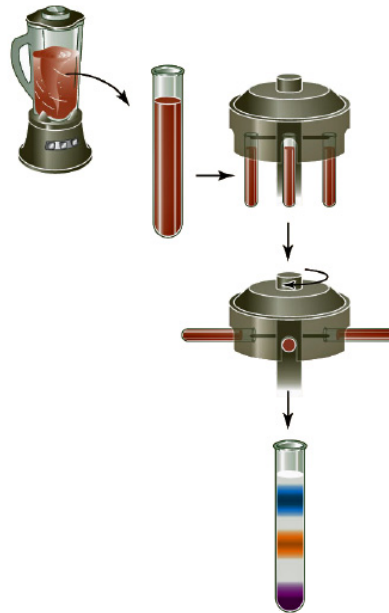


Cell Fractionation

The eukaryotic cell contains many organelles, each of which performs one of more specialized functions, suspended within the cytoplasm and bounded by the plasma membrane. Each of the cell components differs in size, shape, density and chemical composition as well as function. Because of these different features, individual types of organelles can be isolated from cells and studied. One technique for isolating organelles is to homogenize cells in a blender. Homogenization ruptures the cells freeing many of the organelles. To preserve the viability of the organelles cells are homogenized in a phosphate buffered sucrose solution. Under carefully controlled conditions organelles can continue to perform their functions outside of the cell for some time.

In order to study specific organelles, homogenization is followed by some procedure that can isolate one type of organelle from the others. The technique utilized in this laboratory is **differential centrifugation**, a process by which homogenized cells are centrifuged at increasingly higher speeds and for increasingly longer periods of time. Centrifugation tends to isolate the cellular components in order of density and, to some extent, size. The densest cell components and cell fragments settle out as a **residue** during the first centrifuging. Less dense organelles remain suspended in the buffer solution as the **supernatant**. With subsequent centrifugings, more and more organelles settle in layers in the residue, until only the least dense organelles remain in the supernatant.



Materials Required For the Entire Class:

- Blender
- 2 or 3 Fresh spinach leaves
- About 1/2 cup pea seeds (Fresh, frozen or dry peas that have been soaked for several hours)
- 1.5 Liters cold sucrose phosphate buffer
- Note: The buffer should be kept cold during the laboratory period.
- Large funnel
- Several layers of cheesecloth
- 1000-ml Beaker
- Crushed ice
- 7 Multi-speed centrifuges
- 38° C Hot-water bath

Materials For Each Laboratory Table

4 Identical centrifuge tubes
6 Test tubes
2 Test tube racks
China marker (grease pencil)
2 Capillary pipettes
2 10-ml Graduated cylinders
4 Microscope slides and cover slips
50-ml Beaker of homogenized filtrate
100-ml Beaker for sucrose phosphate buffer
Large culture dish filled with crushed ice

Dropper bottles of:
Distilled water
Methylene blue, dissolved in water
Tetrazolium
Iodine
Janus green
Vegetable oil

Preparation of the Homogenate (This will be done in advance or by the lab staff, your instructor or by student volunteers)

- Place the soaked pea seeds, spinach leaves and 400 ml cold sucrose phosphate buffer in a blender and blend at high speed for 1 minute.
- Line the large funnel with several layers of cheesecloth and place the funnel in the 1000 ml beaker, which has been placed in a container of ice. Pour the spinach/pea "juice" through the cheesecloth filter.
- The **filtrate**, comprised of the smaller cell fragments and organelles, passes through the cheesecloth.
- Pieces of pea seed coats, spinach leaf fragments and cell and tissue aggregates will collect in the cheesecloth. These form the initial **residue**. *Set the residue aside for later examination.*

After the filtration has been completed, one student from each lab table should obtain 50 ml of filtrate for use during the laboratory exercise. Keep the filtrate on ice during the exercise whenever possible. Each laboratory table will complete the following steps.

Exercise I: Separation of Cellular Components by Differential Centrifugation

Although the procedure outlined for this laboratory exercise may seem tedious, the steps should be followed exactly as written. Suggestions for improvement are always welcome.

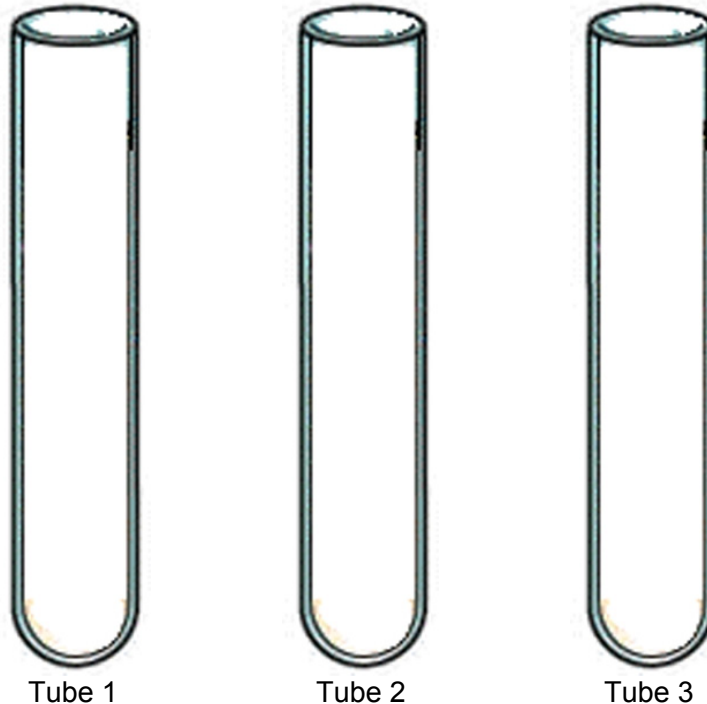
A: First Centrifugation

1. Label your centrifuge tubes "1", "2", "3" and "4".
2. Agitate your small beaker of filtrate to suspend the solid material.
3. Pour filtrate into the centrifuge tubes labeled "1" and "2". Each tube should be 3/4 full. The amount is not critical; however, the fluid in the 2 tubes must be at exactly the same level in order to keep the centrifuge balanced.
4. Place your 2 tubes in opposite holes of a centrifuge. Centrifuges are located on the side counters. Note which positions you have used, since other student groups will be using the centrifuge at the same time. Do not start the centrifuge until the other student groups sharing your centrifuge are also ready.
5. When all the tubes are in the centrifuge, close the top and set the centrifuge to slow speed (1) if a speed control is available.
6. Centrifuge for 3 minutes at the slow setting or 1 minute if no speed control is available. Allow the centrifuge to slow naturally.
7. Remove your 2 tubes and return to your lab table.
8. Using a capillary pipette, transfer the supernatant (the fluid portion) from tube 2 into the centrifuge tube labeled "3".
9. **Do not disturb the residue remaining in tube 2. You will use it later in the laboratory.**

B: Second Centrifugation

1. Compare the volume of fluid in tube 3 with the volume of tube 1. Adjust the volumes with additional sucrose phosphate buffer if needed so the 2 tubes are at the same level.
2. Place tubes 1 and 3 in opposing holes of the centrifuge, and, when the centrifuge is full, close the top.
3. Set the centrifuge for medium speed (2) if a speed control is available.
4. Centrifuge for 8 minutes at medium speed or 3 minutes if no speed control allowing the centrifuge to slow naturally when finished.
5. Remove centrifuge tubes 1 and 3 from the centrifuge and return to your lab table.
6. Compare the sediment (residue) layers in tubes 1, 2 and 3.
7. **After diagramming the layers in each of the three tubes, set tubes 2 and 3 aside for later study.**

Are the layers in tube 3 the same as in the first 2 tubes, or is one or more layers reduced or absent? Why?



C: Third Centrifugation

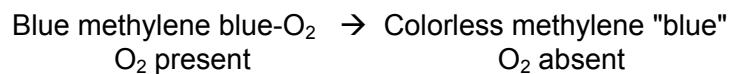
1. Transfer the supernatant of tube 1 to a 10-ml graduated cylinder using a capillary pipette. **Save this supernatant for later.**
2. Add fresh buffer to the residue in tube 1 so that it is 3/4 full. Agitate the contents thoroughly. You may have to loosen the residue from the bottom of the tube with the capillary pipette.
3. Fill centrifuge tube 4 with buffer so that it has exactly the same level of fluid as tube 1.
4. Place tubes 1 and 4 in the centrifuge in opposing holes.
5. Centrifuge for 7 minutes at medium-high speed (4) or 5 minutes if no speed control. Allow the centrifuge to stop naturally.
6. Take tubes 1 and 4 back to your table.

Exercise II: Mitochondria Test

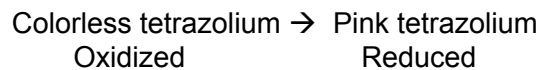
Only the larger of the cell's organelles are readily visible with the compound light microscope. However, many organelles can be detected by chemical means. Since mitochondria are the primary sites for aerobic cell respiration, they can be detected by a variety of methods. We shall use two chemical tests that are capable of detecting the presence of mitochondria in a solution.

Methylene blue and tetrazolium show characteristic color changes in specific chemical "circumstances" that occur during aerobic cell respiration so both can be used to determine the presence of mitochondria in various cell fractions.

Oxygen is consumed during cell respiration. **Methylene blue** is colorless in the absence of oxygen. If mitochondria are mixed with a non-toxic methylene blue solution in a container where additional oxygen is excluded, the methylene blue will turn colorless as the mitochondria consume the existing oxygen within the container as they do aerobic respiration.



Tetrazolium is an indicator of oxidation-reduction reactions (those chemical reactions where electrons are lost and gained). The respiratory enzymes in the mitochondria mediate a series of oxidation-reduction chemical reactions. If mitochondria are in a solution with tetrazolium, the normally colorless oxidized form of tetrazolium will get reduced, turning pink to red indicating the occurrence of the oxidation-reduction reactions of the respiring mitochondria.



Preparing the solutions

1. Remove and discard the supernatant from centrifuge tube 1. Add 6 ml of cold sucrose phosphate buffer to the residue in tube 1 and agitate. If needed, loosen the residue with a pipette.
2. Retrieve the 10-ml graduated cylinder of supernatant from tube 1 that you saved and set aside while preparing for the third centrifugation in Exercise 1C
3. Label 6 test tubes from 1 to 6 and with some identifying mark for your table. Mark the tubes at the top because they will be placed in a hot-water bath.
4. Set up your mitochondria test according to the following directions in the table below. Add the substances listed for each of the test tubes. Be sure that you rinse the graduated cylinder that you use for measuring your liquids after each use.

Tube #	Buffer	Suspended Residue (#1)	Supernatant (#2)	Methylene Blue	Tetrazolium
1	6 ml			Several Drops*	
2	3 ml				3 ml
3	3 ml	3 ml		Several Drops*	
4		3 ml			3 ml
5	3 ml		3 ml	Several Drops*	
6			3 ml		3 ml

* The 3 tubes with Methylene blue should be about the same shade of blue.

5. Agitate all tubes.
6. Add 1 – 2 ml thick film of oil to the surface of the tubes that contain Methylene blue (tubes 1, 3 and 5).

7. Place the 6 test tubes in the 38° C hot water bath. The hot water bath will have racks to hold your test tubes. Leave your test tubes in the hot water bath until the next class period.
8. At the beginning of the next class period remove the 6 test tubes from the hot water bath. Record your observations and conclusions on the table below. When you have recorded your results clean your test tubes and place them in the appropriate container.

Mitochondria Test Results

Tube #	Contents	Observations
1	Methylene Blue/Control	
2	Tetrazolium/Control	
3	Methylene Blue/Residue	
4	Tetrazolium/Residue	
5	Methylene Blue/Supernatant	
6	Tetrazolium/Supernatant	

In which tubes did you expect to find evidence of mitochondria?

Which of the test tubes showed evidence of mitochondria?

Why did you place oil on the surface of the test tubes containing methylene blue?

Exercise III: Observing the Cell Components

The cell components to be observed in this exercise, ideally in isolation, are structures observed in previous laboratory exercises and should be readily identifiable. If necessary, refer to your notes from earlier laboratory work to help identify them. **Draw and label the cell components you observe in each of the four slides.**

You may observe the cell components in this exercise at any time they are available during the laboratory period. However, be sure you allow time to set up exercise II prior to the completion of all observations.

A. Wet mount of the original cheesecloth residue

Make a wet mount of the original residue that remained in the cheesecloth after filtration. Examine the slide first with low power and then with the high power objective. What cell structures are present?

Add a drop of iodine to the edge of your coverslip. What cell components have been stained a "blue-black" with the iodine

B. Wet mount of the white residue from Exercise I, tube 2

Use a capillary pipette to remove a drop of the white residue from the bottom of tube. Make a wet mount of this residue and stain it with iodine. Examine the slide first with low power and then with the high power objective. What cell components are most abundant?

How does the size of these components compare with the size of those in the previous slide of the cheesecloth residue?

Are any other cell structures present?

C. Wet mount of the green residue from Exercise I, tube 3

Carefully remove a drop of the green layer of residue from tube 3. Make a wet mount of this layer and examine it with your microscope, using low power to locate and then the high power objective for detail.

Note the aggregates of green bodies. What are these?

If the nuclei have not been destroyed by the centrifuging you may also see some of these rather large, round and grayish structures.

Add a drop of iodine to the side of the coverslip. If amyloplasts are present in this layer they should now be conspicuous. How does the size of the amyloplasts in this layer compare to those seen in previous slides?

D. Wet mount of the supernatant from Exercise I, tube 3

Make your fourth wet mount using the supernatant from tube 3. Add a drop of Janus green stain to this wet mount. Again, observe first with low power and then focus with your high power objective.

You should see clumps of darkly stained specks. They are mitochondria. The chemical tests in Exercise II are being done to confirm this.