

READING ASSIGNMENT

WHAT IS LIFE?

What can we learn about life using some basic biological techniques?

I. HOW DO WE MEASURE TISSUE MASS AND DENSITY?

II. HOW DO WE PREPARE TISSUES FOR MICROSCOPIC OBSERVATION?

III. WHY SECTION TISSUES BEFORE OBSERVING THEM?

IV. WHAT CAN WE LEARN USING SUBCELLULAR FRACTIONATION AND CENTRIFUGATION?

V. WHAT SIMPLE TESTS CAN BE USED TO DETERMINE PRESENCE AND/OR AMOUNT OF ORGANIC COMPOUNDS IN TISSUES?

VI. WHAT FACTORS AFFECT DIFFUSION?

I. HOW DO WE MEASURE TISSUE MASS AND DENSITY?

After reading this material you should know how to:

- 1) Calculate:
 - a) wet and dry weight of an organism or tissue
 - b) percent dry weight or percent wet weight of that organism or tissue
- 2) Determine organic vs inorganic content of a specimen.
- 3) Calculate:
 - a) what percent organic weight is of total wet weight (and of dry weight).
 - b) what percent inorganic weight is of total wet weight (and of dry weight).
- 4) Determine the density of an object given a scale for weighing the object and a graduated cylinder which measures liquid volume.

A. How do we determine wet versus dry weight and organic versus inorganic content of tissues?

The vast majority of living tissue is water. The remainder is primarily organic content. This includes carbohydrates, proteins, fats and nucleic acids. Inorganic or mineral compounds make up only a very small percentage of living organisms.

It is not surprising that water makes up the bulk of most cells (and organisms) since it is required to facilitate movement of many substances within and between cells as well as across cell membranes. Water is also required in many metabolic reactions.

Wet weight is the live or fresh weight of an organism or tissue. **Dry weight** is obtained by cutting samples of the tissue into small pieces and placing the tissue into a drying oven (80 to 100 °C), usually overnight or until no further change in weight is noted. This drying removes all of the free (chemically unbound) water from the tissues by evaporation.

The dried tissue is burned to determine organic versus inorganic content. This can be done by placing small pre-weighed samples of the dried tissue into covered crucibles. These are placed over a Bunsen burner and heated until no further change in weight is noted (one or more hours depending on the sample size). When burned completely, all of the organic material is oxidized to carbon dioxide and water. The residual ash includes most of the inorganic material. Some may be lost in the escaping smoke.

Organic and inorganic content are determined for the dry tissue only. The water that is removed while drying is not considered part of the inorganic content of the tissue. In other words, living tissues contain organic material, inorganic material and free water.

B. How do we determine tissue density?

The density of an object is measured as its mass per unit volume ($D = M/V$) and is often expressed in terms of kg/m^3 or g/cm^3 . Densities encountered in the universe range from about 10^{-20} kg/m^3 for density of matter in “empty” space (equivalent to about one atom per cubic centimeter) to $2 \times 10^7 \text{ kg/m}^3$ for density of an atomic nucleus (approximately a billion tons per cubic inch). Densities of more common substances are noted below.

SUBSTANCE	MASS DENSITY kg/m^3	RELATIVE DENSITY ¹
Solids:		
Platinum	21,400	Since the mass density of water is 1000 kg/m^3 the relative density of each substance is the value in the preceding column divided by 1,000.
Gold	19,300	
Lead	11,400	
Iron	7,900	
Aluminum	2,700	
Magnesium	1,750	
Ice	917	
Wood(pine)	500	
Cork	240	
Liquids:		
Mercury	13,600	
Sea water	1,030	
Pure water	1,000	
Petroleum	878	
Alcohol, ethyl	790	
Gases (at STP):		
air	1.29	
helium		0.178
hydrogen	0.090	

¹ **Relative density** is a measure of the density of an object relative to the density of water. For example, the relative density of platinum is $(21,400 \text{ kg/m}^3)/(1000 \text{ kg/m}^3)$ or 21.4. This number is not followed by units; the units cancel. Relative density gives you an indication of how dense something is relative to water and indicates whether or not the object will float or sink. For example, objects with a relative density less than 1 float. Those with relative densities greater than 1 sink.

II. HOW DO WE PREPARE TISSUES FOR MICROSCOPIC OBSERVATION?

After reading this section, you should:

- 1) understand why fixation and staining are often required for cell studies.
- 2) have filled out the tables provided for fixation methods and stains. These will provide you ready reference when conducting Lab 2.

Cells can be studied either in their normal, living state or after death. Most methods used to determine cell structure or chemical makeup either kill the cell or require that it be dead to begin with. There are many ways to kill cells. However, only some are appropriate if analysis (rather than death alone) is the objective. Appropriate methods are termed methods of fixation.

A. What do we need to consider when choosing how to fix or preserve tissues on slides?

Good fixation requires that the characteristics of the cell under study be preserved in as normal a state as possible. If we are interested in structure the fixation cannot distort the details we want to see. Alternatively, if we are interested in chemical composition, fixation must not destroy or dissolve these components. Finally, fixation should result in a stable preparation where decomposition due to enzyme action or environmental influence is minimized. Considering all this, it shouldn't surprise you that there is no one ideal fixative or fixation method. Rather, over the past 125 years or so, fixatives and fixation methods have been developed for a wide range of different cell types and for specific purposes. Some examples of fixatives and fixation methods, their uses and limitations follow.

B. When do we use different fixatives and fixation methods and what limitations do each of these have?

The following list provides you with information on some of the more common fixatives and fixation methods.

Acetic acid, usually a 45% aqueous solution, is good for rapid fixation of cells for squash preparations when loss or distortion of proteinaceous structures and loss of acid soluble components are not important. Acetic acid is very commonly used in chromosome studies, for example, in *Drosophila* salivary squashes and root tip squashes.

Acetic alcohol is usually made up of 3 parts of 100% ethanol plus 1 part of glacial acetic acid. It is similar in application and drawbacks to acetic acid alone, but somewhat less rough on membranes and a little less distorting. Use of either acetic alcohol or acetic acid alone results in cells and tissues which are flexible and can therefore be flattened on the slide for study at high light microscope magnifications.

Air drying is a simple method often used for single isolated cells, cells which are not part of a tissue, for example, bacteria or blood cells. This method avoids extraction of even water soluble materials, but leads to extreme distortion and rather unstable preparations. The instability results because the cells' degrading enzymes can be reactivated whenever there is enough moisture available to permit chemical reactions.

Formaldehyde is usually made as a 5% aqueous solution. This results in good preservation of proteinaceous structures and does not in itself remove acid-soluble compounds. However, cells are difficult to flatten because the denatured and cross-linked protein molecules make the formalin fixed cells rigid and resistant.

Freeze-drying permits very rapid "fixation" involving a minimum of distortion, but suffers from the same instability as air-drying unless it is followed by chemical fixation.

Freeze-substitution occurs when cells are rapidly frozen and then brought up to room temperature by passing them through a series of different alcohols at different temperatures. The rapid freezing produces minimal distortion and translocation (movement) of soluble materials. The alcohols permit a gradual removal of the cell's water as it thaws and at the same time they inactivate enzymes and stabilize the remaining cell structure. One drawback of this method is its complexity. Another is the fact that the resulting cells are too rigid for squashing and must be sectioned.

A wide array of variations of these methods exists. In particular, there are many different kinds of chemical fixatives. Chromic acid, picric acid and heavy metal salts are used in a large number of fixative solutions of complex makeup. Most of these more complex fixatives are designed for material which will be sectioned rather than squashed. Many books on cytochemistry, histochemistry and microtechnique are available in the biology and medical libraries on campus.

Fill in the following table for quick reference.

Fixative/Method	Advantages	Disadvantages
Acetic acid		
Acetic alcohol		
Formaldehyde		
Freeze drying		
Freeze substitution		

C. Why does what we see depend on the kind of stain we use?

Almost since the light microscope was invented, dyes or stains have been used to make cells or parts of cells more visible, that is, to enhance the contrast of the microscopic image of the fixed cell. Stains can be used to increase image contrast under the microscope and/or to demonstrate whether specific compounds or classes of compounds are present within the cell. In addition, with some stains, it is possible to determine the amount of a given compound within the cell by measuring the amount of dye bound.

As with fixatives, literally hundreds of staining methods exist, each having a different specificity for cell structures and chemical components. There are specific stains for chromosomes, nuclei, nucleoli, mitochondria, for nucleic acids, proteins, polysaccharides, fats, for acidic compounds and for basic compounds. Some dyes provide color by absorbing certain wavelengths of light and transmitting the rest. Others, the fluorescent dyes, absorb light at one wavelength and emit light at another, longer wavelength. As with fixatives, you will find detailed descriptions of the various methods in reference volumes.

In this lab, we will restrict ourselves to the use of only a few examples of commonly used stains.

Aceto-carmin is a basic dye in acid solution. This dye will stain acidic compounds, such as nucleic acids. It is not a very specific stain and is used mainly for the contrast it produces.

The Feulgen reaction is a staining method developed by Robert Feulgen in 1924. It is used in the Schiff's reaction for the detection of aldehyde groups and is a highly specific stain for DNA. The staining intensity is proportional to the DNA concentration. As a result this can be used as either a qualitative or quantitative stain. For information on a simple Feulgen tissue staining procedure you can access the following web site:

<http://www.nottingham.ac.uk/~mpzjlowe/protocols/feulgen.html>

Cells fixed in the presence of alcohol or extracted with alcohol contain no free aldehyde groups. If such cells are subjected to acid hydrolysis, aldehyde groups are formed on the deoxyribose portion of the DNA molecules as the acid labile purines are removed. Add Feulgen reagent and a fuschia (reddish-purple) color will appear wherever there is DNA. RNA does not interfere with this reaction because:

- a) acid hydrolysis dissociates and removes RNA from the cell and even if it were there,
- b) ribose, unlike deoxyribose, exists primarily as a ring structure, without free aldehyde groups, even if it were freed of attached bases, the ribose would not react with the Feulgen reagent.

Fast Green is a stain which brings us close to the dividing line between chemistry and witchcraft, i.e., the center of the realm of cytochemistry. Used at pH 2, fast green is a general protein stain. At pH 8 it is specific for a particular group of basic proteins, the histones, which are associated with chromosomal DNA in somatic cells. Fast Green cannot be used with predictable results after acid fixation, but only after formalin fixation: i.e., proteins must be preserved in the cell. Moreover, when used at high pH to stain histones, the DNA must be dissociated from the histones so that the latter's basic reactive groups are available to react with the dye. In other words, the conditions under which fast green is used must be strictly controlled.

All of the staining reactions discussed so far are appropriate for fixed cells. There are also a few stains which can be used on living cells. In fact the effectiveness of these stains depends on the cell's capacity to carry out its normal functions. These are called vital stains. One example is **triphenyl tetrazolium chloride**. A 1.5% aqueous solution of **tetrazolium** when heated to 37 °C will turn pink in the presence of reduction reactions, like those that occur in living cells or isolated active mitochondria.

To impress –or depress– you further, all of the techniques described are for use with the visible light microscope. An entirely different set of techniques have been developed for use with the electron microscope.

Stain	Used to detect:	Conditions of use:
Aceto-carmine		
Feulgen		
Fast Green		
Feulgen or Schiff's		
Tetrazolium		

D. What is phase contrast microscopy?

For those more interested in microscopy than chemistry, it may be reassuring to know that considerable information on cell structure and composition can be obtained by purely optical methods, applied to either living or fixed cells. One of these may be available on the microscopes you will use in lab, i.e., phase contrast microscopy.

Phase contrast microscopy can provide adequate contrast and resolution for the study of cell structure without staining. Phase contrast microscopy takes advantage of differences in refractive index between different cell components and between the cell and the medium surrounding it. A phase ring placed between the condenser and the stage allows only a ring of light to pass through the specimen. The objective lens contains a special phase plate. This system is set up to take advantage of the fact that highly refractive structures in the cell bend light more than structures with low refractive index. The more the light is diffracted or bent the more time it takes to get to the objective. Light reaching the center versus the periphery of the lens is therefore about $\frac{1}{4}$ wavelength out of phase. Being out of phase, these light rays can cancel each other out when the image is focused. Where they cancel each other out, the light intensity is reduced and the image appears darker. How much darker various parts of the cell appear depends on the difference that exists in refractive index among them.

III. WHY SECTION TISSUES BEFORE OBSERVING THEM?

After reading this section you should know:

- 1) the basic function of a microtome and its general operation
- 2) the advantage of looking at sections and serial sections of tissues

Many single celled organisms can be visualized easily in whole mounts. Observing the tissues of multicellular organisms often requires more preparation. Thicker structures, e.g., stems, fruits, muscle or liver, must be sectioned into thin transparent slices before being mounted on a slide for observation. Cutting or sectioning the material can lead to distortion. To help maintain structures in appropriate orientation thicker tissues are usually fixed and then embedded in paraffin (or some other embedding medium) or are frozen and then sectioned. Embedding or freezing the tissues also makes them easier to handle. Temporary mounts of leaves or stems, can sometimes be made by inserting the specimen between lengths of elderberry pit or fresh carrots and then sectioning. Such mounts allow you to observe the “natural state” of the tissue section. However, such fresh, unfixed tissue decomposes rapidly.

It is possible, with a great deal of practice, to produce good free hand sections for microscopic analysis. However, it is generally easier and better to use a microtome when sectioning. A microtome is an instrument used to cut tissue into thin sections for microscopic observation. The “knives” or blades used can be of metal, glass or diamond. The microtome can be adjusted for thickness and enables you to cut uniformly thin slices of the material. Producing serial sections of the tissue also allows you to reconstruct the 3-D structure of the tissue from information on the various sections.

IV. WHAT CAN WE LEARN USING SUBCELLULAR FRACTIONATION AND CENTRIFUGATION

After reading this section you should know:

- 1) the general purpose(s) of subcellular fractionation and centrifugation.
- 2) what key factors affect behavior of a subcellular particle undergoing centrifugation.

Cytochemical and autoradiographic techniques have been very useful in studying the distribution of cellular components *in situ* (“in place” or in the cell or organism) at both the light and electron microscope levels. A major breakthrough on the biochemical side came with the realization that the various subcellular structures could be separated from each other on the basis of size, shape and density by centrifugation of disrupted, broken cells. Living, unfixed cells can be disrupted by solubilizing their outer membranes or by mechanical disruption in a blender. Relatively large

samples of specific cell components can be isolated by centrifugation and their biochemical properties can be studied *in vitro* (“in glass” or in a test tube). The information obtained from studies on “fractionated” cells agrees with the data provided by cytochemical analysis of intact cells.

In centrifugation, the degree to which a given type of cellular component, e.g., chloroplasts, separates from other components depends primarily on its mass and the resistance it encounters in moving through the suspension medium. Thus, it is possible to separate crude preparations of organelles, such as nuclei, chloroplasts, mitochondria, etc. from the cytosol by the differential centrifugation of a “homogenate” or suspension of disrupted cells. Differential centrifugation involves repeating centrifugations at successively higher gravitational forces. At a given centrifugal force, large, dense and heavy structures sediment most rapidly. Therefore, at low centrifugal forces only the largest, densest and heaviest of subcellular structures would sediment. At a higher centrifugal force, subcellular structures of midweight, would sediment. Successively higher speeds and longer spin times are required to purify smaller and smaller organelles.

The media used for these cellular separations vary considerably, but they usually contain sucrose at a molarity of about 0.3 – 0.5 M and a buffer in the range of 7.0 - 7.5 pH. The media are designed to maintain the osmotic, ionic and pH conditions appropriate for preserving the organelles in their biochemically functional states. Phosphate and tris (hydroxymethyl) amino methane are frequently used for the buffer. Other common constituents include chelating agents such as EDTA, metal ions such as K^+ , Mg^{++} or Ca^{++} , and protective agents for sulfhydryl groups. Chelating agents prevent specific substances from reacting with others by bonding specifically with the substance.

Differential centrifugation is a very widely used technique for the separation of subcellular components. However, the resulting fractions are usually heavily cross-contaminated. A complementary technique which is often used for further purification of the fractions is equilibrium density (isopycnic) centrifugation. In this technique, the particles are allowed to sediment in a density gradient in bands centered around their own buoyant densities. Using this method, particles of similar size, but different densities can be separated.

To determine relative purity of fractions, they can be examined by electron microscopy or can be tested using enzymes specific for various components.

V. WHAT SIMPLE TESTS CAN BE USED TO DETERMINE PRESENCE AND/OR AMOUNT OF ORGANIC COMPOUNDS IN TISSUES?

After reading this section you should know:

1) what simple tests for organic substances will be available to you in this and future labs.

Four major groups of organic macromolecules are found in living cells: carbohydrates, proteins, lipids or nucleic acids. Many different tests (both quantitative and qualitative) have been developed to determine the chemical composition of cells and cell components. A few of the simpler tests are noted below.

Iodine Test for Starch. This is a qualitative test. It can be used to determine whether or not starch is present or absent in a sample, but it will not provide quantitative information on how much starch is present.

To perform this test: Add a few drops of dilute iodine solution to the sample. A change in color to blue black is a positive reaction for starch.

Benedict's Test for Reducing Sugars. This can be used for qualitative or quantitative testing. A sample is heated in Benedict's reagent (an alkaline reagent containing blue cupric copper). If the sample contains reducing sugars, e.g., glucose or fructose, the copper is reduced to red cupric oxide. Because the original reagent is blue in color, a positive test will tend to go through a series of color changes: green to yellow to orange red. The color and the amount of color can be measured in a spectrophotometer. A spectrophotometer measures the amount of a preset wavelength of light that passes through a sample. To determine concentration in the unknown sample, this value is compared to the values obtained **standard solutions** of known concentration.

Fats and Oils. The simplest qualitative test for fats and oils is to place the substance on unglazed paper. If a permanent translucent spot is produced, fats or oils are present. Water will also produce translucent spots. However, these will dry and disappear. The translucent spots produced by fats and oils will be permanent.

Biuret's Test for Protein. This test can be used for qualitative or quantitative testing. The test relies on the fact that any substance with 2 or more carbamyl groups ($-\text{CONH}_2$) joined directly or through another C or N atom give a purple color reaction in the presence of 2.5M NaOH and a 1% solution of copper sulfate. The amount of color can be measured in a spectrophotometer and compared to known standard samples to determine amount of protein. Caution: Glucose will interfere with this test by causing the copper to precipitate out of solution. This will turn the solution red. If this occurs, the interpretation of the results for protein presence/absence will be

obscured.

Feulgen's Stain for DNA. The Feulgen reaction is the Schiff's reaction for the detection of aldehyde groups (- CHO) and was described earlier under the section titled: Staining and Fixing. As noted, this is a highly specific stain for DNA and, since the reaction is stoichiometric, it can also be used for establishing the quantity of DNA in a cell.

VI. WHAT FACTORS AFFECT DIFFUSION?

A. What is diffusion?

Diffusion refers to the movement of a substance from an area of higher concentration to an area of lower concentration. In biological systems diffusion usually refers to the movement of a solute in an aqueous (water) solution. A solute is defined as a substance that will dissolve in a solvent, for example in water. Diffusion occurring across a membrane is termed either osmosis or dialysis. Osmosis is the term used to describe the passage of water across a membrane. Dialysis refers to the passage of substances other than water, e.g. solutes, across a membrane.

In order to dissolve in water, the surface area of each polar or ionic molecule must interact with free water molecules. In effect, the free water molecules form a ring of hydration around each solute molecule. The greater the total surface area of the solute molecules, the more free water that is needed to form these rings of hydration. As a result, when solute molecules are dissolved in water, the concentration of free water molecules per unit volume is effectively reduced. The greater the number of solute molecules dissolved per unit volume of a solution (i.e. the greater the molarity), the greater the diffusion gradient or potential.

B. What is the force behind diffusion?

The kinetic energy of free water molecules (as evidenced by Brownian movement) is the driving force behind osmosis and diffusion. A solution with a higher concentration of free water molecules per unit volume has a greater kinetic energy per unit volume. As a result, free water tends to move from an area of higher concentration (greater kinetic energy) to an area of lower concentration. Similarly, solute molecules will tend to move from an area of higher concentration to an area of lower concentration.

