**CELL PHYSIOLOGY LABORATORY**

**BIO 121**

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**SECTION 1: THE CELL PHYSIOLOGY LABORATORY**

**Learning Objectives for the laboratory portion of Cell Physiology:**

* Become familiar with the laboratory facilities and four or five general procedures that you may use during the semester.
* Learn to keep a laboratory journal.
* Identify a worthwhile problem that can be investigated using the available facilities and procedures.
* Design experiments that will help solve the problem.
* Perform the experiments and collect valid data.
* Organize and interpret data.
* Evaluate the results in terms of research done by others in the literature.
* Communicate both the preliminary planning stages and the final results of your research both orally and in writing.

**This is a lot of stuff to learn and do! The following several pages contain directions and hints for accomplishing these objectives.**

**MANDATORY RULES OF LABORATORY SAFETY**

***SAFETY ATTITUDES***

**1. USE COMMON SENSE:** Most laboratory accidents start with something simple.

**2. BE AWARE**: Know the hazards before beginning. At a minimum, read the label and the Material Safety Data Sheet (MSDS) for the chemicals you are using.

**3. BE PREPARED**: Answer the following question: “What’s the worst thing that can go wrong?”

**4. BE PROTECTED**: Know what practices and equipment can minimize exposure to hazards in your workplace. “What should I do to be prepared for it?”

***GENERAL SAFETY RULES***

**1. NEVER** work alone in a laboratory/chemical handling area or chemical storage area.

2. **NEVER** drink, eat, chew gum, smoke or apply cosmetics in the laboratory/chemical handling areas.

3. **KNOW** the location of the emergency eye wash station and safety shower before starting work in the laboratory/chemical handling area.

4. **KNOW** the location of all sources of ignition when working with flammable chemicals near enough to cause a fire or explosion in the event of a release or spill.

5. **WASH PROMPTLY** whenever a chemical has contacted the skin and **BEFORE LEAVING** the laboratory with soap and water; do not wash with solvents.

6. **DO NOT “SNIFF TEST”**; Avoid inhalation of chemicals.

7. **DO NOT MOUTH PIPETTE** anything; use suction (pipetting) bulbs.

8. **DON’T EVEN THINK ABOUT** horseplay, pranks, or other acts of mischief – they are especially dangerous in the laboratory and are reason for dismissal.

***WORKING IN THE LABORATORY AFTER HOURS***

1. The Laboratory is available for work outside of your scheduled laboratory session. Once groups are working on their semester projects all Cell Physiology laboratory sessions are open to all students. We also have arrangements with most of the other courses in Sequoia 212 during the semester. You must respect the students and instructors in a scheduled class, however, or you may lose your after hour privileges.

2. Working off hours requires that at least one instructor is available and has agreed to be nearby to assist you. You must also arrange with at least one other classmate to work with you.

3. Working in the Laboratory during off hours increases both yours and the instructors responsibility. You must be particularly careful of all rules. Remember – this is a privilege and it can be taken away!

***CHEMICAL SPILL PROCEDURES***

1. **LEVEL 1:** In the event of a chemical spill of known low toxicity inform the instructor and s/he will help you clean it up.

2. **LEVEL 2:** In the event of a chemical spill of known moderate toxicity move your classmates away and inform the instructor - s/he will help you clean it up.

3. **LEVEL 3:** If the chemical spilled is unknown or extremely hazardous, inform the instructor immediately, evacuate, close doors to the laboratory and wait for emergency personnel.

4. **LEVEL 4:** In case of a medical emergency, summon medical help immediately by contacting the Department of Public Safety; phone from any campus phone.

***RULES FOR THE PROPER USE OF CHEMICALS***

1. **KNOW** the chemical hazards of what you are using before you use it, as determined from the MSDS and other appropriate references.

2. **KNOW** the appropriate safeguards for every chemical, including required or suggested personal protective equipment before using it.

3. **KNOW** the location and proper use of clean-up, safety and emergency equipment.

4. **KNOW** how and where to properly store the chemical when it is not in use.

5. **KNOW** the proper method of transporting chemicals within the facility.

6. **KNOW** appropriate procedures for emergencies, including evacuation routes, spill cleanup procedures, and proper waste disposal.

***RULES OF HOUSEKEEPING***

1. Access to emergency equipment, showers, eyewashes, and exits shall never be blocked by anything, not even a temporarily parked cart.

2. Whenever possible, chemicals will be stored in their original container and label integrity maintained. If chemicals are re-containerized, the label on the new container will include all hazards and safety information as on the original container.

3. Keep all the work areas, especially benches and counter tops clear of clutter.

4. No chemicals are to be stored in aisles or stairwells, on desks or laboratory benches, on floors or in hallways, or to be left overnight on shelves over the workbenches.

5. Do not store extraneous materials in fume hoods. These materials will interfere with the airflow and jeopardize the safe operation of the hood.

6. Store all chemicals in a seismically safe manner. Closed cabinets and lipped or retaining wired shelves are two methods used for seismic bracing.

7. All chemicals shall be placed in their assigned storage areas at the end of each workday.

8. At the end of each workday, the contents of all unlabeled containers are to be considered waste.

9. Wastes shall be properly labeled and kept in their proper containers.

10. Promptly clean up all spills; properly dispose of the spilled chemical and cleanup materials.

11. All working surfaces and floors should be cleaned regularly.

12. Dispose of cracked or broken glassware immediately. Protect your hands when inserting or attaching glassware. Lubricate glassware.

13. Do not dispose of toxic chemicals in the sinks; place in properly labeled waste containers.

***PROTECTIVE CLOTHING AND EQUIPMENT***

1. Eye protection worn while working with chemicals should meet the requirements of ANSI Z87.1. Wear goggles, such as type G, H, or I, at all times. When working with corrosive liquids, also wear a face shield, type N, large enough to protect the chin, neck and ears, as well as the face.
	1. If your vision requires the use of corrective lenses in glasses and you are required to wear eye protection, either: (1) wear corrective glasses that meet the requirements of ANSI or (2) wear approved goggles over regular corrective glasses.
	2. Avoid wearing contact lenses when working with chemicals. In the event of eye exposure, contact lenses may absorb and/or trap chemicals against the eyes. Wear safety glasses instead of contact lenses.
2. When working with corrosives, wear gloves made of material known to be resistant to permeation by the corrosive chemical and tested for the absence of pin-hole leaks.
3. Wear a laboratory coat or a rubberized laboratory apron. Do not wear shorts or short skirts; legs should be covered.
4. When working with allergenic, sensitizing, or toxic chemicals, wear gloves made material known to be resistant to permeation by the corrosive chemical and tested for the absence of pin holes.
5. Do not wear shoes with open toes or constructed of woven material. High heals are discouraged.
6. Whenever exposure by inhalation is likely to exceed the threshold limits described in the MSDS or the OSHA permissible exposure limits (PEL), use a hood; if this is not possible, an appropriate respirator must be worn. Before a respirator may be worn, a physical examination, training and fit testing must take place. Consult with the instructor before doing any such work.
7. Carefully inspect all protective equipment before using. Do not use defective protective equipment.

**THE LABORATORY NOTEBOOK**

***The Laboratory Notebook is a permanent record of your experiments.***

***It is the property of the laboratory – not of the student/scientist.***

The purpose of the notebook is to record the plan for your experiments, your thoughts on the execution and appropriateness of the plan, and the results. The peer-reviewed scientific process requires that you report, not just the results and interpretation of your work, but enough information for any skilled scientist to repeat the experiment independently. This includes ***you***! A well-kept notebook will allow you to monitor your daily efforts, troubleshoot things that don’t work out, and improve your experimental protocols when things go particularly well.

 ***How to keep a Laboratory Notebook:***

1. Write out your plan for each day’s experiment in advance. Each new technique is written out in detail. Things that are repeated and unchanged can be referenced to the latest page in the Notebook on which the protocol is written.

2. On the day of the experiment, record your observations on laboratory conditions, group member tasks, variations from intended times and concentrations, etc. Record your data.

3. After completing the experiment, record your thoughts on the success or failure of the experiment. Was the data what you expected? If not, why do you think it was different? If the data were what you expected, was the protocol followed without deviation? If you deviated from the plan why do you think it might have given good results?

 **\*PLEASE SEE APPENDIX 1 ON PAGE 30**

**SECTION 2: THE LABORATORY EXERCISES**

**EXERCISE 1: CELLS, MICROSCOPY AND MEASUREMENT**

**Microscopy.** The microscopes that we use in this laboratory are relatively inexpensive models of the great workhorses used for centuries to study cell biology. The Compound Microscope is the standard microscope that most people are already familiar with. The conventional model is a binocular microscope that allows you to view your specimen from above the stage with lighting from below the stage passing through the specimen and to your eye. Most of these microscopes have fixed magnification ocular lenses near the eyes (usually 10X) and a series of adjustable objective lenses near the object (usually 4-100X). The focal length of these objective lenses, the distance between the object and the objective lens at which the image is in focus, is very short. This allows for very high magnification lenses. However, these short focal lengths do not allow whole specimens or thick tissues to be viewed but instead require the use of low volume cell preparations or thin sectioned material placed on microscope slides. High magnifications make up for these inconveniences, of course, by allowing for a great deal of close analysis.

 Another feature that we will make use of this semester is having both Conventional and Inverted Microscopes equipped for epifluorescence. Epifluorescence is the use of compounds that emit light in very tight patterns of wavelengths in the visual spectrum. They give off light that is a given color (red, green or blue) whose wavelengths do not overlap. These compounds can be used to show which cells contain given proteins or chemicals without any ambiguity of interference from other fluorescent compounds. A microscope equipped for epifluorescence is the same as one of the microscopes described above with the addition of a high energy light source and a series of light filters that control both the wavelengths of light that hit your sample and the wavelengths of light that reach your eye. These are called the excitation and emission wavelengths, respectively. Your instructor will show you how to use these fluorescent systems as the semester progresses.

**A. Size Does Matter!  *Meters, micrometers, nanometers and ängstroms***

 **1 meter = 39.37 inches**

 **= 1,000 millimeters (mm) (10-3)**

 **= 1,000,000 micrometers (m) (10-6)**

 **= 1,000,000,000 nanometers (nm) (10-9)**

 **= 10,000,000,000 ängstroms (Ä) (10-10)**

**B. The human eye is not enough to work with most cells**

1. The eye can resolve two points 90 m apart

 a. Any closer together, or smaller, they look like one thing

 b. A red blood cell is 7-8 m in diameter

# C. Relative Sizes of Cells and Cell Components for Microscopy

 **Micrometer Ängstrom**

Skeletal muscle cell 10 – 100 100,000 – 1,000,000 eye?

Cardiac muscle cell 15 – 25 150,000 – 250,000

Columnar epithelium 8 - 10 80,000 - 100,000 light microscope

Smooth muscle cell 5 –10 50,000 – 100,000

Red blood cell 7 – 8 70,000 – 80,000

Nucleus ~3 ~30,000

Mitochondria 0.1 – 0.3 1,000 – 3,000 light microscope

Ribosome 0.014 – 0.016 140 – 160

Myosin “thick” filament 0.015 150

Cell membranes 0.004 – 0.010 40 - 100

 Electron microscope

Actin “thin” filament 0.007 70

Cytochrome C 0.0025 25

**D. Microscopes and Optical Resolution**

**1. Light Microscopy: Photon absorption, transmission and/or reflection**

***a. The binocular light microscope.***

a. Physics:

1. bright white light (all wavelengths)

2. glass lenses magnify the image, mirrors bring it to the eye

3. passes through thin cells or pieces of tissue

b. Resolution:

1. objective lens near the object: 4X to about 100X

2. ocular lens near the eye: 10X

3. maximum resolution 1000X relative to the eye

 (eye = 90 m or 90,000 nm; light ‘scope = 90 nm)

c. Uses:

1. “Conventional”: most standard microscopic analysis

2. “Inverted”: live cultures can then be viewed from below

***b. The binocular light microscope equipped for epifluorescence.***

a. Physics:

1. we see different wavelengths of light as different colors

2. fluorescent molecules hit with correct wavelength of light kick one of their electrons into an outer shell, when the electron falls back to its normal shell it releases light in a different wavelength

3. specific filter passes excitation  from a mercury lamp

4. specific filter passes emmission  (seen as color) to the eye

b. Resolution:

1. 100X - 600X magnification

c. Uses:

1. Multiple spectra filter sets now allow more than one

 fluorescent color to be used in a single specimen

***2. Confocal microscopy***

a. light is collected through a pinhole so that light outside

of the exact plane of focus of the microscope is eliminated

b. allows “optical sectioning”: step controlled stages

automatically collect a series through the specimen

c. computers are then used to reconstruct 3D image

**2. Electron Microscopy: Electrons transmitted or absorbed by electron density**

***a. Transmission electron microscopes (TEM)***

a. Physics:

1. electron beam passes through ultrathin sections

2. electron density of tissue components determines how many get

 through – the more electron-rich the tissue the darker it looks

b. Resolution:

1. working resolution is 10A (90,000X the eye)

2. theoretical resolution is 2 A !!!

c. Uses:

 1. subcellular analysis

***b. Scanning electron microscopes (SEM)***

a. Physics:

1. electron beam is scanned along the surface of the specimen

2. the surface is first sputter-coated with electron-rich metal

b. Resolution:

1.same as TEM

c. Uses:

1. the amazing detail comes from the natural shadows from the contours of the surface being drawn out by the uniform coating

**WEEK 1 PROCEDURE: Comparing Tissue Preparations and Live Cell Cultures While Learning to Use and Care for Your Microscopes**

***I. Examine and compare the human breast epithelium slides on the conventional light microscope.***

***A. Please exercise caution with our microscopes; the parts are fragile (and expensive!)***

1. Do not use the 40X (or 100X) lenses with thick preparations. You will probably crack the coverglass and could damage the lens.

2. Do not use the coarse focus knob with the 40X or 100X lenses. This knob moves the slide too great a distance.

3. Use the light condenser only with 40X and 100X objective lenses. Under lower magnifications it will unnecessarily

4. Lenses should be cleaned only if absolutely necessary. Use fresh lens paper on oculars and objectives. Wipe the lens gently. Do not use a piece of lens paper more than once. Do not use any other material on lenses. The stage and slides may be wiped off with a kimwipe.

5. Use lens paper only when cleaning hemacytometers and their engineered cover slips.

 6. Be sure that you wipe all oil off the slide and the lens after using 100x.

 ***B. Learn the microscope parts and their functions.***

1. Place the slide on the stage. Fastenings hold the slide (by the edges). Turn the knobs at the side of the stage to move the slide.

2. Turn on the substage illuminator. Bring the light to medium brightness.

3. The oculars magnify the image 10X. Adjust for the distance between your eyes (also known as the interpupillary distance).

4. The nosepiece contains the objective lenses. Hold the nosepiece and put the 10X objective into position over the slide. (Do not turn the lenses by holding a lens.)

5. The focusing knobs are on the side of the arm. The coarse focus knob is used with the low power objectives. The fine focus knob is used for sharp focus and to focus the high power objectives. Bring an object on your slide into sharp focus.

6. Adjust the illumination. Turn the light up or down and fine-tune the condenser under the stage.

7. Turn the nosepiece and bring the 40X objective over the slide. When you move from one objective lens to the next higher (or lower) power, the object on the slide should remain nearly in focus (parfocal). Bring the object to sharp focus using the fine focus knob. The image also remains fairly centered in the field of view (parcentral).

8. Trade places with the other members of your group; take your slide(s) with you. Compare and contrast the older Zeiss microscopes and the newer Olympus microscopes

***C. Draw the normal tissue in your lab notebook***

1. Select a representative field of view using the 10X objective lens

2. Draw the tissue morphology and the shapes and positions of areas that are not covered by tissue.

3. Within your 100-fold field of view, select a representative field of view using the 40X objective lens

4. Again, draw the tissue morphology and the shapes and positions of areas that are not covered by tissue. At both 10X and 40X place everything that you can see into your drawing, including the cell shape outlined by plasma membrane, the position and size of the nucleus and possibly some of the smaller organelles.

***D. Draw and compare the cancerous epithelium with the normal tissue***

1. Select a representative field of view using the 10X objective lens

2. Draw the tissue morphology and the shapes and positions of areas that are not covered by tissue; note how the tissue differs from the normal tissue you drew earlier.

3. Within your 100-fold field of view, select a representative field of view using the 40X objective lens

4. Again, draw the tissue morphology and the shapes and positions of areas that are not covered by tissue; note how the tissue differs from the normal tissue you drew earlier.

***II. Micrometer Cell Measurements and Microscope Calibration.*** The ability to measure materials on the stage of a microscope is very important to the study cell biology. Healthy cells have a distinctive size and shape in tissues as well as in ex vivo cultures. Many influences from within the organism and its environment can change this morphology and these changes are frequently diagnostic for the conditions under which the cells are living. We will use a stage micrometer to calibrate the occular micrometer found in the occular lenses of some of our microscopes under different magnifications created by different objective lenses. We can then use the occular micrometer to measure whatever is on the stage of our microscope.

***A. Calibrate the occular micrometer to the microscope you are using***

1. Obtain a Zeiss microscope and place in it an occular lens that contains an occular micrometer.

2. Obtain a stage micrometer, determine the dimensions of its measuring ‘bar’ and place it on the stage of the microscope.

3. Using both the 10X and 40X objective lenses, align the stage and occular micrometer ‘bars’ and determine the length of the occular ‘bar’ from the known length of the stage ‘bar’. What is the relationship between the length of the ‘bar’ under the two magnifications?

4. Assuming that both the stage and occular micrometers were cut to highly accurate and standard length, what do the measurements tell you about the accuracy of the light path of your microscope?

***B. Look at Prepared Slides and Measure and Label the Morphology of the Cells.***

1. Using your Zeiss microscope with a calibrated occular micrometer, draw and measure cell shape and size of both normal and cancerous breast epithelial cells.

***III. Examine the live cancerous human breast epithelium cells on the inverted light microscope.***

***A. Learn the microscope parts and their functions.***

1. Note that there are two different inverted microscopes and learn the properties of each.

2. Find the illuminator. Turn it on and bring the light to medium brightness.

3. Place a plate of live cells on the stage.

4. Put the 10X objective into position under the plate using the turret grip (do not turn the lenses by holding a lens!) and bring the cells on your plate into sharp focus.

5. Turn the nosepiece and bring the 40X objective under the slide. Note the parfocality and the parcentrality of each microscope and closely observe the detail of individual cells provided by the higher magnification.

***B. Draw and compare the cancerous epithelial cells with the normal and cancerous tissues you drew earlier***

1. Select a representative field of view using the 10X objective lens

2. Draw the cell and culture morphology and the shapes and positions of areas that are not covered by cells; note how the cell culture differs from the tissues you drew earlier.

3. Within your 100-fold field of view, select a representative field of view using the 40X objective lens

4. Again, draw the cell morphology and the shapes and positions of areas that are not covered by tissue; note how the cell culture differs from the tissues you drew earlier.

**EXERCISE 2: CONTRAST ENHANCEMENT AND IMAGING**

***Contrast Enhancement.*** With the microscopes we have at our disposal a certain type of cell, a molecule of RNA or protein in a given cell, or even which side of a bi-layer membrane our target is in are all within our technical ability to see and capture images. In most cell biology experiments we will first identify the size and chemical make up of the cellular target(s) we wish to investigate. The next question is, “How do we tell our target from all the stuff around it?” This is done by increasing the visual contrast between our target and other things in the tissue so when we look into the microscope our target stands out. These tricks of the trade are advancing as fast or faster than the ‘scopes themselves.

**a. How do we Identify Specific Cell Types or Molecules?**

***1. Classic and Neoclassic morphological methods.***

a. Different cell types can look very different in cultures and in tissues.

1. Many have characteristic shapes or appendages, etc.

b. These techniques are enhanced by basic histochemical methods.

1. We’ll use trypan blue

2. Standard ionic dyes like hematoxylin and eosin (H&E).

3. There are more specific things like protein, lipid and ion dyes.

c. Cells increasingly accessible to study through fluorescent compounds.

***2. The “State of the Art” is molecular specificity.***

a. The molecular revolution strikes again!

1. Has completely changed the study of cell physiology.

2. We now can ask what genes, RNA’s or proteins are involved in

the physiological process of interest?And are they specific to the tissues or cells you’re looking at?

b. Cell-specific marker proteins and nucleic acid sequences are now

 easily the most important and fastest way to identify cell types.

1. Proteins are identified through immunocytochemistry.

 a. Also Western blots and ELISA Assays

2. Nucleic acids are identified through *in situ* hybridization.

 a. Also Southern and Northern blots, PCR

**WEEK 2 PROCEDURE: *Prepare and Analyze Live-Dead Assays from Classical Trypan Blue Contrast-Enhanced Cells and Fluorescently Contrast-Enhanced Cells.***

***I. Prepare Two Live-Dead Assays for Comparison***

***A. Fluorescent Live-Dead Assay.*** In this procedure, living cells are distinguished from dead cells based upon the integrity of the cell membrane. Dead cells are penetrated by water soluble compounds that cannot cross intact plasma membrane and dead cells lose their cytosolic components by diffusion through the compromised membrane.The LIVE/DEAD EukoLight Viability/Cytotoxic kit, supplied by Molecular Probes, Inc., contains calcein acetoxymethylester (CAM) which is virtually nonfluorescent, but readily penetrates healthy cells. Once inside, CAM is rapidly hydrolyzed by intercellular esterases to polyanionic calcein which is intensely fluorescent (green) and is well retained only within living cells. The kit also contains ethidium homodimer (EthD) which only penetrates dead cells and fluoresces (red) only when it binds to DNA. Both fluorophores are excited at 485nm and the light emission can be viewed using a long-pass filter which transmits light above 530nm.

1. Remove a two-well culture slide from the 37oC incubator, select from the shelf labeled for your lab section

2. Aspirate media, rinse with 1ml PBS, aspirate PBS

3. Add 500 µl of the Molecular Probes assay reagent. Mark the well as Well #1.

4. Record the time in your notebook. Cover and incubate for 1 hour at room temperature.

***B. Trypan Blue Live-Dead Assay***. In this procedure, living cells are distinguished from dead cells based upon the integrity of the cell membrane. Dead cells are penetrated by water soluble compounds that cannot cross intact plasma membrane. We will use the membrane exclusionary dye, trypan blue, to assess cell viability

1. On the other side of your two-well culture slide, aspirate media, rinse with 1ml PBS, aspirate PBS

2. Add 500 µl of the 0.04% Trypan Blue reagent. Mark the well as Well #2.

3. Record the time in your notebook. You must finish your cell counts within 30 minutes.

***II. Count the Number of Live and Dead Cells in Your Preparations and Record Images.***

***A. Count the Trypan Blue Live-Dead Assay on the Inverted Light Microscope***.

1. Place the Trypan Blue well over the lenses on the inverted light microscope. Count total viable cells (clear, not blue) and total non-viable cells (blue)*.* Determine percent non-viable.

2. Follow the posted instructions to capture digital images of your cells.

 ***-Write-up instructions for new software and computer***

***B. Count the Fluorescent Live-Dead Assay on the Inverted Epifluorescence Microscope***

1. Turn on the computer hooked to the camera on top of the microscope. Turn it on both the mercury lamp and the microscope illuminator. Bring the illuminator to medium brightness.

2. Place a plate of live cells on the stage. Note the color of the fluorescent probe used in the preparation.

3. Put the 10X objective into position over the slide and bring the cells into sharp focus.

4. Turn off the illuminator and move the filter wheel to provide activation of fluorescent probes in the prepared culture. You may need to remove the shutter from the mercury light path.

5. Determine the percentage of live cells by counting the number of green fluorescent cells *vs.* the number fluorescing red in several fields of view. Red is dead.

6. Follow the posted instructions to capture digital images of your cells.

 ***-Write-up instructions for new software and computer***

**EXERCISE 3: STERILE CELL CULTURE AND CELL COUNTING**

***Growing Cells for Experimentation:*** Tissue culture or cell culture refers to the *ex vivo* growth of cells on artificial (plastic) support. Cell cultures are derived from cells that have been enzymatically, mechanically, and/or chemically disaggregated from the original tissue. Except for the various blood cells, cells of most “normal” animal cells must attach and spread out on a substrate before they can grow. Plastic cell culture dishes can provide an adequate substrate for the attachment of these cells provided that they are, first, “treated.” Cell culture dishes must either be coated with certain proteins, like fibronectin, or irradiated (thereby, adding electrical charges to the plastic). Cells attached to the dish may be removed by gentle treatment with certain proteases (enzymes that cleave proteins); trypsin is often used. Cells grow as a monolayer on a cell culture dish due to contact inhibition. The doubling time of cells in culture is approximately 16 to 24 hours. Culture techniques must be carried out under extreme sterile (aseptic) conditions. Cell culture is an important technique for a variety of different types of studies such as replication and transcription of DNA, protein synthesis, infection, drug action, membrane flux, cell-cell interaction, contact inhibition, limitation of growth, etc. Different polymers, especially proteins and nucleic acids, are often purified from cultured cells.

###### *Cell Counting.*  Direct cell counts on samples obtained from dense populations can be accomplished using a hemacytometer. Each hemacytometer has two separate counting area etched into the metal strips. Each counting area has nine large squares used for counting large eukaryotic cells, and each is made up of smaller squares. The small squares are used for counting small cells like bacteria. Each of the large squares in the four corners has sixteen small squares, while the large square in the center has twenty-five small squares. A special coverslip is supported above the grid such that the volume above each large square is equal to 10-4 ml (0.1µl). The trick in using a hemacytometer is in getting an even distribution of cells over the squares. To do this, first the coverslip is placed on the hemacytometer. A drop of evenly suspended cells is then introduced to the edge of the grid. Fluid is drawn under the coverslip by capillary action. If the volume is too great and fluid overflows into the gutters, the preparation cannot be used. If it takes more than one application to cover the grid, it cannot be used. If the drop is held too long before applying it to the hemacytometer, it cannot be used. To check on accuracy, the numbers from several squares are obtained. If the numbers are, they can be averaged; if they are far apart, the hemacytometer should be cleaned and reloaded.

### *Experimental Methods and Design*

**1. What Exactly Do *In Vitro* and *In Vivo* Mean Anyway?**

**a. This is quite variable and depends on the discipline of the investigator.**

 1. The Ecologist vs. the Cell Biologist.

**b. The hierarchy of *In Vitro* in Cell Biology.**

1. Tissue and organ culture directly from the organism.

a. Allows the cells that normally interact directly to remain in contact.

b. Loses the interaction with the rest of the organism.

2. Primary cell cultures are also taken directly from the organism.

a. Single cell types are very difficult to separate.

b. Usually mixed cell cultures.

3. Cell lines are single-cell types that can be maintained in culture for decades.

a. Immortalized cells are biochemically altered to stay in cell cycle.

b. Naturally perpetuating cells are unaltered and still grow in culture.

4. Free-living single-celled organisms can be used to model animal or plant traits.

a. Pond scum in the Jacuzzi.

b. These are particularly good for studying environmental issues.

5. Cell-free systems and subcellular fractionation.

a. We can separate and study organelles.

b. We can study multi-molecular interactions.

c. Even single molecular activities.

c. Selection of your model organism. The close association in genomic systems allows organisms as seemingly unrelated as bacteria and flies to provide useful information.

1. Prokaryotic bacteria: *Escherichia coli*

2. Eukaryotic yeast: *Saccharomyces cerivisiae*

3. Eukaryotic protists: *Tetrahymena pyriformis*

4. Complex plants: *Lycopersicon esculentum and Arabidopsis thaliana*

5. Nematode worms and fruitflies: *Caenorhabditis elegans and Drosophila melanogaster*

6. Frogs, fish and birds: *Xenopus laevis, Danio rerio, Gallus gallus, Coturnix coturnix*

7. Rats and mouse: *Rattus norvegicus and Mus musculus*

8. Chimpanzees and monkeys: *Pan troglodytes, Macaca mulatta* (rhesus)

9. Humans: Homo sapiens

**2. Standard Conditions of Cell Growth in Culture**

 **a. Epithelial and Mesenchymal Cells.**

**b. The Rate of Growth Curve and Cell Confluency.** Cells will attach and grow at a rate that depends on how much room they have, eg. how full the flask is (% confluence). Judging confluence takes practice. Linear growth occurs when cells are 30-80% confluent. Treatment and data collection must be done within this range or your results will be influenced more by the cell culture then by any treatment.

***Working in the Cell Physiology Cell Culture Hoods***

**1. The Basic Set-Up and Rules:**

1. Equipment placed in the hood should always remain in the hood to help prevent contamination.
2. Any consumable supplies (pipette tip boxes) should only be removed once fully emptied/used.
3. Avoid placing extra materials in the hood as they can clutter your hood and disrupt air flow if stacked too high. Disrupting the airflow can lead to contaminated experiments and more work/time in the lab for you.
4. Large Glass Beaker should be used to contain the end of the vacuum line tubing (when not in use), to collect Pasteur Pipettes and serological Pipets (5mL and 10 mL) while you are working in the hood and should be emptied before you leave the hood.
5. Do not use the beaker for liquids, all liquids should be removed using suction from the vacuum pump.

|  |  |
| --- | --- |
| **Items that should be in Hood (at all times)** | **Quantity** |
| P 1000 pipet | 1 |
| P 200 pipet | 1 |
| P 20 pipet | 1 |
| Large Box of tips for P 1000 (usually Blue but may also be the large white ones) | 1 box |
| Box of tips for P 200/ P20 (usually yellow but may also be the small clear ones) | 1 box |
| Pipet Aid and charger | 1 |
| Serological Pipets (5mL and 10mL) in rack | 1 rack |
| Sterile glass test tube with sterile Pasteur Pipettes | 2 tubes |
| Large Glass Beaker\* | 1 |
| Tubing connected to external flask/aspirator for aspirating liquids | 1 |

|  |  |
| --- | --- |
| **Items that should be on the cart near Hood** | **Quantity** |
| Sharps container (for used Pasteur pipettes) | 1 |
| Kim Wipes | 1 |
| 70% EtOH | 1 |
| Box of small gloves  | 1 box |
| Box of medium gloves | 1 box |
| Box of Large gloves | 1 Box |
| 6-Well plates for experiments | 1 package |
| Centrifuge tubes (50 mL) | 1 filled rack |
| Centrifuge tubes (15 mL) | 2 tubes |

***Suggested Work Flow for the Cell Phys Hoods:***

1. **Plan out your experiment in advance.** Know what cells you're using, amount of plates, flasks, media, TrypLE, PBS, and other reagents/materials you'll need to do your experiments. Often this is the single step that will save students a lot of time.
2. **Turn on hood and allow air to purge for 15 minutes** (per manufacturer’s directions). While hood is purging air:
3. Place media and other solutions (PBS, TrypLE etc.) to be warmed in water bath.
4. Locate/gather rest of the needed materials.
5. **Wipe down all surfaces in the hood** using EtOH and kimwipes.
6. **Wipe down and place extra materials in the hood.**
7. **Begin and complete your entire experiment without exiting the hood.**
8. **Clean the hood and area around it after you have finished**.
	1. Remove everything that you brought into the hood! Dispose of it properly.
	2. Place Pasteur pipettes into the red sharp containers (should be on cart).
	3. Place serological pipettes (1 mL, 2mL, 5 mL, 10 mL etc.) into the pipette container located under the hood. Please do not place them in the red biohazard container.
	4. Remove and properly dispose of any chemical waste (ask your professor for waste labels).
	5. Place any Biohazardous waste into the red Biohazard waste container.
	6. Only broken glass is to go in the broken glass bins! Please do not place gloves, flasks, tubes or papers into the Broken glass bins.
	7. Wipe Down hood surfaces using EtOH.
	8. Ensure Pipet-Aid is plugged in to recharge.
	9. Close sash and turn on UV light.
	10. If you're the last group make sure the water bath is turned off and the light(s) on the inverted scope(s) are off. They can burn out if left on overnight.

***Helpful Equipment Hints/Trouble Shooting:***

* + 1. Using a Pipet-Aid under aseptic conditions is a bit nerve racking when you are just starting out. It is possible to adjust the speed the liquid moves in and out of the Pipet-Aid. Some have lettering on the buttons: S (slow), M (medium), and F (Fast). You also simply press the button lighter. The upper button sucks the liquid up into the pipet. The bottom button pushes the liquid down. (BTW: We can tell if you suck liquid up into the filter, so be sure to pay attention to the liquid rising up the pipette and avoid going higher than the "0" mark on the pipet).
		2. If Pipet-Aid is not working, most likely it's because media was sucked into the filter or was not re-charged. Please inform instructor or lab tech so they can get you a new one.
		3. If the vacuum pump used to aspirate the flasks/plates is not sufficiently removing liquids please check the connections to the pump. Do not turn the dial on the pump! Please instead ask your professor or Lab Tech for assistance.
		4. Please label and keep any left over media, TrpLE and PBS aliquots for you to use later. There are Styrofoam racks in the fridge to hold them and also any other reagents you might have that need refrigeration.. Also, there are plastic racks available in the lab drawers (See professor).
		5. Please use label tape to label anything that was in the lab drawer (beakers, flasks and bottles). These items are reused by future students.
		6. 10% is located by the sink near the inverted microscopes. Underneath the sink is a bottle of bleach in case more bleach is needed.
		7. 70% EtOH should be at each hood. In the event you run out, there should be a spare spray bottle underneath the sink or in the flammable liquids cabinet.
		8. More 5 and 10mL pipettes are located in drawer beneath the inverted microscopes. Additional sterile Pasteur Pipettes/plates are located in 121 Cabinet in the classroom.
		9. Please stay out of the backroom/side prep room because of hazards. Everything you need should be in the classroom. See your professor if you need something that you do not see in the classroom.
		10. Make sure that you spray your hands/flasks with 70% EtOH before going into the hood or incubator to avoid contamination.

**WEEK 3 and 4 PROCEDURE: Sterile Cell Culture I.** Two groups will run this procedure this week while the other two talk about the Cell Phys Project. We will reverse the groups next week.

#### *I. Estimation of the Confluency of a Monolayer Cell Culture.*

 1. Use 10X objective lens (100X total magnification)

 2. Observe 5 different, random fields of view

 3. Estimate the amount of space occupied by all the cells in each field of view

 4. Average the estimates from all 5 fields of view

5. Follow the posted instructions to capture digital images of your cells.

 ***-Write-up instructions for new software and computer***

II. Trypsinization of the Monolayer to Remove Cells from the Container

1. Take a T25 culture flask of human cells from the CO2 incubator.
2. Observe the monolayer morphology of the cells through the Phase-Contrast Inverted Compound Light Microscope.
3. Aspirate growth medium and place in waste beaker.
4. Wash once with 5 ml of phosphate-buffered saline (PBS) by:

a. adding 5 ml of PBS to the bottle (always add solution to the side of the bottle, not directly onto the attachment surface)

b. swirl gently around the bottom of the flask

c. aspirate PBS and place in waste beaker

1. Add 2.0 ml of TrypLE solution and briefly swirl around the flask.
2. Incubate at 37°C (in CO2 incubator) for 10-20 minutes [checking every 2 minutes].
3. Observe the cells through the microscope during the incubation period. When cells have detached from the flask and appear to round up, lean flask to one side and pipette the medium up and down several times to dislodge cells from plate. This technique is called ***trituration*** (to ***triturate***).
4. Transfer your cells to a sterile 15ml conical tube. Spin in the hemotological centrifuge for 5 minutes. Be sure to balance the tube with another group’s or PBS in a spare tube.
5. Very carefully aspirate off the TrypLE solution and add 5ml sterile media to your tube. Resuspend cells into the media to stop trypsin activity.

***III. Preparing Cells from Culture for Counting on a Hemacytometer***

1. Aliquot 1 ml of the cell suspension into each of 2 microcentrifuge (Eppendorf) tubes and centrifuge on the benchtop for 5 minutes at 1,000 x rpm.

2. Discard the supernatant and add 0.2 ml of a 0.04 % Trypan Blue solution to the cell pellet and gently triturate.

3. Count cells [within 30 minutes of adding the Trypan Blue solution] using a hemocytometer. You may have to dilute the cell suspension in growth medium if necessary.

## IV. Hemacytometry

1. Be sure the hemocytometer chamber and its special glass coverslip are clean and dry. Place the cover glass over the chamber.

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2 Suck up a small volume (≈ 20 ul) of thoroughly mixed Cell Suspension in Trypan Blue using a P20 micropipettor. Place the tip of the pipettor in the triangular groove on the metal surface of the hemacytometer. Allow the suspension to flow by capillary action under the coverslip into one side of the chamber until it is just filled; then fill the other side. Do not let the chamber overflow.

3. Place the chamber under the 10X objective of the microscope. Allow the cells to settle for 1 minute. Count all of the cells in each of the four corner squares (1, 3, 7 and 9 below) and the central square (5 below) on one side. Repeat for the second side of the chamber. Include in your count cells that lie on the left and top borders of a square; ignore those that touch the right and bottom lines. You should have 10 counts, 5 on each side of the hemacytometer.

NOTE: You should be able to count 30 to 100 cells per square, which is a good range for accurate calculations of cell concentration in suspension. If you count more, that’s a sign that you need to dilute the cell suspension further.

4. Calculation of the number of cells per milliliter volume of the original cell suspension:

 The dimension of *each square* of the hemacytometer = 1 mm x 1 mm x 0.1 mm = 0.1mm3

 Therefore, the volume of *10 squares* x 0.1 mm3 = 1 mm3

Total number of cells (in 10 squares; i.e. cells per 1 mm3)

 x dilution factor (you are counting 1 ml worth of cells in 0.2 ml solution)

 x 103  (conversion factor: 1ml = 1cm3 = 1000mm3 = 103 mm3)

 **number of cells per ml suspension**

**EXERCISE 4: EXPERIEMNTATION IN CELL CULTURES**

### *In Vitro Experimental Methods and Design.* We are going to do in vitro cell biology studies this semester. The more classic cell biology studies are those that utilize an alternating *In Vitro – In Vivo* combination design. Identify a natural activity *in vivo* that interests you as a researcher. Use *in vitro* system to screen for the possible molecular players. Go back and check for false positives and real possibilities *in vivo.*  Use your *in vitro* system to test your positives. The ultimate goal is to repeat test *in vivo.*

**1. The Concept of the Bioassay**

a. The bioassay uses a natural biological activity as the measurable endpoint to the experiment.

b. Bioassays are models of an “*in vivo*” biological activity.

c. Biological phenomena are never perfectly invariant or repeatable – inherent variablility.

2. Artifact

a. The difference between your experiment and the phenomenon that you are really studying.

1. How much has changed from the normal environment?

2. How has your experiment changed the phenomenon?

3. You must account for all artifact when you interpret your data.

*4. Never “Zero Artifact” just “Less Artifact” – the Heisenberg Principle*

**3. Gain of Function vs. Loss of Function Experimental Designs**

**a. A Gain of Function Experiment** is one in which the experimental model (cell or organism) takes on a new activity that it does not normally perform in its daily routine.

1. Addition of enzyme substrates, chemical activators, gene products that the model doesn’t normally have or at abnormally high concentration.

*a. The key is often knowing the physiological concentration!*

2. These types of studies are usually used if the science is very new or poorly understood since they can be done rapidly and cover a lot of ground

3. The gain of function experiment is easier, but less useful

**b. A Loss of Function Experiment** is one in which a normal daily activity of the experimental model is eliminated in a very specific manner.

1. The loss of a needed component, blocking of a suspected activator.

 *a. The key is knowing that the activity would occur naturally!*

2. These types of studies are usually done to verify a strong hypothesis, when a lot is already known about the mechanism under study

3. The loss of function experiment is more informative, but harder.

#### WEEK 5 PROCEDURE: Sterile Cell Culture II. The goal of this exercise is to learn to control the number of cells that you are working with. Cells will attach and grow at a rate that depends on how much room they have, eg. how full the flask is (% confluence). We want to place enough cells into our experimental culture conditions to produce a known position on the growth curve. To standardize our experiments from week to week we want to start and finish each experiment at a constant confluence. Two groups will be assigned hoods for the semester in Sequoia 212, two in Sequoia 28.

I. Trypsinization of Monolayer Cell Culture

 As described in Steps 1-10 of Procedure II in Exercise 3. Collect 5ml of cell suspension.

***II. Reseeding (Splitting) Cells into Experimental Cultures:***

1. Acquire a six-well sterile culture plate. Use 3.0ml of cells from T-25 flask:

 a. Add 0.5 ml of cell suspension and 2.5 ml of growth medium into one well

 b. Add 1.0 ml of cell suspension and 2.0 ml of growth medium into another

 c. Add 1.5 ml of cell suspension and 1.5 ml of growth medium into a third

2. Incubate the cultures in the CO2 incubator for approximately 24 hours.

***III. Count Your Cells Using a Hemacytometer***

1. Aliquot the final 2 ml of the cell suspension into microcentrifuge tubes and centrifuge on the benchtop for 5 minutes at 1,000 x rpm. Discard the supernatant and add 0.2 ml of a 0.04 % Trypan Blue solution to the pellet and gently triturate. Count and record the cells per ml.

***IV. Determine the number of total cells required to product three known confluences.***

1. Find time in your own schedule (you will need to consult with your lab instructor first) to come back the next day (roughly 24 hours later) to estimate the confluence in each of the three wells of the 6-well plate.

2. Using you cell counts from ‘C’ above, determine how many total cells you placed in each well and compare to your confluency estimates. The ability to find a cell number that produces approximately 30% confluence is optimal.

***V. Alternate means to estimate the transfer volumes by calculating the surface area of all wells and combining with confluency estimates.***

1. For rectangular and square wells the surface area = length X width; for circular wells the surface area = r2

2. Use your confluency estimate for the original flask of cells to estimate how much of the relative surface area should be covered by the proportion of the total cells transferred to that well.

**SECTION 3: WRITING, DESIGNING AND DOING SCIENCE**

***HOW TO DESIGN A PROJECT PROPOSAL***

**Find Something that Interests Your Group as You Perform the Exercises.** As you work through these initial exercises, be thinking of ways that they could be adapted to answer new questions that interest you about the model. What can you learn about the model and its physiology, what might disrupt or enhance some aspect of its physiology, or perhaps you are interested in a similar model that could be approached the same way. ***Talk about possibilities, read up on things!***

**Search the Literature to Refine Your Potential Questions.** Once you have thought up some interesting questions and formulated some hypotheses, it is vital that you do a literature search in the library to locate work that has already been done on the topic. Pertinent scientific review papers, published in the research journals, should give you many more ideas and suggest possible ways to approach your problem. To find a recent review on your topic of interest go to Medline (Pubmed) and type in some key words. Then go to “Limits”, scroll down to “Type of Article”, select “Review”, and set the date for the last 5 years. ***Split up the work amongst your group and go to the library!***

**Decide on Your Project as a Group.** Discuss your ideas and findings with other members of your laboratory group; then meet with them to decide on the project. Write up your version of the project in your laboratory journal and show it to your group for their input. ***When everyone participates, the questions get much better!***

**Go Back to the Literature and Refine Your Proposal.** When you have picked your model and you have a general plan to answer your question go back to the library and find out all the details. How long should the experiment be? How much reagent do we need? Where can we get what we need? Who will do what? Is this really possible in one semester? ***Always work out the details before you begin!***

**Submit Your Proposal by the Due Date in Your Syllabus**. Write up a typed version of your project and submit it to your instructor. Include the model you will use, the question you will test and the background information from the literature. ***Look at the following guidelines to help design your written proposal.***

***HOW TO WRITE A SCIENTIFIC PROPOSAL***

***There is a very specific format for scientific writing – it is a direct demonstration of the Scientific Method. Please get a scientific journal and look at how the articles are written. How are the various sections organized? How are the references organized? A significant portion of the grade for this proposal will come from the proper use of scientific writing.***

**1. The Scientific method**

a. Observe events surrounding a known or unknown phenomenon.

b. Form a theory about its nature based on your knowledge of the world.

c. Generate testable hypotheses that would be support your theory if that theory were true.

d. Test the hypotheses by means of controlled experiments.

e. Closely observe the results and add them to the previous balance of knowledge of the world.

f. Reevaluate the theory in light of the latest observations and modify it, if necessary.

g. Generate more testable hypotheses and off you go again

The **TITLE** is an informative and concise statement of the question or problem which was investigated. Usually the title will be a descriptive phrase rather than a complete sentence. The title should catch the interest of a reader who is looking for an interesting topic to read.

The **ABSTRACT** or **SUMMARY** is the first section the reader encounters. It is very short and to the point, no more than 7-8 sentences. It summarizes the entire project, including the big idea that the work is based on, the hypothesis that is being tested directly, the biological model organism used to study it, the experimental design and the major controls used, and the predicted results. Remember, a proposal is written in future tense!

The **INTRODUCTION** presents your experiment and orients it within the field of cell biology. This section should start with a statement or description of the “big picture”; in other words the theoretical framework that you and your group are working within, and end with a testable hypothesis. The hypothesis should be such that, if the theory is correct, the outcome of the experiment is predictable. The rationale of the experiment(s) and significance regarding cell function should be stated here. A summary of work done by other investigators must be included. There should be no gaps in logic between your theory and your hypothesis. All statements of fact must be credited to their literature sources, and cited numerically or in the “author, year” format. Credit is given to any outside source of information; scientific journals, texts, references books and personal communications. A minimum of four (4) references to the primary (peer-reviewed) literature should be included. Do not copy statements you find in your reference sources. Digest and re-summarize the information and re-tell it in your own words.

The **METHODS** **AND** **MATERIALS** section should be a clear description of the testability of your hypothesis. It should describe your procedures, equipment, and reagents. You need to tell what will be done in the experiment using your own words and in enough detail so that the reader could repeat your work. **Do not** simply list materials or steps in a procedure; incorporate them into the text using complete sentences and paragraph format. If multiple procedures are to be used, be sure to describe each in a separate paragraph. Include a subtitle for each procedure. **Do not** just copy the laboratory manual here. Leave out trivial and repetitive information. **Do not** describe in detail how each instrument is used; simply state which were used and for what purpose. Be sure to cite all references used.

The **PREDICTED RESULTS** section of a proposal lets the reader know that, if your theory is correct, this is what the outcome of the experiment should be. You need to think through your experiment all the way to the end. Generate graphical representations of what untreated controls would be expected to do over the experimental timeline; and then predict changes in the graph that would be expected from your treatments if your hypothesis is correct. In addition, describe the graphical representations using complete sentences and paragraph format. This is an essential step for you to do BEFORE you perform your experiments! Know ahead of time what to expect. Be sure to cite all references used.

The **DISCUSSION** or **POTENTIAL PITFALLS AND ALTERNATIVE APPROACHES** section in a proposal is an effort to account for all the experimental artifact or error that is introduced into the experiment due to the use of model organisms, *in vitro* experiments and human influences. If this section is well thought out and written, the potential for your experiment to be successful is greatly enhanced. Think about the differences between the “big picture” event you are studying and the model you are using ahead of time and you will have a much more error-free experiment! Be sure to cite all references used.

The **REFERENCES** conclude the project proposal. References are listed in alphabetical order (of the last name of the first author for each reference) or numerically in the order that the references were used. Please obtain a peer-reviewed journal and follow an acceptable reference format.

*GUIDELINES FOR THINKING ABOUT PROPOSALS*

* Is the general area of study clearly introduced?
* Has a case been made or why the study is interesting or worthwhile?
* Is the information given from prior studies in the literature which summarizes the present state of knowledge in the area?
* Are the literature sources for all statement of fact referenced in a proper format? (At least four pertinent research journal references should be given)
* Is the question asked by the proposal clearly stated?
* Is the given background information relevant to the question?
* Are independent and dependent variable operational definitions clear?
* Are the proposed experiments summarized in sufficient detail so that supplies and equipment needs can be anticipated?
* Is it clearly explained how each experiment will help answer the question?
* Are the methods appropriate for the question asked?
* Have the requirements for controls and replicates been addressed?
* Is the project feasible? Can the project investigators reasonably expect to complete the project in the time allotted?
* Had jargon and wordiness been avoided?
* Is it possible to read the proposal smoothly from beginning to end, following the author’s logic, without sudden transitions or skipping back and forth?
* Are correct grammar, spelling, and punctuations used throughout the proposal?

***FINAL PROJECT PRESENTATION***

***The final step in the semester’s project will be a formal PowerPoint. PowerPoint presentations make for an engaging and easy to work with format. At scientific meetings and conferences, posters are often used for the presentation of data. Posters are easy to create and remain the predominant way to present scientific information at Meetings and Conferences; however, you will NOT be presenting these. Be aware that the aim of scientific writing is precision, clarity, and economy of words. We’ll discuss this later but don’t hesitate to ask your instructor for help getting started. The following are the components of a PowerPoint presentation. This format is much like that of the proposal, but remember – the presentation of your work is in the past tense! All information should be presented using bullet points, NOT in paragraph form. The slides should be used as a prompt or outline for both the presenter and the audience, NOT used to be read word for word; that is, the presenter will need to elaborate on all points outlined on the slides.***

The **TITLE** is an informative and concise statement of the question or problem which was investigated. Usually the title will be a descriptive phrase rather than a complete sentence. Draw in your audience!

The **INTRODUCTION** should present your experimental subject and be oriented within the field of cell biology. The rationale of the experiment(s) and significance regarding cell function should be stated here. A brief summary of the results of background by other investigators must be included. This should be pretty much the same material as that written for the proposal but should be presented in a succinct and highly organized manner. Stress the key logical connections that make the work an advancement to the field of study.

The **METHODS** **AND** **MATERIALS** section should describe your procedures, equipment, and reagents. Tell what was done in the experiment using your own words and in enough detail so that the reader could repeat your work. As with the proposal, do not just copy the laboratory manual here and leave out trivial and repetitive information. Be sure to use past tense throughout this section. Use a bullet point format of your original paragraphed material. Make it easy for the audience to understand what you did and why.

The **RESULTS** section should contain just that - your results arranged in a unified and coherent sequence. The results might be descriptive observations, measurement arranged in a table, calculations, data manipulations, graphs, figures, or records. All of these must be properly labeled with Table or Figure #, title (for tables), captions (for figures), and units of measurements. Be sure calculations are presented in an organized and easily followed format. Do not use three-dimensional graphics. The discussion should be presented orally.

The **CONCLUSION** should be a brief statement of your results and what can, in general, be concluded from them. Did the experiment you conducted conform to your hypothesis? Did your performance of the experiment, such as your choice of model organism and artifact allow you to be confidant in your study? Several sentences will often suffice – there should be no lengthy discussion or repeat of theory.

The **REFERENCES** conclude the presentation All statements of fact must be credited to their literature sources, and cited numerically or in the “author, year” format in the preceding sections. They are then listed in order in a separate “References” section at the end. Credit is given to any outside source of information; scientific journals, texts, references books and personal communications may be used. A minimum of four (4) references to the primary printed literature should be included in addition to the laboratory manual and any web-based citations. Try not to just copy statement you find in your reference sources. Digest and re-summarize the information and re-tell it in your own words.

***ELEMENTS OF A QUALITY POWERPOINT PRESENTATION***

***Purpose of PowerPoint slides*:**

 1. They should be used as a prompt for both the presenter and the audience.

 2. The presenter can use the slides organize her/his thoughts.

 3. The audience can use the slides as a guide to following the presentation.

***Content of PowerPoint slide presentation*:**

 1. *Order of slide presentation***:**

 a. Title slide

 b. Introduction

 c. State question and/or problem

 d. Hypothesis

 e. Materials and Methods

 f. Results

 g. Conclusion

 NOTE: i. There is a "Conclusion" slide, NOT "Discussion" slide. The discussion is done verbally, by presenter.

 ii. There should NOT be an "Abstract" slide.

***Have good visual effect on each slide*:**

 1. Keep slides simple if at all possible.

 2. Be consistent for all slides,

 a. same font

 b. same font size

 c. same spacing, indentation, etc.

 3. For word slides,

 a. present ideas in sentence fragments or major terms/words

 b. use bullets to delineate thoughts

 c. DO NOT write explanation in the prose form. Explanation should be expressed verbally.

***Rule of thumb for word slides*:**

 1. Present only 1 concept per slide.

 2. List of no more than 3-5 subtopics/ideas per slide.

 3. Write no more than 7-10 lines per slide.

 4. If using Times New Roman, the font size should not be no smaller than 18 points.

 5. Each slide needs a title and the font size of words in the title should be larger than those in the main body of the slide (maybe 32-40 points)

***Rule of thumb for figure slides*:**

 1. Each slide needs a title.

 2. All figures need to be labeled (X-axis and Y-axis, columns and rows, linez, etc.)

 3. Avoid picture-in-picture if at all possible.

 4. Words and figures should stand out against background

**APPENDIX 1: HOW TO WRITE UP AN EXPERIMENT IN YOUR LABORATORY NOTEBOOK**

**Example: CILIAR REGENERATION**

 **IN *Tetrahymena pyriformis.***

#### *INTRODUCTION.* Several ciliated and flagellated cell types have the ability to regenerate their cilia if the cilia are gently removed. Therefore these cells have been used to study factors which can influence ciliary growth. It has been found that regeneration depends upon processes which promote assembly of ciliary tubulin subunits into microtubules and not on protein synthesis following deciliation. Polymerization of microtubules requires exchange of tubulin dimers between the growing microtubule and a cytoplasmic dimer pool. The tubulin monomers are constituitively synthesized by the cell and tubulin heterodimers are assembled in the cytoplasm. The dimers then add at the growing (+) end of elongating microtubules which are located at the tips of regenerating cilia. Various drugs are known to alter the microtubule assembly process. Some act by inhibiting or enhancing tubulin polymerization, while others block tubulin synthesis.

 The experimental cells used in this exercise will be those of the ciliate protozoan *Tetrahymena pyriformis*. This organism had been widely used in cell physiology research. In nature Tetrahymena feed on bacteria; but in the laboratory, they can be grown on a variety of nutrient media in axenic culture (i.e., without other organisms). Historically *Tetrahymena* was the first eukaryotic cell to be cultured in a completely defined medium. It was also the first cell to be artificially synchronized with respect to cell division. *Tetrahymena* has a very short generation time, dividing every three to four hours under favorable conditions; hence, large numbers of cells can be grown in a short time.

###### *LIVE/DEAD Cell Viability Assay.*  In this procedure, living cells are distinguished from dead cells based upon the integrity of the cell membrane. Dead cells are penetrated by water soluble compounds that cannot cross intact plasma membrane and dead cells lose their cytosolic components by diffusion through the compromised membrane. We will use the membrane exclusionary dye, trypan blue, to assess cell viability.

###### PROCEDURE

##### A. Concentrate and Check Your Cells

1. Obtain 100 mL of a late exponential culture of *Tetrahymena*.

2. Pour the culture into two 50 ml conical centrifuge tubes, balance and spin at 200 g (1,100 rpm) for 5 min in the IEC centrifuge (THE SHAPE OF THE TUBE IS CRITICAL!).

3. When the centrifugation is complete, aspirate (or remove) enough supernatant to leave 2.5 mL total in the two tubes, gently resuspend the cells and recombine them into one tube.

4. Prepare a wet mount of the cell suspension and verify that there are cells in the suspension, that the cells are ciliate protozoans, and that they are swimming (i.e., ciliated).

##### B. Deciliation Procedure. In this procedure a balance must be struck between treatment gentle enough to avoid harming the cells, but vigorous enough to remove al of the cilia. The deciliation procedure must be carried out with attention to precise timing. Before beginning, prepare a flow chart of your plan of action and be sure that all materials and solutions are assembled.

5. At time = 0, add 5 mL of **Solution A** (10 mM EDTA, 50 mM Sodium acetate, pH 6.0) to the cell suspension. Mix rapidly.

6. Thirty seconds later (t = 30) add 2.5 mL of distilled water.

7. One minute later (t = 90) add 0.25 mL of o.4 M CaCl2. Use a P-1000 micropipettor for this addition. Mix rapidly.

8. Thirty seconds later (t = 120) draw the cell suspension up into a 10 mL syringe fitted with an 18-gauge needle and expel. This helps shear the weakened cilia away from the cells. Repeat the shearing a second time.

9. Immediately add culture medium to the tube, bringing the volume to 50 mL. Wait 10 min.

10. Centrifuge as in step #2. Carefully aspirate the supernatant and add enough fresh culture medium to the pellet to give a convenient cell density (1-10 mL is correct, start with a small volume 1-3mL). Mix. Transfer the cells to a larger container (for example, a 20-ml beaker) to oxygenate them.

NOTE: The 1-10 ml is determined by the fact that you should be able to count 30 to 100 cells per square on a standard hemacytometer (please see below), which is a good range for accurate calculations of cell concentration in suspension. If you count more, that’s a sign that you need to dilute the cell suspension further.

C. Count the Number of Non-Motile Cells

11. Be sure the hemocytometer chamber and its special glass coverslip are clean and dry. Place the cover glass over the chamber.

12. Suck up a thoroughly mixed suspension

of the cells in a Pasteur pipette (or using a

P200 micropipettor). Working quickly, discard

the first two drops. Allow the next volume to

flow by capillary action under the coverslip

into one side of the chamber until it is just filled;

then fill the other side. Do not let the chamber

overflow.

13. Place the chamber under the 10X objective of the microscope. Allow the cells to settle for 1 minute. The cells are not fixed; but, if the cilia have been removed, they will not be moving. Count all of the cells in each of the four corner squares and the central square on one side. Repeat for the second side of the chamber. Include in your count cells that lie on the left and top borders of a square; ignore those that touch the right and bottom lines.

14. Calculate the mean and standard deviation of the ten counts.

 For each time point, calculate the percentage of non-motile cells:

NM = Nt/N0 X 100 = % non-motile at time t

Where N0 is the average number of non-motile cells counted at time = 0 and Nt is the mean number of cells counted at any given time t.

100 - Nt/N0 X 100 is the percentage of cells which have regenerated their cilia.

D. Prepare the Live-Dead Assay

15. Aliquot 100 l of the cell suspension into a microcentrifuge (Eppendorf) tube.

16. Add 100 l of 0.04 % Trypan Blue solution to the cell suspension and gently triturate.

17. Count total viable cells (clear, not blue) and total non-viable cells (blue) in 10 squares *(5 in each chamber/grid)*.Determine percent non-viable

18. Count cells [within 30 minutes of adding the Trypan Blue solution] using a hemocytometer.

E. Continue Counting Non-Motile Cells

19. \*Thirty minutes after the initial counts, and at 10 minute intervals thereafter load the hemacytometer and count the non-motile cells in the squares. Eventually the number should decrease as cells regain the ability to move. The hemacytometer must be cleaned, dried, and recharged with a fresh sample for each count.

 \*NOTE: Swirl cell suspension before removing samples to count, each time.

20. Plot the percentage of regenerated cells *vs.* time. From this plot, estimate the time at which 50 % of the cells have regenerated their cilia (CR50). The CR50 is then a measure of the rate of cilia regeneration. A higher value of CR50 represents a slower rate of regeneration.

**Example Write-Up for the Laboratory Notebook**

***(To be recorded before starting the experiment!)***

**Description of Laboratory Procedures for the week of 02/05/10:**

***1. concentrate 100 ml of Tetrahymena culture down to 2.5 ml***

 - evenly distribute culture in 2 conical tubes,

- spin at 200 g = 1100 RPM, 5 minutes, IEC centrifuge

 - remove most of the fluid to total of 2.5 ml, resuspend gently, recombine

***2. check on the ‘scope to make sure they are alive and ciliated***

 - prepare a wet mount and view cells under conventional light microscope

***3. deciliate them stepwise as indicated in the lab manual***

 - time ‘0’ sec: add 5 ml solution A, mix rapidly

 - time 30 sec: add 2.5 ml distilled water

 - time 90 sec: add 0.25 ml 0.4M CaCl2, mix rapidly

 - time 2 min: shear the suspension 2x with an 18g needle on a 10 ml syringe

 - immediately add culture media to a volume of 50 ml

 - time 12 min: centrifuge and resuspend to a “convenient” volume (1 – 10 ml)

 - mix gently, transfer to a larger container to oxygenate

***4. count the deciliated cells per unit volume on the hemacytometer***

- calculate mean + sd

- consider this time ‘0’

***5. determine the percentage of live vs. dead cells with the CAM/EthD assay***

 - start it during the 30 minute break

 - mix 50 ul CAM assay solution to 50 ul cell suspension in cover slip dish

 - incubate 1 hour then count on fluorescent ‘scope

***6. 30 minutes after time ‘0’ recount the unciliated cells, repeat every 10 minutes***

 - rewash hemacytometer each time

 - continue until number plateaus

***7. plot the reciliation percentage relative to time and determine the CR50***