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Throwing the Dice: Teaching the Hemocytometer

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One of the concepts taught to our science students is the use of the hemocytometer. Students in Microbiology, Genetics, and Anatomy and Physiology classes use the hemocytometer in a variety of activities, from quantifying yeast cells to counting white blood cells. Students do not always understand that cells on a hemocytometer are in a three-dimensional volume. While they clearly see on the slide a two-dimensional square with measurable length and width, they do not perceive that use of the cover slip adds height, the third dimension. They also do not always grasp the concept that the number of cells counted on the hemocytometer represents only a fraction of the total number of cells in a milliliter and that the number determined by counting on the hemocytometer can be used to estimate the final cell count in a larger volume. In this workshop we present a short lab activity in which students use dice and rulers to understand the hemocytometer. We then present an activity in which students apply their newfound knowledge of the hemacytometer to quantify the number of chloroplasts and thus the amount of chlorophyll in spinach leaves.

Keywords: hemocytometer, dice, spinach, chloroplast, chlorophyll, cell counting

Introduction

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Student Outline

Part I: Teaching the Hemocytometer Using Dice

The Concept

- The black dots on the dice represent cells
- Students count the dots on one die (21 dots)
- They measure the dimensions of the die and calculate the volume (4,096 mm^3)
- They are given the dimensions of the cardboard box (which must be 48 mm in each dimension for the math on this worksheet to work) and calculate its volume (110,592 mm^3)
- They calculate how many dice can fit into the cardboard box using their determined dimensions (110,592 $mm^3 \div$ 4,096 mm^3 = 27)
- They then predict how many "cells" would fit in the cardboard box, knowing that one die has 21 "cells" (27 x 21 = 567)
- They then fit the dice into the box confirming their earlier calculation (27 dice fit).
- They count the dots on the 27 dice (or just multiply 27 x 21) to see if their earlier prediction is correct.
- They then apply this thinking to the hemocytometer
- This workshop has been recently published in the July/August 2010 issue of the *Journal of College Science Teaching,* pg 64, under the same title (Authors Salm, Goodwyn, van Loon and Lind).

Part II: Using the Hemocytometer to Count Chloroplasts in Spinach Leaves

Introduction

The green color of many plant organs (primarily leaves and stems) is due to the presence of the green pigments chlorophyll *a* and chlorophyll *b* in the subcellular organelles called chloroplasts. The remainder of a plant cell is typically colorless. The green organelles lie free in the cytoplasm of the cell, unattached to other cellular components such as the cell wall, the plasma membrane, the nucleus, and the mitochondria. When the cell wall is disrupted, the plasma membrane breaks, and the subcellular components are released as separate particles of various sizes and densities.

In this laboratory, the cells of spinach leaves will be disrupted, freeing the untethered organelles, which can then be sorted out from each other by filtration and differential centrifugation as cell fractions. Filtration will remove large debris (*e.g.*, cell walls) and unbroken cells, providing a filtrate that contains organelles (nuclei, chloroplasts, mitochondria, and ribosomes), small membrane vesicles, and soluble components; most of these will not be visible in the light microscope. Low-speed centrifugation will sediment remaining large bodies from the filtrate, and moderate-speed centrifugation will sediment chloroplasts, leaving most of the mitochondria, ribosomes, and soluble components in the supernatant. (The mitochondrial fraction could be collected by high-speed centrifugation, and ribosomal and membrane vesicle fractions by ultra-high-speed centrifugation.) Repeated rounds of differential centrifugation can be used to further purify the chloroplasts when highly purified preparations are required for experimentation, but one round of low-then-moderate centrifugation will suffice for the purposes of this exercise.

In this laboratory, you will prepare a crude suspension of chloroplasts and determine several characteristics of the preparation. You will measure the chlorophyll *a* content of the suspension, count the chloroplasts per unit volume of the suspension, and use these data to estimate the quantity of chlorophyll *a* per chloroplast.

Procedure (work in pairs)

NOTE: Keep all tissue and fractions ice-cold throughout the procedure.

A. Chloroplast separation by differential centrifugation

1. Obtain spinach leaves and de-vein them (remove the main large stem). Using a balance, weigh out approximately 8 grams of de-veined spinach tissue. (This does not have to be exact). Rinse the tissue in ice water, blot dry, and cut into pieces approximately 1 cm square. (This does not have to be exact).

2. Bring your de-veined spinach to your instructor at the front bench. Your instructor will place the leaf pieces in a pre-chilled blender cup containing 40 mL of ice-cold 0.5 M sucrose / 8 g spinach leaves. You instructor will blend for 15 seconds at top speed, pause about 10 seconds, then blend again for 10 seconds. Receive an aliquot (~40 mL) of blended sucrose/spinach homogenate from your instructor.
3. Obtain a chilled 100-mL beaker and strain the leaf homogenate through four layers of 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 give them to your instructor for centrifugation. Tubes will be centrifuged at 200 x g for 5 min. (Don't forget, that the centrifuge must be balanced.)
5. Using a Pasteur pipet, transfer each supernatant (containing the chloroplasts) to a second centrifuge tube and give them to your instructor for centrifugation at 1,000 x g for 7 minutes. Save the pipet. (*What is in the pellet?*)
6. Using the pipet, remove and discard the supernatants, being careful **not to disturb the pellets**. (*What is in the supernatants?*)
7. Pipet 2 mL of phosphate buffer onto each pellet and gently resuspend the chloroplasts by moving the liquid up and down in the pipet—leave some liquid in the pipet at all times to avoid the formation of bubbles. Combine the two suspensions into one of the tubes, and discard the empty tube.
8. Using a clean Pasteur pipet, add phosphate buffer to a total volume of 8 mL (using the markings on the side of the tube), and mix the diluted suspension by moving it up and down in the pipet.
9. This is your chloroplast suspension. Examine it under the microscope by putting a drop on a microscope slide. (Don't forget to use a coverslip). Record what you see – do you see only green chloroplasts or are there other organelles also? **Use this chloroplast suspension for parts B and C.**

B. Estimation of chlorophyll a concentration of the suspension.

1. Measure 4.75 mL of 80% acetone into a 13 x 100 mm tube. (What will the acetone do to the chloroplast membranes and the pigments therein?)
2. Add 0.25 mL (250 µL) of chloroplast suspension and mix well. (What is the dilution factor?)
3. Using a spectrophotometer, read the absorbance at 652 nm, using a reference blank of (4.75 mL acetone+0.25 mL (250 µL) phosphate buffer—Why use this particular blank?). Don't forget to blank the spectrophotometer after changing the wavelength.

   Record the A value. A_{652} = ____________

C. Determination of chloroplast concentration of the suspension.

1. Measure 4.75 mL of the phosphate buffer into a clean 13 x 100 mm test tube, add 0.25 mL (250 µL)of chloroplast suspension, and mix well. (*What is the dilution factor?*)
2. Prepare the clean, dry hemocytometer with a cover slip in place supported by the frosted-glass shoulders of the chamber.
3. Making certain that the chloroplasts are evenly suspended (not settled or clumped), take up some of the suspension into a clean pipet; let part of a droplet from the pipet tip flow under the cover slip of the chamber. When properly delivered, the liquid will fill the space between cover slip and etched surface of the chamber and will not overflow into the side troughs beneath the cover slip.
4. Using the 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 25 sets of 16 very small squares. Number of chloroplasts in chamber = ______________

D. Student Worksheet

1. (1 pt) Estimate the concentration of **chlorophyll *a*** in your **dilute** suspension. To do this, use the Beer-Lambert Law (A = ECL). Luckily for you, the extinction coefficient (at 652 nm) for chlorophyll *a* is known. It is 44.85 mL/ mg cm. Thus, use the Beer-Lambert relationship to determine the concentration of chlorophyll in your **dilute solution**.
2. (1 pt) What is the dilution factor for diluting your stock chlorophyll a solution in acetone? (See Procedure B, steps 2 & 3.)
3. (1 pt) Now, use the following equation to calculate the concentration of chlorophyll *a* in your **undiluted stock solution**.

   Concentration stock solution = concentration of dilute solution x 1/ Dilution Factor

4. (1 pt) Determine the concentration of **chloroplasts** in your **dilute suspension**. This is possible because the volume in the hemocytometer in which you counted chloroplasts is known. The volume contained in the chamber over the large square in which you counted chloroplasts was 0.1 µL or 10^{-4} mL. Thus, you counted the number of chloroplasts in 10^{-4} mL. Use this knowledge to calculate the concentration of chloroplasts (# chloroplasts/mL) in your **dilute suspension.**
5. (1 pt) What is the dilution factor for diluting your stock chloroplast solution in phosphate buffer? (See Procedure C, step 1.)
6. (1 pt) Now, use the following equation to calculate the concentration of chloroplasts in your **undiluted stock solution**:

   Concentration stock solution = concentration of dilute solution x 1/ Dilution Factor

7. (1 pt) Combine calculations 3 and 6 to determine the amount of chlorophyll *a* per chloroplast:

   $$\frac{\text{mg chlorophyll } a \text{ / mL undiluted solution}}{\text{\# chloroplasts / mL undiluted solution}} = \text{mg chlorophyll } a \text{ / chloroplast}$$

8. (1 pt) Finally, convert mg to an appropriate unit of mass, a unit that will allow expression of the calculated value as a number between 0.1 and 100 (e.g. 20 pg or 0.6 g), using the following relationship as needed:

   10^{12} fg = 10^{9} pg = 10^{6} ng = 10^{3} µg = 1 mg = 10^{-3} g = 10^{-6} kg

About the Authors

Sarah Salm received her PhD at the University of the Witwatersrand in Johannesburg, South Africa. She did her postdoctoral work at New York University, focusing on prostate stem cells, her continued field of research. She currently teaches Microbiology, Anatomy and Physiology, and General Biology at the Borough of Manhattan Community College.

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Sarah Salm and Jessica Goldstein met at their first ABLE conference, and they have collaborated on ABLE presentations ever since.

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