the core, keep it in the cold treatment for 30 minutes.

2. While the cold tube is incubating, start the hot treatments. For each of the 3 hot treatments (20° C, 60° C and 100° C) remove the beet core from its vial, and place it in a separate beaker of water at appropriate temperature.

3. Keep each core in the individual hot treatment beakers for 1 minute.

4. Remove each beet core from the treatment beaker in the hot baths carefully with forceps, being sure not to squeeze the core. Return the beet cores to their respective vials, and add 20 ml room temperature tap water to each tube.

5. After 30 minutes, remove the beet core from the cold-treatment tube, place it in the appropriate vial, and add 20 ml of room temperature tap water.

6. Keep all four temperature-treatment beet cores in their appropriate vials for 20 minutes, allowing any betacyanin to leak out into the water. Gently swirl each vial occasionally.

7. Quantify the amount of leaked betacyanin for each treatment by filling a cuvette with the solution, and using the spectrophotometer to measure the absorbance at 460 nm, the wavelength at which betacyanin absorbance is the highest. Make sure to calibrate or “blank the spec” first with a cuvette of tap water by pressing the button marked 0 ABS/100%T.

8. List your results in a neat, legible table, and graph your results with temperature as the independent variable on the x-axis and absorbance as the dependent variable on the y-axis.
B. Solvent-related treatments

1. Using the remaining five vials with beet cores, add 20 ml of the following solutions of solvents to each vial:
   - Tap water
   - 25% acetone
   - 50% acetone
   - 25% Isopropanol
   - 50% Isopropanol

2. Cap each vial, and keep them at room temperature for 20 minutes. Gently swirl each vial occasionally.

3. After 20 minutes, remove each core from its treatment.

4. Again, quantify your results for each treatment by placing a cuvette of each solution in a spectrophotometer and measuring absorbance at 460 nm. For measurements of solutions of acetone and Isopropanol, you will still “blank the spec” using tap water first.

5. List your results in a clear, legible table, and graph your results with solvent concentrations as the independent variable on the x-axis and absorbance as the dependant variable on the y-axis.

IV. THOUGHT QUESTIONS

1. Compare your results to other lab groups. Do your individual results support or fail to support your hypothesis? Do the compiled results from the entire class support or fail to support the hypotheses?

2. Which treatment damaged the membranes the most? The least? Provide data to support your answer.

3. Relate the results of these experiments to the normal practices of freezing and cooking foods.

4. Many plants, such as trees, over winter in sub-freezing temperatures, without damage to cells. How might plant cells protect themselves from ice damage?

V. CELLULAR HOMEOSTASIS

In this experiment, you will use tissue from a red onion to observe the effects of a different environmental condition on an individual cell’s ability to maintain homeostasis; in this case its ability to control the movement of water into and out of the cell. Let’s put this into the same terms as our previous experiments.
**Observation:** Cells are surrounded by membranes that function to control the internal environment of the cell, or help maintain homeostasis. This includes moderating the movement of water and salts in and out of the cell.

**Questions:** What happens to cells when exposed to salty water? Can we observe changes in water movement?

**Hypotheses:** When scientists investigate a new problem, they often start with observing changes in response to the environment prior to developing a hypothesis, experimental protocol, and predictions. In this case, we will make changes to the environment of the onion cells and see what happens. These initial observations can then be used to develop testable hypotheses and experimental protocols, which is how science improves our understanding of the natural world.

We can consider onions to be “fresh water” organisms since they live in soil wet with fresh water (rain) not salt water, like a marsh. Knowing this, we might wonder:

1. What happens to onion cells exposed to salt water?
2. What happens if we expose onion cells to distilled water?
3. Can we actually observe a single cell as it tries to maintain homeostasis?

First, a little background. Cell membranes have been found to be semi-permeable. They allow some molecules to pass, and restrict the movement of others. They are selective by size and charge of the molecule thereby permitting hydrophobic (uncharged) but not hydrophilic (charged) molecules or ions to pass. When salt (NaCl) is put into solution with water, it separates into 2 charged atoms one of Na\(^+\) and one of Cl\(^-\).

**Experiment:**
Play with some onion cells and salt water, and see what happens when the conditions are changed! (See the procedure below).

**Results:** What did you observe?

**Experimental procedure: Onion cells**

1. Cut a small square out of a layer of the red onion (1-2 cm\(^2\)).
2. Slice off/peel the thin red layer on the outside of the square using a razor. If you are diligent you should be able to peel the layer off. If you resort to cutting, the thinner the layer, the better, so take your time.
3. Place the sample on a slide and try to keep it from folding up. Placing a cover slip over the sample will help.
4. Place the slide on the microscope and focus on the layer of cells that are filled with red pigment. Try to find an area where you can clearly see individual cells. Make a new slide if you need too!
5. Draw the cellular structure of these cells in your notes (note your magnification). If you look closely, you can see cell nuclei too.
6. Without removing the slide from the scope, drip a drop or two of 15%NaCl onto the edge of the cover slip. It should be pulled under the coverslip by capillary action, and cover your onion layer.
7. Continue to watch the cells for the next few minutes. You may want to move your slide around to see other areas of the slide since some parts of the tissue may react differently. Record your observations!

8. If you successfully observed interesting changes in an area of cells, now let’s try some distilled water. Add a few drops to the side of the coverslip again. Now apply the edge of a Kimwipe to the opposite side of the coverslip carefully so that you don’t move the coverslip. You should be able to draw the distilled water under the coverslip, flushing out the salt water you added earlier. Repeat the process with another application of distilled water. What do you observe?

Questions:
1. Did the cells gain, lose, or maintain volume in each solution?

2. What do the results of these experiments tell you about the cellular membrane?

3. Can you develop a hypothesis based on these observations?

4. What do you think would happen to a single-celled freshwater organism if it were suddenly thrown in the ocean? Refer to experimental evidence to support your answer.

5. Early life evolved in the sea before moving onto land. What are the challenges faced by a single-celled aquatic organism that tries to live on land? Please explain with reference to the following terms: osmosis, diffusion, salt concentration, and semi-permeable membrane.