**Procedure for chloroplast isolation:**

Keep the tissue and all fractions ice cold throughout steps 1 - 6 and 10.

1. Obtain 8 grams of de-veined leaf tissue rinsed in ice water, blotted and cut into pieces about 1 cm square.
2. Place the leaf pieces in a pre-chilled blender cup containing 40 ml of ice-cold 0.5 M sucrose. Blend for 15 sec. at top speed, pause about 10 sec., then blend again for 10 sec.
3. Remove the ice from the 100-ml beaker, then squeeze the leaf homogenate through four layers of pre-chilled cheesecloth into the cold beaker by twisting the top corners of the cloth around each other.
4. Pour 14 ml of the homogenate into each of two centrifuge tubes and centrifuge at 200g for 5 min.
5. Using a Pasteur pipet, transfer each supernatant (containing the chloroplasts) to a second centrifuge tube and centrifuge at 1000g for 7 min. (Save the pipet.)
6. Using the pipet, discard the supernatant but be careful not to disturb the pellet. Pour 2 ml of phosphate buffer onto the pellet and gently resuspend it by moving it up and down in the pipet. Using a clean Pasteur pipet, add buffer until you have a total volume of 8 ml and mix the diluted suspension using the pipet.

**This is your chloroplast suspension. You should examine it in the microscope.**

Use the suspension for steps 7, 8, and 9, and then prepare the chloroplasts for storage in step 10.

1. Estimation of chlorophyll a concentration of the suspension.

a. Measure 4.75 ml of 80% acetone into a 13 x 100 mm tube.

b. Add 0.25 ml of chloroplast suspension, mix well, and read the Absorbance at 652 nm against 80% acetone as the reference blank.

8. Determination of chloroplast concentration of the suspension.

a. Measure 4.75 ml of the phosphate buffer into a 13 x 100 mm tube, and add 0.25 ml of chloroplast suspension, and mix well.

b. Prepare the clean, dry hemacytometer with a cover slip in place supported by the frosted-glass shoulders of the chamber.

c. Making certain that the chloroplasts are evenly suspended (not settled, not clumped), take up some of the suspension into a clean Pasteur pipet; let part of a droplet from the pipet tip flow under the cover slip of the chamber. When properly delivered, the liquid fills the space between cover slip and etched surface of the chamber and does not overflow into the side troughs beneath the cover slip. (Hint: have a tissue ready, folded to a point; if the chamber fills, but there is still a droplet standing at the side of the cover slip, pull it up gently into the point of the tissue, taking care not to pull any liquid from under the cover slip.) If overflow nevertheless occurs, clean and dry the chamber again and cover slip, wiping finally with alcohol and a Kim-wipe, and begin again with another sample of suspension.

d. Using the high-dry (40x) objective, count the total number of chloroplasts in the large central square of the counting chamber - the square that is bounded by a triple-line border and is itself subdivided into 20 sets of 16 very small squares.

9. In vivo absorption spectrum. Using the dilution of chloroplasts in buffer prepared in step 8, and distilled water as the reference blank, record the absorption spectrum at 10-nm intervals between 400 and 700 nm. When an absorption peak appears, read Absorbance at 5-nm intervals near the peak to determine its location more precisely. Observe the suspension visually at frequent intervals to be sure that the chloroplasts are not settling. If necessary, mix by inversion against Parafilm in order to keep the chloroplasts as evenly suspended as possible.

10. Storage of the chloroplast suspension. Centrifuge the remainder of the suspension at 1000g for 7 min. Using a Pasteur pipet, aspirate and discard the supernatant. Submit the chloroplast sediment to the instructor for storage (frozen) at - 20oC for use next week. Note and record the color of the pellet.