Chapter 4

Cell Fractionation in *Tetrahymena*

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Contents

Materials

Live Material**:** *Tetrahymena thermophila* cultures, grown in 250 ml flask
Microscopes, compound - 1 per pair of students
Vortex mixers, 3 per lab
Hemacytometers, bright-line, plus cover slips - 1 per pair of students
Table top centrifuges, head to hold 15 ml tubes - 3
Spectronic 20, 600 nm - 3
Boiling water bath for diphenylamine - hot plate with water bath and test tube rack, foil cover
Temperature blocks - set at 90°–95° C
Ice buckets
Sharps containers
Test tube holders
Lab mat and scissors
Each pair of students will need the following:

- Plastic (polypropylene) conical centrifuge tubes, 1 ml, with ml markings - 2
- Pasteur pipettes and bulbs
- Slides, coverslips
- Pipumps, 1 ml size
- Pipettes - 5 ml, 10 ml
- P1000 Pipetman and tips
- Beakers, 125 –250 ml size
- Parafilm, sharpies, scissors
- Test tubes, for diphenylamine reaction (hold 4 ml)
- Spec 20 tubes or disposable 13 × 100 mm tubes
- Test tube racks

Solutions in laboratory room

1. Medium A - 50 ml per bench, 3 per lab
2. Methyl green pyronin - 1 dropper bottle per bench
3. Medium B: 150 ml *exactly* of medium A in a 200 ml squirt bottle.
   Label *Medium B, shake well.*
   Under hood: n-Octanol - 10 ml in a dark bottle. Add 0.95 ml of n-Octanol to 150 ml of medium A just before use; shake well before each use.

Under hood

4. TCA 5% - 75 ml per lab. Label CAUTION.
5. DNA Stock, 100 µg/ml - 25 ml per lab.
6. Diphenylamine Reagent - 50 ml per lab. Label CAUTION.

Solution Directions:

1. Medium A
   Dispense in 50 ml aliquots

	1 liter	3 liters
0.1 M Sucrose	34.23 g	102.69 grams
4.0% Gum arabic	200 ml of 20%	600 ml of 20%
0.0015 M $MgCl_2$	0.142g	0.43 grams
0.01% Spermidine HCl	0.1 g	0.3 grams

   Adjust the pH to 6.75 with 1N NaOH. (If you use $MgCl_2 \cdot 6H_2O$ - mol. wt. 203.3, use 0.304 g/l or 0.9145 g/3 liters; mol. wt. of $MgCl_2$ is 95.23). This can be prepared in advance and frozen in aliquots. For ordering: Spermidine Trihydrochloride, Sigma Chemicals S-2501.
   Gum Arabic, 20% stock solution: (Acacia Powder, Mallinckrodt H173-61).
   Centrifuge at 16,000× g for 10 minutes to remove particulate material which interferes with the nuclear preparation. Store in frozen aliquots in plastic bottles.
2. Medium B
   150 ml of Medium A + 0.95 ml of n-octanol, added just before use. Place 150 ml of Medium A in a 200 ml squirt bottle with a sidearm. Put 10 ml of n-octanol in a dark bottle. Store under hood. The instructor can add octanol just prior to use in the laboratory.
3. Methyl green pyronin—1 dropper bottle per bench
   Dissolve 1g of the pure dye *methyl green* (Harleco) in 200 ml hot distilled water. With a batch of methyl green that has the dye content given as 89% on the label, use 1.12g. When cool, transfer to a separatory funnel and extract repeatedly with 30 ml aliquots of chloroform until the chloroform is colorless or nearly so. The chloroform removes the contaminant, methyl violet. Add 0.1 gram of the pure dye *pyronin Y* (Eastman Kodak Co.) to 200 ml of the chloroform-extracted methyl green solution. Shake well and dispense in dark dropper bottles. The staining solution can be stored for several months in a dark bottle but it loses intensity after 6 months.
4. Culture Media

		1 liter of 10×
Proteose peptone, Difco	1%	100 g
Dextrose (glucose)	0.2%	20 g
Yeast extract, Difco	0.1%	10 g
Sequestrine (Ciba-Geigy Chem.)	0.003%	0.3 g

Make media 10×, centrifuge 7000× g for 30 min. to remove insoluble particulate matter that contaminates the nuclei preparation. Store in appropriate sized aliquots at –20°C, or make fresh. Thaw, dilute and sterilize prior to use. Each flask is inoculated with a 10 ml tube of *Tetrahymena*. Distribute the media in test tubes, 18 × 125 mm, screw capped, containing 10 ml medium, for inoculum, and 250 ml Erlenmeyer flasks, screw capped, with 125 ml media, for class use.

A note about *Tetrahymena* media: Proteose peptone is the basic growth medium; the additional ingredients are used to enhance growth and if not available can be omitted. Sequestrine is used to provide iron. The original recipe is 2% proteose peptone but we have been using 1% successfully.

5. Trichloroacetic Acid (TCA) 5% —make 1500 ml.
   Dissolve 75 g TCA in 1300 ml water, with CAUTION. Bring the volume to 1500 ml. Dispense 75 ml per lab and label 5% TCA CAUTION on the bottle. Store in hood. (Note: Fisher sells 10% TCA w/v, dilute in half for 5%.)
6. DNA Stock, 100 µg/ml in 5% TCA
   Add 10 mg DNA (e.g., Deoxyribonucleic Acid from herring testes, Sigma Chemical Co. D-6898) to 100 ml 5% TCA. To dissolve, place the mixture in a 90°C water bath for several minutes and shake well. Dispense 25 ml per lab. *Do this in the hood.*
7. Diphenylamine Reagent
   In a hood, dissolve 2.0 grams fresh diphenylamine in 200 ml glacial acetic acid and 5.5 ml concentrated sulfuric acid. Make within 1–2 hours of lab. Dispense 50 ml per lab, label Diphenylamine CAUTION, and put under hood. *Alternate storage directions:* Stable 6 months at 2°C; warm to room temperature, and swirl to remix before use.
8. *Tetrahymena thermophila* culture.
   (a) Log cultures of *Tetrahymena*
   Inoculate one 10 ml culture (3–7 day culture, grown in liquid media on a slant for aeration) into 125 ml medium in a 250 ml Erlenmeyer flask. Grow at 26–28°C, shaking, for approximately 21 hours. *Tetrahymena thermophila* can be obtained from the author or American Type Culture Collection (http://www.atcc.org).
   Equipment needed: Shaker or shaking water bath with adapters to hold 250 ml Erlenmeyer flasks at 26–28°C.
   (b) Stock Cultures of *Tetrahymena thermophila*.
   Using sterile technique, transfer 0.2–0.4 ml into 5–10 ml of 1% Proteose Peptone in a 15 ml screw-capped culture tube. Screw the cap on almost tight to retard growth. Store upright at room temperature. Transfer every 4–6 weeks.
   (c) Long term storage
   Autoclave a soy bean in 10 ml of distilled water. Inoculate with several drops of an actively growing culture. Look for organisms at the liquid interface. Reinoculate these into 1% proteose peptone. Active cultures were obtained after 6 months. *Tetrahymena* must be grown in an enriched medium (as 1% proteose peptone) before being inoculated into soy bean liquid.

Notes for the Instructor

This laboratory was designed to give Introductory Biology lab students experience with cell fractionation. Students isolate nuclei from a ciliated protist and determine the amount of DNA per nucleus. The isolation steps are monitored with the stain methyl green pyronin which stains DNA a blue-green (nuclei) and RNA a rose-red (cytoplasm).

Comments on the Nuclear Isolation Procedure:

Initial Cell Count

To save time the instructor can fix and prestain *Tetrahymena* (2 ml cells + 1.6 ml 70% ethanol + 0.4 ml methyl green pyronin = 2× dilution). Shake gently to resuspend the cells for counting.

Comments on Selected Steps

2. Observe live cells, follow with staining. Methyl green pyronin takes about 10 min. to stain. Under 10× objective look for cells that are a deeper purple color, most cells are pink. Once some good cells are found, examine using 40× objective.
   The macro/micronuclei are blue and the cytoplasm purple-pink. (Don't confuse the contractile vacuole for the nuclei.) The organisms are swollen because they are hypertonic to the medium + dye solution. They are isotonic in the growth medium.
3. Spills: Methyl green pyronin stains the lab benches and everything else. Have students put their slides on lab mat to absorb spills.
4. Resuspend the firm pellet (whole cells) in 5 ml Medium A by pipetting up and down with a pasteur pipette. This step washes out the growth medium; cells are in a medium that will be used in the next step.
5. **CRUCIAL STEP** Medium B
   [UNDER HOOD, add 0.95 ml octanol to 150 ml Medium A (in a sidearm wash bottle) just before it is used by the students. Octanol floats to the top, so SHAKE THOROUGHLY BEFORE USE.]
   Resuspend the firm pellet of whole cells in 5 ml Medium B (SHAKE MEDIUM B VERY WELL, possibly discard first few drops.) Resuspend cells by vigorous pipetting with the pasteur pipette then *either* vigorously shake (40+ times) or intermittent vortexing for 1.5 min. Check to see that most cells are broken (10× objective); switch to 40× objective to observe the blue nuclei & debris. *If the cells are not broken* then shake or vortex some more. If this doesn't work, add 1–2 ml of additional Medium B, or 1 drop of octanol to the tube.

Note: if it is difficult to lyse the cells, slightly increase the concentration of octanol in medium B.

   MEDIUM B: The *octanol* (alcohol that solubilizes the membranes) is not in solution but forms an emulsion. Shaking the cells is a form of homogenization and breaks up the cells. Upon centrifugation, the octanol emulsion floats the nonnuclear cell constituent (lipids-membranes) away from the nuclei and forms an octanol skin on the top.

6. Centrifuge 5 min at the highest speed. Remove the supernatant and skin at the top with the Pasteur pipette but DON'T DISTURB THE NUCLEAR PELLET. Resuspend the SMALL pellet in 1 ml medium A, examine with the microscope and if there are too many whole cells repeat the Medium B step. (The SMALL tightly packed white pellet is rich in macronuclei. Materials which don't pellet (macronuclei, micronuclei, nonnuclear cell components) are found in the cloudy supernatant or in the skin at the top of the tube. The size and tightness of the skin varies with culture age, degree of blending, octanol concentration, etc.)

The supernatant can always be shaken again, centrifuged to bring down the macronuclei that didn't come down. There will always be whole cells and debris along with macro/micronuclei. Emphasize that this procedure is not 100% perfect the first time.

Nucleic Acid Extraction

Centrifuge the combined nuclei for 5 min. Polypropylene centrifuge tubes withstand the 90°C treatment. Resuspend pellet in 3 ml TCA, then 90°C for 15 min. in temperature block, centrifuge 2 min. and decant supernatant.

Diphenylamine Reaction for DNA

Turn on boiling water bath, cover with foil (replace evaporated water as needed). Follow lab directions.

FLOW CHART FOR EXPERIMENT

Tetrahymena Nuclear Isolation ***Initial cell count*** on fixed cells

2 tubes of 10 ml cells
examine, stain drop
↓
*×g highest speed 2 min.
discard supernatant
↓
resuspend pellet in 5 ml Medium A
(washes cells)
↓
×g highest speed 2 min.
discard supernatant
↓
CRUCIAL STEP: Resuspend pellet in 5 ml Medium B, shake/vortex
examine under microscope so most cells broken (stain a drop)
↓
×g highest speed 5 min.
discard 'skin', supernatant
↓
resuspend small pellet in 1 ml Medium A
(can pool both tubes)
stain drop, examine. Count on hemacytometer. ***Nuclear count***

DNA Extraction

2 ml of nuclear preparation
↓
pellet nuclei: ×g highest speed 5 min.
discard supernatant
↓
3 ml TCA, 90°C, 15 min.
↓
×g 2 min., highest speed
↓
Decant supernatant and KEEP

Diphenylamine Reaction *use caution*

Standard Curve
2 ml of Nucleic Acid Extract + 2 ml diphenylamine
↓
boil 10 min., then into ice
↓
Spectronic 20 @ 600 nm

*×g = centrifuge

Cell Fractionation

Introduction

One approach to analyzing the composition and function of the formed elements (organelles) in cells involves isolating and purifying them and studying their properties. By this means, specific cellular components can be localized to specific organelles and/or specific functions can be ascribed to specific parts of cells. This approach to studying cells is referred to as cell fractionation. Unfortunately, cell fractionation is not without its pitfalls. Removing organelles from their normal, *in situ*, environment can change their properties. Often the cell fractionation procedures itself causes redistribution of cellular components. Undetected trace contaminants in a seemingly pure cell fraction can cause misinterpretations in the properties ascribed to the major component of that fraction. Any change in structure, composition or function of an isolated organelle caused by the method employed in studying it is referred to as an artifact. In spite of the fact that cell fractionation (like most other methods of studying cells) produces artifacts, this approach has played a very important role in furthering our understanding of cells.

In this exercise, nuclei will be isolated from the ciliated protozoan, *Tetrahymena thermophila* (Figure 4.1). It is a small fresh water ciliate, longer than it is wide (50 × 30 µm), with oral cilia at the anterior end. *Tetrahymena* can be cultured under sterile conditions and using optimal growth conditions, each cell divides in 2.5 hours. *Tetrahymena* has been used as a research organism for studies on eukaryotic nuclear structure and function, cilia formation, genetics and physiology.

Like most species of ciliates, this organism contains two types of nuclei, a larger macronucleus (8–10 µm) and a smaller micronucleus (1–2 µm). The large macronucleus contains many copies of DNA, synthesizes RNA, controls the growth and phenotype of the cell and does not participate in sexual reproduction. The micronucleus undergoes meiosis and fuses with another micronucleus during sexual reproduction eventually giving rise to a new micro and macronucleus. The nuclear fraction contains 90–95% macronuclear DNA. Both types of nuclei will be isolated and the total amount of DNA per macronucleus will be determined. The nuclear isolation will be monitored microscopically using the stain methyl green pyronin. Methyl green stains DNA, blue-green, and pyronin stains RNA, rose-red.

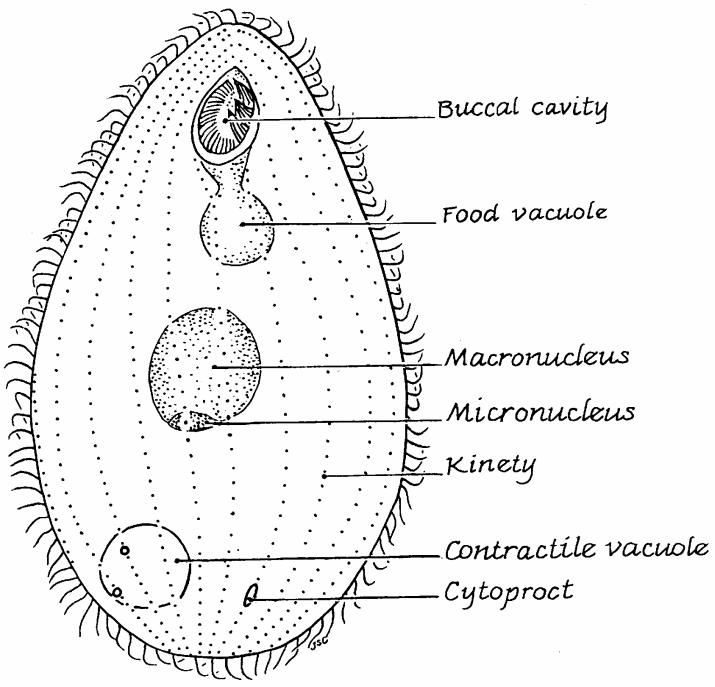


Figure 4.1. *Tetrahymena thermophila.*

A. Nuclear Isolation

Prepare a flow chart of the nuclear isolation procedure before the laboratory. *Tetrahymena* has been growing in a sterile medium, shaking, for 24 hours at 28°C. Swirl the cells to get an even distribution before taking an aliquot. Cells are heavier than the liquid and tend to sink to the bottom.

Cell Count:Determine the initial cell count by counting cells that have been previously diluted 2× in methyl green pyronin. Shake the tube to resuspend the cells. Put a sample on a hemacytometer and determine the #cells/ml and the total number of cells. Counting directions are outlined on page 10.

When using methyl green pyronin, put your slide on several layers of paper towels or lab mat on the lab bench so spills will be absorbed and NOT stain the benches.

1. Prepare two conical tipped centrifuge tubes, with 10 ml of *Tetrahymena* culture in each.
2. Place a small drop of the culture fluid on a clean microscope slide. Examine the cells under low power. Put a coverslip on the drop and examine the cells with the 40× objective (adjust illumination to give maximum detail). Can you see the macronucleus? Can you see the micronucleus? What other parts of the cell can you see? Place a drop of methyl green pyronin staining solution on the slide so that it can seep under the coverslip (removing a small amount of solution from the opposite edge of the cover slip with an absorbent paper), or mix a fresh drop of cells with a drop of the methyl green pyronin solution. Examine cells that have been killed and stained by methyl green pyronin. Most cells are stained in 10 minutes. The macronucleus and micronucleus appear attached; the micronucleus is set in a depression of the macronucleus. Using the fine focus, distinguish between the two nuclei. The nuclei are stained blue, the cytoplasm is stained a pinkish-purple color and the contractile vacuole is stained a deeper pink. The contractile vacuole pumps water to the exterior and helps maintain the cells osmotic pressure. Additional organelles as food vacuoles, pinocytic vacuoles and mitochondria are seen in the cell. Are the cells swollen in the methyl green pyronin dye? If so, explain.

SAFETY: Discard all slides, coverslips and Pasteur pipettes in the SHARPS container .

3. Separate the cells from the growth medium by centrifuging for two minutes at the highest speed in the table top centrifuge. *Make sure the tubes are balanced.* A centrifuge should always be balanced by placing equal volumes in equal sized tubes opposite one another. Spin the tubes for 2 min. There will be a *pellet* of cells and a clear *supernatant* with few or no cells. Immediately transfer the supernatant, either by decanting (pouring) or with a Pasteur pipet, from above the cell pellet to a clean tube.
4. Carefully resuspend the pellet of cells in 5 ml of medium A. Medium A is made of
   0.1M sucrose
   4% gum arabic
   1.5 mM $MgCl_2$
   0.01% spermidine HCl

   Magnesium chloride helps stabilize DNA, positively charged spermidine stabilizes the nuclear membrane, and sucrose and the polysaccharide, gum arabic, are included to provide an osmotically balanced medium. Study a drop of the resuspended cells under the microscope. Do the cells look normal? Centrifuge the cells 2 minutes at the highest speed on the table top centrifuge. Remove the supernatant; the pellet of washed cells will be used in step 5.
5. Medium B consists of 0.95 ml of N-octyl alcohol per 150 ml of Medium A and must be made immediately prior to use. The octanol floats on top so thoroughly *shake this solution before using it*. Octanol is a detergent used to solubilize the cell membrane. Medium B is in a squirt bottle; shake the bottle and squirt Medium B into the centrifuge tube up to the 5 ml mark. Resuspend the pellet in 5 ml of Medium B by vigorously pipeting up and down with a Pasteur pipet. Place a small square of Parafilm over the top of the centrifuge tube and homogenize the cells by either (a) putting your thumb

over the top and shaking the tube vigorously up and down about 20 times or (b) intermittent mixing on the vortex mixer for 1 minute.

Check the suspension, using methyl green pyronin stain, to see that most of the cells are broken and the macronuclei and micronuclei are released. Can you distinguish the two types of nuclei (stained blue)? Note whether the macronuclei appear to be intact or damaged. If there are few or no whole cells, proceed to the next step. If many whole cells are present, repeat the homogenization. The optimum level of homogenization occurs when all of the cells are lysed but none of the nuclei are damaged; it is rarely achieved. Make a reasoned decision about when to stop homogenizing.

6. Centrifuge the homogenate at high speed for 5 minutes. Carefully remove the cloudy supernatant and floating "skin" on the top while not disturbing the nuclear pellet. Usually the more compact the "skin" the better the nuclear preparation. Resuspend the nuclear pellet in 1.0 ml of Medium A, examine a drop under the microscope with stain. How does its composition compare to the original homogenate and to the nuclear supernatant. If there are too many whole cells repeat the Medium B step.
7. Combine the two nuclear preparations of 1.0 ml each for a total of 2.0 ml and following the counting directions on page 10 use a hemacytometer to determine the total number of macronuclei/ml. Remember to account for both a concentration and dilution factor. Put the cleaned hemacytometer on the front bench - DO NOT DISCARD.
8. Examine the nuclear supernatant. Are there any nuclei remaining in the supernatant? What kinds? If the time permits, you might wish to re-centrifuge the supernatant. Why?

B. Nucleic Acid Extraction

Pellet the nuclei (5 min., highest speed), discard the supernatant and resuspend the nuclei in 3.0 ml of 5% TCA (Trichloroacetic acid) with a Pasteur pipette. Nucleic acids are soluble in TCA, proteins are insoluble. Place the tube in a 90°C temperature block for 15 min., agitate the tube every few minutes. This treatment dissolves out and partially degrades the RNA and DNA. Centrifuge the tube at highest speed for 2 min., and then carefully decant the supernatant containing the degraded nucleic acids into a clean test tube.

C. Diphenylamine Reaction for DNA

The diphenylamine reaction is specific for 2-deoxypentoses and will be used to determine the amount of DNA in the nuclei of *Tetrahymena*. The diphenylamine reagent contains acetic and sulfuric acid as well as diphenylamine.

$$\text{DNA} \xrightarrow[\text{heat}]{\text{acid}} \text{2-deoxyribose + purines, pyrimidine nucleotides, pyrimidine nucleosides, } H_3PO_4$$

2-deoxyribose is converted to w-hydroxylevulinyl aldehyde which reacts with diphenylamine to form a blue color that is read at 600 nm. A standard curve will be obtained from the four known DNA concentrations and from this the nuclear DNA concentration determined.

Procedure

1. Label 6 test tubes and add the indicated amounts of each solution.

Tube	Final DNA Concentration µg/ml	DNA Stock (100 µg/ml)	*Tetrahymena* Nucleic Acid Extract	5% TCA	A 600 nm
Blank	0	–	–	2.0 ml	
1	25	0.5 ml	–	1.5 ml	
2	50	1.0 ml	–	1.0 ml	
3	75	1.5 ml	–	0.5 ml	
4	100	2.0 ml	–	–	
5	–	–	2.0 ml		

CAUTION: Use caution when handling TCA, the DNA stock which is dissolved in 5% TCA, and diphenylamine. Immediately rinse any spills. Goggles should be worn.

2. Add 2 ml diphenylamine reagent to each tube. Mix contents carefully.
3. Place tubes in a boiling water bath for 10 minutes.
4. Remove tubes and immediately immerse them in ice to cool quickly.
5. Turn on the Spectronic 20, set the wavelength to 600 nm.
6. Transfer the tube contents with Pasteur pipets to Spectronic 20 tubes. Read the absorbance at 600 nm. Plot the absorbance versus the known DNA concentrations (µg/ml). Determine the concentration of DNA per *Tetrahymena* nucleus. Use the nucleus count that was determined in step A7.

D. Counting Chamber Directions for Counting Cells or Nuclei

1. Obtain a sample of the 2× diluted cells or for the nuclear count, mix 0.1 ml of the nuclear suspension with 0.1 ml of methyl green pryonin stain (2× dilution).
2. Place the clean, dry cover slip on the clean and dry hemacytometer.
3. Load both sides of the hemacytometer with a long-tipped dispo pipet. Place a drop in the "V" groove at the edge of the cover slip. Both sides should be filled independently (not with the same pipet full). The cells or nuclei should be well suspended before putting on the hemacytometer. The hemacytometer should be filled just to completion; if the coverslip is floating or there is an air bubble you must start over.
4. Place the hemacytometer on the microscope stage. Using 10×, find the ruled areas to make certain that cells are evenly distributed. Use the 10× objective for the initial *Tetrahymena* count and the 40× objective for the nuclear count. Both cells and nuclei can be counted on the 1 mm square. Ideally, count at least four of the 1 mm squares on each side of the hemacytometer and at least 50 cells or 100 nuclei.
5. Determine the cell or nuclear count.

$$\frac{\#\text{ cells}}{\#\text{ of 1 mm squares}} \times \text{dilution} \times (1 \times 10^4) = \#\text{ cells/ml}$$

For example:

In two 1 mm squares, 60 cells were counted. The cells were diluted 2× with methyl green pyronin.

$$\#\ \text{cells/ml} = \frac{60\ \text{cells}}{2\ \text{squares}} \times 2\times \text{dilution} \times (1 \times 10^4)^*$$

$$= 60 \times 10^4\ \text{cells/ml}$$

$$= 6 \times 10^5\ \text{cells/ml}$$

$$\text{Total cells} = \text{(initial) 20 ml of cells} \times 6 \times 10^5\ \text{cells/ml} = 12 \times 10^6\ \text{cells}$$

*Multiplication factor: the volume for a 1 mm square = 1 mm × 1 mm × 0.1 mm depth = 0.1 mm^3. The 1 mm square volume (0.1 mm^3) times 1×10^4 = 1.0 ml

Dilutions and concentrations:

a. If you have to count more than one square, divide by the number of squares counted.
b. Remember to account for any dilutions you have made. Multiply the

$$\frac{\#\ \text{cells}}{\text{ml}} \times \text{dilution factor}$$

c. If you have concentrated the cells, divide by the concentration factor.

E. Additional Information About Counting Chambers

A counting chamber is a microscope slide which is designed so that the cover slip rests a known distance above a surface ruled with tiny squares of a known area. Thus, counting all the cells that appear inside a square gives the number of cells in a known volume, and multiplying by an appropriate number gives the number of cells/milliliter. Counting chambers are sometimes called "hemacytometers" (Figure 4.2).

There are several kinds of counting chambers, and each kind has different dimensions. Therefore, the volume measured within each square, and the multiplication factor used to obtain cells/ml is different for different chambers. It is very important then, to note the following:

A. The name of the counting chamber you are using. A "Fuchs-Rosenthal" chamber, for instance, has different dimensions than an "Improved Neubauer" chamber, but all "Improved Neubauer" chambers are the same.
B. The type of square you are counting. Most chambers have big squares, divided into smaller squares, divided into even smaller squares. You can count the cells in any of these, but make sure the number you multiply by to get cells/ml is appropriate for that size square, not a larger or smaller one.
C. The dimensions of the counting chamber from which the multiplication factor is determined. Written on the chamber is usually a set of dimensions: one is the depth of the chamber, and one is the area of the smallest square on the chamber. Multiplying area (mm^2) × depth (mm) gives the volume (mm^3).

Directions for converting cell counts to #cells/ml using an Improved Neubauer hemacytometer:

1. The large 1 mm squares have a volume of 0.1 mm^3 (1 mm × 1 mm × 0.1 mm depth). There are nine 1 mm squares.

$$\frac{\#\ \text{cells}}{\text{ml}} = \frac{\#\ \text{cells}}{\text{largest square}} \times (1 \times 10^4)$$

2. Medium squares are one of the 25 medium squares (E, F, G, H, I) found in the center millimeter square.

$$\frac{\#\ \text{cells}}{\text{ml}} = \frac{\#\ \text{cells}}{\text{medium square}} \times (2.5 \times 10^5)$$

3. The central square is divided into 25 groups of 16 small squares. The volume over each of the 16 small squares is 0.00025 mm^3 (× 4000 = 1 mm^3). Since 1 ml = 1000 mm^3, then

$$\frac{\#\ \text{cells}}{\text{ml}} = \frac{\#\ \text{cells}}{\text{smallest square}} \times (4 \times 10^6)$$

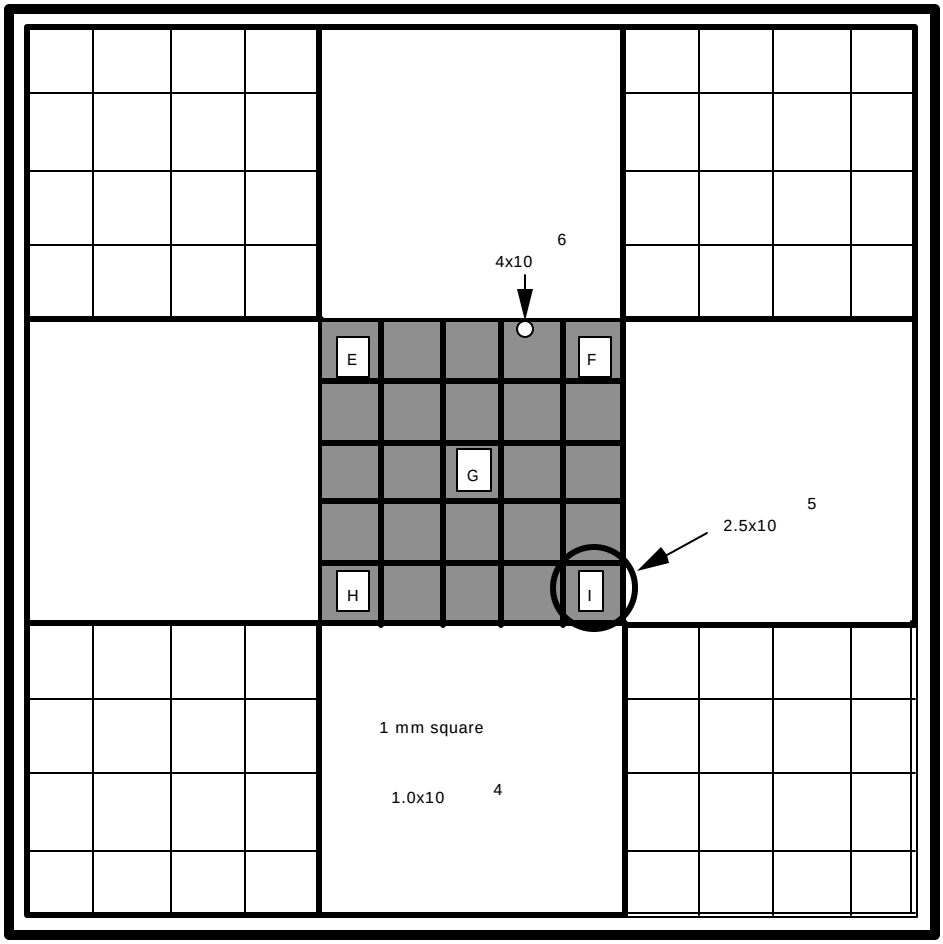


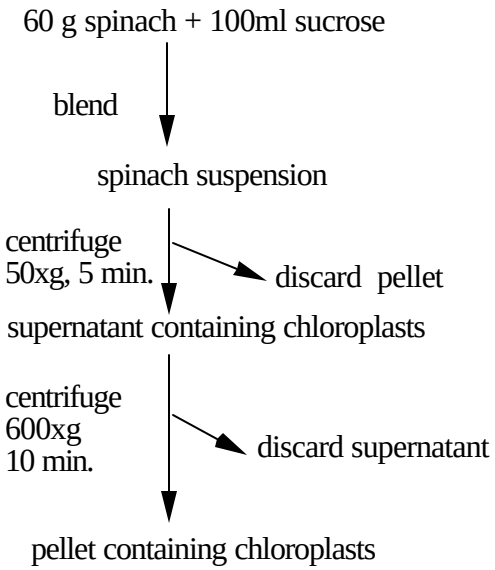
Figure 4.2. Hemacytometer.

Each ruled area covers 9 square millimeters made up of nine 1.0 mm squares; the central square millimeter is ruled into 25 groups of 16 small squares. Each group is separated by triple lines, the middle one of which is the boundary. The ruled surface is 0.10 mm below the cover glass, so that the volume over a square mm is 0.1 cubic mm.

Laboratory Report

Your lab report should include (but not be limited to) the following questions.

1. Include a flow chart and indicate what is in each pellet and supernatant.
   An example of a flow chart for the isolation of chloroplasts from spinach follows:



2. Determine the original # of cells/ml and total # of cells at the beginning of the experiment.
3. Determine the concentration of nuclei (# nuclei/ml) and total # of nuclei in the nuclear preparation (see step A7 and Counting Chamber Directions).
4. Compare the total number of cells at the beginning of the experiment to the total number of nuclei recovered. What % of nuclei were recovered? Which steps contributed to loss of nuclei and why?
5. Discuss the effectiveness of each step, artifacts produced, differential losses, purity, etc. Include observations you made in A3, A6 and A8. Discuss shortcomings you have noted in the procedure and methods of improving the procedure.
6. In what types of experiments could isolated nuclei be used and for what reason?
7. Plot the DNA standard curve (A 600 nm vs µg/DNA) and determine the total DNA concentration of your nucleic acid extract. Calculate the amount of DNA per nucleus.

Acknowledgments

This laboratory is adapted from one devised by Dr. Martin Gorovsky, Biology Dept., University of Rochester, Rochester NY. Julia Swope Child, Woods Hole, MA illustrated *Tetrahymena*.

Literature Cited

Gorovsky, M.A. 1973. Macro- and Micronuclei of *Tetrahymena pyriformis*: A Model system for Studying the Structure and Function of Eukaryotic Nuclei, J. Proto. Zool 20: 19–25.

Gorovsky, Martin A., M. Yao, J. Keevert and G. Pleger. 1975. Isolation of Micro- and Macronuclei of *Tetrahymena pyriformis*. Methods in Cell Biology, Vol. IX, D. M. Prescott, ed., Academic Press, New York.

APPENDIX
Expected Results

Methods to calculate cell and nuclear counts: based on cell/ml or on total volumes. Students have a much easier time with calculations using total numbers of cells and nuclei.

a) Cell Count: 20 cells in 1 mm square

20 cells × 2× dil. × 1×10^4 = 4×10^5 cells/ml

4×10^5 cells/ml × 20 ml = 8×10^6 total cells

b) Nuclear Count: 50 nuclei in 1 mm square

$$\frac{50 \text{ nuclei}}{10 \text{ conc.}} = \times 2\times \text{dilution} \times 1 \times 10^4 = 1 \times 10^5 \text{nuclei/ml}$$

1×10^5 nuclei/ml × 20 ml = 2×10^6 total nuclei

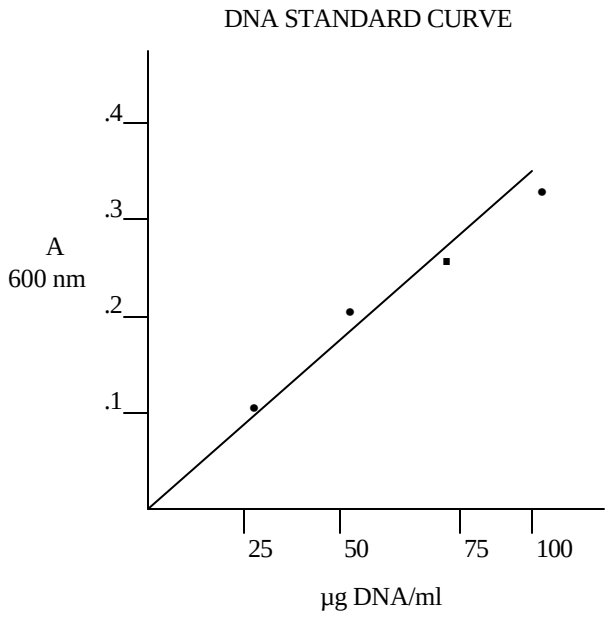
[Another way to calculate total number of nuclei:

50 nuclei in 1 mm square × 2 × dil. × 2 ml total volume × 1 × 10^4 = 2×10^6 nuclei]

c) % yield based on total counts = $\frac{\text{nuclei}}{\text{cells}} = \frac{2 \times 10^6}{8 \times 10^6} = 25\%$

% yield based on nuclei and cells per ml = $\frac{\text{nuclei}}{\text{cells}} = \frac{1 \times 10^5}{4 \times 10^5} = 25\%$

d) To compute the amount of DNA per nucleus:



Typical data:

	$A_{600\ nm}$
25 µg DNA	0.11
50 µg DNA	0.22
75 µg DNA	0.26
100 µg DNA	0.34
Tetrahymena DNA (2 ml)	0.12

Reaction is done in 2 ml: read directly from graph.

Tetrahymena DNA $\text{Absorbance}_{600\ nm} = 0.12 = 33$ µg DNA/ml

Total DNA:

From standard curve, sample reads 33 µg/ml × 3 ml = 99 µg DNA

(The nuclei are dissolved in 3.0 ml TCA).

Total Nuclei: 2×10^6 nuclei

$$\text{DNA/nucleus} = \frac{99 \times 10^{-6}\text{ g DNA}}{2 \times 10^{6}\text{ nuclei}} = 50 \times 10^{-12}\text{ g DNA per nucleus}$$

Actual amounts of DNA: Macronucleus $0.8–0.9 \times 10^{-12}$ g DNA

Micronucleus $10–12 \times 10^{-12}$ g DNA