Basics of Cell Culture

A student laboratory manual

By Golnar Afshar PhD

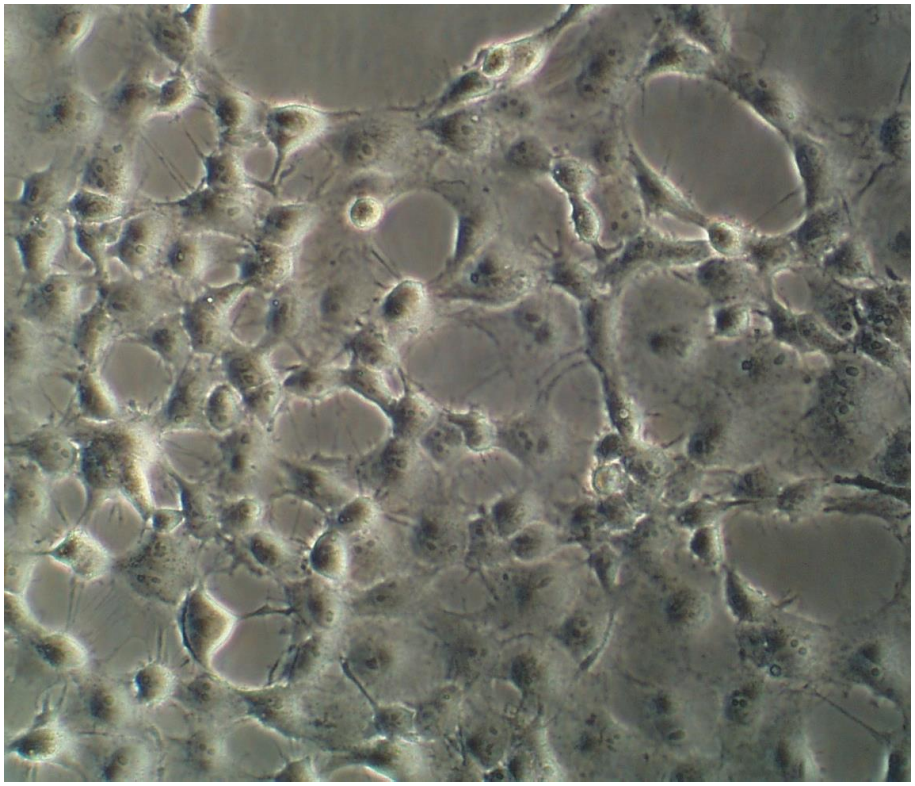


Table of Contents:

Edited by: Deborah Blancas, Carin Zimmerman Ph.D., and Edie Kaeuper Ph.D.

Revised 2019 by G. Afshar Ph.D

Note: Terms bolded in the paragraphs of this text may be found in the glossary.

Basics of Cell Culture

Cell culture--a method of multiplying cells under controlled, laboratory conditions--is used by scientists to study cellular functions, structures and behaviors. Cell culture is rooted in tissue-culturing techniques first developed in the early twentieth century. Initially, tissue cultures were made by using fragments of tissue or organs called explants. However, this form of culture yielded tissue with very limited growth capabilities.

One of the first significant advances in tissue growth was the ability to isolate individual cells from tissue for use in the laboratory. But, these individual cells were also limited by a finite number of divisions, a phenomenon known as Hayflick's Limit (Hayflick and Moorhead, 1961). This limitation was overcome by the discovery in the 1950s of special cervical carcinoma cells, known as HeLa cells, which have an *indefinite lifespan* (Gey et al. 1952). Due to modern advances, cells can now be easily grown in large numbers and stored indefinitely.

In addition to studying cellular structure and functions, cell cultures are used for a variety of applications, including toxicology studies, drug development, recombinant protein production, diagnosis, treatment and more.

This course is designed to expose students to the cell culture laboratory environment and to introduce the fundamental concepts and techniques of mammalian cell culture.

The class meets once a week for nine weeks. Each class lasts four hours with a brief lecture, followed by a laboratory exercise.

Laboratory safety and professional conduct

Safety policies are essential for this class and must be followed not only for your own protection but for the protection of others as well. Failure to follow these procedures will not only severely affect your grade, but may also lead to health complications. The following policies must be adhered to while in the laboratory.

1. Eating, drinking or chewing gum is prohibited.

2. Keep your hands away from your mouth during the laboratory class.

3. Horseplay or running in the laboratory is not allowed.

4. You must wear a laboratory coat during the laboratory session.

5. You must disinfect your laboratory work area prior to and after each class.

6. You must wash your hands before starting your work with the cells and after completion of the laboratory class. Gloves are required and will be provided for procedures that use potentially hazardous material.

7. Laboratory accidents, spills and injuries must immediately be brought to the attention of the instructor.

8. Friends or visitors are not permitted in the laboratory.

9. Closed-toe shoes and long pants must be worn.

10. Biohazard material and contaminated cultures must be disposed of according to the instructions.

11. Dispose of the glass Pasteur pipettes and micropipette tips in the biohazard sharp pouches. Dispose of serological pipettes into the dedicated disposal trays beside your work area; the pipettes will be sterilized before disposal.

12. Aspirate liquid waste into waste bottles, before throwing flasks and plates into trash.

13. Cell phones must be turned off for the laboratory period.

14. Familiarize yourself with the locations of emergency safety equipment in the laboratory, such as first aid kit, eye-wash station, and fire extinguisher.

Laboratory Notebooks

Accurate record keeping is essential to the progress of science. Scientists place great importance on recording the procedures and data during the experiment. The original hand-written notes and records are of great importance for accuracy since rewriting notes or relying on memory can increase the risk of introducing errors to the records. Laboratory notes need to be accurate, readable and organized so that another scientist, who is unfamiliar with the experiment, can follow the procedures and make sense of the data.

It is very important that the mistakes made during a procedure or changes made to the protocol are recorded in the notes. Mistakes often lead to new questions and discoveries; and knowing that a procedure was changed due to mistakes or unexpected circumstances help to interpret the data.

Laboratory notebooks are often used as legal records for patenting discoveries and for verification of compliance with regulations. Therefore, it is important that the records are properly documented in a way that makes it difficult to change, add or delete the data that has been recorded. In some industries and research institutions the notebooks are signed by the supervisors and kept in a safe.

Rules for laboratory notebooks:

Student notebooks will be collected at the end of the semester and graded, based on organization and completeness of the notes.

1. All laboratory notebooks must be permanently bound. Binders and spiral-bound notebooks are unacceptable. This will prevent the addition and/or removal of pages.

2. All entries must be made in black or blue ink, except drawings or sketches, which may be in pencil.

3. You must start your notebook with a table of contents that lists titles of lab exercises and their corresponding page numbers.

4. Every page should be numbered sequentially with no pages missing or torn out.

5. If you are repeating a protocol or part of an experiment, it is acceptable to write "as done previously on page A" without rewriting the entire entry.

6. Do not leave any pages blank. Cross out blank spaces in your notebook. This will prevent adding notes to previously recorded data.

7. Entries must be made in a chronological order. If an experiment runs over for several days, you can write at the bottom of your entry "continued on page B". When you continue an entry on another page start with "continued from page C". Remember to note the page numbers in your table of contents.

8. Figures and graphs must have a title and be clearly labeled.

9. Graphs or material written on a separate piece of paper must be glued to your notebook. Do not use staples or tape.

10. Do not use white-out. If you make a mistake, just cross the incorrect words with a single line so that it is still readable.

11. Leave a space at the bottom of every page for the student and the instructor to sign and date.

12. The date must be in the international scientific format, “day/month/year”.

13. Your name must be on the cover of your notebook and on the three side edges.

14. If you follow the steps outlined above, you will have an entry detailing the lab work you performed on that day.

15. Each entry (Fig. 1) should include:
-Title
-Purpose or Objectives (may be kept simple 1-2 sentences)
-Materials (a list of items that you need to perform the experiment.)
-Methods or Protocol- (step by step procedure. Can be in form of diagrams.) The protocols provided in this manual are general procedures that may have to be altered for your specific cell line or purpose. Use your own words to simplify the steps in a way that is easy to follow.
- Observations of the status of your cells
-Data and Results
-Answers to analysis questions which forms your discussion and a conclusion

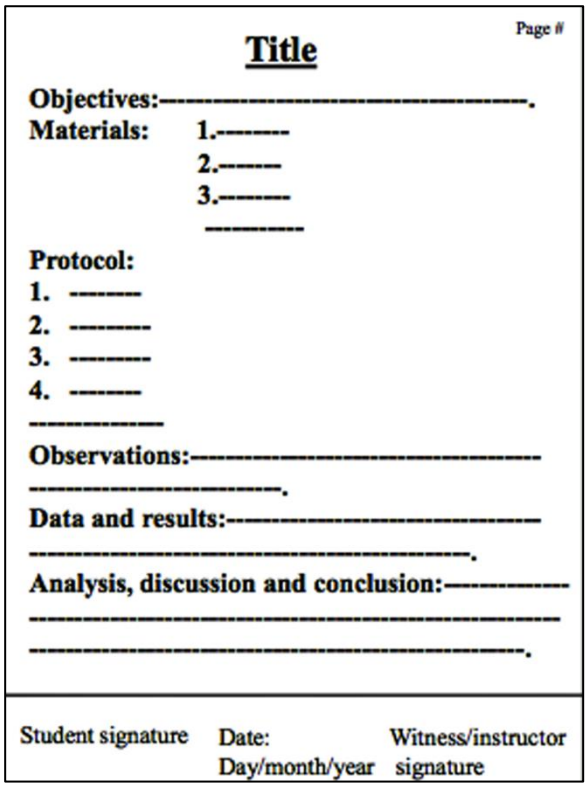


Figure 1- An example of a laboratory notebook entry.

Cell Culture Laboratory Equipment

Equipment commonly used in a cell-culture laboratory:

Laminar-flow hood

Most cell culture procedures are performed inside laminar-flow hoods (Fig.2). Laminar-flow hoods, or biological safety cabinets, provide a clean working environment to prevent contamination of cell cultures. The air is filtered and cleaned of particles before blown into the cabinet. Additionally, the flow of air in the hood is in smooth parallel lines which creates a "curtain" to separate inside from outside. Some laminar hoods are equipped with a UV-germicidal lamp to sterilize the contents inside while not in use. The UV lamp must be turned off before working in the hood to prevent exposure to hazardous UV light.

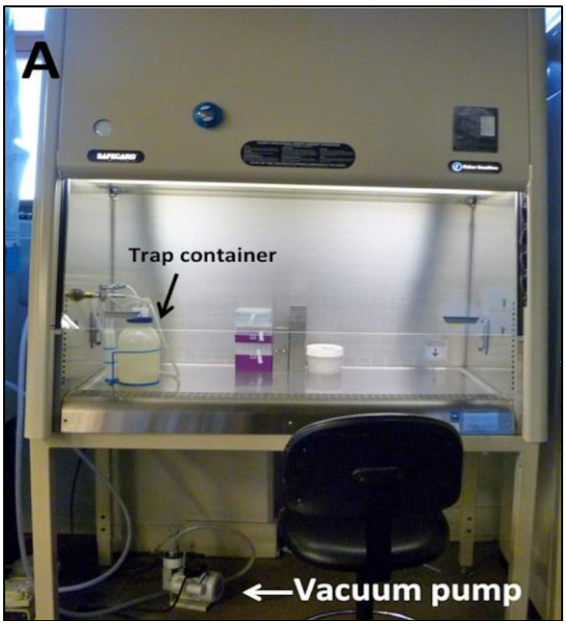


Figure 2- A laminar flow hood.

The following guidelines must be followed while working inside the hood:

1. Make sure that the UV-germicidal lamp is turned off.

2. Open the glass shield to the allowable level and switch on the blower. Wait about 10 minutes before starting work to allow for the air inside the cabinet to be filtered.

3. Wipe down the inside surface of the hood with alcohol before starting work.

4. All equipment and supplies used inside the hood must be clean and wiped with alcohol.

5. Do not overcrowd the hood. Keep your space clean and organized.

6. Wipe any spills immediately with alcohol.

7. Do not block the air filters and blowers.

8. Try to work towards the middle of the hood while being careful not to block the front area where the air filter is located.

9. Do not make rapid movements since it will disrupt the laminar flow of the air.

10. Clean the inside of the hood and wipe down with alcohol after your work is finished.

11. Turn off the blower and close the glass shield before you leave.

Inverted microscopes

Inverted microscopes are used to observe the cells in culture (Fig. 3). Inverted microscopes are a type of microscope with the objective lenses below the stage and the light source and the condenser above the stage. These microscopes are especially suitable for observing cells that are attached to the bottom of the plates and/or flasks.

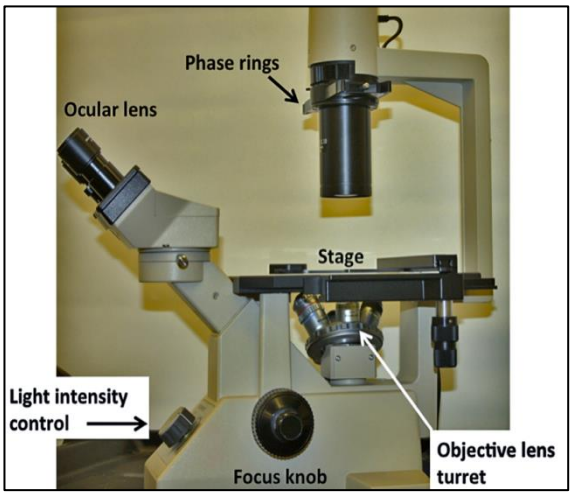


Figure 3- An inverted microscope.

The flask/plate is placed on the stage and the image is focused by turning the focus knobs. The objective lenses under the stage can provide 4X, 10X, 20X and sometimes more magnification of the image. The objective **turret**, which holds the objective lenses, can be turned to place the appropriate objective lens in place. The phase rings above the stage are placed in the light path to change the phase of the light when going through different structures of the cells in order to make the transparent structures more visible to the eyes. Different phase rings are located on a slider and can be placed in the light path for a clearer image. The condenser above the stage concentrates and focuses the light from the light source.

When observing your cells:

1. Wipe the stage with alcohol.

2. If you need to clean the lenses, only use lens paper to avoid scratching any of the microscope's lens.

3. Place your plate or flask of cells on the stage.

4. Turn on the light.

5. Turn the objective lens turret to the smallest magnification (4X).

6. Look at the cells by using the ocular lenses while moving the flask slightly and focusing to find the cells.

7. Turn the turret to a higher magnification. Usually 10X is sufficient for routine observations.

8. Adjust the focus knobs for the best possible image. To focus for both of your eyes, close your right eye and focus with the focus knob first. Then close your left eye and focus by turning the right ocular lens.

9. You may want to slide a phase ring in the light path for a clearer image. For observing some cell types, phase rings may be helpful.

10. Turn off the microscope after you are finished.

Fluorescent inverted microscopes

Fluorescent microscopes are inverted microscopes, used to observe cells and molecules that have been labeled with fluorophores. **Fluorophores** are molecules that can absorb energy of light at specific wavelengths and emit less energetic fluorescent light. Fluorescent microscopes are equipped with filters that will separate the absorbed light from the emitted fluorescent light. A set of filters are mounted on a block called the **filter cube**.

Fluorescent microscopes usually have several filter cubes with different sets of filters appropriate for observing fluorophores that emit light at different wavelengths. The filter cubes are conveniently located on a turret that can be rotated in order to place the appropriate cube in place to observe a specific fluorophore. Use of fluorophores and fluorescent microscopes has enabled scientists to view cellular structures and to study molecular functions and interactions.

Your instructor will demonstrate the proper use and care of the fluorescent microscopes in detail. The following are some general rules to be followed when using a fluorescent microscope:

1. For better view, turn off the lights in the room.

2. Turn on the high-intensity light source (usually a xenon-arc or a mercury-vapor lamp) and allow about 10 minutes for warm up. **Do not turn off the lamp until you are completely done with your work.** You cannot turn the light back on again until it has cooled off completely, which may take up to 1-2 hours.

3. Block the light path to prevent your cells from overexposure to the high intensity light. Fluorophores that are exposed to continuous light will eventually lose their fluorescent properties, a phenomenon called "**photobleaching**".

4. Place your plate or flask of cells on the stage and turn on the regular microscope light.

5. Using the lower magnification objective lens, find your specimen and focus.

6. Turn to the appropriate higher magnification objective lens and adjust the focus.

7. Turn off the regular microscope light and unblock the high-intensity light path.

8. Place the appropriate filter cube in place.

9. Observe the cells and make notes.

10. Remember to provide protection against photobleaching by blocking the light path when not observing the cells.

11. Turn off the light source when your work is finished.

12. Do not put the plastic cover on the microscope until the light source has cooled down.

Clinical Centrifuge

Clinical centrifuges (Fig. 4) are used to concentrate the cells and to separate the cells from the media or other reagents. Slow-speed clinical centrifuge must be used in order to prevent damage to the cells. For routine spinning of the cells, speed of 80-100g (gravitational force) is sufficient. Higher speeds may damage the cells. Proper care and operation of the centrifuge will be explained in detail by your instructor.

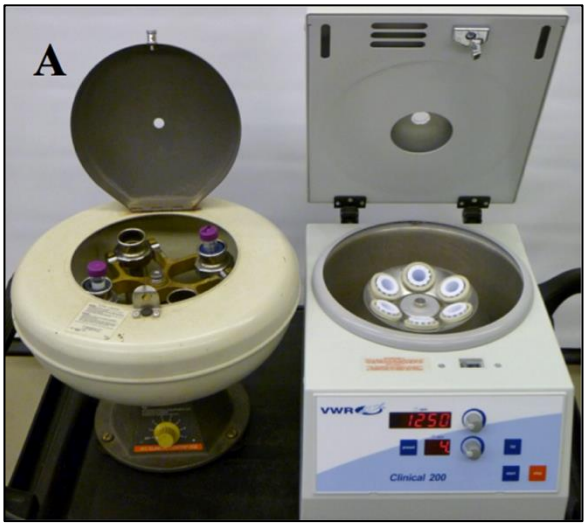


Figure 4- Two different models of clinical centrifuges.

General rules for using a centrifuge:

1. Transfer the liquid suspension to the appropriately sized centrifuge tubes. Not all tubes will be able to survive the forces of the centrifugation, so please use the tubes specifically manufactured for centrifuge you are using.

2. Weigh your tubes with their contents on a pan-balance to make sure that the tubes are of equal weight. Fig. 5 depicted two tubes that are properly balanced, i.e. weigh the same amount.

3. Sometimes you must prepare a separate balancing tube of the same size by filling it with tap water. The balancing tube must be of the same weight as the tube that needs to be centrifuged.

4. Place the two tubes that have been balanced into two opposing slots of the centrifuge.

5. Close the safety lid.

6. Set the centrifuge to the appropriate speed and time, then turn it on.

7. Stay close to the centrifuge for the first minute to make sure the centrifuge is running smoothly. If the centrifuge is not balanced properly, it will vibrate. Some centrifuges will turn off automatically if unbalanced. You need to turn off the older model centrifuges manually as soon as you sense the imbalance and vibrations. Continuation of the spin while the centrifuge is imbalanced will damage the centrifuge.

8. Do not open the safety lid while the motor is running.

9. Wipe any spills that might have occurred after centrifugation.

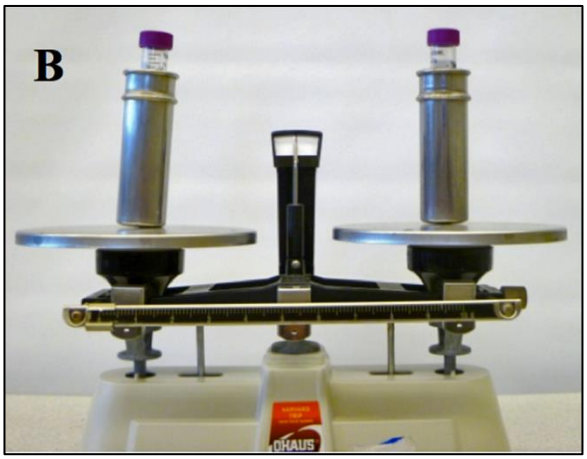


Figure 5- Use a scale to balance your tubes for centrifugation.

Incubator

The incubators (Fig. 6) provide the appropriate environment for the cells to grow. Cell culture incubators have three main functions:

1. Constant temperature- Incubators can be set to a specific temperature appropriate for the cells. For mammalian cells the temperature is kept at 37^{o}C, which is the optimal temperature for their growth.

2. Humidity- Although cells are kept in liquid media, smaller dishes that hold less liquid require a humid environment to prevent evaporation of the media. Usually a container filled with sterile, distilled water is placed in the incubator to provide humidity. The water needs to be replaced with fresh sterile distilled water regularly to prevent growth of microorganisms and reduce the possibility of contamination.

3. CO_2 gas is needed to keep the pH balanced; that is why cell culture incubators are connected to a CO_2 gas tank. CO_2 gas is injected inside the incubator and distributed by a fan or natural convection. CO_2 levels are usually maintained at 5%. CO_2 interacts with the **bicarbonate** buffer in the cell culture medium. This interaction stabilizes the pH at about 7.4. Uncorrected changes in medium pH can damage the cells.

$$\mathbf{H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-}$$

It is important to periodically check the tank gauges and take care that CO_2 levels are maintained in your incubator. Keep the incubator closed at all times and avoid frequent opening of the door to prevent loss of the CO_2 gas.

Figure 6- A cell culture incubator connected to the CO_2 tank.

Some incubators are also able to control the amount of oxygen available to the cells.

The optimal temperature and humidity provides an environment suitable for the growth of bacteria and other

microorganisms. Therefore, it is necessary to clean the incubators frequently to prevent growth and spread of contamination.

37^0C water bath

The media and most of the solutions used for cell cultures are kept in the 4^oC refrigerator. The cells, however, are kept in the 37^oC incubator. In order to prevent shocking the cells with cold temperature, the media and reagents are warmed up in the water bath before use. The warm water in the water bath is the ideal environment for the growth of microorganisms and contaminants. Therefore, the water bath needs to be cleaned and the water replaced with fresh distilled water routinely. Bottles and containers that have been warmed up in the water bath must be wiped down carefully with alcohol before being transferred to the hood.

Refrigerator and freezers

Most reagents and solutions used for cell culture are kept in the refrigerator for short-term storage. Some of the reagents can be kept in the -20^oC freezer for longer term storage. Cell-culture facilities often have a -80^oC freezer for storage of some of the reagents and short-term storage of frozen cells.

For long-term storage, cells are kept in **liquid nitrogen tanks**. The temperature of liquid nitrogen is $-196\ ^oC$. Cells can be kept frozen in liquid nitrogen for many years. The very cold temperature of liquid nitrogen is hazardous; therefore, thick gloves must be worn when opening the tank with great care.

Biohazard waste containers

Potentially hazardous material must be disposed of properly in biohazard waste containers and sterilized before disposal. Biohazard material must be handled according to federal, local and institutional regulations. Your instructor will explain the proper disposal of material in class. It is very important to comply with the rules to prevent the spread of potentially hazardous material to the environment.

Cell culture vessels

Most cells in culture need to attach to a substrate in order to divide and grow. These cells often form a **monolayer** and cover the surface available to them. Cells that require attachment for growth are said to be **anchorage-dependent**. Hematopoietic cells and a few other cell types can grow in liquid suspension without attachment. These cells are said to be **anchorage-independent** cells. The vessels used to grow anchorage-dependent and anchorage-independent cells need to be designed in a way to support the proper growth of these two cell types.

In most laboratories, disposable polystyrene plastic vessels are used to grow anchorage-dependent cells (Fig. 7). The vessels are flat at the bottom to provide a surface for cell growth. The bottom surface of the culture vessels are coated by molecules such as poly-L-lysine, laminin, gelatin, or

fibronectin. These mimic the natural **extracellular matrix** and allow the cultured cells to attach. There are three types of commonly used culture vessels used for anchorage-dependent cells: flasks, dishes, and multi-well plates. All three types can be of different sizes with different surface area. The choice of the vessel depends on the nature of the procedures and personal preference.

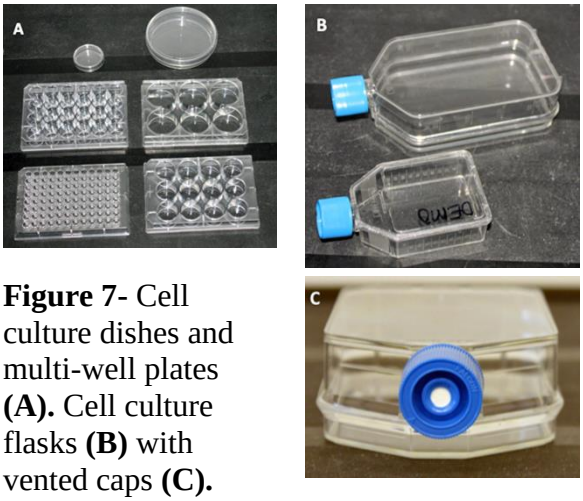


Figure 7- Cell culture dishes and multi-well plates **(A).** Cell culture flasks **(B)** with vented caps **(C).**

The vessels used to grow anchorage-independent cells do not need to be treated for cell attachment. Sterile, stirrer bottles are normally used for agitation of the culture and to keep the cells in suspension.

Both cell types need exchanges of gases (O_2 and CO_2) for growth. Therefore the cell culture vessels must allow gases to enter. Dishes and plates have loose fitting lids and the caps of the flasks must be closed loosely to allow gases to go in. Some flasks have vented caps with an opening that is covered by a filter to allow gases in, but prevent entrance of contamination (Fig. 7C).

Pipettes

Pipettes are used for transfer of specific volumes of liquid. Two kinds of pipettes are commonly used in laboratories:

1. **Serological pipettes** can measure liquids between 0.1 to 50 mls. You must choose the appropriate size pipette for the volume that you are transferring. Serological pipettes are marked with calibrated lines to allow measurement of accurate volumes. The maximum volume that can be transferred and the size of the pipette's subdivisions are printed close to the top of the pipette. Serological pipettes require the use of electrical or manual pumps to draw and release liquid.

2. **Micropipettes** are used to transfer small volumes of liquid, between 1-1000µls (Fig.8). You must choose the micropipette of the appropriate size to use for the volume you are transferring. The range of volumes that can be transferred is written on the micropipette. It is important to stay within the allowable range. Setting up the pipette to volumes above or below the allowable range will damage the pipette and will result in inaccurate measurements. Micropipettes fit tips that are specific for the specific volumes. The tips are kept in color-coded sterile boxes.

When using a micropipette:

1. Choose the appropriate size micropipette.

2. Set the dial to the desired volume to be transferred.

3. Open the appropriate size tip box in a sterile environment (inside the hood).

4. Pick up a tip using the pipette. Do not use your fingers to fit the tip on the pipette. Remember to shut the box after the tip is removed to maintain sterility.

5. Press down gently on the plunger using your thumb to the first point where you feel resistance.

6. Place the tip inside the liquid just below the surface.

7. Gently release the plunger to draw the liquid in. If the plunger is released too rapidly the liquid will aerate into the micropipette and will increase the chances of contamination.

8. To expel the liquid, hold the tip inside the vessel, touching against the side of the vessel while holding it slightly tilted. Press down on the plunger, all the way to the bottom (past the first resistance point).

9. To dispose of the used pipette tip, hold the pipette above the disposal bucket and eject the tip into disposal by pressing the ejection button.

If used properly, micropipettes can measure accurate small volumes of liquid. They are very sensitive and expensive tools and must be handled with great care.

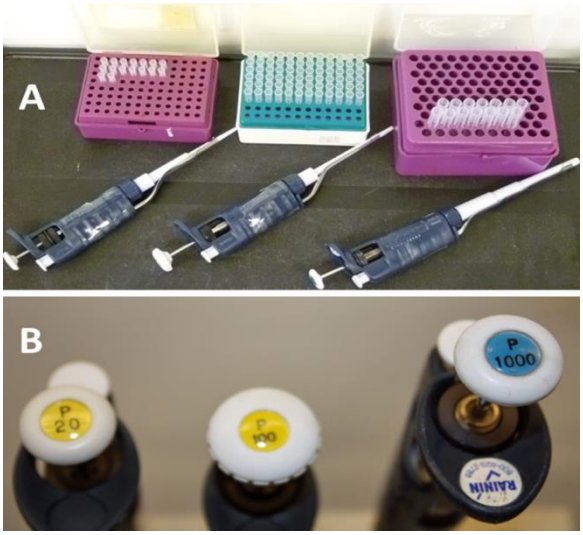


Figure 8- Three different sized micropipettes (p20, p100 and p1000) with their appropriate tips **(A).** View of the micropipette tops **(B).**

Contamination

Cell-culture contamination is a very common problem in all cell-culture facilities. Even the most experienced scientist encounters contamination problems once in a while. Contamination can be frustrating and will lead to the loss of the culture, time, effort, and of course is expensive. Although eliminating the problem of contamination is not completely possible, it can be very well be managed and reduced. Careful handling of the cultures and reagent preparation and following good laboratory practices can reduce the contamination problem significantly.

Cell-culture contaminants can be divided into two major categories; chemical and microbial. Chemical contamination occurs when an unwanted chemical substance is present in the media or any of the reagents. The unwanted chemical may be harmful to the cells or may affect their behavior and, therefore, affect the results of the experiment. Chemical contamination is usually not visible and difficult to detect. Common sources of chemical contamination are the media components, water and other reagents. To prevent this kind of contamination, care must be taken when measuring and preparing the media and other solutions. Presence of alcohol in cell cultures is a common chemical contamination problem, since it is routinely used to clean the work environment. When spraying alcohol you must make sure that there are no open cell cultures or reagent bottles/tubes in the area.

The warm temperature of the incubators and the rich media in cell cultures provide an ideal environment for the growth of various **microbial contaminants**. Some of the most common microbial contaminants in cell cultures are bacteria, fungus, viruses, mycoplasma and other microorganisms. Some microbial contaminations such as bacteria and fungus are visible and can be detected easily. Bacterial growth in the cell culture will often acidify the media. The **phenol red** component in the media is a pH marker and will turn yellow at low pH. Therefore, a yellow-colored media may be an indication of bacterial contamination. The bacterial growth at later stages looks cloudy and can easily be detected with the naked eye. Examining the cells under the microscope can also help in detecting bacterial contamination (Fig. 9). Bacteria cells are much smaller than mammalian cells and look like tiny dots that cover the surface of the cells and cover the empty spaces of the flask/plate in between the cells. Use of antibiotics in the media can prevent growth of some bacteria. However, routine use of antibiotics is not recommended, since it encourages the growth of more aggressive, antibiotic-resistant bacteria.

Mold contamination is also easily detected. The fuzzy-looking mold growth can be seen with the naked eye. Under the microscope you may be able to see the fungi hyphae strands and the spores in the cell culture.

Yeast is another form of fungus that may contaminate cell cultures. Yeast cells are

circular and smaller in size than mammalian cells and can be detected under the microscope.

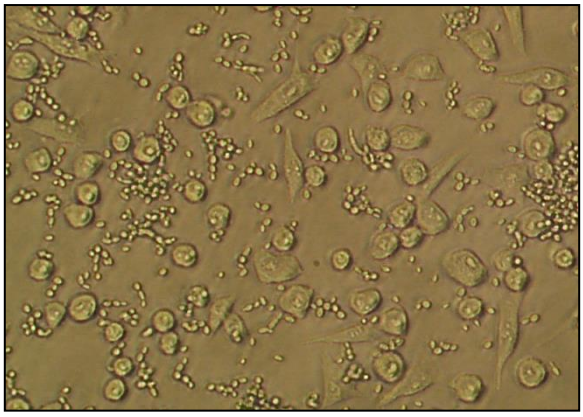


Figure 9- Mammalian (CHOk1) cells contaminated with yeast.

Other types of microbial contamination such as viruses and mycoplasma, are invisible to the eyes and are, therefore, more difficult to detect. **Mycoplasma** are very small microorganisms that can grow rapidly in cell cultures without being visible. Their presence affects cellular growth and metabolism. Viruses are very small particles that often infect cells and destroy them. Most laboratories perform routine molecular tests to detect the presence of mycoplasma and some of the common viruses. Cultures that are found contaminated are destroyed immediately.

Another form of cell culture contamination is cross-contamination by a different cell line. Scientists often may grow more than one cell line in the laboratory. If careless, a worker may mistake one cell line for another; and two or more cell lines can easily become mixed up. This can be a serious problem, and will invalidate all of the results obtained from a particular cell line. Proper labeling of the culture dishes and paying attention to the labels is critical and can significantly reduce this problem.

Prevention of contamination and aseptic technique

Aseptic techniques are a series of techniques and practices used to reduce the chances of contamination of the cultures by microorganisms and to protect laboratory workers from contamination by cell cultures and other potentially hazardous material.

Some of the common sources of cell culture contamination in a laboratory are non-sterile solutions and supplies, air-borne dust, laboratory personnel and unclean equipment, such as the water bath, incubator and laminar hood. Below is a list of some practices and aseptic techniques that can reduce the chances of contamination:

1. Maintain and clean the equipment routinely. Wipe down the laminar hood with alcohol before and after every use. The hood should also be cleaned more extensively once a month. The incubator shelves need to be cleaned and sterilized routinely and the water container in the incubator must be replaced every week. The 37^{o}C water bath has to be cleaned once a week. All other equipment must also be cleaned routinely. In addition, the equipment must be cleaned when spills have occurred or contamination had been detected.

2. All equipment and supplies must be wiped down with 70% Ethanol, if possible, and be cleaned before being used for cell-culture handling. Remember, wiping down with alcohol cleans, but does not sterilize the equipment.

3. Do not put your notebooks, papers and pens inside the hood. Everything in the hood must be clean.

4. Do not cough or sneeze while facing the hood.

5. All solutions and reagents used for cell culture must be sterile.

6. To keep from contaminating your cells wash hands before and after handling your cells. Also you should wear a clean lab coat. A clean lab coat covers your clothes that may be harboring bacteria and other microorganisms.

7. Always have back up cultures and freeze your cells frequently. It is wise to freeze some of your cells when possible, so that in case of contamination, you have frozen cells as back-ups that can be defrosted and used.

8. Aliquot (distribute) solutions and reagents into smaller volumes in several sterile containers, and use one aliquot at a time. This way, if one aliquot gets contaminated, you can use the other.

9. NEVER use other people's media and solutions. This is how contamination is spread among cultures.

10. Label your reagents and cultures properly to prevent mix-ups.

11. Open the wrapped and sterile serological pipettes inside the hood. You can only touch the upper part of the pipette with your fingers. Be careful not to touch the lower portion of the pipette to anything that is not sterile.

12. Use new sterile serological pipettes and micropipette tips in between solutions and cells.

13. Do not open the tops of the culture vessels, the sterile solution containers or the pipette and tip boxes outside of the laminar hood.

14. While working, be careful not to touch the rims or the interiors of tubes, flasks and their caps.

15. Micropipettes can be wiped clean with alcohol before use, but they are not sterile. Only the tip is sterile, therefore, only the tip should touch the solutions and the interiors of the containers. Be careful if you use a micropipette inside of a tall tube. It is preferred to use sterile serological pipettes instead, if possible.

16. Always write a protocol ahead of time and get organized before starting work. Disorganization and confusion increase the chances of contamination.

17. Do not overcrowd your hood and do not block the flow of air in the hood.

18. Always observe your cells before starting a procedure and destroy contaminated cultures immediately (see below).

19. Do not leave the tops of culture vessels or reagent bottles open when they are not in use.

20. Wipe the spills on the hood surface or the exteriors of bottles and flasks immediately with alcohol before it dries out and you forget.

21. When in doubt, do not use. If you are not sure if a solution or pipette is sterile or not, just assume the latter and do not use it for cell culture. You can put aside the non-sterile solutions and pipettes to be used for other procedures in the laboratory.

What to do in case of a microbial contamination

Contaminated cultures must be destroyed and disposed of immediately to prevent further contamination.

1. Add a 10% bleach solution to the contaminated culture vessel and wait for 10 min. The 10% bleach solution will be clearly labeled for ease of use.

2. Discard the bleached culture in the sink with running water. Dispose of the flask/plate in trash.

3. Clean your work area and equipment that has been in touch with the contaminated culture.

4. Inform other lab personnel who are sharing the incubator, so that they are aware of the possible contamination.

5. If the contamination problem is widespread among your cultures, discard all media and reagents that you have been using and start new.

Common Laboratory Procedures:

Important notes- For all procedures remember to:

- Practice good aseptic techniques.
- Always wash your hands and clean your work area before and after a procedure.
- Always observe your cells before any procedure, to make sure the cells are healthy and not contaminated.
- For most routine procedures you may have to sacrifice extreme accuracy for the sake of speed. It is important to work fast when cells are outside of the incubator and especially when cells are trypsinized (enzymatically removed from plates or flasks) since they are under a lot of stress and may die. Therefore, it is important to be organized and do one procedure with one culture at a time, if possible.

I. Media Preparation

Cells in culture are missing the tissue and systemic interactions that normally occur in the body of an organism to support their growth. Therefore, cell cultures have additional needs to those of cells in a whole animal. Cells need an abundant source of easy to use nutrients to enable them to divide rapidly in culture. Formulations for cell culture media are designed to mimic natural conditions as much as possible with additional supplemental nutrients and building block components required for cellular growth. Most cells are grown in a **basal media**, containing nutrients, vitamins and minerals supplemented with animal **serum**, containing additional nutrients, growth factors and hormones. This type of media is said to be "**undefined-media**" since the exact components of the supplemental serum are unknown. The serum is normally taken from different types of animals such as calf, fetal bovine, horse or human.

Different formulations of basal media are available for purchase such as Eagle's Minimal Essential Medium (MEM) [Eagle, 1959], RPMI 1640 [Moore et al., 1967] or Dulbecco's Modification of Eagle's Medium (DMEM) [Dulbecco and Freeman, 1959]. Different cell types may prefer different formulations for optimum growth. Although the exact recipes may be slightly different, all basal media contain the following components:

- Carbon source (glucose / glutamine)- source of energy
- Amino acids, which are necessary building blocks of proteins.
- Vitamins, which are needed to promote cell survival and growth.
- Balanced salt solution, which is an **isotonic** mixture of ions to act as cofactors for enzymatic reactions, cell adhesion, etc.
- Phenol Red dye, which has no nutritional value but is a pH indicator. The color of phenol red changes from orange/red at pH 7-7.4 to yellow at acidic lower pH and purple at basic higher pH environments. The color change is used to monitor the pH of the media.
- Bicarbonate or HEPES buffer, which is used to maintain a balanced pH in the media.

To prepare, a "**complete media**" serum is added to the basal media. Antibiotics may be added to the complete media to prevent bacterial growth. Continuous use of antibiotics, however, is not recommended since it encourages the growth of more aggressive, resistant bacteria.

Alternatively, individual growth factors, proteins and other components can be added to the basal media, without the use of serum, in order to prepare the complete media. This type of a media is said to be "**defined media**", since the exact components of the factors added are known.

Defined-media can be customized and selective for a specific cell type or experimental conditions. The disadvantage of using the defined media is that the preparation can be more time consuming, since each component must be added at proper concentrations, separately. A number of serum replacements, containing known concentrations of some of the serum components, are also available commercially.

For the cells used in this class we will prepare undefined media using fetal-bovine serum. To prepare 500ml of complete media:

1. Wash your hands before starting.

2. Turn on the laminar hood and clean it with 70% ethanol.

3. Gather the materials you need and wipe them down with alcohol before putting them in the hood:

- Serological pipettes
- 50ml sterile tubes
- Bottle of basal media containing 450 ml. Most manufacturers include 450 ml of basal-media in each bottle to make the preparations easier.
- Bottle of 200 mM L-Glutamine (optional)
- Bottle of fetal-bovine serum
- Tube of antibiotics at 100X concentration [penicillin and streptomycin (Pen-strep)]. (Optional)

4. Thaw the fetal-bovine serum, L-Glutamine and Pen-strep bottles that are kept in the freezer in the 37^{o}C water bath. Wipe them down carefully with alcohol and mix each of them well by swirling the bottles a few times, before opening them in the hood.

5. Read the formulation of the basal media. Eagle's MEM formulation is appropriate for growth of most cell types; however, you may have a different, but similar, basal media available to you for your cells. Check to see if there is any L-Glutamine already included in the basal media.

6. Uncap the basal media bottle. To the 450 ml, add 50 ml of fetal-bovine serum, using a serological pipette.

7. Using a new pipette, add 5 ml of L-Glutamine to the bottle so the final concentration of L-Glutamine is 2 mM. If L-Glutamine is already included in the basal media, you do not need to add additional L-Glutamine. Depending on the cell type, the final concentration of L-Glutamine may be different (0.5-10 mM).

8. If using antibiotics, add 5 ml of 100X pen-strep to the bottle. Remember to change to a new serological pipette for every different solution.

9. Recap all bottles.

10. Mix the bottle by swirling.

11. All reagents used for media preparation must be sterile. (You can reassure yourself by filtering the mixed complete media through a 0.2μm filter.)

12. Using a new serological pipette, aliquot the complete media into 50 ml sterile plastic tubes.

13. Label each tube of complete-media with the date and your initials. If antibiotics are used, note that as well.

14. Cap the tubes and keep them in the refrigerator. Use one tube at a time. Complete media can be kept in the refrigerator for up to a month.

II. Routine cell-Culture Maintenance (Feeding and Subculturing)

Once the newly plated cells have attached to the flask/plate, they will start dividing and the cell culture will grow. The cell culture media should be changed every 2-3 days to provide nutrients to the cells. Changing of the media is called **feeding.** When feeding, you need to remove the old media from the culture and replace it with fresh media.

The rate of growth is usually slow at first after plating, but once the cells have become accustomed to their new environment, they will divide rapidly. As cells divide, they will cover the bottom surface of the culture vessel. The percentage of the surface covered by cells is referred to as **confluency**. Anchorage-dependent cells keep dividing until the entire 2-dimentional surface available to them is covered (100% confluent) and they form a **monolayer**. The cells will stop dividing as space, nutrients and oxygen become insufficient for them, a phenomenon known as **density-dependent growth inhibition.** Most cancerous cells however, may continue to divide on top of each other and form a multilayer (above 100% confluent). These cultures become very unhealthy if they are allowed to continue dividing when they have insufficient access to nutrients and oxygen. For all cultures, in order to keep the cells healthy, it is best to keep them at about 50-80% confluent. This will assure that cells have enough space, nutrients and oxygen available to them. Once a culture is above 75% confluent, it needs to be subcultured. Subculturing, splitting or **passaging** is when cells in a culture are divided into two or more subcultures. Depending on the cells' growth rate, cultures may need to be passaged every 3-7 days.

Anchorage-dependent cells in culture are attached to each other and to the coating on the bottom surface of the flask/plate via cell surface glycoproteins. The adhesion molecules are often dependent on Ca^{2+} for attachment. Subculturing involves removal of the media, detachment of cells from the surface by use of dissociating enzymes and removal of Ca^{2+} ions by a chelator. **Trypsin** is the most commonly used dissociating enzyme that digests the attachment proteins on the surface of the cells. There are other types of dissociating enzymes available that may be suitable for a particular cell line, such as collagenase and dispase. Dissociating enzymes are often mixed with EDTA (Ethylene diamine tetra acetic acid) which is a **chelator** and removes Ca^{2+} ions. Long exposure to trypsin is harmful to the cells, so cells need to be separated from trypsin by centrifugation.
The following is the protocol for routine subculturing of a culture growing in a T25 flask using trypsin:

1. Turn on the hood and wipe down with alcohol.

2. Gather the material you need and wipe down with alcohol before placing them in the hood:

- Serological pipettes
- Sterile centrifuge tube
- Tube rack
- Trypsin/EDTA [trypsin is stored in the freezer for long term storage and kept in the refrigerator for short-term storage. Trypsin is an enzyme, and similar to other proteins, it should not be re-frozen and thawed and should not be kept out of the refrigerator for too long. Cold trypsin needs to be warmed to 37 ^{o}C before use.]
- Complete media; warmed
- Ca^{+2}/Mg^{+2} -free Phosphate-buffered saline (PBS); warmed. [PBS is an isotonic solution that is often used for rinsing cells and for routine dilutions.]
- Fresh flasks

3. Observe your cells using an inverted microscope and note the confluency and status of your culture.

4. Inside the hood, aspirate the media from the flask.

5. Transfer about 3 ml of PBS to the flask.

6. Swirl gently to rinse the cells.

7. Aspirate out the PBS. This washing will remove the residual serum from the flask. Some components of the serum inhibit the activity of trypsin.
8. Add 1ml of trypsin to the flask.

9. Lay down the flask and make sure all the cells are covered by trypsin.

10. Wait 2-3 minutes. You may want to transfer the flask to the incubator to speed the process. Trypsin's enzymatic activity is more efficient at 37^{o}C. It is more difficult to trypsinize some cell lines as compared to others. You may have to increase the amount of trypsin and incubate the flask in the incubator for longer time.

11. Observe your cells under the microscope. Trypsinized cells round up and are loosened.

12. Tap or rock the flask gently to detach all of the cells.

13. Once the cells are floating, deactivate trypsin by adding 4 ml of complete media to the flask. Trypsin is harmful to the cells, so as soon as cells are detached you should dilute it by adding media and separate the cells from trypsin. Do not leave the cells in trypsin for too long.

14. Transfer the floating cells in trypsin and media to a sterile centrifuge tube and label.

15. Centrifuge the tube at 100g (about 1000-1500 rpm) for 3 minutes.

16. Wipe the tube clean and gently transfer it back to the hood.

17. Using a sterile glass pipette, aspirate out the supernatant. Be careful not to remove the cells (pellet).
18. Resuspend the cells in 2-5ml of fresh complete media. Cells that have been trypsinized and centrifuged tend to clump together. It is important to separate the cells by pipetting up and down a few times. Try to avoid creating too many bubbles.

19. Transfer the appropriate amount of the cell suspension into new flasks. (Never re-use the old flasks).

20. Add enough complete media to the flask to bring up the volume to 5 ml.

21. Swirl the flask to ensure uniform distribution of the cells.

22. Observe your cells again to make sure there are cells floating and there are no big clumps.

23. Label the flask with your name, date and the cell-line's name and place in the incubator.

24. If there are any left-over cells, aspirate them into the waste bottle and discard the old flask.

Notes: The reason behind centrifugation, is to separate the cells from the harmful trypsin. Alternatively, you may deactivate trypsin by diluting it sufficiently by adding enough media. For most cell lines, if the volume of trypsin in the media is diluted enough that it is less than $1/20^{th}$ of the total volume, then there is no need to spin the cells. For example, after initial 5X dilution of trypsin (step #13 above) by addition of media and mixing, take out 1ml of the mixture and transfer it to a fresh flask. Add 4 mls of media to the flask to bring up the volume to total of 5 mls. So you have diluted the cell mixture an additional 5X. The total dilution factor of trypsin, therefore, is (5X) x (5X) = 25X. Twenty five times dilution of trypsin is sufficient to deactivate it and make it harmless to the cells.

III. Counting Cells Using a Hemocytometer

Hemocytometer is a device invented by Louis-Charles Malassez (Verso, 1971) to count cells. A hemocytometer is made of a glass slide with a grid on each half (Fig. 10). Each grid is made of nine squares. Each square is subdivided into smaller squares. The grid is covered by a cover slip and creates a chamber. Each of the larger squares in the grid can hold 0.1µl of liquid.

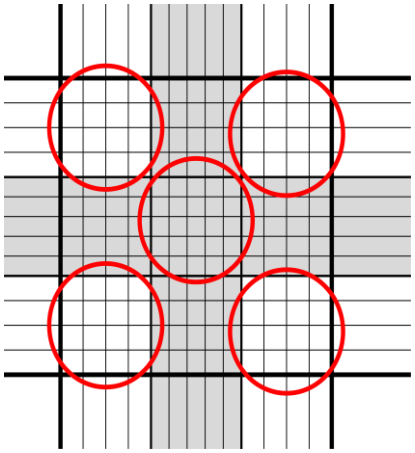


Figure 10. Diagram of a grid of a hemocytometer. Each square marked by a circle holds 0.1 µl of liquid.

In order to count, cells are trypsinized first and a small sample is placed in the hemocytometer chamber. The cells are visible under the microscope and can be counted. Since the volume in each square is known the concentration of cells can be calculated.

To count cells in a T25 using a hemocytometer:

1. Turn on the hood and wipe down with alcohol.

2. Gather the material you need and wipe them down with alcohol before placing them in the hood:

- Serological pipettes
- Hemocytometer
- Micropipettes and tips
- Trypsin; warmed
- Ca^{+2}/Mg^{+2} – free PBS; warmed
- Complete media; warmed

3. Remove the media from your flask.

4. Rinse the cells with 3ml of PBS once. Remove the PBS.

5. Add 1 ml trypsin to the flask and allow 2-3 minutes for the cells to detach from the flask. Tap the flask on the side to loosen the cells.

6. Observe the cells under the microscope to make sure the cells are all floating.

7. Add 4 ml of media to quench trypsin. Mix well by pipetting up and down a few times.

8. Using a micropipette transfer 12 µl of the cell suspension to each chamber of the hemocytometer. Hold the coverslip on the chamber loosely with your thumb, place the pipette tip on the edge of the cover slip and release the liquid. Repeat for the other half.

9. Place the hemocytometer on the stage of a microscope. Find the grids using the low magnification lens (4X). Change to higher magnification (usually 10X is good) to see each square and the cells easily.

10. Count number of cells in 5 squares of the grid (shown in circles on figure 10), that is, the four corner squares and the one in the middle. (You may repeat counting five squares for the other grid-10 squares total for more accurate counting). For each square, count the cells resting on the two of the border lines and exclude the cells resting on the other two border lines. Decide on which borders you want to count and which ones are excluded before counting and be consistent for all your counts.

11. In order to get an accurate count you should count approximately 10 to 50 cells per square. If you have too many cells, the chances of making mistakes are high so you need to dilute your sample and repeat counting. If you have too few cells, the number is not statistically valid, and therefore, you need to concentrate your cells by spinning your cells and resuspending the pellet in less volume of the media before counting again.

12. Calculate cell concentration (cells/ml) using the formula below:

$$\frac{(\text{Total \# cells counted})(10^4)(\text{dilution factor})}{(\text{Total \# squares counted})}$$

.

For an explanation of dilution factor see examples 1-4 on page 29.

13. Clean the hemocytometer and its cover slip by wiping down with alcohol.

Notes:

- Hemocytometers are fragile and expensive. Please be careful not to drop and break them.

- Floating cells settle to the bottom of the flask or tube rapidly and they tend to aggregate together and form clumps. Clumps distort the count. It is very important to mix the cells well by pipetting up and down immediately before counting to dissociate the clumps and to ensure a homogenous cell suspension.

- Cells that are outside of the incubator and trypsinized are under stress and may die if not plated soon. Therefore, sometimes it may be more important to speed up the procedure rather than being accurate. If you are short on time or want to speed up the process, you may count cells in five squares rather than ten.

- Observe your cells in culture before trypsinizing for counting. If you have very low confluency, you may not get a large enough count to be accurate. So you can take out a small sample of cells after they have been trypsinized before quenching and set aside in a tube for counting. Then quench the rest of the cells in the flask with media. Count the cells in the tube; since they are more concentrated, you are more likely to get a large enough number for your count.

You can always dilute your sample appropriately if the concentration is too high (see the examples on page 29). Alternatively, after quenching trypsin if you count too little cells, you can concentrate the cells and count again. Transfer your cells to a centrifuge tube, spin for 3 minutes, remove the supernatant and resuspend the cells in less volume of media. Count again.

- If you have a very confluent flask, you may want to quench trypsin with a larger volume of media to dilute the cells further. The amount of media depends on the confluency of your culture. Note that if you add too much media the cells will become too dilute and obtaining an accurate count becomes difficult. Keep in mind, it is always easier to dilute than to concentrate.

- Depending on conditions, every cell culture contains a low or high number of dead cells. Dead cells are usually detached from the surface and are floating. When media is removed and cells are washed with PBS for trypsinization, most dead cells are removed from the culture. However, for some procedures it may be important to collect and count the number of dead cells as well as the live ones. To collect the floating dead cells, the culture's media and the PBS used for washing the cells are transferred to a sterile tube, before cells are trypsinized. Live cells are then trypsinized and pooled together with the floating dead cells in the same tube, before counting.

- To distinguish between dead and live cells a stain solution named **Trypan-blue** may be added to the cells, before counting. Transfer 50μls of your cells to a small tube and add 50μls of 0.4% trypan blue. After mixing, pipette 12 μls to each half of a hemocytometer and count.

  Trypan-blue is a blue stain that can get inside the dead cells through their porous membranes. Live cells don't allow the stain to get in. The dead cells therefore look blue as opposed to the clear live cells. Once the cells are mixed with trypan blue they must be counted immediately. Live cells will eventually die in the solution and will turn blue.

- To calculate the concentration of the cells that have been mixed with trypan blue you must note that the dilution factor is 2, since the cells were diluted 2X with trypan blue solution.

Example 1:

A student counted 80 cells in ten squares of a hemocytometer. Since he didn't dilute his culture further the dilution factor is 1. His culture's cell concentration is:

$\frac{(80 \text{ cells})(10^4)(1)}{(10 \text{ squares counted})} = 8 \times 10^4$ cells/ml

Example 2

A student has mixed 50μls of her cells with 50μls of trypan blue solution. She counts 120 cells in 5 squares of a hemocytometer. Since she has diluted her cells 2X with trypan blue, dilution factor is 2. The cell concentration of her culture is

$\frac{(120 \text{ cells})(10^4)(2)}{(5 \text{ squares counted})} = 4.8 \times 10^5$ cells/ml

Example 3:

A student counts more than 1000 cells in 5 squares of a hemocytometer. In order to get a more accurate count, he decides to dilute his sample before recounting. He takes out 100 μls of his cells and mixes with 900 μls PBS in a separate tube. After mixing, he pipettes 12 μl in the upper chamber of a hemocytometer. This time he counts 200 cells in 5 squares. The cell concentration for his original culture in the T25 flask is

$\frac{(200 \text{ cells})(10^4)(10)}{(5 \text{ squares counted})} = 4 \times 10^6$ cells/ml

Example 4:

A student has very little cells in her T25. She trypsinizes the cells with 1ml trypsin. After the cells are floating she mixes them well and takes out 50 μl and sets aside in a tube. She then adds 4 ml of media to the rest of the cells. She puts 12 μls from the tube into each chamber of a hemocytometer and counts 50 cells in 5 squares.

$\frac{(50 \text{ cells})(10^4)(1)}{(5 \text{ squares counted})} = 10^5$ cells/ml

The cell suspension in the T25 flask is five times less concentrated:

$10^5 / 5 = 2 \times 10^4$ cells/ml

See appendix B for more practice problems.

Laboratory Exercises

Lab Exercise 1: Pipetting and Aseptic Technique

Learning Objectives

The students will practice and become familiar with:

- Proper aseptic technique
- Concept of serial dilution
- Morphology of cells in culture
- Proper use of inverted microscopes

The objective of this exercise is to practice transferring liquids of specific volumes accurately and aseptically using pipettes (refer to pp. 13-19). When working with the cells, you are often required to transfer cell suspensions, media or other reagents from one container to another. It is important to be as accurate as possible in measuring the volumes while paying special attention to using good aseptic technique to prevent contamination of your cells.

When preparing identical liquid mixtures to be transferred to multiple containers, it is always wise to make one big master-mix and aliquot the desired volumes into each container rather than preparing the mixture for each container separately. This way, there are less chance of errors and more consistency. To account for possible pipetting errors and to make sure, that there is enough mixture for all of the containers, you should always prepare a master-mix that is slightly higher in volume than the final desired volume.

In this exercise you will use different size pipettes to prepare 4 identical mixtures in four wells of a 12-well cell culture plate. First you will prepare each well separately. Second you will make a 4-ml master-mix and aliquot it into 4 wells. Finally, you will make a 5-ml master-mix and aliquot 1 ml into each well. Your partner will then check, to confirm the accuracy of your measurements.

Protocol

1. Turn on the laminar flow hood and wipe it with alcohol.

2. Gather the material you need and wipe them down with alcohol before placing them in the hood:

    - Serological pipettes of different sizes
    - Micropipettes an tips of different sizes
    - An electrical or manual pipette pump
    - One sterile 12-well cell culture plate
    - Solutions A, B, C and D
    - Sterile tubes

3. Open the culture plate inside the hood carefully. Label the wells 1-12 on the top of the lid.

4. Using the appropriate sized pipette transfer the following solutions to well #1.

Solution	Volume (µl)	What pipette size did you use?
A	15	
B	115	
C	370	
D	500	

5. Repeat for wells #2, #3, and #4. **Pay attention to your aseptic technique.**

6. In a sterile tube prepare a **4-ml master mix** of the above mixture:

Solution	Volume	What pipette size did you use?
A		
B		
C		
D		

7. Using an appropriate sized pipette transfer 1-ml of the mixture into each well #5-8.

Did you have enough mixture for all four wells?

8**.** In a separate sterile tube prepare a **5-ml master mix** of the above mixture:

Solution	Volume	What pipette size did you use?
A		
B		
C		
D		

9. Using an appropriate sized pipette transfer 1-ml of the mixture into each well #9-12.

Did you have enough mixture for all four wells?

10. You should have 1ml of the mixture in each well of the 12-well plate. Using a 1-ml serological pipette draw the liquid in each well and check the volume. Be careful, not to have any bubbles at the tip of the pipette. Record the observed volume and calculate the % error.

Up to +/- 5% error is normally acceptable for most routine cell culture exercises. Often times consistency and speed are more important than precision. However, for more sensitive experimental procedures accuracy may be more important.

Well #	Observed volume	Percent Error: **Expected - Observed X 100** **Expected**
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		

11. Place the 12-well plate in the incubator until next week.

12. Clean the hood and wipe with alcohol before turning it off.

13. Record the experimental procedure, your observations and the data in your note-book.

14. Discuss your observations in your note-book. Are the percent errors within the acceptable range? How can you improve accuracy? Which method is preferred when dispensing into multiple wells (prepare each well separately, prepare a master-mix or prepare a master-mix with a little extra volume)?

15. At the next class session check your plates. Do you have any contamination? How can you improve your aseptic technique?

Lab exercise 2: Aseptic Technique and Serial Dilution

Learning Objectives

Practice and become familiar with

- Proper aseptic technique.
- Proper use of micro and serological pipettes.
- Proper use of the laminar flow hood.
- Concept of serial dilution

.

In this exercise you will pipette the less expensive bacterial media into cell culture plates, practicing the use of aseptic techniques and the use of serological and micropipettes. You will dilute water in LB (Luria Bertani) media $10^3$and 10^4 times (refer to Appendix A for a review of serial dilutions). Note that water is being diluted in LB. You will pretend that water is the cell suspension of 10^6 cells/ml and bacteria growth medium (LB) is the cell culture medium.

1. Turn on the laminar flow hood and wipe it with alcohol.

2. Gather the material you need and wipe them down with alcohol before placing them in the hood:

- Serological pipettes
- 10 mls of LB
- 1ml of sterile dH2O
- Tube rack
- 24-well cell culture plate

3. Pretend that the water you are given is a cell suspension at the concentration of 10^6 cells/ml. You are asked to plate 3 wells of a 24-well plate with 1 ml of cells at the concentration of 10^3cells/ml in each well and 3 wells at the concentration of 10^2 cells/ml. Use 1ml for each well.

Think about your strategy and plan before starting. You can use the extra wells in the plate for your dilution steps. Remember, for more accurate measurements, not to pipette volumes less than 50 µls.

4. After plating, label the plates and place them in the 37^{o}C incubator.

5. Clean the hood and wipe with alcohol before turning it off.

6. Record the procedure in your notebook.

7. At the next class session, observe your plate. If there are bacteria or fungus growing, it means that you did not use good aseptic technique. How can you improve your aseptic technique?

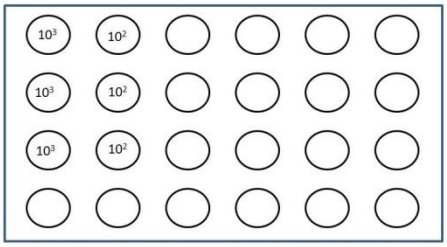


Figure 11- Diagram of the 24-well plate.

Lab exercise 3: Plating Cells from Frozen Stocks

Learning Objectives

Practice and become familiar with

- Plating cells from frozen vials
- Proper use of clinical centrifuges.

When cells are frozen they are kept in compete media plus 5-15% DMSO (dimethyl sulfoxide). DMSO prevents crystallization and breakage of the frozen cellular components. Although beneficial to preserve the frozen cells, DMSO is harmful to cells while growing in culture. Therefore, after defrosting, cells are centrifuged to separate them from the DMSO before they are plated. When cells are defrosted they must be plated immediately. You should organize your work area and be ready to proceed with the plating before you take out a frozen stock.

1. Turn on the laminar-flow hood and wipe down with alcohol.

2. Gather your material and wipe down with alcohol before putting them in the hood:

- One 15-ml sterile centrifuge tube
- Tube rack
- Serological pipettes
- Complete media warmed up at 37^{o}C
- One T-25 cell culture flask

3. Take out a frozen vial of cells from the -80 ^{0}C freezer or the liquid nitrogen tank. Always look at the label of the vial to ensure you have the correct cell line and make a note of the freezing date and other information on the vial. **Note:** wear protective mittens.

4. Place the frozen vial in a small container in the 37^{o}C water bath. Be careful that the cap does not get wet to avoid possible contamination.

5. As soon as the vial is defrosted (about 1-2 minutes) take it out of the water bath and wipe down well with alcohol. Don't let the defrosted cells sit in DMSO for too long.

6. Mix the contents of the vial by inverting the tube several times, and take it to the hood.

7. Transfer 9 mls of the complete media to the 15ml tube.

8. Using a sterile serological pipette or a micropipette, transfer the contents of the vial (usually 1ml) to the same 15ml tube. This will dilute the amount of DMSO ten times (10X dilution).

9. To wash and remove any remaining cells in the vial, pipette 1ml of the contents from the tube back into the vial and then pipette it back in the tube.

10. Close the top of the tube and label.

11. Using a balanced tube (see page 10), centrifuge the cells at about 100g (about 1000-1500 rpm for most clinical centrifuges) for about 3 minutes.

12. The heavy cells will form a **pellet** and are separated from the media (**supernatant**). The pellet may not be visible to the eyes. The pellet may be found on the side of the tube close to the bottom.

13. Take out the tube gently. Shaking the tube will resuspend the cells and you will need to re-centrifuge. Wipe down the tube with alcohol and put it back in the hood.

14. Using the aspirator in the hood and a sterile glass pipette (or a serological pipette), remove the supernatant from the tube carefully. To avoid sucking up the pellet, you may leave a small amount (~500µl) of the supernatant behind.

15. Transfer 3mls of complete media to a T-25 flask.

16. Transfer 2mls of complete media to the pellet and resuspend the pellet by pipetting up and down a few times. Although it is not possible to avoid bubbles, pipette gently to reduce the formation of the bubbles.

17. Transfer the resuspended cells to the T25. Mix by pipetting up and down a few times. Close the flask's cap.

18. Swirl the flask gently. Be careful not to get the flask's cap wet.

19. Observe the cells using an inverted microscope. You should have plenty of cells floating in the media.

20. Place the flask in the incubator. (Remember to loosen the cap if the flask is not vented).

21. Clean the hood and wipe with alcohol before turning it off.

22. Record your procedure and observations in your notebook.

Cells my take up to about a day to attach to the bottom of the flask and start dividing. Most of the cells may not survive the process of freezing and defrosting and may never attach to the flask. However, if the number of cells is high enough, even a small percentage of cells that survive are sufficient to start a new culture.

At the next class session, observe your cells and record your observations. What is the confluency of the culture? Are there many cells floating? What is the color of the media? Does the culture need to be fed or subcultured?

Lab exercise 4- Serial Dilution and Morphology

Learning Objectives

The students will practice and become familiar with:

- Proper aseptic technique
- Concept of serial dilution
- Morphology of cells in culture
- Proper use of inverted microscope

Day 1

Preparing the 24-well plates

1. Clean your work area thoroughly by wiping the work surface area and everything on it with 70% alcohol.

2. Gather the material you need and wipe down with alcohol before placing them in the hood:

- Serological pipettes
- Micropipette tips
- Ca^{+2}/Mg^{+2} –free PBS, warmed
- Trypsin, warmed
- Complete media, warmed
- 24-well cell culture plate

2. Un-wrap the 24-well cell culture plate in the hood.

3. Loosen the cap of the media and trypsin tubes but leave the cap on. Remove the cap only when you need.

4. Carefully pipette 0.5 mls of media per well into wells A1 and C1 (Figure 12).

5. Pipette 0.9 mls of media in the wells A2-A4 and C2-C4.

6. Place the top back on the plate. Using the permanent marker label the plate with your name and the date on the top.

Trypsinizing the Cells

This procedure may be performed by the instructor.

1. Take out a flask of cells from the incubator and observe the cells growing in the flask under the inverted microscope. Take a note of the confluency and health of the cells. The flask needs to be about 60-85% confluent. If contaminated, the cells need to be bleached and discarded and not be used.

2. Transfer the flask to your hood. Wipe the flask with alcohol.

3. Open the cap, but leave the cap on loosely until you are ready.

4. Aspirate out the media by using the vacuum aspirator or by pipetting with a 10 ml pipette. Discard the media.

5. Add 3 mls of PBS to the flask.

6. Close the cap and swirl the flask gently to wash the sides.

7. Remove the PBS from the flask using vacuum or by pipetting and discard in the bleach bucket. This washing step will remove the remaining serum.

8. Add 1 ml of Trypsin to the flask.

9. Close the flask's cap tightly and place it in the 37C incubator or room temperature so trypsin covers all the cells. Wait 1-3 minutes.

10. Observe the cells under the inverted microscope. If the cells are loose, tap the plate to dislodge all of the cells.

11. If the cells are all floating, add 9 mls of media to the cells. (If the cells are still attached, return the flask to the incubator and wait another minute).

12. Resuspend the cells by pipetting up and down 5-8 times using a 5/10 ml serological pipette. Avoid getting too many bubbles.

13. If you are given two cell lines. Proceed to trypsinizing the second flask by repeating steps 1-12. Label the tubes carefully to avoid mixing them up.

Note: Trypsinized cells need to be plated as soon as possible.

Serial Dilutions and plating of the cells in 24-well plates:

You will plate the cells in wells of the 24-well plate and dilute the cells by serial dilution to lower concentrations. Use row A for the first cell line and row C for the second cell line.

1. Working with one cell line at a time, open the cap in the hood and mix the cells by pipetting up and down 2-3 times (avoid creating bubbles).

2. Trasnsfer 0.5 mls to the wells in column 1 (Well A1- Figure 12).

3. Using the same pipette mix the cells in the wells by pipetting up and down 2-3 times.

4. Using a smaller serological pipette or a micropipette, transfer 100 µl (0.1 ml) from well 1 to well 2 (A1 to A2).

5. Mix the cells in well 2 by pipetting up and down.

6. Transfer 100 µl from well 2 to well 3.

7. Mix well 3 by pipetting.

8. Transfer 100 µl from well 3 to well 4.

9. Mix well 4 by pipetting.

10. Remove and discard 100 µl from well 4.

11. Repeat the serial dilution for the second cell line in row C.

Note: Every well will have 0.9 mls of cells. You may use the same pipette for transfers within each cell line. You must use a different pipette for the two cell lines to avoid mixing.Place the plate in the cell culture incubator for 1-2 day.

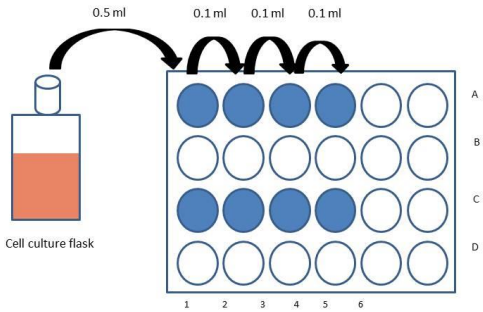


Figure 12- Diagram of the 24-well plate

Serial dilution questions:

1. In the above procedure how much was each cell type diluted in the wells of the first column as compared to the original flask?

2. How much was the last well (column 4) diluted compared to the original flask?

3. In this procedure you are not asked to count the number of cells. Assuming there are 5 x 10^6 cells **total** in the original flask.

   A. What is the **concentration** (# cells/ml) of cells in the flask (10-mls)?
   B. What is the **concentration** of cells in each well?
   C. What is the **total number** of cells per well? (Note that each well has 0.9 mls of cells).

4. Assuming 5 x 10^6 cells total in the original 10-ml flask.

   A. How will you prepare a well containing 10^4 cells in 1 ml?
   B. How will you prepare a well containing 100 cells in 1 ml?

Day 2

Observe your cells by using the inverted microscope:

1. Take out your plate from the incubator and place it on the stage of the inverted microscope.

2. Turn on the microscope. Adjust the light intensity for your eyes.

3. Set the 10X objective lens in place.

4. Select the corresponding phase contrast ring for the 10X objective.

5. Move the plate on the stage to place the well with cells in the light path.

6. Adjust the focus by using the focus knobs and turning the ocular lens.

7. Move the plate on the stage to look at all of the wells.

8. You may change the objective lens to higher magnification for more detailed observations.

14. Record your observations.

Observations-What do you see?

1. Check the clarity of the media. Is there any contamination?

2. Check the color of the media. The media turns yellow when the environment is too acidic and purple when it is too basic. Contamination and/or overcrowded cultures

usually turn the media yellow (can you explain why?).

3. Estimate the confluency of each well by looking at the percentage of the surface covered by cells?

4. The cells were diluted by the factor of 10 in each consecutive well during plating. Do you see the 10 times difference in cell density?

5. Draw a typical cell that you see for each well at high and low confluency. Point to the structures that you see (ex. Nucleus, nucleoli, cytoplasm and cell membrane) How does the shape of the cell differ as the confluency changes?

6. How do the two cell types differ in shape? Compare the relative size of the nucleus to the surrounding cytoplasm for each cell type.

7. Anchorage- dependent cells round up and loosen up as they are going through mitosis. After mitosis the cells spread out again. Do you see any cells going through mitosis?

Day 2-Continue:

Giemsa Staining:

Giemsa is a commonly used stain for observing cells. Giemsa stains the nuclei purple, cytoplasm blue or pink with granules of varying colors.

You do not need to work in the hood for this procedure. **Wear gloves.**

1. Remove the media.
2. Add 1 ml PBS to each well.
3. Remove and discard the PBS in the bleach beaker.
4. Add 1 ml PBS/methanol mix to each well. Wait 2 min.
The alcohol is a **fixative**. It kills the cells but keeps the structure of the cells intact.
5. Remove and discard the PBS/methanol.
6. Add 1 ml methanol to each well. Wait 10 min.
7. Remove the alcohol.
8. Add another 1 ml of methanol to each well. Remove and discard. There is no waiting time for this step.
9. Add 0.2 mls of Giemsa stain per well. Wait 2 min.
10. Add 0.8 mls of distilled water per well. Mix the plate gently for 2 min.
11. Over a sink or a container, gently pour water into the wells and allow the stain to spill over, Repeat 3-5 times to wash the cells.
Note: It is important to remove the excess stain by floating upward to avoid any precipitates that may remain on the cells.
12. Observe the stained cells under the microscope. Answer the observation questions # 5-7 on page 38.

Lab exercise 5: Count and Plate 10^4 Cells in a T25

Learning Objectives

Become familiar with and practice to:

- Subculture cells
- Count and calculate cell concentrations
- Proper use of a hemocytometer
- Serial dilution of cells for plating

Please read all of the protocol before starting.

1. Turn on the hood and wipe down with alcohol.

2. Gather the material you need and wipe down with alcohol before placing them in the hood:

- Serological pipettes
- Micropipette tips
- Microfuge tubes
- Ca^{+2}/Mg^{+2} –free PBS, warmed
- Trypsin, warmed
- Complete media, warmed
- Trypan blue

3. Observe your cells and make sure they are healthy. Note the confluency of your flask.

Trypsinize

4. Inside the hood remove the media from your flask.

5. Add 3 mls of PBS to the flask.Swirl the flask once. Remove PBS. This washes the serum away.

6. Add 1ml trypsin to the flask. Make sure all of the cells are covered by trypsin.

7. Wait about 2-3 minutes for the cells to detach.

8. Tap the side of the flask and observe under the microscope to make sure all of the cells are floating.

9. After cells are detached, quench trypsin by adding 4 ml of media.

10. Mix well by pipetting up and down a few times.

Count with a hemocytometer

11. Take out 50 µl of the cells and transfer to a microfuge tube.

12. Add 50 µl of trypan blue solution to the tube and mix.

13. Transfer 12µls of the cell suspension in the tube to one half of the hemocytometer.

14. Count the number of live cells in 5 squares. If the number is too high (>500), you need to dilute your cells appropriately and count again. If the number is too low

(<25), you need to concentrate your cells by transferring to a centrifuge tube, spinning, removing the supernatant and resuspending the cells in less volume of complete media.

15. Calculate cell concentration and total number of cells.

# of cells in 5 squares	
Dilution factor for mixing with Trypan blue	
Further dilution factors, if any	
Total dilution factor	
Concentration of cells in the flask (cells/ml)- C1	
Total volume in the flask	
Total number of cells	

Plate

16. You are planning to plate 10, 000 cells in a T25. Normally 5 ml of media is sufficient for a T25 flask. So you need to make a suspension of 10,000 cells in 5ml or 2000 cells/ml (final concentration). Calculate the volume that you need from your culture using the formula $(C_1)(V_1)=(C_2)(V_2)$, where C_1 is your culture's concentration that you have calculated. V_1 is the unknown volume containing 10,000 cells. C_2 is the final concentration of 2000 cells/ml and V_2 is the final volume that is 5 mls. Solve for V_1.

C1 (initial concentration, calculated above)	
C2 (final desired concentration)	
V2 (final desired volume)	
V1 (initial volume of culture to start with, unknown)	

17. If V1 is less than 50 µl, then go back to your original culture and dilute some of it appropriately in order to get a larger V1. (Refer to appendix A for serial dilutions).

18. Mix your culture well since cells might have settled and might have formed clumps.

19. Calculate the amount of fresh media for the final cell suspension and pipette it inside a fresh T25 flask.

V1 (calculated above)	
Volume of fresh media	
Total volume	**5 mls**

20. Take out the correct volume (V_1) of your culture and add it to the media inside the new flask.

21. Mix by pipetting.

22. Label the flask (date, cell type, your initials), observe it under the microscope and place it in the incubator.

23. You may plate more than one plate for future use or discard the remaining cells by aspirating inside the waste bottle and disposing of the flask appropriately.

24. Observe the cells after they are settled next time you are in class and note the status of the cells.

Analysis questions:

1. What was the status of your cells before starting? (% Confluency, color of the media and general health of the culture.)

2. Did you have enough cells to plate 10^4 cells?

3. Did you have to dilute your culture further or concentrate it for more accurate count? If so how did you do it? Show your calculations.

4. For step 17, did you have to dilute your culture to increase the V_1 value? How did you do it?

5. What is the status of your cells after settling (step 24)?

Lab Exercise 6: Survival Assay- Sensitivity to UV Exposure

Learning Objectives:

Practice and become familiar with:

- Survival (clonogenic) cytotoxicity assay
- Staining and counting colonies
- Construction of a survival curve and determination of the IC_{50} value

After plating, cells attach to the surface of the culture vessel and start dividing. A single cell divides into two daughter cells which divide into four and then eight and so forth, thus forming a **colony**. A colony refers to a population of cells that are all descendents of a single parental cell. Cells in a colony sit close to each other and if there are enough cells in a colony, they can easily be seen without the help of the microscope.

When plating, many of the cells die in the process and never reattach to the flask. Among those who do attach, some may die later. Some of the cells may stay alive but will not divide enough times to make a colony. **Plating efficiency (or cloning efficiency)** is a measurement used to identify the percentage of cells that survive and are able to divide and form colonies after being plated. Plating efficiency is a value that measures cell survival when cells are plated at low densities (2-50 cells/cm^2). When cells are plated at lower densities, they are far apart and are not able to assist each other for survival. These cells are on their own, and if they survive they will divide and form colonies. Each colony represents a single cell that has managed to survive. To measure plating efficiency, cells are plated at low densities and allowed to grow 7-10 days. During this time the cells that have survived will divide and form colonies. The colonies are then counted and plating efficiency (PE) is calculated:

$$PE = \frac{Number\ of\ colonies}{Number\ of\ cells\ plated}\ x\ 100$$

Plating efficiency is used to study differences in cell survival and growth rate within cells of the same population or among cells of different populations. The number of colonies represents cell survival and the size of colonies represents growth rate. The bigger colony size can be interpreted as faster growth rate.

Plating efficiency is one of the techniques often used for **cytotoxic studies**. Cytotoxic studies involve measurement of altered metabolism or loss of viability due to a toxic factor. *In vitro* cytotoxicity studies are often used by environmental scientists and pharmaceutical companies to screen for potentially toxic drugs and environmental factors. Plating efficiency is a form of a cytotoxicity assay that measures cell viability several divisions after exposure to the toxic reagent. To study the effects of a toxic reagent or drugs on cell survival, cells are plated at low densities in separate vessels and exposed to different dosages of the reagent. After several divisions, the number of colonies is counted. PE and **surviving fraction (SF)** values are compared among cells that were exposed to different dosages and control cells with no exposure.

$$SF = \frac{Number\ of\ colonies\ for\ each\ dosage}{Number\ of\ colonies\ for\ control\ cells}$$

The lower SF values represent less survival as a result of exposure. In addition, average colony size can reveal the effect of the toxic reagent on growth rate. The SF values can be plotted against dosages to generate a survival curve.

In the following exercise you will determine the PE and SF values for cells that have been exposed to different dosages of UV (ultra violet) light. The objective is to determine the level of sensitivity of your cells to UV light. You may do the experiment with two or more cell lines and compare their UV sensitivity levels.

Ultraviolet light exposure causes formation of covalent bonds between two adjacent **pyrimidines** (C and T) in cellular DNA forming **pyrimidine dimers** (Goodsell, 2001). Pyrimidine dimers block progression of replication forks and, if left unrepaired, may lead to base deletions or substitutions and cause mutations. The mutations are subsequently propagated as the cell goes through more divisions and can have disastrous effects for the organism (for example, skin cancer). Fortunately, our cells are equipped with a number of proteins that are specialized in recognition and repair of pyrimidine dimers. Mutations in the genes that code for these proteins can make the repair mechanism inefficient and cause devastating diseases. People who are affected by these mutations are very sensitive to light exposure and develop severe sunburns and tumors and die at young ages.

In the following exercise you will compare the sensitivity levels of two cell lines to UV radiation. The following exercise will be done over a week. On the first day, you will plate your cells at low density in multi-well plates. After one or two days, the cells are exposed to several dosages of UV light. The cells are allowed to divide and form colonies for one week. The colonies are then stained and counted. Finally, you will draw a survival curve and determine the sensitivity of your cells to UV light. Some of the steps may be performed by the instructor or the lab aids.

Plating of the cells

1. Turn on the hood and wipe down with alcohol.

2. Gather the material you need and wipe them down with alcohol before placing them in the hood:

- Serological pipettes
- Micropipette tips
- Sterile centrifuge tubes
- Ca^{+2} /Mg^{+2} free PBS-warmed
- Trypsin- warmed
- Hemocytometer
- Five 6-well plates

3. Observe the cells under the microscope and note the confluency of your cultures. Work with one cell line at a time.

4. Remove the media, wash with Ca^{+2} /Mg^{+2} free PBS and trypsinize cells with 1 ml trypsin. (Refer to the page 35 for detailed protocol of trypsinizing.)

5. After cells are detached and floating, add 4 mls of media and mix by pipetting.

6. Using a hemocytometer count the number of cells and calculate cell concentration. (Refer to page 26, counting cells using a hemocytometer, for a detailed protocol).

7. Plate 200 cells into one row or 3 wells of the six-well plate (density of about 20 cells/cm^2) according to the diagram in figure 13. Repeat for the other four plates. (The number of cells that you need to plate may be different depending on the plating efficiency of your cells). When plating cells

in multi-well plates, it is best to prepare a master-mix first and then aliquot into the wells rather than plating each well separately. This is to reduce the chances of mistakes in plating each well and keeping the wells consistent. For this experiment we will plate three wells per plate for each cell line, which is a total of 15 wells. You can use 2 ml of media for each well. You must always prepare a master-mix with slightly larger volume than you need to account for pipetting errors. Therefore, make 32 ml of the master-mix with 2 ml extra. Calculate the volume of cells you need to take out in order to make your master-mix (V_1). Use the $(C_1)(V_1)= (C_2)(V_2)$ formula.

	Cell line 1	Cell line 2
# of cells counted in 5 squares of the hemocytometer		
Dilution factor for counting		
Cell concentration, C_1		
Final cell concentration of the master-mix (cells/ml), C_2		
Final volume of the master mix (ml), V_2	32 ml	32 ml
Initial volume of the culture, V_1		
Volume of complete media to be added		

8. Prepare the master-mix by mixing the appropriate volume (V_1) of cells from your culture to a large tube with enough complete media to bring up the volume to 32 ml.

9. Mix well by pipetting.

10. Aliquot 2 ml into each well.

11. Label the plates as shown in (Fig. 13) and put them in the incubator.

12. If you are using more than one cell line, repeat the above procedure for the other cell line, using the second set of three wells on the six-well plates.

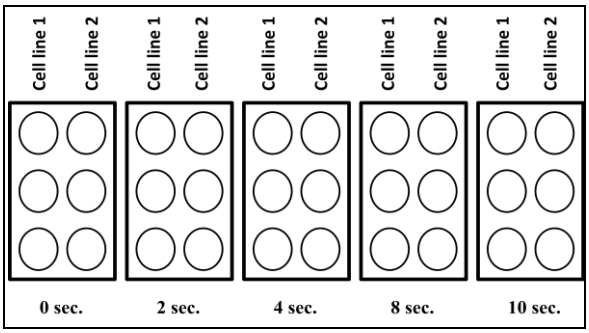


Figure 13- Diagram of the six-well plates. Seed 3 wells (or a row) of each plate with each cell line.

UV exposure

One day after plating:

13. Turn on the hood and wipe down with alcohol. Gather you material and wipe down with alcohol:

- Serological pipettes
- PBS (with Ca^{+2} /Mg^{+2})
- Complete media, warmed
- UV protection goggles

14. Remove the media from your wells.

15. Wash the wells with 0.5 ml of PBS per well. Remove the PBS such that the wells are dry.

16. Expose each of the plates to one of the following doses: 0, 2, 4, 8 or 10 seconds of UV light. To expose the plates:

- Wear protective goggles.
- Remove all other cells that you are NOT treating with UV from the hood.
- Place the plate to be treated under the UV light.
- Remove the lid from the cells that you are going to treat with UV.
- Turn on the UV lamp for 0, 2, 4, 8 and 10 seconds.

17. After UV treatment, quickly add 2 ml of fresh complete media per well.

18. Put the plates back in the incubator and do not move for 7-10 days.

Counting colonies

In order to see the colonies clearly, you will stain the colonies with a blue/violet stain called **crystal violet**. Crystal violet stain is mixed with methanol, which is a fixative. Methanol will kill the cells but keeps the cell structures intact and prevents disintegration of the cells.

19. Aspirate out the media.

20. Wash the cells once with about 0.5-1 ml per well of PBS (with Ca^{+2} /$Mg^{+2)}$. Remove the PBS.

21. Add 0.5 ml of Crystal violet/Methanol to each well.

22. Allow the cells to stain at room temperature for about 30-60 min.

23. Transfer the stain to the waste bottle (the stain can be filtered and re-used). Do not use the aspirator: crystal violet will stain the tubing of the aspirator, which is difficult to clean.

24. Gently pour some tap water into each well and wash the excess stain 2-3 times. Dump the water waste in the sink with running water.

25. Invert the plates and let them dry.

26. Count number of colonies for each well. Colonies that are too tiny (less than 50 cells) are not counted. Different sizes of colonies represent variations in growth rates among cells in the same culture.

27. Calculate the average number of colonies for each set of three wells with the same treatment.

28. Calculate PE and surviving fraction values for each dosage and each cell line.

29. Graph a surviving curve and analyze your data.

Analysis questions:

1. Count the average number of colonies for each set of three wells and calculate the PE value for each dosage? Repeat for each cell line. How do the PE values change with different dosages?

	Cell-line 1		Cell-line 2	
Dosage (sec)	**Ave. # of colonies**	**PE**	**Ave. # of colonies**	**PE**
0				
2				
4				
8				
10				

2. Calculate the SF for each dosage and each cell line. How do the SF values change with different dosages?

Dosage (sec)	**SF Cell-line 1**	**SF Cell-line 2**
0	1	1
2		
4		
8		
10		

3. How do the average sizes of colonies differ with different dosages of UV? What do the differences in colony size indicate about the effect of UV on your cells?

4. Draw a survival curve for this experiment. Survival curves are drawn in a semi-log graph format, with the SF values on the Y-axis in the log format and dosage on the X-axis in standard format. Draw both curves for the two different cell lines on the same graph.

5. What is the **IC_{50}** value for each cell line? IC_{50} is the dosage at which there is 50% inhibition of colony formation (SF value of 0.5). IC_{50} values are often used to compare sensitivities to toxic reagents. Higher IC_{50} values indicate lower sensitivity and lower IC_{50} means higher sensitivity. Which cell-line is more sensitive to UV radiation?

6. If you were able to repeat the experiment, how would you do it differently?

7. Discuss your results further in your notebook.

Lab exercise 7: Viability Assay

Learning Objectives

Become familiar with and practice:

- Concept of the viability assay
- Construction and analysis a viability curve

Viability assay is a form of cytotoxicity assay that can be used to test for the effect of a toxic agent on the short-term viability of the cells. Cytotoxic studies involve measurement of altered metabolism or loss of viability of cells due to exposure to a toxic factor. *In vitro* cytotoxicity studies are often used by environmental scientists and pharmaceutical companies to screen for potentially toxic factors and drugs. Cytotoxicity assays measure the toxicity effects of a factor by detecting cell death. Alternatively, toxicity can be measured by viability assays which are designed to detect cellular survival and viability rather than death. In order to do a viability assay, cells in culture are exposed to a toxic reagent, and shortly after the survival rate is measured by detecting an activity in the cells that is characteristic of live cells or simply counting number of live cells Viability assays are often quick and easy to perform. However, the disadvantage of such assays is that only the short-term effect of the drug is being studied. The assay is usually performed only 1-3 days after the drug exposure and so the longer time effects that the drug may have on cells are missed. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay is a form of viability assay that is often performed in science laboratories. MTT is a yellow water soluble dye that is reduced to a purple insoluble product by reductase enzymes produced by live cells. Cells are cultured in microtitration plates and exposed to a wide range of toxin dosages. After 2-3 population doublings, MTT is added and the change of color from yellow to purple is measured for each dosage using a spectrophotometer. The more intense purple color indicates higher reductase activity which is indication of higher number of live cells. The results of the assay are shown in a viability curve where percentage of live cells is graphed against the toxin dosage (Figure 14). The dosage at which viability is 50% is called IC_{50} (half maximal inhibitory concentration). Lower IC_{50} values represent lower viability and so higher toxicity. High IC_{50} indicates higher survival and higher tolerance to the toxic reagent.

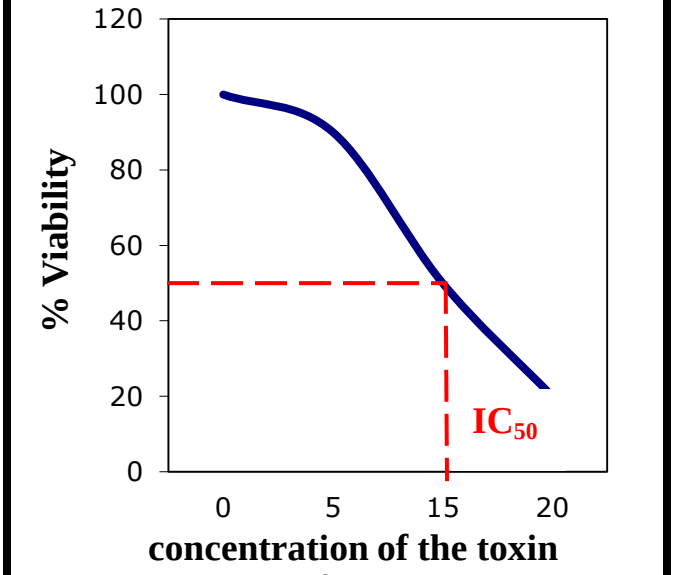


Figure 14- Example of a viability curve.

You will perform a modified version of the viability assay to study the effects of caffeine on cell viability. Studies have shown that at high concentrations, caffeine inhibits cell proliferation and increase cell death by apoptosis in some cancer cells (Ku *et al* 2011). The cells are cultured in multiwell plates and exposed to different

dosages of caffeine. After a few days of exposure, all living cells in the wells will be fixed with alcohol and stained purple with crystal violet (instead of MTT). The darker purple color represents presence of more cells and lighter purple is evidence of less cells present. You will compare the intensity of the purple color in the wells exposed to different concentrations of the toxic reagent.

Day 1

You will be using aseptic technique in the procedures below.

1. Place your tube of cell culture media. PBS (without Ca/Mg) and trypsin in the 37^{o}C water bath/incubator to warm up.

2. Turn on the hood and wipe down with alcohol. Clear the space from anything that is not needed and keep it organized.

3. Gather the material you need and wipe down with alcohol before placing them in the hood.

- Serological pipettes
- Ca^{+2}/ Mg^{+2} Free PBS
- Trypsin-warmed
- Complete media- warmed
- Hemocytometer
- 96-well cell culture plate

4. Get a flask of cells and observe the cells under the microscope. Take a note of the confluency and health of the cells. Your culture should be about 70-95% confluent. If contaminated, the cells need to be bleached and discarded and not be used.

5. Transfer the flask back to the hood. Remove the media. Use the aspirator (or use a serological pipette to remove the media and transfer to a liquid-waste beaker containing bleach).

6. Add 3 mls of PBS (Ca/ Mg free) to the flask. Swirl the flask gently to wash the cells.

7. Remove and discard PBS from the flask

8.Add 1 ml of trypsin to the flask. Make sure trypsin is covering the bottom surface of the flask, where the cells are attached.

9. Wait 2-3 minutes for the cells to loosen up. (You may need to incubate the flask in the 37 ^{o}C incubator. The enzyme works more efficiently at 37^{o}C).

10. Observe the cells under the microscope. Cells that are about to detach are rounded. If the cells are round, tap the flask a couple of times to detach the cells. If the cells are not round, return the flask to the incubator and wait another 1-2 minutes.

11. Transfer the flask back in the hood. Quench trypsin by adding 4 mls of media to the flask.

12. Mix the cells by pipetting up and down a few times. Use a 1 or 5 ml serological pipette. Try to avoid getting bubbles.

13. Using a micropipette transfer 50 µls of the cells to a small tube. Be careful, only the tip is sterile and should touch the inside of the flask.

14. Add 50 µls trypan blue to the tube. Mix well by pipetting up and down a few times.

15. Transfer 12 µls of the mix to a hemocytometer.

16. Count the cells in 5 squares. (Do the proper dilutions if needed). Calculate cell concentration in the flask. **Note:** Since the cells were diluted 1:2 in trypan blue, the dilution factor is 2. If you diluted the cells further, you need to calculate your total dilution factor.

# of cells in 5 squares	
Dilution factor	
Concentration cells/ml	

17. Prepare a master-mix of cells. You are planning to plate cells in a 96-well plate in columns 2-10 of rows A-C as is shown in figure 15.

Note:

- The number of cells may vary between 5,000-10,000 cells/well depending on cell type and number of days you are planning for the cells to grow.
- It is very important for all the wells to have equal number of cells plated in them.
- There are no cells plated in the first column.
- Plate 200 µls in each well.
- Use the C1V1=C2V2 formula to calculate the initial volume (V1) of cells needed for preparing the master mix.
- The volume of the master-mix (V2) should be slightly more than what you need to account for possible pipetting errors.

Concentration of cells calculated above (C1)	
Final concentration (C2)	
Volume of the master mix (V2)	
Initial volume (V1)	

18. Prepare and mix the master mix by pipetting up and down a few times.

19. Transfer 200 µls of the master mix into each well (rows A-C, columns 2-10). Mix the master mix frequently to keep a homogeneous suspension.

20. Add 200 µls of media (no cells) to the first column (wells A1, B1 and C1). The first column will be a negative control.

21. Label the plate, and transfer to the incubator.

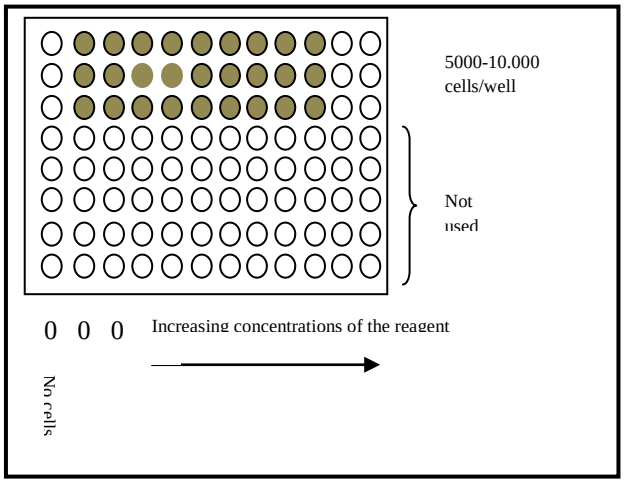


Figure 15- Diagram of the 96-well plate used for the viability assay. Cells are plated in the wells shown in gray.

Day 2

Caffeine exposure (This may be done by your instructor or laboratory technicians.)

22. Turn on the hood and wipe down with alcohol.

23. Gather the material you need and wipe down with alcohol before placing them in the hood.

- Serological and micro pipettes
- Multi-channel micropipettes
- Complete media- warmed
- Caffeine stock solution [50 mM (10 mg/ml)]

24. Prepare 7 different concentrations of sterile caffeine in media.

- First prepare 2 mls of 5 mM Caffeine using the stock solution
- Next use serial dilution to prepare 2 mls of each concentration. Note that you may end up with 1 ml in the tube (you only need 0.6 mls) Show your calculations and strategy in your notebook.

Concentration	Media	Caffeine
0.075 mM		
0.15 mM		
0.31 mM		
0.62 mM		
1.25 mM		
2.5 mM		
5 mM		

25. Remove the old media from all the wells in the plate. Be careful not to scratch the bottom of the wells where the cells are.

26. Add 200 µl of the media plus reagent mixture to the appropriate wells (columns 4-10). Add 200 µl media with no caffeine to the first three columns.

Column #	Caffeine Concentration (mM)
1	0 mM
2	0 mM
3	0 mM
4	0.075 mM
5	0.15 mM
6	0.31 mM
7	0.62 mM
8	1.25 mM
9	2.5 mM
10	5 mM

27. Keep the remaining caffeine solutions in the refrigerator if you plan to feed the cells in 3-4 days.

28. Incubate the plate in the incubator for 2-7 days.

29. If you are planning to keep the cells exposed to caffeine for a week, you should feed the cells with fresh media plus the appropriate Caffeine concentration at least once during the week (Repeat steps 22-28 in 3-4 days after the first plating).

Day 3-7

Viability assay

30. You will fix the cells in the plate. Therefore, there is no need to work in the hood from this point forward. Note the solutions used outside of the hood are no longer sterile.

Gather the material

- Micropipettes (Multi-channel micropipettes if available)
- PBS with Ca^{+2}/ Mg^{+2}
- Crystal violet/methanol
- A plate reader with a 590 nm filter (spectrophotometer)

31. Remove the media from all of the wells of the 96-well plate.

32. Wash each well with 0.1 ml PBS (with Ca^{2+} and Mg^{+2}). Remove PBS.

33. Add 100 µl of crystal violet solution to each well (except for column 2- control). Use multi-channel pipettes. Wear gloves.

34. Incubate at room temperature for 30 min. The alcohol in the crystal violet solution will kill and fix the cells. Dead cells become porous and the dye can go inside the cells.

35. Remove crystal violet by pipetting and transfer to a waste tube. Do not aspirate, the aspirator tubing will turn blue.

36. Using a beaker filled with tap water, pour some water over all of the wells. Dump the water in the sink with lots of running water. Repeat the wash 3-5 times

37. Invert the plate on a paper towel to dry.

38. Using a spectrophotometer (plate reader), read absorbance at 590 nm.

Note: If you do not have access to a plate reader. Take a picture of your plate from above. Use Photoshop or Image J programs to quantify the intensity of the purple color in each well.

Viability Curve and Analysis

1. Look at the cells under the microscope. Do you see a difference in morphology of the cells that are exposed to different concentrations of the drug?

2. Calculate average absorbance (or color intensity) for each drug concentration triplicate. Then calculate % viability.

$$\% \text{ Viability} = \frac{\text{Average absorbance for each concentration}}{\text{Average absorbance at 0 mM}} \times 100$$

Column #	Concentration	Average Absorbance	% Viability
1	0 mM		
2	0 mM		
3	0 mM		
4	0.075 mM		
5	0.15 mM		
6	0.3 mM		
7	0.6 mM		
8	1.25 mM		
9	2.5 mM		
10	5 mM		

3. Are the three absorbance values for each triplicate similar? If not explain what might have gone wrong?

4. The first two columns are control experiments. Column 1 has no cells and column 2 has cells but the cells were not stained. Do you observe the expected results from the control experiments? What do we learn from the control experiments?

5. Graph the percent viability (Y-axis) values for each concentration (X-axis). Use a graph paper or Excel.

6. What is the IC50 value, dosage of drug that reduces absorbance to half or caffeine concentration at 50% viability?

7. Explain what you have learned about the toxicity of caffeine from this experiment?

8. If you could repeat the experiment, what would you do differently?

Lab Exercise 8: Live and Dead Cell Identification by Using Fluorophores

Learning Objectives

Become familiar with and practice:

- The use of fluorescent microscopes.
- Identification of live and dead cells by using fluorophores.

In this exercise you will use fluorescent molecules to distinguish between live and dead cells. Fluorescent molecules, also known as **fluorophores**, can absorb the energy of light at specific wavelengths and emit less energetic fluorescent light. When a fluorophore absorbs the energy of light it becomes excited by elevating to a higher energy level (excitation). The excited fluorophores go back to the ground state by releasing the energy in form of heat and emitted light (emission). A large variety of fluorophores have been made and modified to interact with specific cellular structures in order to study them.

Propidium Iodide (PI) is a fluorescent molecule that absorbs light most efficiently at 500-550 nm and emits bright red fluorescent light at 600-650 nm wavelengths. Live cells are impermeable to PI, so PI can only enter dead cells that have porous membranes. Once inside the cell, it intercalates between nucleic acids (both RNA and DNA). PI is considered hazardous and you must **wear gloves** when working with PI.

Calcein AM is a derivate of calcein and is not fluorescent. Calcein AM is permeable to both live and dead cells. Once inside the live cells, esterase enzymes convert calcein AM into calcein. Calcein is a bright green fluorescence compound that absorbs and emits light at 495/520 nm respectively. Dead cells do not have functional esterases and so will not be green.

Day 1

plating: (This can be done by your instructor or the lab technicians)

1. Plate cells in 24-well plates (Figure 15) so the wells are 60-80% confluent for the day of the experiment. Use 1ml of media per well.

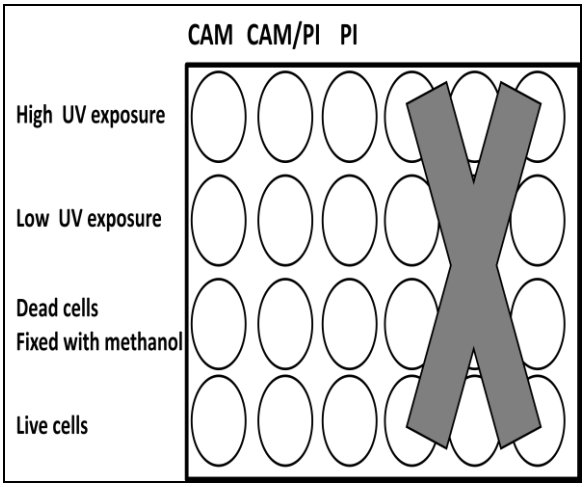


Figure 15- Diagram of the 24-well plate. One half of the plate marked by X is not used for the experiment.

Day 2

2. Your instructor will expose the first two rows of the cells to UV radiation (or other toxic reagents) prior to the start of the class.

The following steps are performed by the students:

3. Turn on the hood and wipe it down with alcohol.

4. Gather your material and wipe down with alcohol before placing them in the hood:

- Micropipettes and tips
- Serological pipettes
- 70% methanol in PBS
- PBS (with Ca^{+2}/Mg^{+2}), warmed
- Complete media, warmed
- 10 μg/ml Propidium Iodide (PI)
- Calcein AM (8μM)

5. Observe the cells under the microscope. Do you see a difference between the rows of cells that were exposed to UV (or other reagents) and cells in the other rows?

6. Remove the media from the third row of cells.

7. Rinse the cells in the third row with 0.5 ml of PBS (with Ca/ Mg) and remove PBS.

8. Add 0.5 ml per well of 70% methanol to the third row and incubate for 30 min. Methanol will kill and fix the cells so the cellular structures remain intact after dying.

9. Remove the methanol from the third row.

10. Remove the media from the wells in the first two columns of the plate labeled CAM and CAM/PI.

11. Add 1 ml of media to the third well of the third row.

12. Rinse all of the cells in the first two columns with 0.5 mls/well of PBS.

13. Remove PBS from all of the wells in the first two columns.

14. Add 200 μl per well of 8μM Calcein AM per well of the first and second column of cells.

15. Put the plate back in the incubator for 30 minutes.

16. Remove the Calcein AM and media from all of the wells in all the rows and columns.

17. Rinse all of the wells with 0.5 mls of PBS once. Keep the PBS in the first column but remove it from the second and third columns.

18. Add 200 μl per well of 10 μg/ml PI to the wells of the second and third columns.

19. Cover the plate with aluminum foil to keep it in the dark and prevent photobleaching of the fluorophores. Incubate at room temperature for 1 min.

20. Examine the cells under the fluorescence microscope. Use the green filter cube to see

the red color of PI and the blue filter cube to see the green color of Calcein.

21. Compare the fluorescent colors in different wells and write your observations in your notebook.

Analysis questions:

1. Expectations: What color fluorescence do you expect to see for each row and each column? Why?

2. Observations: What color fluorescence do you see in each well? Is what you see the same as you expected? Explain.

3. Is there a difference between the row of cells exposed to high level of UV and the row of cells exposed to the low level of UV?

4. Where do you see the red color? Nucleus or the cytoplasm? Explain

5. Where do you see the green color? Nucleus or the cytoplasm? Explain.

5. Discuss the results in your notebook.

Lab Exercise 9: Transfection

Learning Objectives

Become familiar with and practice:

- Transient transfection of mammalian cells with plasmids.
- Practice using fluorescent microscopes.
- Observe and identification different cellular structures (mitochondria and cytoplasmic actin filaments) that have been labeled with fluorophores.

Transfection is a method used to transfer non-viral recombinant DNA into mammalian cells. Transfer of viral genetic material into eukaryotic cells is referred to as **infection**. Transfected cells are used to study gene expression and regulation, protein expression and function, gene therapy and more. DNA sequences of interest are inserted into carrier DNA molecules; commonly known as vectors, by the use of **recombinant DNA** techniques. One of the most commonly used vectors for transfection is a **plasmid**. Plasmids are small circular DNA molecules that naturally exist in bacteria and some eukaryotic cells. Plasmids are engineered and designed to carry sequences that enable the plasmids to be replicated by the host cell's replication machinery. There are various methods used to introduce the vector, which is carrying the DNA sequence of interest into the cells. Examples of these methods for introducing DNA into cells include the use of electric currents, microinjection, shooting of DNA coated particles into cells, and forming a complex of DNA and cationic molecules that can enter the cell by endocytosis or membrane fusion. If there are regulatory sequences that are recognized by the host cell present, after entering the nucleus, the genes that are carried on the foreign DNA may be transcribed and expressed into proteins by the cell. For most transfections, the foreign DNA molecule stays independent of the host genome and is eventually lost during subsequent cell divisions. This type of transfection in said to be transient. In order to study **transient transfections**, the cells are assayed 48-72 hours post transfection, before the loss of DNA. Occasionally, the introduced DNA is inserted into the host genome. The DNA becomes part of the host genome and is passed to the next generations. This type of transfection is known as **stable transfection.** The cells that are stably transfected need to be selected for and separated from the transient or non-transfected cells.

In the following exercise, you will transfect your cells transiently by using two different plasmids:

1. pDsRed2-Mito vector (from Clontech Laboratories Inc. http://www.clontech.com/), carrying the sequence coding for a **fusion protein** of a red fluorescent protein and a mitochondrial targeting sequence.

2. pEGFP-Actin vector (from Clontech Laboratories Inc.), carrying the sequence for a fusion protein, consisting of green fluorescence protein (GFP) and cytoplasmic β-actin.

In the following protocol Fugene® HD transfection reagent (from Promegahttps://www.promega.com/resources/protocols/technical-manuals/101/fugene-hd-transfection-reagent-protocol/) is used to insert the plasmid DNA into the cells. You may have different plasmids and/or transfection reagent available and need to follow the manufacturer's protocol. Note that you may need to optimize the protocol for your cell line and DNA preparations.

Two days after transfection, the cells are fixed with 4% paraformaldehyde in order to keep the cellular structures intact. The cells are then observed by using a fluorescent microscope.

Day 1

plating: (This can be done by your instructor or the lab technicians).

1. Plate cells in 24-well plates, so the wells are 70-80% confluent by the day of the experiment. Use 1ml of media per well.

Day 2

Transfection:

2. Turn on the hood and wipe down with alcohol.

3. Gather the material you need and wipe them down with alcohol before placing them in the hood:

- Pipettors
- Pipette tips
- Basal media (serum-free)
- Complete media, warmed
- Fugene® HD
- Plasmid DNA (1µg/µl), mix before use
- Sterile microfuge tubes

4. Observe the cells before the start of the procedure. The cells should be healthy and actively dividing.

5. Mix the vial containing Fugene® HD by vortexing or manually inverting and bring it to room temperature.

6. Dilute your DNA in serum-free media. Prepare 4 tubes:

	1	2	3	4
	Control	**Mito**	**Actin**	**Mito/ Actin**
Serum-free media	100 µl	98 µl	98 µl	98 µl
pDsRed2-Mito (1µg/µl)	0	2 µl	0	1 µl
pEGFP-Actin (1µg/µl)	0	0	2	1 µl

7. Add 8 µls of Fugene® HD to each of the four tubes (8:2 ratio of Fugene: DNA). Mix by vortexing and incubate at room temperature for 15 minutes.

8. While waiting, change the media in your plate. Add 1mls of fresh media per well.

9. Add 25 µls of the transfection mixture to each well drop-by-drop, according to the diagram in figure 13. Note that there are

three wells for each mixture. Discard the extra mixture.

10. Mix the plate by moving it back and forth or left and right gently, without spilling the media.

11. Place the plate in the incubator for 2 days.

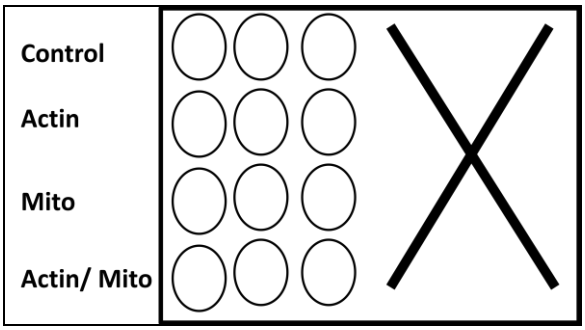


Figure 16- Diagram of the 24-well plate. Half of the plate marked by X is not used for the experiment.

Day 4

Fixing

12. 48 hours after transfection, turn on the hood, wipe down with alcohol and gather your material (wipe down with alcohol):

- Serological pipettes
- PBS (with Ca^{+2}/Mg^{+2}), warmed
- 4% paraformaldehyde in PBS, wear gloves
- Paraformaldehyde waste bottle

13. Remove the media from your cells.

14. Add 0.5 ml of PBS per well and remove PBS.

15. Add 0.5 ml of 4% paraformaldehyde per well.

16. Wait 15 minutes at room temperature.

17. Transfer the paraformaldehyde to the waste bottle (Paraformaldehyde is toxic and should **not** be discarded in the sink).

18. Wash the cells with 0.5 mls of PBS per well. Remove PBS.

19. Add another 0.5 ml of PBS per well and, this time, keep the PBS in to prevent the cells from drying out.

20. At this point you can store the plates in the refrigerator for future viewing. Fluorescent molecules are photosensitive, so the plate should be kept in the dark.

View the cells

21. Place the plates on a fluorescent microscope and observe the results. You need the green filter-cube to see the red fluorescence and the blue filter cube to see the green fluorescence.

22. Note your observations in your notebook.

Analysis questions

1. Do you see a difference in fluorescence between the control cells and the transfected

cells? Are your observations as expected? Explain.

2. What percentage of cells do you estimate is transfected with pDsRed2-Mito? Approximately what percentage of the cells is transfected with pEGFP-Actin? Which plasmid has a better transfection efficiency?

3. Do you recognize any pattern in fluorescence of the cells transfected with pDsRed2-Mito (where do you see the fluorescence)? How about pEGFP-Actin?

4. Discuss your results in your note book.

Lab Exercise 10: Freezing Cells

Learning Objectives

Become familiar with and practice

- Freezing and storage of cells
- Counting cells

When freezing, cells are resuspended in freezing media which is complete media plus 10% DMSO (Dimethyl sulfoxide). DMSO prevents crystallization and damage to the cells. To keep the cells viable, they need to be frozen slowly. The cells are transferred to cryo-vials and placed in freezing boxes. Freezing boxes are filled with isopropanol to keep the cells from freezing too fast (Figure 17). When the box is placed in the -80^{o}C freezer, the temperature goes down at the rate of 1^{o}C per minute. For long-term storage, frozen vials are transferred from the -80^{o}C freezer to a liquid nitrogen tank.

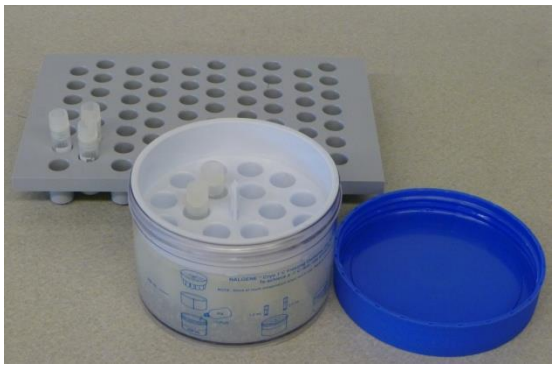


Figure 17. Cell culture freezing box and cryovials.

Many of the cells die in the process of freezing and defrosting, therefore, usually cells are frozen at very high concentrations so, when defrosted, there is a good chance that some cells will survive and make a culture. A concentration of 1 x 10^{6} cells/ml is usually used for freezing. **Wear gloves when working with DMSO.**

To freeze cells:

1. Turn on the hood and wipe down with alcohol.

2. Gather the material you need and wipe them down with alcohol before placing them in the hood:

- Serological pipettes
- Centrifuge tubes
- Tube rack
- Trypsin, warmed
- PBS (Ca^{+2}/Mg^{+2}- free), warmed
- Complete media, warmed
- DMSO, wear gloves
- Cryovials
- Hemocytometer

3. Observe your cells first. Your culture must be healthy and more than 60% confluent to be suitable for freezing.

4. In a sterile tube, prepare freezing media by mixing 4.5 mls of complete media with 0.5 ml of DMSO.

5. Remove the media from your culture.

6. Wash the cells with 3mls of PBS and remove the PBS.

7. Add 1ml trypsin to the flask. Make sure the cells are covered by trypsin.

8. Wait 2-3 minutes for the cells to detach.

9. Tap the side of the flask, and observe under the microscope to make sure all of the cells are floating.

10. Quench trypsin by adding 4mls of complete media and mix well by pipetting up and down.

11. Pipette 12µls on each side of a hemocytometer and count cells.

12. Calculate cell concentration in your culture.

13. Calculate total number of cells in your culture:
(Total cell #)= (cells/ml)(total volume, mls)

# of cells counted	
# of squares counted	
Cell concentration	
Total number of cells	
Volume of the freezing media for final concentration of 10^6 cells/ml	

14. Transfer the cells to a sterile centrifuge tube.

15. Spin the cells at about 1000-1500 rpms for 3 min.

16. Wipe the tube down with alcohol and place it back in the hood.

17. Aspirate out the supernatant. Be careful not to aspirate the cells in the pellet.

18. Add the appropriate volume of freezing media to the pellet to get concentration of 1×10^6 cells/ml.

19. Resuspend the cells in the freezing media by pipetting up and down a few times.

20. Transfer to cryotubes (up to 1ml per tube).

21. Label the tubes with your name, cell type and date.

22. Place the tubes in isopropanol freezing boxes (see the note below).

23. Put the box in the -80^{o}C freezer. (Cells may be kept in the -80^{o}C freezer for up to a few weeks, depending on the cell type.)

24. After one day you can transfer the tubes to a liquid Nitrogen tank to keep indefinitely.

Notes:

- You can use Styrofoam boxes instead of the isopropanol boxes for freezing the cells. Place the tubes in Styrofoam boxes. Make sure they are covered on all sides. Label with your name, date and cell type. After you are finished put it in the -80^{o}C freezer.

- For routine freezing you can estimate the number of cells without counting. For a 100% confluent T25 you can generally use 2-4mls of freezing media. (Note that for different cell lines this number may be different).

- Freezing media is kept in the refrigerator.

- DMSO is light sensitive. Cover the tube of freezing media with aluminum foil to keep it in the dark.

Lab Exercise 11: Stem Cell Differentiation into Cardiomyocytes

Learning Objectives

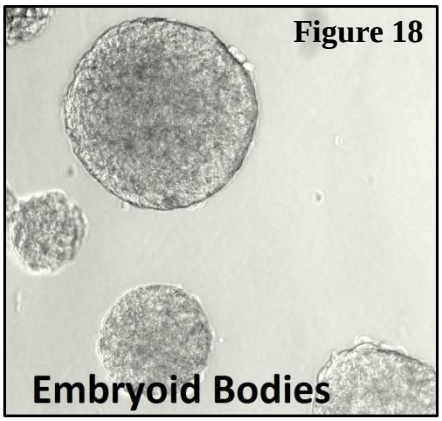
Become familiar with and practice:

- Growing embryoid bodies in culture and initiate spontaneous differentiation of mouse embryonic stem cells.
- Identify differentiated cardiomyocytes

Stem cells are cells that are able to renew themselves (**self-renewal**) indefinitely, while producing cell progeny that can mature into specialized cells (**differentiation**). **Adult stem cells** in our body are able to regenerate cells and renew damaged tissues. **Embryonic stem (ES) cells** are a type of stem cells that are formed very early during embryonic development. Embryonic stem cells are said to be **pluripotent**, meaning that under proper conditions they can generate all three cell layers (ectoderm, mesoderm and endoderm).

ES cells are maintained as a monolayer adhered to the culture vessel. The recipe for stem cell culture media contains important components to keep the cells from differentiation and to preserve the pluripotent characteristic of the stem cells (pluripotent media).

To trigger differentiation, ES cells are grown in the differentiation media that allows the cells to differentiate. The cells are plated on uncoated plates to prevent adhesion to the plates. The cells in suspension form aggregates (spheroids) called **embryoid bodies (EB);** and differentiation initiates spontaneously upon aggregation of cells (Figure 18). The cells can potentially differentiate into all cell types spontaneously. Several methods have been developed to direct differentiation into specific cell types by culturing stem cells under appropriate and controlled conditions. In the following exercise (adopted from a protocol by Dr. J. Ng) you will differentiate ES cells into beating cardiomyocytes (heart muscle cells).



The cells are grown in low attachment plates (or uncoated Petri dishes) to prevent adhesion of the cells to the plate. The cells growing in suspension form embryoid bodies (EB). After growing in suspension for a few days, the EBs are then plated on gelatin-coated plates to adhere to the plates and continue differentiation.

Since you are not in the laboratory every day, some of the steps will have to be done by the lab technicians.

Day 1

Plating cells in suspension

1. Turn on the hood and wipe it down with alcohol.

2. Gather your material and wipe down with alcohol before placing them in the hood:

- Serological pipettes
- PBS (Ca^{+2}/Mg^{+2}-free), warmed
- Trypsin, warmed (you may use Ca^{+2}/Mg^{+2}-free PBS or Versene solution instead. ES cells are attached loosely and can easily be detached without Trypsin)
- Embryonic stem cell differentiation media, warmed
- A hemocytometer
- Centrifuge tube and tube rack
- Sterile low attachment plate (or petri dish)

3. You will be given a T25 flask of pluripotent ES cells.

4. Observe the cells using a microscope.

5. Inside the hood, aspirate the medium off.

6. Wash by adding 5 ml of PBS (No Ca/ Mg).

7. Rock the flask gently and aspirate PBS.

8. Add 1 ml of trypsin (or 1 ml PBS) to cover the cells.

9. Incubate the flask in 37^{o}C incubator for 1-3 minutes or until cells are uniformly dispersed into small clumps.

10. Tap sides of flask to dislodge the cells.

11. Back in the hood, add 5 ml of ESC differentiation media to the flask.

12. Count the cells with a hemocytometer.

13. Calculate your total number of cells

Concentration (cells/ml) x Volume in the flask (ml) = Total # of cells

14. Transfer all of your cells to a sterile centrifuge tube

15. Spin the cells at 100 g (about 1000 rpm) on a clinical centrifuge.

16. Remove the supernatant. Be careful not to remove the pellet (your cells).

17. Resuspend the cells in the appropriate volume of differentiation media in order to get you want final concentration of 300,000 cells/ml concentration.

Volume of media for resuspension (ml)= Total number of cells/ 3×10^{5} cells/ml

18. Mix the cells well by pipetting up and down a few times.

19. Transfer the cells to the low attachment plate. If using a 6-well plate, add 2 mls per well. (For a 100 mm plate add 7-12 mls per plate)

20. Label and incubate the plate in the 37°C incubator.

Day 2 or 3

Feeding the embryoid bodies

21. Turn on your hood and wipe it down with alcohol.

22. Gather your material and wipe them down with alcohol before placing them in the hood:

- Serological pipettes
- Sterile conical tubes
- Tube rack
- Differentiation media, warmed
- Low attachment plate (or a petri dish)

23. Observe your cells under the microscope. By now the cells must have aggregated together and formed floating embryoid bodies (EB).

24. Using a 5 or 10-ml serological pipette, carefully transfer all the media plus embryoid bodies from your plate into a sterile tube.

25. Wash each plate/well with 2mls of differentiation media and add it to the tube.

26. Close the cap and transfer the tube to the incubator inside a clean rack.

27 Allow the embryoid bodies to settle to the bottom of the tube for 10 minutes.

28. Transfer the tubes gently back to the hood and carefully remove the media, leaving the EBs undisturbed.

28. Slowly, add 10mls (the volume may differ depending on how much cells you started with and the capacity of the dish you are using) of fresh ESC differentiation media to the tube. (Do not pipette up and down more than 2-3 times, it may break the EBs)

29. Using a serological pipette, gently transfer the EBs back to new low attachment plate/ Petri dish. If the EBs are clumped together, gently pipette up and down to separate them. Be very careful not to break the spheres.

30. Place the dishes back in the 37°C incubator.

Day 4 or 5

Repeat feeding (Steps 21-30)

31. Feed the EBs as above every 2-3 days with fresh differentiation media.

Day 7

Plating EBs on gelatin

32. Turn on the hood and wipe down with alcohol.

33. Gather your material and wipe them down with alcohol before placing them under the hood:

- Serological pipettes
- 2ml or 5 ml serological pipettes
- 24-well tissue culture plates
- Sterile 0.1% gelatin in ddH_2O
- Differentiation media, warmed

34. Add 0.5 ml of 0.1% gelatin per well of the 24-well plates and wait for 5 minutes.

35. Aspirate off the gelatin. This will gelatin coat your multi-well tissue culture plate.

36. Add 1ml of ESC differentiation media to each well of the 24-well plate.

37. Using a serological pipette, mix the EBs in the Petri dish by pipetting up and down very gently 2-3 times.

38. Gently, transfer 0.3-0.5 mls of the EBs into each well of the 24-well plate. DO NOT use a micropipettor, since the opening of the tips is narrow and will crush the EBs, unless you have wide-bore pipette tips. Note, since the EBs are heavy, they tend to settle to the tip of the pipette fast. It may be helpful to mix the EBs by pipetting gently in between dispensing to each well to get a more homogeneous mix.

38. Label your plate and place it into the 37°C incubator.

Day 9+

Observing the differentiated cells

51. Observe the cells under the microscopes. Beating cardiomyocytes and other differentiated cell types should be visible.

Analysis questions

1. What percentage of the cells are differentiated into cardiomyocytes? How efficient is the above procedure in directing cardiomyocyte differentiation?

2. Count the number of beats per minute for one or more of the cardiomyocyte cell clusters in your culture. Compare your numbers with other students in class. (Average number of beats per minute is about 72 for human heart and 500-600 for mouse heart.)

3. Can you recognize any other cell types in your plates?

4. Write your observations in your notebook and discuss your results.

Lab Exercise 12: Stem Cell Differentiation into Neurons and Immunocytochemistry

Learning Objectives

Become familiar with and practice:

- Growing embryoid bodies in culture and initiate guided differentiation of mouse embryonic stem cells into neurons.
- Identify differentiated neurons.
- The process of immunocytochemistry.
- Use of fluorescent microscopes.

To trigger differentiation, ES cells are grown in suspension and embryoid bodies are formed before adhering to gelatin coated plates as it was discussed previously (lab exercise 11). The cells can potentially differentiate into all cell types spontaneously. However, under appropriate and controlled conditions the cells may be directed to differentiate into specific cell types. In order to direct cells into neuron development, retinoic acid is added to the media. Retinoic acid is a signaling molecule that has an important role in neural development and activity at the embryonic stages (Dhara and Stice, 2008).

In the following procedure, you will differentiate mouse embryonic stem cells into neurons by following the same procedure as exercise 11 but adding retinoic acid to the differentiation media. Following differentiation, you will use **immunocytochemistry** techniques to visualize neurons using fluorophores. Immunocytochemistry is a technique that utilizes **antibodies** that target specific molecules (**antigens**) inside the cell. The antibody-antigen complexes can be detected using fluorophores and visualized under a fluorescent microscope.

Antibodies are proteins that are naturally secreted by the immune system and bind to specific molecules that are foreign to the animal's body known as antigens. The specific binding of antibodies to antigens marks the invading antigens for elimination. Many biotechnology techniques use antibodies as tools to detect the presence of specific antigens.

In the following procedure you will use an antibody that binds to a neuron-specific protein named **MAP2** (Microtubule Associated Protein 2). Map2 is essential for microtubule assembly in neurons, an important process during neurogenesis. Anti-Map2 antibody is then detected by using a fluorescent-labeled secondary antibody that recognizes the primary anti-Map2 as its antigen. The labeled secondary antibody can be visualized using a fluorescent microscope. To visualize the nuclei of the cells in culture, you will counter-stain the cells with **Hoechst** fluorescent dye which can bind to DNA and therefore label the nuclei fluorescent blue. The nuclei of all cells in culture will be stained blue, but only the cytoplasm of the differentiated neurons will fluoresce with the labeled secondary antibody.

Day 1
Plating cells in suspension

Follow the steps 1-20 from lab exercise 11.

Day 2 or 3
Feeding the embryoid bodies

Follow the steps 21-30 from lab exercise 11.

Day 4 or 5
Add retinoic acid

31. Turn on and wipe your hood down with alcohol.

32. Gather and wipe down your material with alcohol:

- Sterile conical tubes
- Tube rack
- Serological pipettes
- Differentiation media, warmed
- Retinoic acid (5mM)
- Low attachment plate (or petri dish)

33. Using a serological pipette, carefully transfer all the media plus embryoid bodies from the dish into a conical tube.

34. Wash the wells of the plate or the dish with 0.5-3 of differentiation media and add it to the tube.

35. Close the cap and place the tube in a rack inside the 37°C incubator.

36. Allow the embryoid bodies to settle to the bottom of the tubes for 10 minutes.

37. Carefully remove the media, leaving the EBs undisturbed.

38. Prepare 10 ml of the differentiation media (the volume of media may differ depending on how much cells you started with and the type of plate you are using). Add retinoic acid to the media to the final concentration of 5 μM.

39. Add the differentiation media plus retinoic acid mixture to the tube to direct the cells into neuron differentiation.

40. Gently transfer the EBs plus the differentiation media and retinoic acid to a new low attachment/ petri dish.

41. Label the dish and incubate it back in the 37°C incubator. Feed the cells every 2-3 days with fresh media with 5 μM RA (follow steps 31-40)

Day 7
Plating EBs on gelatin

42. Turn on the hood and wipe down with alcohol.

43. Gather your material and wipe them down with alcohol before placing them under the hood:

- Serological pipettes
- 2ml or 5 ml serological pipettes
- 24-well tissue culture plates
- Sterile 0.1% gelatin in ddH_2O
- Differentiation media, warmed
- 5mM retinoic acid (RA)

44. Add 0.5 ml of 0.1% gelatin per well of the 24-well plates and wait for 5 minutes. Aspirate off the gelatin. This will gelatin coat your multi-well tissue culture plate.

45. Prepare 25 mls of fresh ESC differentiation media with 5µM Retinoic acid for neuron differentiation.

ESC Diff. media	25 mls
5 mM RA	

46. Add 1ml of media with RA to each well of the plate. Label your plate.

47. Using a serological pipette, mix the EBs in the Petri dish by pipetting up and down very gently 2-3 times.

49. Gently, transfer 0.3-0.5 mls of the EBs into each well of the 24-well plate. DO NOT use a micropipettor, since the opening of the tips is narrow and will crush the EBs, unless you have wide-bore pipette tips. Note, since the EBs are heavy, they tend to settle to the tip of the pipette fast. It may be helpful to mix the EBs by pipetting gently in between dispensing to each well to get a more homogeneous mix.

50. Label the plates and place them into the 37°C incubator.

Day 9+

Observing the differentiated cells

51. Neural cells with long axonal projections should be visible under the microscope.

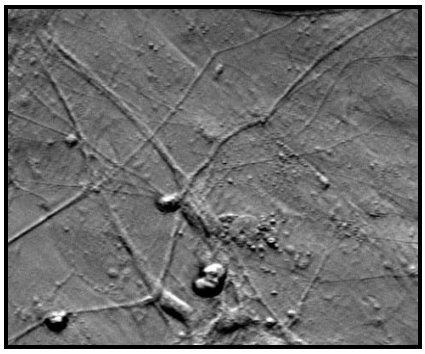


Figure 19. Differentiated neurons in culture.

Immunocytochemistry (ICC)

52. After observing the cells choose 3 wells to perform the ICC on.

52. Gather your material and wipe them down with alcohol before placing them under the hood:

- 4% Paraformaldehyde
- PBS (with Ca^{2+} & Mg^{2+})
- PBS with 0.1% Triton X-100
- 10 mM Glycine in PBS, pH 7.2
- Blocking solution (PBS with 0.1% Triton, 2% Fetal calf serum, 3% BSA)
- Rabbit anti-Map2 (diluted to the appropriate concentration in the blocking solution)
- Serological pipettes
- Paraformaldehyde waste collecting tube

53. Remove the media from the wells. Do not scratch the bottom surface of the wells with the pipette tips.

54. Wash the wells with about 0.5 mls of PBS (with Ca^{2+} & Mg^{2+}) per well. Remove PBS.

55. Add 0.3 mls of 4% Paraformaldehyde to each well. **Wear Gloves.** Paraformaldehyde is a fixative that will kill the cells but maintains the structure of the cells intact.

56**.** Wait for 10 minutes at room Temperature.

57. Transfer paraformaldehyde to a waste tube with a pipette. Paraformaldehyde is toxic and should not be discarded in the sink.

58. Wash the wells with 0.5 mls of PBS (with Ca^{2+} & Mg^{2+}) per well. Remove PBS.

59. Add 0.3 mls of PBS/0.1% Triton X-100 per well. Wait for 20 minutes at room Temperature. This will permeablize the cells further allowing the antibodies to enter the cells.

60. Remove PBS/0.1% Triton X-100.

61. Add 0.3 mls of 10 mM glycine in PBS per well. Wait for 20 minutes at room temperature.

62. Remove glycine/PBS solution.

63. Add 0.3 mls of blocking solution. Wait for 1-3 hours at room temperature. (At this step you may keep the cells in the blocking solution over night in the refrigerator.)

Blocking solution will block non-specific binding of the antibody to cellular proteins.

64. Remove the blocking solution.

65. Add 0.2 mls of the primary antibody solution per well to two of the wells. Add 0.2 mls of blocking buffer to the third well to be used as a negative control.

66. Keep the plates in the 4 ^{o}C refrigerator. You may keep the cells in the primary antibody in the refrigerator over-night or longer up to one week.

Day 10+

Secondary antibody detection

67. Gather your material. At this point the cells are dead and so you may work outside of the hood with non-sterile but clean material.

- PBS (with Ca^{2+} & Mg^{2+})
- Fluorescent labeled secondary antibody (diluted to the appropriate concentration in the blocking solution).
- Hoechst stain (Diluted to the appropriate concentration in PBS)
- Pipettes

68. Remove the primary antibody.

69. Wash the wells with 1 ml of PBS per well 2 times. Remove PBS.

70. Add 0.2 mls of the secondary antibody per well (all three wells including the negative control).

71. Cover the plates with aluminum foil to prevent photobleaching of the fluorophore by exposure to the light. Keep at room temperature for 1-3 hours. (At this time you may keep the cells in the secondary antibody in the refrigerator for up to 1 week.)

72. Remove the secondary antibody. Wash the wells two times with 1 ml of PBS per well. Remove PBS.

73. Add 0.3 mls of Hoechst stain per well. Keep the plate covered. Wait for 3 minutes.

74. Remove the Hoechst and Repeat step 73.

75. Wash each well with 1 ml PBS two times. Keep the plate covered. Remove the PBS.

76. Add 0.3 mls of PBS per well. Keep the cells in PBS.

77. Observe the cells under the fluorescent microscope and take notes.

Analysis Questions

1. What percentage of the cells is differentiated into neurons? How efficient is the above procedure in directing neuron differentiation?

2. A. What percentage of the cells is blue?
B. What does the blue color represent?
C. Where in the cell is the blue color localized?

3. What is the difference between the negative control and the other wells? Are the results as expected?

4. Did you see any cell types other than neurons? Explain your observation.

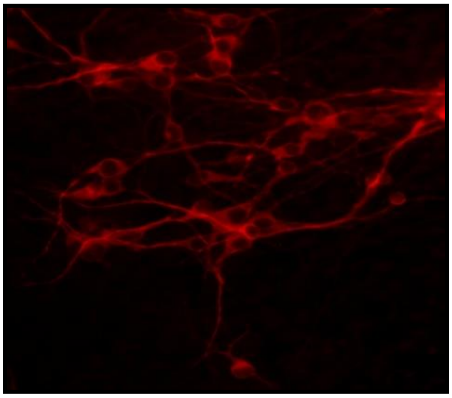


Figure 19. Localization of MAP2 (red) in neurons.

Appendix A

Serial Dilution

Serial dilutions are commonly used in microbiology, chemistry and biotechnology. Serial dilutions are performed to prepare solutions that are less concentrated than the original solution. Serial dilutions are usually made in increments of 1000, 100 or 10. A small amount of the original solution is removed and added to the appropriate amount of buffer.

For example, if you remove 10 μl of your original solution and add it to a tube containing 990 μl of water, you have made a 1:100 dilution. Meaning that your new solution is 100 times less concentrated than the original solution. If you remove 1μl of your diluted solution and add it to a third tube containing 999 μl of water, then you have made another 1: 1000 dilution. So the concentration of the third tube is 1000 times less than the second tube and 100,000 (10^5) times less than the original tube.

In cell culture techniques, if we are going to measure a volume of liquid containing cells, it is more accurate to avoid measuring amounts less than 50-100 μl (there may be times that we do not have enough cell solution or not enough buffer solution for multiple dilution steps, and so we would have to measure less than 50 μl).

For example, if we have a cell-culture solution containing 5 x 10^8 cells/ml and need to prepare a solution of 5 x 10^3 cells/ml (Fig. 20), we need to first add 100 μl of the original culture to a tube containing 900 μl of buffer (1:10 dilution). In the second step, add 100 μl of the diluted sample to 9.9 ml (9900 μl) of buffer (1:100 dilution). In the third step, take out 100 μl of the later tube and add it to a new tube with 9.9 ml of buffer (1:100 dilution). Our total dilution factor is 10 x 100 x 100 = 10^5. So the final solution is 10^5 times less concentrated than the original solution and it contains 5 x10^3 cells/ml.

Note, if you were to dilute the original solution 10^5 times in only one step, we would need to add 100 μl of solution to a tube containing 9, 999, 900 μl (9, 999.9 ml) of buffer. This would require a very big container that could hold large volumes of liquid. It would also be impractical since a lot of buffer would be wasted. Therefore, serial dilution is the best method used for large dilutions.

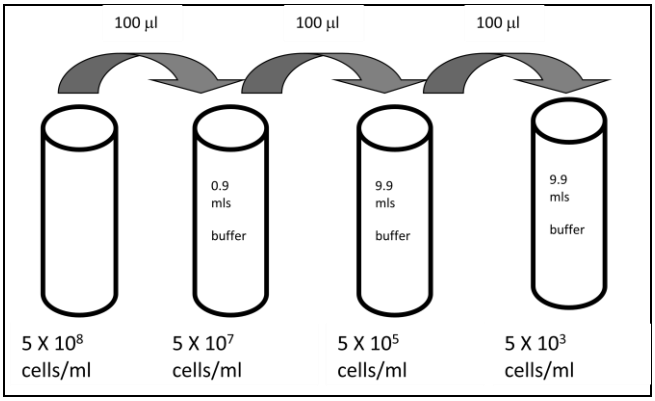


Figure 20- 10^5 times dilution of a 5 x 10^8 cells/ml suspension completed in three steps.

Appendix B

Practice problems

1. A student has a cell suspension of 10^5 cells/ml. How is he going to prepare 10 ml of a 10^2 cells/ml suspension?

2. A student has a cell suspension of 10^5 cells/ml. She needs to plate 5 ml of 5000 cells/ml culture into a T25 flask. How is she going to plate her cells?

3. A student has a cell suspension of 10^5 cells/ml. She needs to plate 5 ml of 100 cells/ml culture into a T25 flask. How is she going to plate her cells?

4. A student has trypsinized his cells with 1 ml of trypsin. He then adds 4 ml of media to quench trypsin. Using a hemocytometer he counts 150 cells in five squares. What is the concentration of his cells? How many total cells does he have in his culture?

5. A student is interested in counting both live and dead cells in a culture. After collecting the floating cells and pooling with the trypsinized cells from his culture, he takes out 50 μl and mixes it with 50 μl of Trypan blue. He then uses a hemocytometer for counting. He counts 60 dead cells and 300 live cells in five squares. What is the concentration of live and dead cells?

6. A student counts more than 600 cells in five squares of a hemocytometer. To get a more accurate count, he dilutes his cells by taking out 100 μl of cells and mixing them with 900 μl of PBS. He then takes out 50 μl of the diluted sample and mixes it with 50 μl of Trypan blue. Using a hemocytometer he counts 60 cells in ten squares. What is the concentration of cells in his original culture?

7. A student is asked to plate 1000 cells per well of a 24-well plate with 1ml of media in each well. He first trypsinizes his cells in a T25 flask and quenches the trypsin with media. He then counts the cells using a hemocytometer. He counts 100 cells in five squares. How is he going to plate the cells?

8. A student is asked to plate 100 cells per well of a 6-well plate using 2 ml of media per well. After trypsinizing she counts 200 cells in 5 squares of a hemocytometer. How is she going to plate the cells?

9. A student is asked to plate cells in a T25 (25 cm^2) at the density of 1000 cells/cm^2. She first counts the cells using a hemocytometer. She counts 240 cells in five squares. How is she going to plate the cells?

10. A student is asked to seed a 12-well plate with 2000 cells/cm^2 using 1 ml of media per well. Each well of a 12-well plate is 3 cm^2. He counts 30 cells in five squares of a hemocytometer. How is he going to plate the cells?

11. A student is asked to freeze his cells from a T25 flask at the concentration of 10^6 cells/ml. After trypsinizing his cells with 1ml trypsin, he adds 4 ml of media to quench the trypsin. Using a hemocytometer, he counts 260 cells in 5 squares. How much freezing media does he need to freeze his cells in and how is he going to freeze his cells?

Glossary:

Adult stem cells- Stem cells that are found in adult tissue in the body and are able to regenerate specific types of cells (multipotent).

Anchorage-dependent cells- Cells that need to attach to a solid support to survive and divide.

Anchorage-independent cells- Cells that can survive without attachment to a solid support and grow in suspension.

Antibodies- Molecules that are secreted by the immune system and bind to specific antigens to mark them for elimination.

Antigen- A molecule that is recognized as non-self by the immune system.

Aseptic techniques- Series of techniques and practices used to reduce the chances of contamination of the cultures by microorganisms and to protect laboratory workers from contamination by cell cultures and other potentially hazardous material.

Basal media- Media containing basic nutrients, vitamins and minerals necessary for cells to grow in culture. Basal media needs to be supplemented with additional growth hormones, growth factors and proteins for efficient cell growth.

Bicarbonate buffer- A buffer used in cell culture media that interacts with CO_2 to keep the pH balanced at about 7.4.

Chelator- Molecules that bind to certain metal ions and inactivate the ions so that they cannot react with other elements.

Colony- A population of cells that are all descendents of a single parental cell. Cells in a colony are located close together.

C**omplete-media-** Basal media supplemented with additional proteins, growth factors and components necessary for efficient cell growth.

Confluency- The percentage of the culture vessel's surface covered by cells.

Cytotoxic studies- Studies that involve measurement of altered metabolism or loss of viability of cells due to a toxic factor.

Defined media- Basal media plus additional growth factors, proteins and other components that are added without the use of serum. Since the exact components of the factors added are known, this type of media is can be defined.

Density-dependent growth inhibition- When the cells stop dividing due to insufficient space, nutrients or oxygen available to them.

Differentiation- A process when cells mature and become specialized.

Embryoid bodies (EB)- Spheres made of aggregates of embryonic stem cells.

Embryonic stem (ES) cells- A type of stem cell formed very early during development. ES cells are pluripotent.

Extracellular matrix- Collection of molecules outside of cells that provide structural support for the cells in a tissue in addition to having other important functions.

Feeding- When the old media is removed from the culture and replaced with fresh media.

Filter cube- An optical block with a set of filters that allow selection of certain wavelengths, used in fluorescence microscopes.

Fluorophores- Molecules that can absorb energy of light at specific wavelengths and emit less energetic fluorescent light.

Fusion protein- A protein made by fusion of two or more different proteins.

Hoecsht- A fluorescent molecule that binds to DNA and emits blue light.

IC_{50}- The dosage of a toxic reagent at which there is 50% inhibition of colony formation.

Immunocytochemistry- A technique in which specific proteins are targeted and visualized by labeled antibodies.

Infection- Transfer of viral genetic material into eukaryotic cells.

Isotonic solutions- Solutions with the same concentration of solutes as the inside of the cells.

Hayflick's limit- A phenomenon of normal cells in culture when they divide for a limited number of times before they stop dividing.

Liquid nitrogen tanks- Tanks filled with liquid nitrogen, used for long-term storage of cells at a very cold temperature.

MAP2 (Microtubule Associated Protein 2)- A protein involved in microtubule assembly and cytoskeleton stability of neurons.

Microbial contaminants- Microorganisms that may grow in cell media and contaminate the culture.

Micropipettes- Pipettes that are used to transfer small volumes of liquid between 1-1000 μl.

Monolayer- Refers to cells growing in a single layer, attached to the bottom of the culture vessel.

Mycoplasma- Very small bacteria-like microorganisms that can grow rapidly in cell cultures without being visible.

Passaging- Splitting the cells in a culture into subcultures.

Pellet- The components of a mixture that are precipitated to the bottom after centrifugation.

Phenol red- A component of the cell culture media that is a pH marker and will change color with the changes in the pH.
Photobleaching- the loss of fluorescent properties of a fluorophore due to

continuous exposure to light at any wavelength.

Plasmids- Small, circular DNA molecules that naturally exist in bacteria and some eukaryotic cells. Plasmids are used as tools in biotechnology to carry foreign DNA sequences into cells.

Plating efficiency (or cloning efficiency)- The percentage of cells that survive and are able to divide and form colonies after being plated.

Pluripotent- A characteristic of embryonic stem cells, which is their ability to generate all cell types.

Propodium Iodide- A fluorescent molecule that intercalates between nucleic acids (both RNA and DNA) absorbing light most efficiently at 500-550nm and emitting bright red fluorescent light at 600-650nm wavelengths.

Pyrimidines- Cytosine (C), Thymine (T), and Uracil (U) nucleic acids.

Pyrimidine dimers- Molecular lesions formed from adjacent thymine or cytosine bases in DNA by formation of covalent bonds caused by exposure to UV radiation.

Recombinant DNA- A DNA molecule that is made artificially by combining two or more different DNA sequences together.

Self-renewal- A characteristic of stem cells, which is their ability to divide and make more stem cells.

Serological pipettes- Pipettes that are used to measure and transfer larger volumes of liquid. Serological pipettes need the help of the "pipette aid" pumps to draw and release liquid.

Serum- Blood without the cellular components.

Stable transfection- Transfection when the foreign DNA becomes part of the host genome and is passed to the next generations.

Supernatant- The liquid component of a mixture that stays on top of the pellet after centrifugation.

Transfection- A method used to transfer non-viral recombinant DNA into mammalian cells.

Transformed cells- Mammalian cells that have been immortalized.

Transient transfection- Transfection of cells when the foreign DNA molecule stays independent of the host genome and is eventually lost during subsequent cell divisions.

Trypan blue- A blue dye that is impermeable to live cells but can go through the porous membranes of dead cells and turn them blue.

Trypsin- A commonly used dissociating enzyme which digests the attachment proteins on the surface of the cells. Used to

detach the cells from the surface of the culture vessel.

Turret – Part of a microscope that holds the objective lenses and can be rotated to put an objective lens in the light path.

Undefined-media- Basal media plus serum. Since the exact components of the serum are unknown, this type of media is called undefined.

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