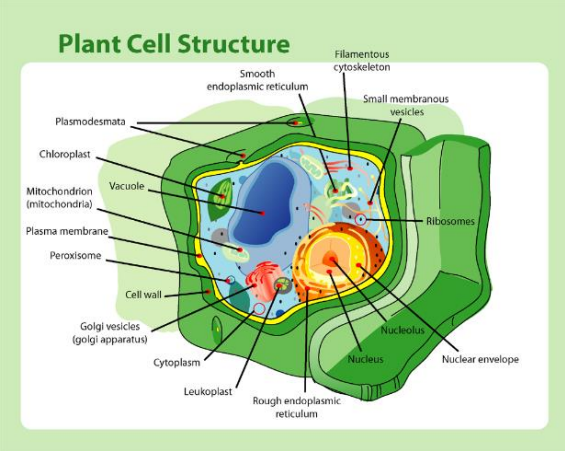
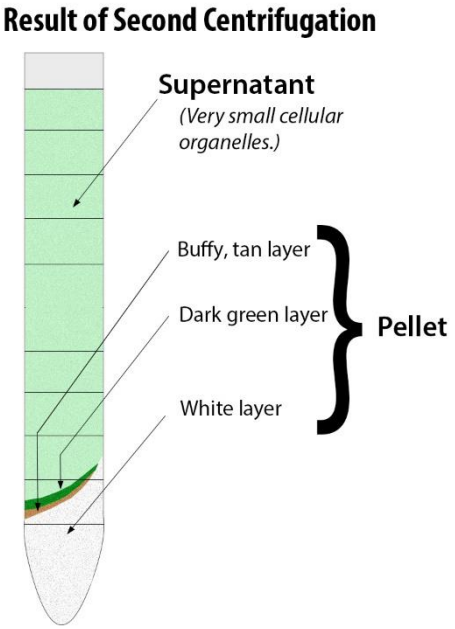
Cellular Respiration: Peas

This activity uses germinating pea seeds. Seeds are the 'offspring' of plants. While to most of us they look relatively inert or dead, seeds are actually viable organisms. Because seeds are generally NOT photosynthetic they must acquire the energy needed for growth and development from stored molecules like proteins, oils and carbohydrates. Under less than favorable conditions, they metabolize at a very low rate, but they do metabolize including generating ATP via aerobic cellular respiration. Once the seed is hydrated (imbibition), taken in water, germination begins. Germination includes the production and activity of many enzymes. This is a period of astounding growth for the newly developing plant.



The image to the left is a generic plant cell. Plant cells contain most of the same organelles as animal cells. Plant cells also contain some organelles that are only found in plants such as the chloroplast and central vacuole. When biologists study the function of organelles they can study the organelle *in situ* or in place. However just as often they want to study the action or manipulate a particular organelle or structure without the clutter of the rest of the cell components. In this latter case, the biologist may fractionate the cells to separate the organelle of interest from the rest of the cell contents or to simply enrich a sample with the organelle of interest.

Fractionation of cells begins with macerating ('chewing') the tissues, then filtering the macerated slurry to remove large particles and finally differential centrifugation to separate cell components. Maceration is frequently done with a blender. It breaks open cells and releases the components of the cytoplasm into the solution. The macerating solution is isotonic and pH balanced to the contents of the cell to protect the cell organelles from lysis. Filtration is usually accomplished by pouring the macerated solution through cheesecloth. The filtered cell extract is then poured into centrifuge tubes and centrifuged. Centrifuges are machines that spin the sample. As the centrifuge spins particles in the cell extract settle to the bottom of the centrifuge tube. At slow centrifuge speeds large particles settle to the bottom of the tube. As the centrifuge speed, measured in RPMs increases, smaller and smaller particles settle to the bottom of the tube. Differential centrifugation is the technique where more than one speed is used to fractionate a sample. So when examining a centrifuge tube, the largest components settle to the bottom first. The components that centrifuge to the bottom of the tube make up the semi-solid pellet. The liquid cell extract above the pellet contains the smallest cellular organelles and molecules. This liquid portion of the tube is called the supernatant.



In this activity you will be fractionating cells from pea seeds that have been hydrated over night to begin germination and identifying the components of different fractions in the centrifuge tube. You will be testing fractions of pea extract for the presence of mitochondrial activity and the presence of starch. Iodine will be used to indicate the presence of starch. 2,3,5 Triphenyl tetrazolium chloride is one member of a family of molecules called tetrazolium. Tetrazolium compounds when dissolved in water are colorless. If they become reduced by acquiring hydrogen ions and electrons, they turn colors. 2,3,5 Triphenyl tetrazolium chloride when reduced turns a red color, strawberry red. So where can tetrazolium acquire hydrogen ions and electrons? Tetrazolium intercepts hydrogen ions and electrons that are stripped from glucose during aerobic cellular respiration. Hydrogen ions and electrons removed during the Kreb's cycle would normally be transported by carrier molecules such as NAD^+ or FADH, however when tetrazolium is present it intercepts these ions and electrons. A red color in a tissue when exposed to tetrazolium is indirect evidence of functioning mitochondria. Why do we consider this indirect evidence and not direct evidence?

Materials

Conical centrifuge tubes – 4, 15 mL
Centrifuge - clinical
Sucrose buffer
Iodine
Pipette – Pasteur - 4
Marker
Microscope - 2
Microscope slide - 2
Microscope coverslip - 2
Beaker – 250 mL, 2
Beaker – 50 mL, 1
Graduated cylinder – 100 mL
Graduated cylinder – 25 mL
Cheesecloth
Peas – hydrated, 24 hours
Dixie cup, small disposable cup
Glass rod
Cleaning brushes
Test tube rack
Test tubes – 3
Iodine (optional)
2,3,5 Triphenyl tetrazolium chloride – 1.5 %

Procedure

1. Dispense 90 mL of sucrose buffer into the graduated cylinder (100 mL).

2. Fill a Dixie cup with peas from the common supply table. Invert the cup to pour out any excess water.

3. Pour the sucrose buffer and peas into the blender. Blend for 5 minutes. Because of the limited number of blenders available, your instructor may ask groups to combine samples.

4. Pick up 4 centrifuge tubes, 2 beakers and cheesecloth from the supply bench.

5. Pour ~ 100 mL of pea juice into a beaker and return to your bench.

6. Place 4 layers of cheesecloth over the top of the remaining empty 250 mL beaker. Slowly pour the pea juice onto the cheesecloth. Use the glass rod to stir the slurry in the cheesecloth. Once all of the extract has filtered through the cheesecloth, throw away the used cheesecloth.

7. Pour the pea extract into 3 of the 4 conical centrifuge tubes. Fill the tubes to the 14 mL level. Return any remaining extract to your instructor.

8. Fill the 4th conical centrifuge tube to the 14 mL mark with water.

9. Place the 3 centrifuge tubes containing pea extract into the centrifuge. Once the centrifuge is full your instructor will spin the samples at 1500 RPM for 5 minutes.

10. After the 5 minute spin, remove one centrifuge tube and replace it with the centrifuge tube containing water from your bench. Your instructor will restart the centrifuge for 20 minutes at 3500 RPM.

11. While the centrifuge is spinning, examine the low speed spin tube that you removed. What do you see? _____

Draw the tube below or insert a photograph.

12. Pinch the bulb of the Pasteur pipette and insert the pipette into the white pellet at the bottom of the tube. Release the bulb. Some of the white materials should now be in the pipette. Replace the centrifuge tube in the test tube rack.

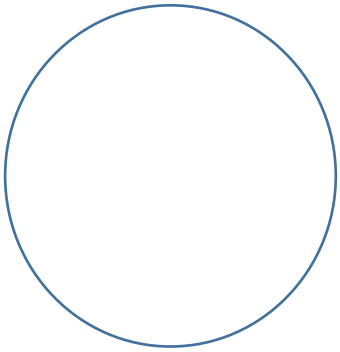
13. Place a microscope slide on your desk. Add a drop of the white material in the pipette to the slide.

14. Cover the drop with a coverslip. Add a drop of iodine to the slide, next to the coverslip. Draw the iodine across the slide by placing a paper towel on the opposite side of the coverslip.

15. Place the microscope slide on the stage of the microscope. Examine the slide, begin with the 4X objective and increase magnification until you reach 400X. What do you see? Draw the field of view below. Label your drawing. What is the composition of the white material? _____

White material of the pellet, stained with iodine.

Magnification: _____



16. At the completion of the centrifugation, remove **all 3** (2 tubes containing extract, 1 tube containing water) of your centrifuge tubes and return to your bench. Place the tubes in the test tube rack at the bench.

17. Each tube should contain a pellet with three distinct layers. The bottom layer is white, the layer above this is a buffy tan color and the upper most layer of the pellet should be a dark green. The supernatant above the pellet is a lighter green color. Draw or insert a photograph of one of your centrifuge tubes below. Label the supernatant and the individual layers of the pellet.

18. Carefully pour the supernatant from 1 centrifuge tube into the 25 mL beaker. Do not disturb the pellet. Return the centrifuge tube to the test tube rack.

19. Pick up 3 test tubes (15 x 150 mm) from the supply table. Label the tubes as follows: buffer, supernatant, pellet.

20. Use the graduated cylinder to dispense the following solutions into the test tubes. Add 5 mL of sucrose buffer to the test tube labeled 'buffer'. Pour 5 mL of the supernatant in the beaker into the test tube labeled 'supernatant'. Rinse the graduated cylinder to remove the supernatant.

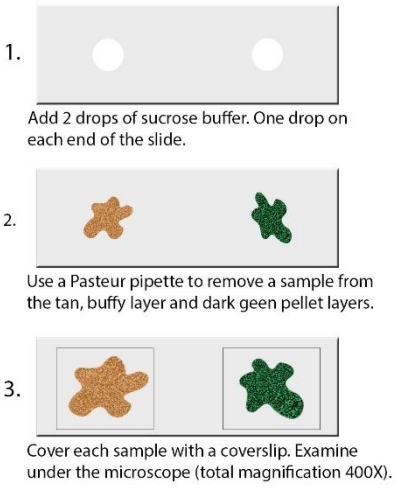
21. Pour out any remaining supernatant from the centrifuge tube used in step 18. Add 5 mL of sucrose buffer to the pellet in the centrifuge tube. Stir the pellet with a glass rod to re-suspend the pellet in the sucrose buffer. Pour 5 mL of this suspension into the test tube labeled 'pellet'. Wash the graduated cylinder.

22. Add 5 mL of tetrazolium to each tube. Swirl gently. Tetrazolium degrades quickly. You instructor will not make the solution until everyone in the class is ready to perform the test.

23. Place the test tubes in a warm water bath (40^{o} C) for 30-45 minutes or until color development occurs. While the test tubes are incubating in the water bath continue with the microscopic examination of the pellet layers.

24. Use one slide to examine the contents of both layers (buffy brown layer and green layer). Add one drop of sucrose buffer to one end of a slide and one drop to the other end of the slide (see below). Use a Pasteur pipette to sample the green layer. To sample a layer, squeeze the pipette bulb, insert the tip of the pipette into the green layer and gently partially release the bulb. You only need a small sample, so release the pipette bulb only enough to pull some of the green layer up into the thin end of the pipette. Once you have the sample, place the tip of the pipette over a sucrose drop on the slide. Squeeze the bulb to add the sample to the drop.

Microscopic Examination of Pellet Layers



25. Use a new pipette to sample the tan, buffy layer. Follow the procedure given in the previous step.

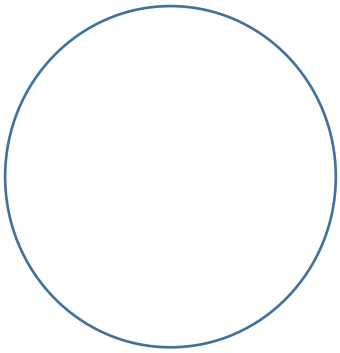
26. Place a coverslip over each drop. **Optional: While staining is not necessary, it may add some additional contrast to the specimen. Iodine can be used as a stain to enhance the specimen contrast.

27. Place the slide on the microscope. Start your examination with the 4X objective. Increase magnification until the total magnification of the specimen is magnified 400 X. Can you identify the predominant organelle in each sample? What organelle do you observe in the dark green layer? _____ Which organelle is present in the tan, buffy layer? _____ Draw what you see under the microscope in the circles below.

Green material of the pellet.

Magnification: _____

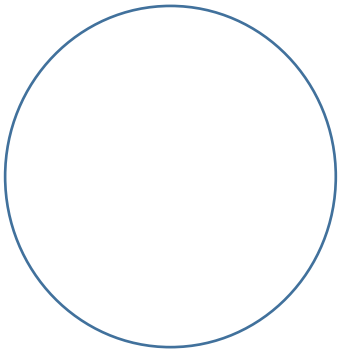
Identification of organelles: _____



Brown material of the pellet.

Magnification: _____

Identification of organelles: _____



28. Once you observe red color in your test tubes you can remove them from the water bath and place them in the test tube rack on the bench. Record your results below.

Test tube contents	Color of tube after incubation with tetrazolium	Are mitochondria present?
Sucrose buffer only		
Supernatant		
Pellet		

29. Clean and return all materials to the supply table. Special conical brushes have been provided for cleaning centrifuge tubes.

There are 2 reasons sucrose buffer was used in macerating the peas in this experiment. What are they? (Hint: Consider the process being studied.)

Which centrifuge fraction contained mitochondria? How do you know?

How does tetrazolium function in this experiment?

Why is a positive reaction with tetrazolium considered **indirect** evidence for the presence of mitochondrion?

List in order from heaviest to lightest the identifiable cell components from the second centrifugation tube.

Would this activity have worked if distilled water were used instead of sucrose buffer? Why or why not?