Chapter 1
Introductory Remarks, Assisted Viewing and Preservation Techniques

I. What is Expected of You in this Laboratory

A. Preparation: Each week before entering the laboratory you are expected to have read and understood the exercise for the current week's laboratory meeting. The amount of information that must be covered each week is considerable and you will absorb it more effectively if you come to class prepared. If you need help, see your instructor during office hours.

You are expected keep all of your laboratory exercises in a loose-leaf laboratory notebook or binder and to bring it to class each week. You are also expected to purchase (unless you already own one) a laboratory dissection kit containing *at least* (a) one pair of surgical scissors, (b) one scalpel or single-sided razor blade, (c) one blunt probe, (d) one sharp probe, (e) one pair of fine forceps, and (f) one metric ruler. These materials are available as a package for this class at the NAU Bookstore for about $25. You will find this kit valuable for other courses in biology, so consider this purchase an investment in your future career. **Bring your kit to class each week**.

B. Written Assignments: Each week you may be asked to take a quiz, diagram animals, tabulate your observations, perform and interpret the results of simple experiments, or answer study questions. *Usually*, the assigned work will be due at the beginning of the next laboratory; *occasionally* it will be due at the end of the laboratory period. The purpose of these assignments is to help you focus your thinking and your observations as you complete your laboratory work. Points will be taken off for late assignments.

C. Quizzes: A 10-point quiz will be given in the first 15 minutes of most laboratory periods. The material covered will consist primarily of material from the previous laboratory, but will also include at least two questions from the current laboratory exercise. Quiz scores will make up 80 points of the 350 total points contributing to your laboratory score. There will be 9 total quizzes. You may drop your lowest score.

D. Diagrams: Use this manual as your laboratory notebook if you like. Notice that its pages are punched so that the entire volume may be placed in a three-ring binder. There will be space for diagramming specimens in most chapters under the descriptions of each animal. However, if you need more space for your diagrams or notes, feel free to add additional pages. If you do this, use plain white 8 1/2 x 11" paper of a texture that will take pencil drawings and/or ink notes. **Do not use odd-sized, lined, colored or tear-out pages, or soft, expensive drawing paper.** For your drawings, use a fairly hard (3-4H) drawing pencil, or if you are brave, use ink, but if you do, use only black and a high quality pen. *Smudgy ball-point drawings are unacceptable and will be returned without a grade!*

The pages of your notebook containing your laboratory drawings and notes will usually be turned in at the end of each period. Your work during the laboratory is of particular interest because this is the only way for your instructor to "see through your eyes," and determine how well or poorly you are comprehending laboratory assignments. Your work will be inspected, marked with comments or suggestions and returned to you before the next laboratory. This exercise is also incentive for students to stay for the entire laboratory period. Most laboratories cannot be completed in less than three or four hours, so plan to stick around, and seriously consider scheduling additional time to review laboratory materials at times other than the scheduled laboratory period.

Since drawings are the "language of anatomy," it is important to learn how to make simple, interpretive drawings, of reasonable scale, properly labeled, and containing as much or as little detail as the subject requires for clarity. This usually takes some practice and that is why you are asked to do a fair amount of drawing for this class. *Draw what you see*, and resist the temptation to embellish your diagrams or render them "artistically." Simply show the spatial relationships of the structures you explore in a clear and diagrammatic way. If you add sheets to your notebook use one side of each page only. *Avoid the use of color unless it is absolutely necessary for clarity*. Do not bother with shading or perspective. Make simple line drawings whenever possible.

You will often be referred to diagrams available in your copy of Brusca & Brusca or in references available in laboratory. Be sure to bring your textbook to laboratory, but use the illustrations therein only as a guide to identify structures and their relative anatomical arrangements. *Do not COPY these diagrams!* In order to learn anatomy you must explore it for yourself.

E. Notes: You will often be asked to tabulate simple notes or to group small diagrams for comparative purposes. You may also be asked to write out the experimental protocol and results of certain experiments. Learn to keep your notes organized and to group your observations and drawings in a planned way. Clear, well-organized notes and diagrams are invaluable when you review your work.

F. Study Questions: The answers to your study questions (as well as your laboratory assignments and exams) are expected to be *legible, correctly spelled and grammatically correct*. Use complete sentences. If you cannot communicate clearly in writing, your instructor may be unable to understand what you mean and will be forced to count your answers wrong. The study questions are designed to help you relate laboratory and lecture material, and will give you practice in answering essay and short answer questions that are likely to appear on exams.

Note: Written exercises are described in outline font. Unless otherwise instructed, you are to hand these in at the beginning of the next laboratory period.

G. Evaluation of Laboratory Work: Your instructor will soon learn that you work conscientiously (which is not synonymous with "quietly"), or tend to waste your time and that of others. Most laboratories will require the entire period to complete, so consider each session an exercise in time management as well as one of Invertebrate Zoology. If you finish

early, there will always be more to see, so if you do complete the exercise, don't leave, stick around and do some exploring. Compare your observations of specimens with those of your classmates. Talk about what you understand as well as what has puzzled you. Ask your instructor questions. Your grasp of the material will increase considerably and you'll have a lot more fun.

Each of your written assignments (other than quizzes) will be rated on a scale of 1 to 5 with increasing points awarded for careful observations with clear drawings and descriptions. Note that this is *not* an art class. However, careful renderings are a necessary skill in Invertebrate Zoology. You may discover a new structure or creature some day, or simply require detailed notes for your records or publications. Strive to improve your notebook-keeping skills throughout the semester. It is important to remember that a well-organized laboratory plan (requiring preparation before class), combined with curiosity and industry during laboratory will enhance your comprehension of lecture and reading material immeasurably. These skills will support the total process of your learning and understanding invertebrates now, and will translate well to your further studies with other organisms. Your laboratory notebook will make up 45 points of the total points for the laboratory.

H. Practical Exams: There will be two practical exams worth 50 points each and a final exam worth 100 points at the end of the semester. *You must take all three exams*. Although each exam will concentrate on material presented since the previous exam, information presented in lecture as well as laboratory will accumulate. All exams will therefore be cumulative. Practical exams will be given during class periods and you will have the entire period to complete your work. Since exams will be given on the same day there will undoubtedly be some overlap in material between exams. Students from the Wednesday morning lab are honor-bound to keep their mouths shut about their exam's content. Lack of honor on your part (or "friendship" to afternoon lab students) will undoubtedly increase *their* overall score at *your* expense. Avoid such altruism! The same rules for missed practical exams are the same as for missed lecture exams. See the course syllabus for details.

I. Field Trip to Puerto Peñasco, Mexico: A field trip to Mexico is tentatively scheduled for late February 2010. This trip is will be part of a separate, one-credit course (BIO 227, *Intertidal Invertebrates of the Sea of Cort*ez) in which students will have the opportunity to examine living marine invertebrates in their natural habitats.

This course will be open *only* to the 12 most outstanding students in the class electing to go (based on lecture and laboratory scores accumulated by mid February 2010). If you are among these students but elect not to participate, your space will be awarded to the next highest-ranked student. If you decide to participate, you must register for this course and pay the $50 laboratory fee *as well as* contribute approximately $130 for food and housing *by 5:00* on 23 February (5 days in Mexico *plus* an hour of Biology credit for $180 is not a bad deal). **Your fees will not be refunded if you withdraw from the course**. Participation or nonparticipation in this course *will not* affect your grade in the lecture or laboratory portion of BIO 221, although you will very likely find it a most rewarding experience.

J. Summary of Laboratory Points: A total of 350 points will be awarded for laboratory activities. This amount is *slightly less than one-half* of the total points for this course (750). This makes sense because at least half of your classroom time will be spent in the laboratory. Information obtained in laboratory will overlap extensively with that obtained in lecture, and vice versa. Your ability to integrate this information will enhance your performance overall. Lab points will be awarded as follows:

Quizzes	80
Laboratory notebook	45
Practical exam 1	50
Practical exam 2	50
Final practical exam	100
Overall participation	25
Total	350

K. A Note on Memorization: A common complaint about survey courses such as BIO 221 is that students are forced to commit huge chunks of information to memory that are unlikely of use to them later in life. This is in some cases true, depending on the career of the student. However, there are two very good reasons for this apparent wanton misuse of power on the part of instructors.

The first is that *nearly all students taking this course are pursuing careers in science*. A skill that **all** scientists must develop (and this especially includes future *medical scientists*, a.k.a., doctors, dentists and veterinarians) is **the ability to organize, store and recall large bodies of factual information, rapidly and accurately**. This is more than simple memorization. It also involves the ability to link and summarize disparate pieces of information, AND, a large mental database IS an essential part of this process. Your accurate diagnosis of disease, or a statistical problem, or species relationships can save money, time and lives. This will be *your job* as a scientist. Therefore, the more practice any future scientist has in training his or her mind to do this, the better scientist he or she will be.

The second reason for developing sound information-handling skills has to do with the nature of biological species. As students of living organisms (including human ones), you are *all* biological scientists. The process of speciation as we know it inevitably leads toward hierarchical relationships among organisms. For this reason, we can best identify and understand organisms by identifying characteristics they share and arranging taxa into more or less distantly related groups. To do this, one must have a sufficiently broad understanding of species characters to recognize closely as well as distantly related groups. Again, the species you study in your careers may not be invertebrates, but the ability understand and skillfully reconstruct biological hierarchies of all kinds will serve you well throughout your scientific careers.

L. Planning your time: As mentioned above, another skill you will learn in this course is time management. There is much to accomplish in each laboratory and you are unlikely to cover it all unless you read and plan your activities before each laboratory. Read each

chapter well before each lab begins. Be sure that you understand what you are being asked to do. Make an outline of all of the required activities and decide how much time you are likely to have available for each one. Schedule weekly meetings with your lab T.A.; his or her job is to help you. Making these kinds of organizational changes may take some practice, but you will find yourself absorbing much more of the material with less effort. Prepare for class ahead of time because providence favors the prepared mind.

M. Concluding remarks: So there you have it. Work hard. Study hard. Attempt to really *learn* the material, not simply memorize it. Immerse yourself in this course, and you will find your appreciation of invertebrate animals and biology in general increasing by the week. You'll also have a great time. Let yourself get behind or attempt to "gut" your way through and you will likely have a truly miserable experience.

II. Laboratory Skills

A. An Introduction to Assisted Viewing

The function of a microscope is to form high-quality, enlarged images of objects that are ordinarily difficult to observe with the naked eye. Three aspects of image formation must be considered in order to understand how microscopes work: ***magnification***, ***resolution*** and ***contrast***. An understanding of these factors will enhance your ability to use this important piece of laboratory equipment.

1. Magnification: Magnification is defined as the ratio of the image size to the size of the object. Magnification is achieved in light microscopes by the refraction of visible light through glass, fluorite or quartz lenses. In a lens system composed of more than one element (as most compound microscopes are), the total magnification equals the arithmetic product of the individual magnifications. Consequently, if an image is formed by two superimposed lenses each having a magnification of 10X, the total magnification is 100X.

2. Resolution: A microscope has a certain limit of resolution that may be expressed as the minimum distance that can separate two objects while still permitting their visualization as separate entities. This means that the smaller the resolution is, the better the microscope is for viewing minute objects. The resolution of an optical system restricts the useful degree of magnification because once the resolution limit is reached, further magnification will not produce images of greater detail.

The limit of resolution is determined by (1) the physical properties of the radiation used to illuminate the object, and (2) the physical properties of the lens system, plus the geometry of illumination (collectively described by a constant called the "numerical aperture"). Thus, the expression:

$$R = \frac{.6\mu}{A}$$

describes the influence radiation wavelength (μ) and numerical aperture (A) have on resolution (R). As wavelength gets shorter (smaller), resolution gets smaller, and objects can be very tiny and still be successfully viewed. Electron microscopes have better (i.e., smaller) resolution than light microscopes in part because objects are illuminated with a beam of electrons whose wavelengths are shorter than those of visible light. Similarly, as numerical aperture increases (usually due to the ability of the lens to focus light in a precise way), resolution decreases, again permitting successful viewing of small objects.

3. Contrast: Assuming that an object is sufficiently magnified and that the resolution is adequate, there must be contrast between the object and its surroundings in order for it to be visible. The human eye perceives contrast through differences in color or differences in intensity (brightness). Few biological objects suitable for microscopy have sufficient inherent color contrast to be useful. In fact, most specimens are so uniformly transparent that cellular structures are nearly equivalent in brightness. In light microscopy, contrast is usually obtained by staining, in which cells are impregnated with dyes. Alternatively, phase contrast microscopy may be used to vary the quality of illumination so that it reflects differently from cellular components and their surrounding environments. We will primarily use stains for contrast.

B. Use of the Binocular Dissection Microscope

1. General: Dissection microscopes differ from the compound microscopes available in this laboratory in having two objectives to give a stereoscopic view of the object. There are a limited number of these 'scopes in this lab so you may have to work in groups of three or four. Remove the 'scope from the cabinet ***with both hands***; one grasping the arm and the other supporting the base. Have a partner carry the light source or make a second trip to the cabinet to get this equipment. Haste in this case, can make you sadder, wiser and *poorer*! You will be held responsible for carelessly broken equipment. Thus, after you have carefully positioned the 'scope at your bench, obtain a slide or other specimen you'd like a closer look at from the demonstration bench.

2. Illumination: Place the 'scope firmly on the lab bench with the light source directly in front of it. Make sure the light source is turned ***off*** before you plug it in. This saves light bulbs. You may use transmitted light via the mirror (if one exists) under the stage or light the object directly, or use a combination of both. Try to position the light source so that each eye tested separately sees the same illumination.

3. Objectives and Oculars: With an object on the stage, rotate the nosepiece so that the smallest objective is toward the front of the 'scope. It should lock firmly into place. Looking from the side, move the focusing knob down until it reaches its limit. Now look through the scope and ***focus upward***. Always focusing upward will prevent you from mashing the objective into the object (a bad thing). Close your left eye and focus on the object using your right eye. Now close your right eye and move the knurled part just below the left ocular left or right until you can see clearly. Focusing each ocular independently will keep your eyes from fatiguing with prolonged 'scope use. Finally, adjust the interpupillary distance of the scope by pushing or pulling gently on the ocular tubes. The scope is now customized for

your face and eyes. *Do this every time you use a dissection microscope*. You may have to make subtle changes in illumination or ocular focus as you change objectives.

4. Care and Cleaning: With dissection 'scopes, be especially careful to remove all saltwater spills, followed by wiping with fresh water, and then drying with paper towel. *Saltwater is very corrosive!* Periodically clean the oculars and objectives with lens paper and a drop of xylene or ethanol to remove eyelash oil. ***Do not use*** Kleenex, kimwipes or your shirttail! Before putting your scope away be sure that it is clean and that the nosepiece is rotated with the smallest power objective forward. Move the focusing knob downward to its limit. When any microscope is not in use, it should be protected by a dust cover or plastic bag. Collapse the movable arm of the light source and gently wrap the cord around the base. Return both the scope and the light source to their proper place in the cabinet at the back of the room. Report any mechanical difficulties to your instructor immediately, and above all, *be gentle*. The dissection scope is the most essential tool in Invertebrate Zoology. Treat it well and your work will be much easier and more satisfying.

Note: You will be assigned particular microscopes to use in this laboratory. It is *your responsibility* to make sure these pieces of equipment are properly cared for, properly stored and IN THIS ROOM after each lab and at the end of the semester. If they are damaged from misuse or missing, you will be held financially responsible.

C. Notes on Use of the Dissection Microscope

Notes:

D. Use of the Compound Microscope

1. General: Microscopes when not in use are kept in the cabinet at the back of the room. Because of the dust problem in BS 328, when not in use, microscopes must at all times be covered by plastic dust hoods. Use the microscope numbered according to your assigned seat (if possible). This means that only you and one or two others will use the same 'scope and ensures responsibility for its care. Laboratories are often wet and we will use much fresh material. As with dissection microscopes, always be sure that your 'scope is clean and free of dust or water before putting it away. Carry the scope with both hands as previously described and use only lens paper to clean the lenses.

2. Illumination and Low Magnification Viewing: Place the microscope firmly in front of you, unwrap the power cord and plug it in. Looking from the side, be sure that the lowest objective is forward and using the coarse focusing knob, move the objectives downward to their limit. Be sure that the condenser is moved all the way up beneath the stage, and move the lever of the iris diaphragm all the way to the right.

Place a prepared slide on the stage and secure it in place with the mechanical stage clip. Turn the light source on. Move the lever of the iris diaphragm to the left until a comfortable amount of light is permitted into the scope, and using the coarse focus, ***focus upward*** until the object is visible. Use the fine focus to bring the object clearly into view. Try to keep both eyes open when viewing the object. With a little practice you'll be able to ignore (or not ignore) what your nonmagnified eye sees. This will help prevent eyestrain and will come in handy for measuring objects.

Shift the position of the diaphragm lever back and forth and notice its effect on the quality of the object's image. Skillful use of this lever is especially important at high magnification. Try the next higher power objective and see how the image changes. Recalling the introductory remarks at the beginning of the exercise, consider some possible explanations for the changes you see. How would you test your hypotheses?

3. Higher Magnification: When you switch to the "high dry" or 40x objective you should find that the object on the slide is very nearly in focus. If adjustment is needed, use only the fine focus and remember which direction you must usually move this knob to relocate the image. You have undoubtedly discovered by now that higher magnification requires more light. Adjust your diaphragm as needed. The highest power objective (100X) is for oil immersion viewing and requires the use of a high quality oil that improves the transmission of light from the object to the objective. **DO NOT USE THIS OBJECTIVE UNTIL ITS PROPER USE HAS BEEN DEMONSTRATED BY YOUR INSTRUCTOR!** After you use oil immersion, be sure to clean the objective off with lens cleaner before putting the microscope away.

4. Making a Wet Mount: Obtain a glass slide and cover slip from the demonstration bench. Also obtain a toothpick, a dropper bottle containing tap water and a dropper bottle containing 0.5% methylene blue. Clean the slide and the cover slip with paper towel to remove any dried material or dust that may obscure your view. Place a drop of water onto the slide and scrape (gently!) the toothpick along the inside of your cheek. Swirl the business

end of the toothpick in the drop of water and cover the drop with the cover slip. ***Note:*** You can avoid air bubbles if you place one edge of the cover slip at the left extreme of the drop and lower the other edge of the slip toward the right (southpaws may prefer to reverse this procedure). Place a drop of methylene blue at the left (or right) edge of the cover slip and a piece of paper towel at the opposing edge. The stain will be drawn under the cover slip and will increase the contrast of whatever it is you extracted from your cheek. Add an additional drop of water to the stain side if necessary. Place the slide on the microscope stage and follow the focusing procedure described above.

5. A Technique for Measuring Objects: In some cases, microscopes are not equipped with ocular micrometers. However, the following method can be used instead to obtain an accurate estimate of the size of objects you'll be viewing. This technique comes in remarkably handy since you will find that often even the most sophisticated dissection or light microscopes may lack ocular micrometers. To begin, place your millimeter ruler *at the level of the stage* next to and perpendicular to the long axis of the slide (*not* under the objective). Keep both of your eyes open and focus on the object (probably a cheek cell) and the ruler *at the same time*. Use one eye for each object. Record the size of the object in metric equivalents. Calculate the total magnification of the image (object x objective) and divide the metric image size by this value. This is the actual size of the object. This takes some practice so don't be frustrated if at first you don't succeed. To check your technique, measure the actual size of a small object with your ruler (1mm or less) and then measure it again at lowest power using the above technique.

6. Calibration of the Ocular Micrometer on a Microscope: Microscopes are often used to measure small objects. For instance, forensic scientists use microscopy to measure the distance between microscopic tool marks or the diameter of fibers. As you can see in figure 1 below, compound microscopes have ocular micrometers, or rulers that can help you measure items under the microscope.

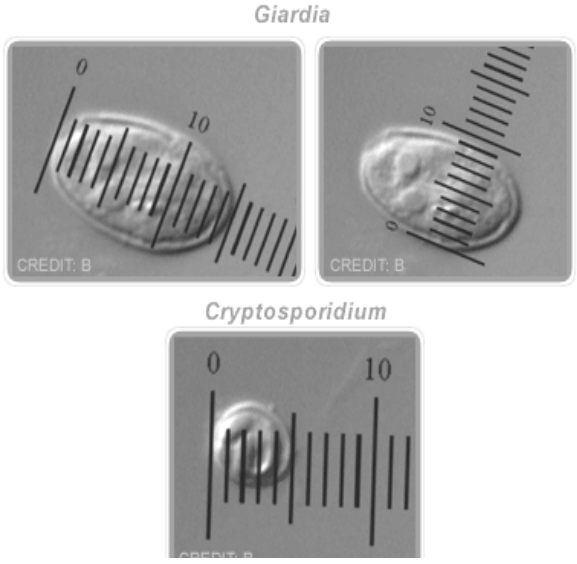

Figure 1: Ocular micrometer superimposed over *Giardia* and *Cryptosporidium* cysts under the microscope (source: http://216.54.19.111/~corp2002/epa/sb/crypto/gandcrypto/crypto0170.html).

However, the scale on the ocular micrometer changes with total magnification, and thus has no absolute value. Therefore, the ocular micrometer does not have units and it needs to be calibrated prior to use. In this activity, you will learn how to calibrate the ocular micrometer, a skill that will help you in the coming weeks.

You will use a stage micrometer to calibrate the ocular micrometer; alternatively at low magnification you can use a metric ruler. A stage micrometer is essentially a ruler that is mounted on a microscope slide that does have units (millimeters (mm) or micrometers (◎m)). When calibrating, you will line up the stage micrometer with the ocular micrometer and count the number of divisions on the ocular micrometer per millimeter or micrometer on the staged micrometer. The number of divisions will change as the magnification changes.

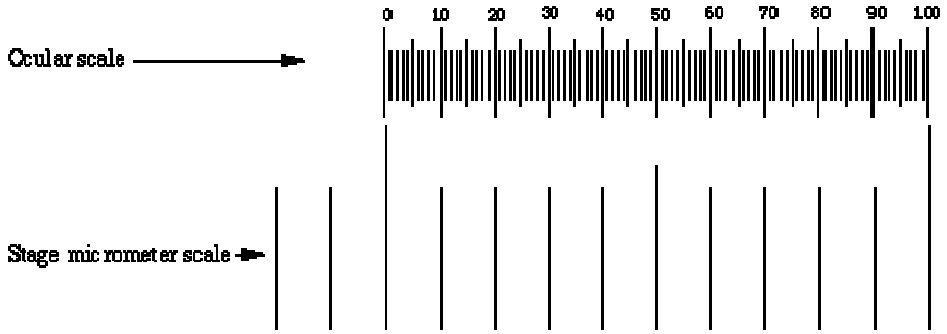

Figure 2: Comparison of the ocular micrometer and the stage micrometer.

Example: At a total magnification of 40x, a student measured 42 ocular micrometer divisions per millimeter. What is the distance in micrometers per ocular unit?

$$\frac{42 \text{ occular units}}{1 \text{ mm}} = \frac{1 \text{ occular unit}}{x} \text{ ; x = 1/42 = .024 mm/ocular unit}$$

Problem 1: At a magnification of 40x, a student measured 41 ocular micrometer divisions per millimeter. What is the distance, in micrometers, per ocular unit at 40x?

Problem 2: At a total magnification of 100x, a student measures 16.4 ocular micrometer divisions per millimeter. What is the distance, in micrometers, per ocular unit at 100x?

Problem 3: At a total magnification of 400x, a student measures 4.1 ocular micrometer divisions per millimeter. What is the distance, in micrometers, per ocular unit at 400x?

Problem 4: Notice the pattern in the above 3 problems. What do you think the distance in micrometers per ocular unit at 1000x would be?

Problem 5: It was found that the units on the ocular micrometer and the units on the stage micrometer matched up 40 units to 1.0 mm at 100 power. (So 40 ocular units equals 1.0 mm.) If the wing of a dead fruit fly was measured to be 2.5 ocular micrometer units at a magnification of 100, what is the length of the wing in mm?

Once you get the hang of it, measure the diameter of 10 cheek cells and their nuclei (choose ones that are relatively flat on the slide). Write each of the 10 values for your cell sizes in a column on one piece of paper, and on another sheet of paper, write out the 10 values for the sizes of their nuclei. Beginning with the cell sizes, add all of the values up and write their **sum** below the column. Now divide this sum by the number of values in the column (10). Your result is the **average** cell (or nucleus) size. Next calculate the **square of the average** and write it below the average cell size. Now calculate the **square of each of your 10 values** and write each value in a column to the right of the first column. Calculate the **sum of the squared values** and write it below the column. Lastly, divide the sum of the squared values by the number of values in the column (10), to get the **average of the squared values**. Write this value below the sum of the squares.

What do you have at this point? You have calculated (1) the average of your observed cell sizes, (2) the square of your average cells sizes, and (3) the average of the squares of your cell sizes. If you subtract quantity (3) from quantity (2) [that is, you subtract the average of the squares from the square of the average], you have calculated the **variance** in the size of your 10 cheek cells. The **variance** (symbolized s^2), is an important statistical parameter in biological science. This parameter provides an estimate of the degree to which a population is dispersed around its average. Thus, whereas the population average provides one bit of information that can be used to describe a population, i.e., a measure of central tendency, the variance provides another, often more useful way to describe a population, i.e., a measure of the spread of the population around the average.

The square root of the variance is called the **standard deviation** of the mean (symbolized s). This value gives a standardized estimate of the variation around the mean. Finally, the standard deviation divided by the square root of the sample size is called the **standard error** (symbolized S.E.). This term is useful for comparing distributions. Record these values in your laboratory notebook. Toward the end of the period, your laboratory instructor will

compile everyone's scores on the board. Notice the shape of the distribution. Discuss the significance of the shape of this distribution from a biological point of view.

If time permits, calculate the **average of the variances** in cell size for each student's distribution of 10 cells. Call this quantity, ***V****within*. It equals the variance in cell sizes *within* the measured distributions of cell size. Next, calculate the **variance of the averages** in cell size across all of the cell size distributions for each student. Call this quantity, ***V****among*. It equals the variance in cell sizes among the measured distributions of cell size. The sum of ***V****within* and ***V****among* equals the total variance in cell size in the entire class, ***V****total*. What is the ratio of ***V****within* to ***V****total*? What is the ratio of ***V****among* to ***V****total*? Discuss what these ratios may indicate with respect to how similar or different the constituent cell size distributions are to each other.

E. Notes on Use of the Compound Microscope:

III. Preservation Techniques

The preservation of invertebrate animals for use in teaching collections or as museum specimens is a necessary skill for every invertebrate zoologist. While excessive collection and preservation of animals is to be discouraged, carefully preserved and well-maintained specimens can provide valuable "hard copies" of animals for examination by students, morphologists and systematists. Preserved specimens, if properly labeled, provide a wealth of information for population analyses, and even the DNA from well-preserved museum specimens can be amplified and sequenced for molecular systematic analysis, tens or even hundreds of years after the animals were originally collected! Clumsily prepared or poorly labeled specimens, however, are useful for none of these endeavors and serve only to decrease invertebrate species diversity and clutter museum shelves. When collecting, take the time and effort to either return specimens to their natural habitats intact, or when necessary, fix, preserve and label them properly.

In this laboratory we will summarize the general techniques necessary for effective preservation of the animal phyla covered this semester. You are required to become familiar with the preservatives and techniques introduced here for next week's quiz. In successive laboratories you will see these techniques used to preserve live specimens for our collection. You may also have the opportunity to practice these techniques on field trips. For further reading on invertebrate collection and preservation, see Brusca (1980) and references therein.

A. Handling Specimens

Scientific specimens are valuable and irreplaceable items. They must be handled with great care to avoid breaking off structures or damaging soft tissues. Wearing gloves, carefully remove specimens from containers if you must (and often you **must** to get a decent look at them!). Rinse them gently if their preservative is noxious (as is often true if specimens are kept in formalin), and view them on a wax-filled pan or other washable surface. Specimens preserved in ethanol will dry out easily, so cover them with water during examination. When your observations are complete, be sure to replace specimens in their original container, replace any lost preservative and *close the lid securely*.

B. A Cautionary Note

Fixatives and preservatives are solutions used to transform living tissue into inert substances that are resistant to bacterial invasion and decomposition. This is accomplished by rapid denaturation and/or tanning of proteins, removal of water, or other radical modification of cell and tissue structure. *Whatever happens to invertebrate tissues when placed in preservative can also happen to YOURS!* In small quantities, many of these substances can be carcinogenic. In larger quantities or in prolonged doses, nearly all of these substances are *lethal*. **Keep this in mind while handling fixatives and preservatives.** Whenever possible, use gloves, protective clothing and protective eyewear when pouring raw solutions. Dispose of waste chemicals in proper waste containers, not down the sink! Be sure to avoid contaminating glassware used for live animals (usually labeled **LM**) with preservatives. Special preserved material

glassware (usually labeled **PM**) will be designated for containing preservatives and preserved specimens. Always wash your hands after handling preserved materials or specimen containers. When handling formalin preserved specimens, wear gloves.

We will mainly use formalin (formaldehyde solution) as a fixative. To avoid osmotic difficulties, marine specimens should be fixed in a 10% solution of formaldehyde in seawater. Freshwater or soft-bodied terrestrial specimens should be fixed in 10% formalin made with tap water. After fixing, most specimens will be preserved in 70% ethanol. If you find a container that contains a specimen preserved only in formalin (do not sniff the jar directly!) contact your instructor. We are attempting to remove all such materials from the lab.

Special note: The procedure of fixation (especially with formalin) is currently under debate because it can also fix DNA and therefore prevent future molecular analysis of museum specimens. Samples for such analysis should always be preserved in ethanol, preferably 95%.

C. Labels and Records

When collecting specimens it is important to record as much information as possible on the location and physical conditions in which the live animals were found. This is one of the reasons why you will also learn to keep detailed field notes (see Chapter 6). When you collect a specimen, you should record *at least* the following bits of information: (1) **country, state and county**; (2) **locality**, the exact geographical location if possible, or at least approximate distances from landmarks or towns; (3) **habitat**, terrestrial or aquatic, with a description of the general conditions of the area; (4) **method of capture**, which may permit comparison with other samples; (5) **date**; (6) **time of day,** including information on tides or lunar phase; (7) **original preservative**; (8) **collector's name**. If detailed notes are kept, a **collection number** is also essential since this number can be written on a piece of white paper in India ink or pencil and placed in the container with the specimen. If a field notebook is not available (and you should hang you head in shame if it is not!), write down *all* of the above information on a card and insert it into the container with the specimens. This information can later be written down in a notebook and filed where the specimens are stored. Even if a field notebook is available, *at least* record information items 1, 2, 5, 7 and 8 on a card and place it with the specimen.

D. Specific Techniques

1. Relaxation

Most organisms are a bit put off by being dumped directly into preservative, and quite understandably can contract into agonized lumps of tissue that are difficult to identify. To make this transition somewhat easier for the specimen, and to salve your life scientist's conscience, seriously consider relaxing your specimens in an anesthetic before fixing and preserving them. Relaxation techniques may vary depending on the specimen and the facilities available. In general, however, it is first necessary to make your specimen comfortable. This is accomplished by placing it (if it is aquatic) into a clean container with its

usual medium (seawater or freshwater) and setting it in a cool, quiet place with reduced light. This will allow the animal to extend lophophores, tentacles, mantle, or other appendages whose observation may be useful in a preserved specimen.

Next you need to introduce some form of anesthetic. For many organisms, simple refrigeration works quite well. Bodily functions slow down to the point where animals simply don't respond when placed into fixative. For aquatic species, 7.5% magnesium sulfate (epsom salts) or magnesium chloride solution, 15% chloretone solution, 10% ethanol, or even just fresh water (for marine species) may be slowly added to the container until the animal becomes unresponsive to mechanical stimuli. A battery of other narcotizing agents and techniques are described on p. 130 of Brusca (1980). Regardless of the method, relaxation may take several hours, so be patient.

2. Phylum Porifera

Sponges are best fixed by immersion for 24-48 hours in 10% buffered seawater formalin solution. After fixing, specimens should be gently rinsed and placed in 70% ethanol. Occasionally, brightly colored sponges or those with proteinaceous spicules may be preserved in formalin (ethanol tends to extract pigments from tissues). Calcareous sponges, however, should never be preserved in formalin, as spicules and other $CaCO_3$ containing structures will deteriorate. If histological examination is to be performed, fix tissues in Bouin's solution or other histological fixative (such as alcohol-formalin-acetic acid; AFA). For examination of calcareous or silicious spicules, soak or boil small bits of sponge in bleach (sodium hypocholorite) until soft tissues are dissolved. Rinse the remaining material in distilled water and store in 95% ethanol. Spicules may be observed by making a wet mount of the preserved material. Proteinaceous spicules are best observed in recently collected specimens.

3. Phylum Cnidaria

Cnidarians should be relaxed in 7.5% magnesium chloride solution for 24 hours before fixation and preservation. A variety of other techniques are described in Brusca (1980), but $MgCl_2$ is usually available in most biology stockrooms. Medusoid forms may be preserved in 5% formalin, or preferably 70% ethanol. Gorgonians and stony corals, having calcareous tests should never be preserved in formalin. *Carosafe*, manufactured by Carolina Biological Supply Company (https://www2.carolina.com/) is also a good, relatively benign preservative for soft tissued animals. Ctenophorans may also be preserved as described above.

4. Phylum Platyhelminthes

Flatworms require special care to collect and preserve. They should be gently lifted or washed off of the substrate on which they are found and placed in separate containers for transport to the laboratory. As with all collected specimens, observations from living specimens should be recorded in a laboratory notebook before preservation is begun. Specimens should first be relaxed in chloretone, chloral hydrate or methanol, then fixed in

10% formalin and preserved in either 5% formalin or 70% ethanol. Many specimens are small enough that mounting on slides is the most effective method for preservation and observation. We may not have time to perform mounting procedures, but techniques will be described in laboratory for later reference. Brusca (1980) recommends collection of at least three specimens of each type, mounting two specimens on slides, and then relaxing and fixing the third specimen in 5% formalin.

5. Phylum Echinodermata

Sea stars (Asteroidea), brittle stars (Ophiuroidea), sea urchins and sand dollars (Echinoidea) may be dried and stored in Riker mounts, or fixed in 10% formalin and preserved in 70% ethanol. Brightly-colored specimens will retain their pigmentation best if preserved in buffered formalin, but if the buffer is not maintained, calcareous structures will deteriorate. Drying can cause animals to contract into painfully unnatural positions. This condition may be prevented, however, by covering the animal with fine sand as it dries. Brusca (1980) recommends the following technique by E. Segal (unpubl.) for drying echinoderms with pigmentation intact: (1) place the specimen in 70% ethanol for 0-2 hours depending on how rapidly pigmentation is extracted, (2) gently rinse the specimen in tap water, (3) place the animal on its aboral surface and sprinkle the oral surface with enough 10% formalin to fill the ambulacral grooves. Place in a covered dish or container for 4-5 hours, (4) cover the specimen with a 1:1:1 solution of 95% ethanol, glycerine and water for 3-4 days, (5) rinse in tap water, and (6) allow to air dry.

Holothuroideans (sea cucumbers) are extremely difficult to preserve, so much so that many collectors simply resign themselves to not including them in their collections. These animals must be first relaxed in a narcotizing solution such as chloretone or epsom salts over a sufficiently long duration (several hours or even days) so that their tentacles remain extended. Brusca (1980) recommends simply letting the animal die in putrid seawater, although this approach can strain relations with other individuals on collecting trips. Once animals are relaxed, however, they can be fixed for 1-2 hours in 10% buffered formalin. In some cases it helps to inject fixative into the body of the animal. The specimen may then be rinsed and preserved in 70% ethanol.

6. Pseudocoelomate Phyla

Pseudocoelomates, if small, are best preserved and mounted as described above for flatworms. Extremely tiny specimens such as kinorhynchs, gnathostomulids, rotifers and gastrotrichs must be fixed and stained on slides using techniques for protozoans. We will not cover these methods in this laboratory. Larger nematodes, priapulans, acanthocephalans and nematomorphs should be fixed in formalin or AFA after relaxation, and preserved in 70% ethanol.

7. Notes on Preservation of Invertebrates

IV. A Key to the Major Invertebrate Phyla

A dichotomous key is a biological tool used for the classification of known taxa. Most keys consist of a list of "couplets" which each describe the physical characteristics of an organism. The couplets begin very generally and proceed toward increasingly specific descriptions, in much the same way that children play the game "20 questions." Just as a skilled player in this game chooses questions which eliminate possibilities, the couplets of a well-prepared key will divide the taxa to which the organism in question may belong into two groups, i.e., the organism either has thus-and-so a characteristic, or it does not. If the organism *does* have the characteristic, the organism belongs to taxon A; if not, it must be something other than taxon A and the biologist moves to the next couplet.

Biological keys, as well as the game, "20 questions" are both exercises in Scientific Method. This approach to knowledge, as discussed in lecture, seeks to provide the simplest explanations for the complex phenomena which make up in the observable universe. A process that identifies what an object ***is not***, as well as what it ***might be***, reduces the possible explanations for what the object ***actually is***. Eventually, if the process is applied systematically, the nature of the object can be simply explained. This is what biological keys help us do. Apply the key to the specimens you have observed and demonstrate for yourself how this method works.

1. Body with radial symmetry or irregular 2
   Body with bilateral symmetry, solitary or modular (comprised of many individuals). . . 4
2. Body with highly porous surface, not gelatinous . . . . . .Phylum Porifera
   Body surface smooth, with calcareous projections or gelatinous 3
3. Body with pentamerous symmetry and tube feet . . . . Phylum Echinodermata
   Body with radial symmetry, tentacles with nematocysts . . . . . Phylum Cnidaria
4. Body modular with feeding units (zooids), each ‹ 0.5 mm in size . . . . . Phylum Bryozoa
   Body solitary or colonial with zooids › 0.5 mm in size 5
5. Body surface gelatinous 6
   Body surface not gelatinous 7
6. Solitary individuals with 8 longitudinal rows of ciliated plates . . . . . Phylum Ctenophora
   Solitary or modular with incurrent and excurrent siphons and/or notochord . . . . Phylum Chordata
7. Body segmented 8
   Body lacking segmentation 9
8. Exoskeleton with jointed appendages . . . . . Phylum Arthropoda
   No exoskeleton . . . . . .Phylum Annelida
9. Body soft with muscular foot, radula, tentacles, and/or calcareous shell. . . . . .Phylum Mollusca
   Lacking all of the above 10
10. Body dorsoventrally flattened . . . . . .Phylum Platyhelminthes
    Body rounded 11
11. Anus near mouth 12
    Anus terminal 16
12. Feeding tentacles arranged in as U-shaped lophophore 13

Lophophore lacking 14
13. Body elongate, without a shell . . . . . .Phylum Phoronida
Body not elongate, with shell . . . . . Phylum Brachiopoda
14. Solitary individuals 15
Modular individuals, usually encrusting . . . . . .Phylum Entoprocta
15. Body large (> 2 mm) . . . . . Phylum Sipuncula
Body small (< 2 mm) . . . . . .Phylum Cycliophora
16. Body surface with thin cuticle 18
Cuticle lacking 17
17. Body when expanded approximately equal in diameter throughout . . . . . .Phylum Nemertea
Body greatly expanded at one end . . . . . .Phylum Echiura
18. Body elongated (>5x longer than wide) 20
Body not elongated (<5x longer than wide, usually microscopic) 19
19. Anterior end ciliated, modified as trochi, marine and freshwater. . . . . .Phylum Rotifera
Anterior end spiny, exclusively marine. . . . . Phylum Loricifera
20. Anterior end simple (or if modified, with 3 lips) 21
Anterior end complex 22
21. Body highly elongated (>20x longer than wide, parasitic in insects) . . . . . Phylum Nematomorpha
Body not so elongated, mouth with 3 lips and/or muscular pharynx . . . . Phylum Nemata
22. Anterior end eversible 24
Anterior end non-eversible 23
23. Posterior end with adhesive 'toes' . . . . . Phylum Gastrotricha
Pharynx with unique jaws . . . . . .Phylum Gnathostomulida
24. Adults parasitic . . . . . Phylum Acanthocephala
Adults free living, usually in marine sand 25
25. Body microscopic, usually with posteriorly projecting spines . . . . . Phylum Kinorhyncha
Body macroscopic, usually with annuli . . . . . Phylum Priapula