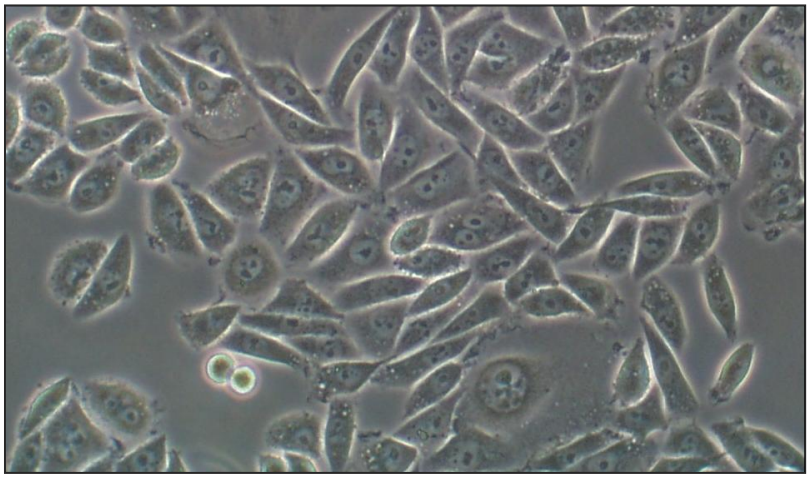
Basics of Cell Culture

An instructor manual

By Golnar Afshar PhD



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Basics of Cell Culture

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

The class meets once a week for nine weeks. Each class meeting is four hours long. Each class period starts with a brief lecture followed by a laboratory exercise.
The lab exercises are designed to introduce the students to basic cell culture techniques and attract their interest to the field.

Because so many high school students have a full schedule of classes and activities, the class assignments are kept to a minimum.

Laboratory safety:

Safety policies listed in the student manual are essential for this class and must be followed for the students' protection. The students must wear a laboratory coat at all times when they are in class. Although the materials used for this class are low risk and injuries are rare, a laboratory classroom must be equipped with basic emergency equipment such as eye wash station, emergency shower, fire extinguisher, and first-aid kit. When designating a classroom for this class you must consider the following:

- Classroom size- The room must be large enough to accommodate the required equipment and to allow students to move around freely. Space must be designated for students to keep their backpacks and personal belongings away from sterile activities. The lecture part of the class may be held in a separate room. Many states have specific rules regarding laboratory classroom size that must be followed.
- An emergency evacuation route must be planned and clearly labeled.
- Safety equipment must be easily accessible, properly maintained and clearly labeled.
- A laboratory classroom must be accessible to students with disabilities.

For detailed guidelines and links for laboratory safety refer to:
http://mdk12.org/instruction/curriculum/science/safety/equipment.html

Cell culture laboratory design and equipment

Since contamination of cultures is normally a major problem, the cell culture classroom should be in the area with a lower amount of traffic and use. The most preferable situation is to designate a room only for cell culture activities and keep the room clean and dust free as much as possible. Windows must be kept closed to keep dust from entering the room. No yeast or bacteria culture activities should be allowed in the same room as the cell cultures. The more sterile activities should be kept away from the main entrance, towards the back of the room where there is less traffic.

The following are the equipment required for a cell culture class. Refer to Appendix A for the list of equipment and supplies and suggested vendors.

Laminar flow hood

Most cell culture procedures are performed inside laminar-flow hoods (Fig.1). 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 being blown into the cabinet. Some 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. Free-standing hoods can be placed close to the back of the room with less traffic. Allow at least 2 feet of space in between the hoods for access to the back and prevent air flow interference (Freshney, 2005). The number of hoods used depends on the space available and the number of students. Two students can work as a group in one station. Some of the bigger hoods have two stations. Alternatively, the students may take turns using the space in the hood.

An aspirator pump is connected to the hood for removing and pumping liquids directly into the disinfectant. A container, filled to approximately 1/5th capacity with a disinfectant such as hypochlorite, is used as a trap. (Figure 1B) The liquid goes through the tubing into the container, not into the pump. In addition, a second trap must be used, between the first container and the pump to prevent overflow of liquid from reaching the pump. The traps and lines must be composed of materials that the disinfectant will not corrode. Always spray some alcohol into the aspirator line at the end of the day to clean the residual liquid left inside the lines. When 2/3^{rds} full, the trap must be disinfected and emptied before being placed back in the hood.

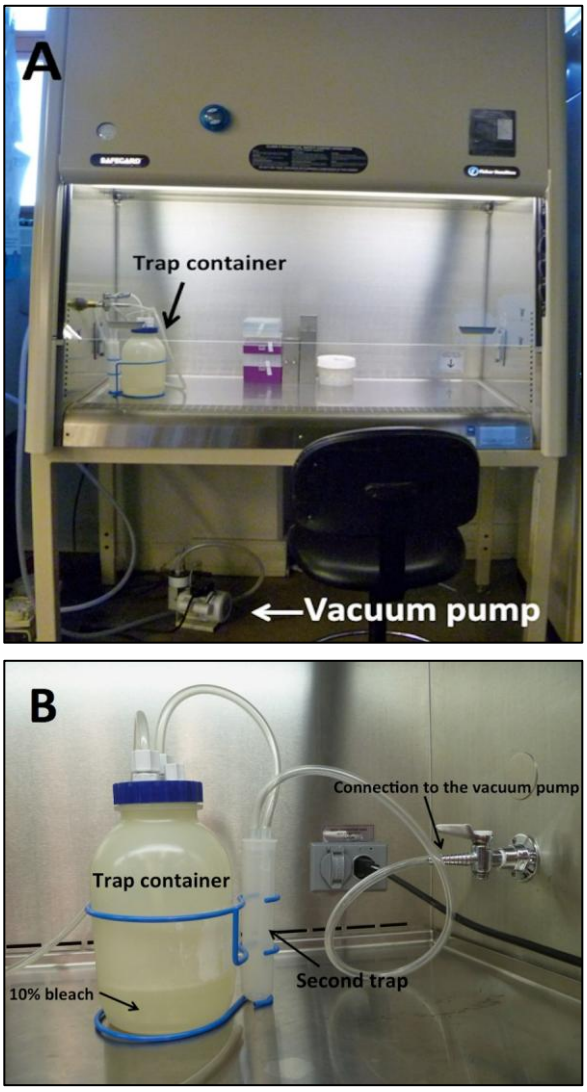

Figure 1- A laminar flow hood **(A)** with a trap **(B)** connected to the vacuum pump.

Some hoods can also be connected to a gas line for the use of flames. Flames are rarely used in cell culture hoods because they, in fact interfere with the air flow. Flames can be used to clean the air only in the event a laminar flow hood is not available. But, use of flames is still generally not recommended. If gas connections to the laminar flow hoods exist, make sure they are turned off and advise the students not to touch them. Finally, the hoods must be cleaned regularly and serviced to ensure they work properly.

Inverted microscopes

Inverted microscopes are used to observe the cells in culture (Fig. 2).

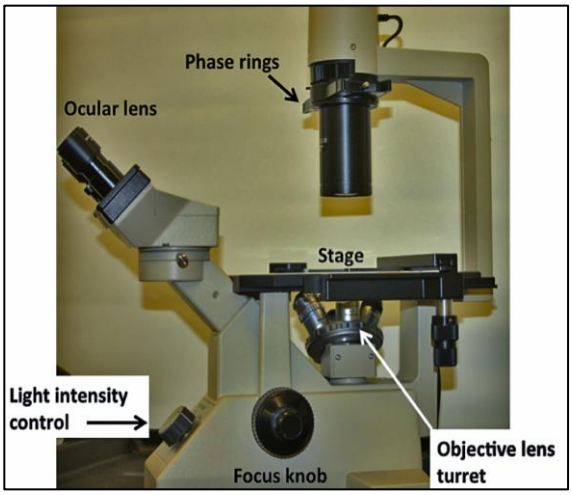

Figure 2- An inverted microscope.

Most inverted microscopes used for cell culture are equipped with 4X, 10X, 20X and sometimes larger, magnification objective lenses. At least one inverted microscope is essential for the class. Multiple microscopes are preferred, depending on the number of students. Some microscopes are equipped with a photo tube and can be connected to a camera for taking pictures of the experiments.

Fluorescent inverted microscopes

Fluorescent microscopes are inverted microscopes that are used to observe cells and molecules that have been labeled with fluorophores. 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 a filter cube. Fluorescent microscopes usually have several filter cubes with different sets of filters appropriate for observing different fluorophores. Fluorescent microscopes are connected to a high-intensity light source (usually a xenon-arc or a mercury-vapor lamp). Refer to the Manufacturer's manual for proper care and use of the fluorescent microscope.

The flurophores used for the experiments for this class are: Propidium Iodide, Ex 540/ Em 615 nm, Calcein, Ex 495/ Em 520 nm, Green Fluorescent Protein, Ex 395/ Ex 475 nm and *Discosoma sp.* Red Fluorescent Protein, Ex 558/ Em 583. The filter cubes used in the fluorescent microscope must be appropriate for observing the above fluorophores. Alternative fluorophores may be used that are appropriate, for the available filter cubes.

Clinical centrifuge

Clinical centrifuges (Fig. 3) are used to concentrate the cells and to separate the cells from the media or other reagents. A slow-speed, clinical centrifuge must be used in order to prevent damage to the cells. For routine spinning of the cells, speeds of 80-

100g (gravitational force) are sufficient. Higher speeds may damage the cells. At least one clinical centrifuge is required for the class. Important: centrifuge tubes must be properly balanced to prevent damage to the centrifuge. A balance should be located next to the centrifuge for balancing the tubes. Refer to the manufacturer manual for proper care and use of the centrifuge.

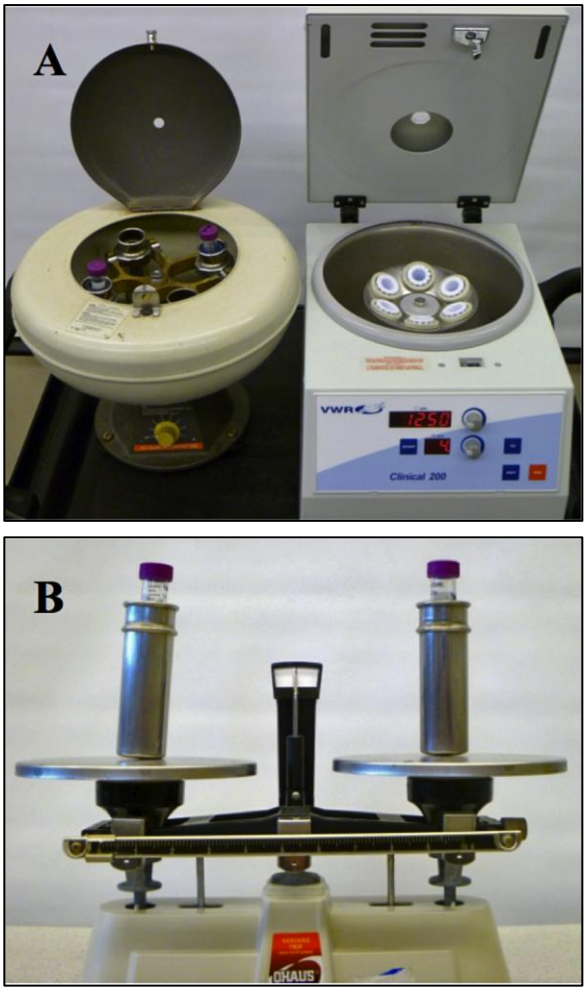

Figure 3- (A) Two different models of clinical centrifuges. **(B)** A balancer used to balance the tubes before centrifugation.

Incubator

The incubators (Fig. 4) provide appropriate environment for the cells to grow. One small or medium sized cell culture incubator is sufficient for one class. The incubator must have temperature control. For optimum growth of mammalian cells, the incubator should be set at 37°C. Some incubators have a water jacket that circulates the heat. The water must be drained regularly and replaced with clean distilled water. However, the water also makes the incubator very heavy and difficult to move. Therefore, an incubator with good insulation is preferred to eliminate the need of the water jacket.

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 to stabilize the cell-culture medium at about a pH of 7.4. Uncorrected changes in the medium pH can damage of kill the cells. Important: check the incubator's tank gauges periodically to ensure that the CO_2 levels are properly maintained. Keep the incubator closed at all times and avoid frequent opening of the door to prevent loss of the heat and the CO_2 gas.

Although cells are kept in liquid media, the smaller dishes that hold less liquid require a humid environment to prevent the media from drying out due to evaporation. 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 water regularly to prevent growth and spread of contamination.

Figure 4- 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. Incubators must be cleaned frequently to prevent growth and spread of contamination.

37^0C water bath

A 37°C water bath is required for a cell-culture facility to warm up the media and other reagents used for cells. 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.

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°C freezer for long-term storage. Frost-free freezers are preferred to prevent repeated defrosting of the reagents. Cell-culture facilities often have a -80°C 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°C. Cells can be kept frozen in liquid nitrogen for many years. If liquid nitrogen tanks are used, the tank must be filled on a regular schedule.

If long-term storage is not possible, cells can be ordered fresh for every semester before the start of the class and continuously kept in culture for the duration of the session.

Water purification system

In addition to rinsing the glassware, purified water is used for filling the water bath and the water container in the incubator. If used to make solutions and reagents for the cell culture, ultra pure water should be used. Most solutions and media used in cell culture can be purchased already prepared to eliminate the need for an ultra purification system.

Sterilizing oven or autoclave

All of the glassware, glass pipettes, pipette tips and tubes used for cell culture must be sterilized. Most of the materials can be purchased already sterilized and disposed of after use. A cheaper alternative is to purchase the material in bulk, non-sterile, and sterilize them on site, as needed. The

tubes and glassware may be washed, rinsed with pure water and sterilized for re-use. A small- or medium-sized oven or autoclave is sufficient to sterilize the material needed for the class. A sterilizing oven or an autoclave must be operated and maintained following the manufacturer's instructions. A less expensive alternative is using a kitchen pressure cooker for sterilizing smaller items.

Biohazard waste containers and disposal

Potentially hazardous material must be placed in biohazard waste containers and disposed of properly. The handling of biohazard material must follow federal, local and institutional regulations. Complying with the rules is very important to prevent the spread of potentially hazardous material to the environment.

Liquid waste from the cell-culture media can be aspirated directly into the disinfectant inside the vacuum trap container. The liquid must be in the disinfectant for at least 20 minutes before being disposed. Pipette tips, disposable glass pipettes, and any other sharps must be disposed of in cardboard biohazard pouches or plastic biohazard containers, not in trash cans. If glass serological pipettes are used, they need to be kept in cylinders filled with disinfectant, before getting washed and sterilized for re-use. Used, disposable serological pipettes must also be kept in boxes or cylinders, and sterilized before disposal.

Pipettes and pipette aids

Individually-wrapped, sterile serological pipettes of different sizes can be purchased. Alternatively, glass pipettes can be washed, plugged with cotton, sterilized and reused. Serological pipettes require the use of electrical or manual pumps to draw and release liquid (Fig. 5).

Figure 5- Manual (two on the left) and electrical (one on the right) pipette pumps.

Micropipettes are used to transfer small volumes of liquid between 1-1000 µls. A set of micropipettes should be kept in clean boxes exclusively for the cell culture procedures. Micropipettes need to be calibrated routinely to ensure precise measurements. Micropipette tips are kept in color-coded, sterile boxes and must be sterilized before use.

Important: students should practice using both serological and micropipettes before using them for actual procedures.

Hemocytometers and Coulter counter

A hemocytometer is a device invented by Louis-Charles Malassez (1971) to count cells (Fig. 6). Multiple hemocytometers are required for the class. Hemocytometers are fragile and break easily. Remind students to handle them with care. If the hemocytometer cover-slips are lost or broken, regular

microscope cover-slips, large enough to cover the chambers, may be used.

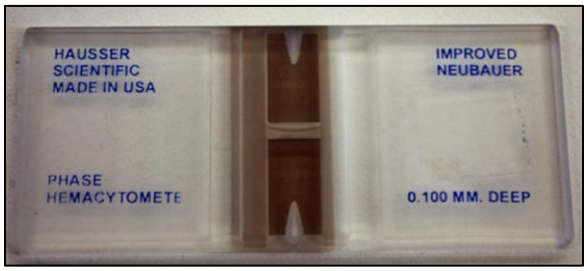

Figure 6- A hemocytometer.

An alternative way to count cells is using a Coulter counter. One Coulter counter is sufficient for a class. Refer to the manufacturer's instructions for care and use of the coulter counter.

Other required items

Glass Pasteur pipettes

Disposable glass Pasteur pipettes are used for aspirating liquids. Pasteur pipettes need to be sterilized before use. They may be kept in metal cans to aid in autoclaving.

Cell culture vessels

The cells used for this class are anchorage dependent cells; therefore, flat-bottom and coated, cell culture vessels are needed for their growth. 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 polylysine, laminin, gelatin, fibronectin, etc. to mimic the natural extracellular matrix and allow cell attachment. Three types of culture vessels are commonly used for anchorage-dependent cells: flasks, dishes, and multi-well plates. All three types are available in different sizes with different surface areas. The choice of the vessel depends on the nature of the procedures and personal preference. For the procedures in this class, T25 flasks, 6-well and 24-well plates are used. The instructor may use the larger flasks (T75 or T175) to grow cells as a backup culture for the class. Flasks with vented caps are preferred, since non-vented caps need to be loose when the flasks are in the incubator to allow for exchange of gases and students have a hard time mastering this technique. For the growth of the embryoid bodies in the stem cell experiment, low-adhesion bacterial Petri dishes (10-cm) are used.

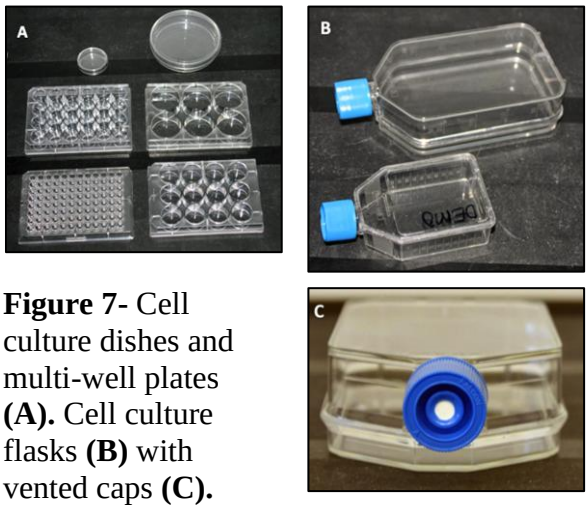

Figure 7- Cell culture dishes and multi-well plates **(A).** Cell culture flasks **(B)** with vented caps **(C).**

Sterile tubes

Sterile, capped tubes or bottles are needed for keeping the media and other reagents. The tubes may either be washed and

sterilized before use or sterile packs of tubes can be purchased. The tubes that are used for centrifugation must be the appropriate type for the centrifuge. Small microfuge tubes are appropriate for storage of some reagents and may be used for dilutions. Store microfuge tubes inside covered, plastic boxes or in aluminum foil-covered cans or glass beakers. Sterilize tubes before use.

Disinfectants

All material and equipment used in the laminar hood must be wiped clean with 70% alcohol. A spray bottle filled with 70% ethanol needs to be at each working station.

Before discarding, the cells and biological contaminated waste needs to be disinfected with chlorine-based disinfectants. One or more squirt bottles filled with 10% chlorine bleach must be present in the laboratory for disinfection. Also, the vacuum traps are to be cleaned with the disinfectant, before discarding the liquid.

Wipes

Wipes are used to clean the surfaces of the hood and the material that have been sprayed with alcohol. Lint-free paper towels or tissues (such as Kim wipes) are preferred.

Sterilization filters

Sterilization filters are used to sterilize media and other reagents that cannot be heat sterilized. Bottle-top or flask filters are available for purchase. Low protein binding filters of 0.2 μm size are suitable.

Contamination

Cell culture contamination is a very common problem at all cell culture facilities. Although the elimination of the problem of contamination is not completely possible, it can be managed and reduced. Careful handling of the cultures, proper reagent preparation, and organized laboratory procedures can reduce the contamination problem significantly. 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 often will 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. Looking at the cells under the microscope can also help in detecting bacterial contamination. Bacteria cells are much smaller than mammalian cells and look like tiny dots covering the surface of the cells and the empty spaces of the flask/plate in between the cells. Use of antibiotics in the media can prevent the 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 a microscope (Figure 8).

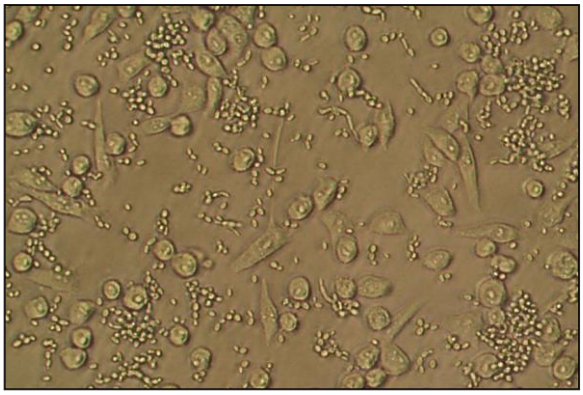

Figure 8- Mammalian (CHOK1) cells contaminated with yeast.

Other types of microbial contaminations, such as viruses and mycoplasma are invisible even with microscopes and are, therefore, more difficult to detect. Mycoplasma are very small microorganisms that can grow rapidly in cell cultures. Their presence affects cellular growth and metabolism. Viruses often infect cells and destroy them. Most laboratories perform routine molecular tests to detect the presence of mycoplasma and some of the common viruses. You can purchase PCR or ELISA based kits to test for the presence of mycoplasma. Cultures that are found contaminated need to be destroyed immediately. To lower the probability of getting contamination:

1. Emphasize the importance of using good aseptic techniques to the students.

2. Disinfect and discard contaminated cultures immediately.

3. If contamination is wide spread among all of the students, discard the stock media, PBS and other solutions that all students are sharing and prepare new solutions.

4. You may occasionally add antibiotics to the media to lower the chances of contamination growth. However, routine use of antibiotics is not recommended.

5. Aliquot all media and reagents into smaller volumes, so if one gets contaminated, the others stay clean. Each student group should get their own aliquot. Do not allow the groups to share media and other solutions.

6. Use sterile containers for storing the media and other reagents.

7. Maintain the sterilizing equipment properly.

8. Store the supplies, such as pipettes, tip boxes, tubes, flasks etc. in closed containers or cabinets, if possible, to prevent dust from settling on them.

9. Have a routine cleaning schedule for the lab:

    - Clean the water bath and replace the water with fresh distilled water once a week.

    - Remove the incubators' shelves and other removable parts for cleaning, wrapping and sterilizing once every semester or sooner, if contamination is detected to contain the spread. Wipe the incubator with alcohol before putting back the shelves and as needed in case of a spill.

    - Wipe the hoods and the hood filters with alcohol routinely. Maintain the hood to make sure it works properly. If your hood is equipped with a germicidal lamp, turn it on and close the glass shield at the end of each day. Make sure the UV lamp is turned off before opening the shield.

    - Keep the laboratory classroom clean and dust free as much as possible.

Cell Lines, Media and Reagents

Refer to Appendix A for the list of reagents and suggested vendors.

Cell lines

Any non-human transformed mammalian cell line that grows well in culture works for the purpose of this class. Cells may be purchased from American Tissue Type Collection (http://www.atcc.org/). Choose a cell line that is relatively easy to grow. Examples are CHO (Chinese hamster ovarian cells) and COS7 (African green monkey kidney fibroblasts).

For the stem-cell differentiation experiment, use mouse embryonic stem cells (mESC). Feeder-independent mESCs are preferred but some cell lines may require feeder cells for their growth. Feeder cells secrete growth factors and hormones essential to mESCs. Mitotically inactivated mouse embryonic fibroblast (MEFs) cells are commonly used as feeders. Important: inhibit mitosis in feeder cells to prevent their proliferation in culture. One way to inactivate feeder cells is to treat the cells with Mitomycin C. Mytomycin C inhibits DNA synthesis and stops the cell cycle. Warning: mytomycin C is poisonous. Read the Material Safety Data Sheet (MSDS) and wear gloves when handling it.

To inactivate MEFs:
(Adopted from Thermo Fisher Scientific Inc., protocol SC 00003, 2009)

1. Plate MEFs in a T175 flask until 80-90% confluent.
2. Remove the media.
3. Replace the media with fresh media plus 10 μg/ml Mitomycin C. While wearing gloves, wash the pipette tip with 10% bleach before discarding it.
4. Incubate the cells in the incubator for 2-2.5 hours.
5. Pipette off the Mitomycin C media into a waste bottle. Add 10% bleach to the waste media for 10 minutes before discarding. Also, wash the pipette with 10% bleach before discarding.
6. Wash the cells with 20 mls of PBS (with Ca^{2+}, Mg^{2+}) five times. Wait for 1 minute for each wash before removing it.
7. Wash one more time with Ca^{2+}-, Mg^{2+}-free DPBS. Remove DPBS quickly before the cells are detached. (Lack of Ca^{2+} and Mg^{2+} loosens the attachments of the cells).
8. Add 7 mls of trypsin to the flask to cover the cells.
9. Wait 2-3 minutes for the cells to detach. Tap the side of the flask to detach the cells.
10. Add 18 mls of media to the flask to inactivate trypsin.

11. Transfer the cells to a centrifuge tube.

12. Spin the cells at about 1000-1500 rpm for 3 minutes.

13. Remove the supernatant.

14. Resuspend the cells in 10-20 mls of freezing media (about 1-2 x 10^6 cells/ml).

15. Transfer the cells to freezing vials (1 ml/vial).

16. Freeze the cells in the -80 ^oC freezer. Transfer to a liquid nitrogen tank for long-term storage.

17. Defrost and plate one vial in a T25 as a test to make sure healthy cells are covering 85-100% of the surface, but are not proliferating.

18. Inactivated MEFs that are plated in culture, need to be used within 5 days. Quality of the feeders is very important for the growth of healthy embryonic stem cells.

Media

The media is prepared by addition of animal serum to the basal media. 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]. Although the recipes are very similar, different cell types may prefer different formulations for optimum growth. The serum is normally taken from different types of animals such as calf, fetal bovine or horse. Additional L-Glutamine may be added to the media depending on the cell type to the final concentration of 0.5-10 mM. 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. Since the class meets for a limited time period, repeating the experiments may not be an option. Therefore, for the purpose of this class, use of antibiotics is recommended, to avoid failure of the procedures from repeated contaminations. Below is a suggested recipe for the growth and maintenance of immortalized cells. For optimum growth of different cell lines, the recipe may be slightly different.

Complete media for immortalized cell lines (500 mls)	
Basal media (MEM)	450 mls
L-Glutamine	2 mM
Fetal bovine serum	10 %
Penicillin	100 U/ml
Streptomycin	100 μg/ml

Mouse embryonic stem cells need additional components to be included into their complete media. To maintain the pluripotent characteristic of the stem cells and prevent spontaneous differentiation, LIF (Leukemia Inhibitory Factor) is routinely added to the pluripotent media. For the purpose of differentiation, LIF is excluded from the differentiation media.

Complete pluripotent media for mESCs (500 mls)	
DMEM High Glucose	450 mls
L-Glutamine	2 mM
Sodium Pyruvate	1 mM
Non-Essential amino acids	0.1 mM
Streptomycin	50 μg/ml
Penicillin	50 U/ml
β– Mercaptoethanol	0.1 mM
Fetal bovine serum (Characterized)	10 %
LIF	1000 U/ml

Media should be aliquoted into smaller bottles or tubes after preparation. Each student group will get its own small bottle of media. The bottle needs to be label with the group name and kept in the refrigerator for that groups use. Important: each student group must have its own media and reagents, not to be shared with others, in order to reduce the chances of contamination spread.

Freezing media

Freezing media is prepared by adding Dimethyl Sulfoxide (DMSO) to the complete media at the final concentration of 10 %. Each student group should prepare its own freezing media and keep it in the refrigerator.

For freezing mESCs use 10% DMSO, 90% FBS plus 1000 U/ml LIF.

Phosphate Buffered Saline (PBS)

Calcium- and magnesium-free Dulbecco's PBS (DPBS) is used for rinsing the cells and for dilutions (Dubelcco and Vogt, 1954). Sterilized modified DPBS either can be purchased for cell culture procedures or prepared and sterilized in the laboratory.

Modified Dulbecco's Phosphate Buffered Saline (1X) (1 liter)	
NaCl	8000 mg
KCl	200 mg
Na_2HPO_4 (dibasic)	1150 mg
KH_2PO_4 (monobasic)	200 mg

For procedures that require the cells to remain attached to the plate, use PBS with Ca^{2+} and Mg^{2+}. Add 100 mg $CaCl_2$ and 100 mg $MgCl_2$. $6H_2O$ to total of 1 liter DPBS. Aliquot PBS into smaller tubes. Each student group needs its own aliquot.

Trypsin

Trypsin is a dissociating enzyme that is used to detach the cells from the surface of the culture vessels. EDTA is sometimes added to trypsin to chelate calcium and magnesium and to help in destabilizing the interactions between cells and the extracellular matrix. A balanced salt solution of 0.05-0.25% trypsin can be purchased for cell culture. Aliquot trypsin into smaller volumes and keep in the -20 ^{o}C freezer for long-term storage. After defrosting you can keep trypsin in the refrigerator to avoid repeated freezing and thawing. Each student group needs to get its own aliquot of trypsin.

Crystal violet dye

Crystal violet is a dye that stains the cells purple and makes them visible to the eye. Methanol is added to the Crystal violet solution to act as a fixative and to keep the cellular structures intact.

Crystal violet / Methanol dye	
Methanol	70 %
PBS (with Ca^{+2}/ Mg^{+2})	30 %
Crystal violet	0.1 %

Trypan blue dye

Trypan blue is a blue dye used to distinguish viable cells from dead cells. A bottle of 0.4% trypan blue can be purchased and aliquoted into smaller tubes. Cells are diluted into trypan blue (1:1) before counting. Important: dilute the cells immediately before counting, since live cells will eventually absorb the dye.

Gelatin

To grow embryonic stem cells in culture, the culture vessels are coated with 0.1% gelatin for adhesion of the cells to the bottom surface. To coat, add enough 0.1% gelatin to the culture vessel to cover the bottom surface. Wait 5 minutes and then remove the gelatin. The cells can be plated immediately after coating the culture vessel. To prepare, dissolve gelatin in ddH_2O by heating. Sterilize by autoclaving. For convenience, you may also purchase sterilized gelatin solutions.

Laboratory Exercises

The class meets once a week for nine weeks. Each class meeting is four hours and starts with a brief lecture, followed by a laboratory exercise. A suggested schedule of laboratory activities for 8 weeks is shown in the table below. The final written and practical exams are scheduled for the ninth week. One or two of the lab exercises may be eliminated, if more time is needed for classroom discussions. Some of the lab exercises require additional attention during the week and some of the procedures may need to be performed by the instructor or the laboratory technicians on the days that the students are not present.

Suggested Laboratory Schedule	
Week	Student activities
1	-Aseptic technique
2	-Plate cells from frozen stocks
3	-Count and plate 10^4 cells
4	-Survival assay-UV irradiation -Live and Dead Cell Identification
5	-Count colonies -Transfection
6	-Stem cell differentiation-Plate EBs
7	-Observe transfected cells -Plate EBs on gelatin
8	-Observe differentiated cells -Freeze cells
9	-Written and practical exams

The students should maintain one or two T25 flasks every week. In addition to the lab exercises for each week, they need to feed or subculture their flasks to maintain a healthy culture. The students' cultures may need to be fed or subcultured during the week by the instructor or lab aids if the students are unable to do so.

The students work in groups of two. Each group needs its own set of media and reagents. Each group needs a work station in the hood. Next to each work station should be a shelf or a cart, supplied with the following:

- Sterile serological pipettes of different sizes
- Micropipettes and sterile tip boxes
- Sterile glass pipettes in boxes or cans
- Sterile box of microfuge tubes
- A spray bottle, filled with 70% ethanol
- A squirt bottle, filled with 10% bleach
- A box of wipes
- A biohazard waste pouch
- A biohazard waste bin
- A regular trash can
- A box or a cylinder for collecting used serological pipettes

Lab Exercise 1: Aseptic Technique Exercise

Learning objectives for exercise 1 are:

- Become familiar with proper aseptic techniques.
- Become familiar with the concept of serial dilution.
- Become familiar with proper use of the laminar flow hood.
- Become familiar with the proper use of serological and micropipettes.

List of materials	
LB (Luria-Bertani) media	10 ml per student group in sterile tubes
ddH_2O or PBS (Phosphate buffered saline)	1 ml per student group
24-well cell culture plates	1 per student group
Serological and micropipettes, micropipette tips, racks, glass pipettes	

Activities:

1. Tour of the facility

Show the students where the safety equipment is located and review the emergency exit plan. The students should become familiar with the locations of the equipment and supplies they will need to perform their experiments.

2. Practice using serological and micropipettes

Demonstrate the proper way to use pipettes. Have the students practice pipetting with tap water.

3. Serial dilution and plating of the pretend cultures

In this exercise the students will pipette the less expensive bacterial media into cell culture plates, to practice aseptic techniques and use of serological and micropipettes. The students will dilute sterile water into sterile LB (Luria Bertani) media 10^3 and 10^4 times. Review serial dilutions (refer to appendix A of the student manual). Note that water (or PBS) is being diluted in LB. In other words, water (or PBS) is used instead of cells and LB is used in place of cell culture media.

Allow the students to figure out their own strategy for the dilutions. Check each groups plan before it starts the dilution exercise.

The plates are incubated in a bacterial 37^{o}C incubator for a week until the next class meeting. Do not use the cell culture incubator for this exercise to prevent contamination of the incubator by the bacteria that may be growing in the cultures. If there are no available incubators, just keep the plates at room temperature for a week.

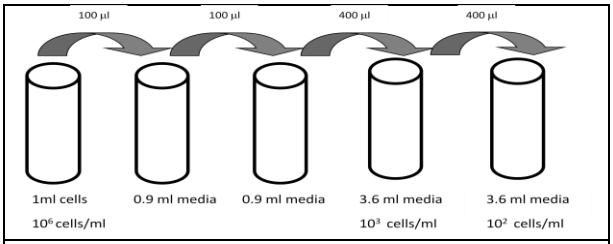

Figure 9- Example of a dilution process.

4. Observe the cultures

On the next class meeting, the students observe their cultures. If the cultures are contaminated, it means the students need to practice their aseptic techniques further. No contamination is the indication of good aseptic technique procedures.

Lab exercise 2: Plate Cells from Frozen Stocks

Learning objectives for exercise 2 are:

- Ability to plate cells from frozen vials.
- Become familiar with the proper use of clinical centrifuges.

List of materials	
T25 flasks	1-2 per student group
Complete media	1 small bottle per group
15-ml centrifuge tubes	1-2 per group
Frozen vials of cells	1-2 per group
Already plated cells in a T25	1 flask for the whole class
Serological and micro pipettes, micropipette tips, racks and glass pipettes	

Activities:

1. Observe the LB cultures

The students will observe the LB cultures from the previous week to see if any bacteria or fungus is growing indicating poor aseptic techniques.

2. Observe cells

Place some already plated cells under the inverted microscope for the students to observe and become familiar with.

3. Plate cells from frozen stocks

Defrost and plate a frozen vial of cells in a T25 flask, while students are watching. Emphasize the aseptic techniques. Demonstrate the proper use of the clinical centrifuge and the aspirator in the hood. The students can then get their own frozen vials and plate the cells. Each group may plate cells from one or two frozen vials.

After plating, the cells need to be fed every 2-4 days. Since the students are not in the laboratory, the cells should be fed by the instructor or laboratory aids. Subculture the cells, if the confluency of the flasks is above 75%.

Lab exercise 3: Count and Plate 10^4 Cells in a T25

Learning objectives for exercise 3 are:

- Ability to subculture cells
- Ability to count cells and become familiar with the proper use of a hemocytometer
- Become familiar with the concept of serial dilutions

List of materials	
T25 flasks	1 per group
Complete media	1 small bottle per group
DPBS	1 small bottle per group
Trypsin	1-2 mls per group
Trypan blue	50-100 μl per group
Hemocytometer	1 per group
Serological and micro pipettes, micropipette tips, racks, glass pipettes and sterile tubes	

Activities:

1. Observe the cells

The students should look at the cells, plated the previous week, and note their status. Note that the cultures might have been subcultured during the week.

2. Trypsinize the cells

The students follow the procedure for detaching the cells from the flask surface, using trypsin.

3. Count the cells

The students mix the cells with trypan blue dye and count the cells, using the hemocytometer. Students may need individual help if this is the first time they are using a hemocytometer.

4. Plate 10^4 cells in a T25

Review the calculations with the students. They may have to dilute their cells before plating. Use DPBS instead of media for routine dilutions since it is less expensive.

The cells are kept in the incubator over the week. Feed the cells every 2-4 days and subculture the cells, if needed.

Lab exercise 4: Survival Assay- Sensitivity to UV Exposure

Learning objectives for exercise 4 are:

- Become familiar with plating certain number of cells in multi-well plates
- Become familiar with survival (clonogenic) cytotoxicity assay
- Ability to stain and count colonies
- Ability to construct a survival curve and determine the IC_{50} value

List of materials	
6-well plates	5 per group
Hemocytometer	1 per group
Complete media	1-2 small bottles per group
PBS	1 small bottle per group
Trypsin	1-2 mls per group
Freshly made Crystal violet/ Methanol	20 mls per group
Crystal violet/ Methanol waste bottle	1-2 for the entire class
Serological and micro pipettes, micropipette tips, racks, glass pipettes, sterile tubes and fresh cell culture flasks.	

Activities:

Either the plating of the cells or the irradiation procedures may be performed by the instructor or the lab aids.

1. Plating of the cells

Trypsinize, count, and plate cells in multi-well plates. Emphasize the importance of observing cells before use to make sure the culture is healthy. Review the concepts of making a master mix and making proper dilutions of cells.

The number of cells to be plated depends on the plating efficiency of the cells. For cells with very low plating efficiency, increase the number of cells per well. Plate enough cells, so that even after radiation there are a few colonies in the wells to be counted. Remind the students to re-plate some cells in a fresh T25 for the next experiment.

2. UV exposure

One or two days after plating, the cells are exposed to the UV light. Wear protective goggles while irradiating the cells. Place the plates directly under the UV light in the hood with the lid off and expose them to the appropriate dosage of UV (refer to the procedures in the student manual). Replace the media and return the plates to the incubator.

3. Count colonies

A week after plating, the students will count the number of colonies, calculate surviving fraction values and graph a survival curve. Demonstrate how to count colonies. Tiny colonies that are less than 50 cells are not counted. There may be colonies growing on the periphery of the wells that should be included in the count.

Colonies that have been fixed and stained can be kept in a dark place at room temperature for a long time. If time is limited, the students can keep the plates to be counted at a later time.

Modifications to the experiment

The experiment may be performed with one, two or more cell lines to compare the responses of the different cell lines to the toxic reagent.

Toxic reagents other than UV light may be used for this experiment. Dilute the reagent in media at 4 or more different concentrations and add to the cells one or two days after plating. Examples of reagents that can be tested are caffeine (at 0.5, 1, 2 and 4 mM) and acetaminophen (at 1, 5, 10 and 20 mM).

Combination of two toxic reagents may also be tested to study the additive effects of different reagents. For example, there is some evidence suggesting that caffeine affects DNA repair mechanisms (Itoh *et al.* 2000 and Engstrom and Kmiec, 2005). Cells may be plated in 1mM caffeine on Day 1, and exposed to different dosages of UV one or two days after plating. Control cells are plated without caffeine and compared to the experimental cells.

Lab exercise 5: Live and Dead Cell Identification by Using Propidium Iodide and Calcein AM

Learning objectives for exercise 5 are:

- Become familiar with the use of fluorescent microscopes.
- Identify live and dead cells by using fluorophores.

List of materials	
24-well plates	1 per group
Complete media	1 small bottle per group
PBS	1 small bottle per group
Trypsin	1-2 mls per group
70 % Methanol	2 mls per group
8 μM Calcein AM	2 mls per group
10 μg/ml Propidium Iodide	2 mls per group
Serological and micro pipettes, micropipette tips, racks, glass pipettes, sterile tubes, gloves and fresh cell culture flasks	

Activities:

1. Plate cells on day 1

This task is performed by the instructor or the laboratory aids. Two days before the class meeting, plate cells in 12 wells (3 columns and 4 rows) of 24-well plates so the wells are 60-80% confluent on the day of the experiment. The number of cells to be plated depends on the cell line's growth rate. Each student group gets one plate. Remember to plate some cells in a fresh T25 to be used in the next experiment.

2. UV exposure

The first two rows of the cells are exposed to UV light 6-20 hours, before the class meeting, by the instructor or laboratory aids. The exposure time may be different for different cell lines. The objective is to kill some cells by the time the class meets so the students can see both live and dead cells in the same well. They should see high numbers of dead cells and low numbers of live cells in the first row of cells and low numbers of dead cells and higher numbers of live cells in the second row. Note: if the cells have been dead for a long time, they are probably floating in the media and will be washed away by the washing steps. Therefore, it is important to expose the cells to the UV light only a few hours before the start of the class.

- Remove the media from the first two rows of cells.
- Wash the wells with 0.5 mls of PBS per well. Remove PBS.
- Place the plate directly under the UV light in the hood.
- Take off the lid, and put it back on, in a way to cover all of the rows, except for the first row. Cover the lid with aluminum foil to protect the last three rows from the UV exposure.
- Turn on the UV light for 20 seconds.

- Slide the lid slightly to uncover the second row of cells.

- Turn on the UV light for another 10 seconds. (that is, the first row is exposed to the total of 30 seconds, while the second row is exposed to 10 seconds).

- Replace the media in the first two rows and place the plate back in the incubator.

3. Stain the cells with the fluorophores

Prepare and aliquot the following before the start of the class:

- 70% Methanol in DPBS- store at room temperature.

- 10 μg/ ml Propidium iodide in PBS- Wear gloves. Keep in the refrigerator and away from light.

- 8 μM Calcein AM in PBS- Keep in the refrigerator and away from moisture.

The students will fix and kill the cells with 70% methanol in the third row. They will then add Propidium iodide and Calcein AM to the cells. Remind the students to wear gloves while working with PI and cover their plates with aluminum foil to prevent photobleaching of the fluorophores.

4. Observe the cells using a fluorescent microscope

Demonstrate the proper use of the fluorescent microscope. The students will observe the results of their experiment and take notes.

Modifications to the experiment

Other toxic reagents may be used instead of the UV light.

Different rows of cells may be exposed to different dosages of UV light or an alternative toxic reagent the day before the class meeting. The cells are then stained with the fluorophores by the students on the day of the experiment. The students observe the cells under the fluorescent microscope and estimate the percentage of live cells to study the sensitivity of the cells to the toxic reagent. Two or more cell lines can be plated and their sensitivities compared.

The cells can be counter-stained with Hoescht fluorophore to visualize the nuclei.

Lab exercise 6: Transfection

Learning objectives for exercise 6 are:

- Ability to follow the instructions for transient transfection of mammalian cells with plasmids.
- Practice using fluorescent microscopes.
- Observe and identify different cellular structures (mitochondria and cytoplasmic actin filaments) that have been labeled with fluorophores.

List of materials	
24-well plates	1 per group
Complete media	1 small bottle per group
PBS	1 small bottle per group
Trypsin	1-2 mls per group
Serum-free basal media	0.5 mls per group
pDsRed2-Mito vector from Clontech Laboratories http://www.clontech.com/	5 µls of 1 µg/µl per group
pEGFP-Actin vector from Clontech Laboratories	5 µls of 1 µg/µl per group
Fugene® HD transfection reagent from Promega http://www.promega.com/tbs/tm328/tm328.html	35 µls per group
4% Paraformaldehyde in PBS	6 mls per group
Paraformaldehyde waste bottle	1 bottle for the class
Freshly made Crystal violet/ Methanol	20 mls per group
Crystal violet/ Methanol waste bottle	1-2 for the entire class
Serological and micro pipettes, micropipette tips, racks, glass pipettes, sterile tubes, gloves and fresh cell culture flasks	

Activities:

1. Plate cells on day 1

This task is performed by the instructor or the lab aids one or two days before the class meeting. Plate cells in 12 wells (3 columns and 4 rows) of 24-well plates, so the wells are 70-80% confluent by the day of the experiment. Use 1ml of media per well.

2. Transfection

One or two days after plating, the students transfect the cells according to the protocol.

3. Fixing of the cells

Forty eight hours after transfection, the instructor or lab aids need to fix the cells with 4% paraformaldehyde. The plates should be kept in the dark and in the refrigerator for the next class meeting. Paraformaldehyde is hazardous and should be handled with gloves. The waste is collected in a waste bottle and discarded properly according to regulations.

4. Observation of the results

On the next class meeting the students will observe the results, using a fluorescent microscope. In lecture refresh the students on the proper use of the microscope. The plates may be covered and kept in the dark in the refrigerator for 2-4 weeks. If time is limited, the cells may be observed on a later date, when the activities are lighter.

Modifications to the experiment

Other types of expression plasmid coding for different fluorescent fusion protein may be used in this experiment. Alternatively, a non-fluorescent protein can be observed, using fluorescent labeled antibodies. After the cells are fixed, the students can stain the cells with fluorescent-labeled antibodies on the next class meeting and observe the results on the same day or on the following week.

Alternative transfection methods (for example, Calcium phosphate or lipids) are available as kits for purchase and may be used. It is wise to test the kit and optimize the procedure before using it for the class. You may also instruct the students to try different concentrations of the plasmids and the reagents, optimizing the procedure on their own by comparing the results.

The cells can be counter-stained with Hoescht fluorophore to visualize the nuclei.

Lab exercise 7: Freezing Cells

Learning objectives for exercise 7 are:

- Ability to freeze cells
- Practice counting cells with a hemocytometer

List of materials	
Dimethyl sulfoxide	0.5 mls per student group (wear gloves)
DPBS	1 small bottle per student group
Trypsin	1-2 mls per student group
Complete media	10 mls per student group
Hemocytometer	1 per student group
Cryo-vials	2-4 per student group
Freezing or Styrofoam box	1 for the entire class
Serological and micro pipettes, micropipette tips, racks, glass pipettes, sterile tubes, and gloves	

Activities:

1. Freeze cells

The students use the instructions in the student manual to prepare freezing media (10% DMSO). Then the students freeze all of the cells that they have in culture from previous weeks (not mESCs).

The cryo-vials containing the cells are transferred to the freezing box and placed in the -80^{o}C freezer. For long-term storage, after 1-2 days, the vials are transferred to a liquid nitrogen tank. However, it is recommended that the students' frozen vials be discarded and purchased cells (or cells that have been frozen by the instructor) be used for the next class session. This will cut down on contamination and cell survival problems.

Lab exercise 8: Stem Cell Differentiation

Learning objectives for exercise 8 are:

- Become familiar with growing embryoid bodies in culture and initiate spontaneous differentiation of mouse embryonic stem cells.
- Identify differentiated cardiomyocytes and neurons

List of materials	
Mouse embryonic stem cells growing in a T25	1flask per group
Pluripotent media	1 bottle for the instructor
Differentiation media	1bottle per group (about 150 mls per group)
DPBS	1 small bottle per group
Trypsin	1-2 mls per group
Sterile bacteria Petri dish	6 per group
Hemocytometer	1 per group
Wide bore p200 pipette tips	1 box per group
5 mM Retinoic acid	25-40 µl per group
0.1% Gelatin in ddH2O	15-30 mls per group and 1 bottle for the instructor
24-well plate	1 per group
Serological and micro pipettes, micropipette tips, racks, glass pipettes, sterile tubes, and fresh cell culture flasks	

Activities:

Recommendation: the instructor or lab aids should perform the entire differentiation procedure a week ahead of the students to have a set of back-up cells on hand, in case the procedure performed by the students fails.

1. Plate and subculture mouse embryonic stem cells (mESC)

This activity is done by the instructor starting about 10 days before the class meeting.

Plate and expand mESCs in order to prepare one T25 flask for each group.

<u>If using feeder-dependent cells:</u>

Plate feeders and mESCs

1. Coat a T25 flask with 0.1% gelatin.

2. Wait 5 minutes then remove gelatin.

3. Defrost and plate one vial of inactivated feeder cells (refer to page 13) in complete media. Follow the instructions on plating cells from frozen stocks (refer to the student manual).

4. Allow the feeders to grow overnight in the incubator.

5. Defrost and plate a vial of frozen mESCs on top of the feeders. Remove the media from the feeders and replace it with stem cell pluripotent media. Note: the LIF in the

media together with the feeder cells keep the mESCs from differentiating.

6. Incubate in the incubator.

7. The culture needs to be fed (replace the old media with fresh media) every day.

Subculture

In order to prevent differentiation, mESCs need to be subcultured every 2-3 days, when the cells are about 50% confluent. When splitting, divide the cells into 2-5 subcultures.

1. Prepare 2-5 T25 flasks with inactivated feeders, as before.

2. Remove the media from the feeders and replace with 3 mls of pluripotent media in each flask. Put the feeders back in the incubator.

3. Wash the T25 flask containing mESC cells with 5 mls of Ca^{2+}, Mg^{2+}-free DPBS, twice. Remove DPBS.

4. Add 1 ml of Trypsin.

5. Incubate for 2-3 minutes.

6. Tap the flask gently and make sure the cells are floating.

7. Transfer the cells to a centrifuge tube.

8. Add 9 mls of media (less expensive complete media may be used rather than the stem cell media).

9. Spin at 1000 rpm for 3 minutes.

10. Remove the supernatant.

11. Resuspend the cells in 4-10 mls of pluripotent media.

12. Pipette the cells a few times.

13. Transfer 2 mls of the cells into each flask with the feeders.

14. Return the flasks to the incubator. Split the cells a few more times until there is one T25 of mESCs per student group.

15. Allow the student flasks to grow to about 80% confluency.

<u>If using feeder-independent cells:</u>

Plate and expand mESCs

1. Coat a T25 flask with 0.1% gelatin.

2. Wait 5 minutes then remove gelatin.

3. Defrost and plate one vial of mES cells in pluripotent media. Follow the instructions on plating cells from frozen stocks (refer to the student manual).

4. Feed the cells every day with fresh pluripotent media.

5. Split the cells into 3-5 subcultures every 2-3 days when the confluency of the culture is about 60%. Remember to use freshly gelatinized flasks every time.

6. Allow the student cultures to grow to about 80% confluency.

2. Plating cells in suspension

On the day the class meets, the students trypsinize, count and plate cells without LIF and feeders in suspension, using low adhesive Petri dishes in order to initiate differentiation.

If using feeder-dependent cells, after trypsinizing and quenching the trypsin with media, transfer the cells to a T25 and incubate for 15-30 minutes. Most of the feeders will attach and get separated from the mESCs. Transfer the floating mESCs to a tube and count.

3. Feeding the embryoid bodies

Cells that have been growing in suspension aggregate and form embryoid bodies (EB). Two or three days after plating the cells in suspension, the EBs are fed with fresh differentiation media. This task is performed by the instructor or the lab aids. Follow the instructions in the student manual for feeding the EBs. Repeat feeding the EBs in two days. This time Retinoic acid is added to the final concentration of 5 μM to one of the Petri dishes for each student group.

4. Plating EBs on gelatin

One week after plating the cells in suspension, the EBs are plated on gelatin-coated, 24-well plates by the students. Provide wide-bore pipette tips so the large EBs can be transferred without being squeezed into tight opening of the regular pipette tips. Alternatively, 1ml serological pipettes can be used. Aliquot retinoic acid into small microfuge tubes for each student group. Students add retinoic acid to the final concentration of 5 μM to the differentiation media for neuron formation.

5. Observing the differentiated cells

Between 2-10 days after plating the EBs on gelatin, the ES cells differentiate into cardiomyocytes, neurons and other cell types. The students observe the differentiated cells under the microscope. To see the beating cardiomyocytes, the students may have to look at many cells in each of wells, since the chances to see them clearly is relatively low.

Neurons may be easier to find and recognize due to their clear axonal extensions.

The differentiated cells may be kept in culture for 2-4 weeks by changing the differentiation media (with or without retinoic acid) every 2-3 days. If the process of differentiation is not complete, the students may need to look at the cells on the following week.

Modifications to the experiment

An alternative protocol uses the hanging-drop method (Hopfl *et al.* 2004):

Day 1:

1. The students are given an about 80% confluent T25 flask of mESCs.

2. Aspirate the medium.

3. Wash the cells with 5 mls of DPBS twice.

4. Remove DPBS.

5. Add 1 ml of Trypsin. Wait 2-3 minutes and tap the flask to detach the cells.

6. Add 9 mls of differentiation media.

7. If using feeder-dependent cells, transfer the cells to a T25 with no gelatin coating and incubate for 15-30 minutes. Most feeders will attach to the bottom surface and the ESCs will be floating.

8. Transfer the cells in suspension to a centrifuge tube.

9. Count the cells using a hemocytometer.

10. You need 2-5 mls of 50,000 cells/ml suspension. Dilute or concentrate the cells to adjust to the correct concentration.

11. Transfer 20 μl (1000 cells) to each well of a 96-well V-bottom plate.

12. Place the lid on the plate and label.

13. Invert the plate upside down and place it in the incubator.

Day 3:

14. Two days later, invert the plate back and add 200 μls of the differentiation media to each well. Be careful not to touch the EBs at the bottom of the well with the pipette tip.

15. Return the plate to the incubator (<u>not inverted</u>) for an additional two days.

Day 5:

16. Remove 100 μl media from each well and replace with 100 μl of fresh media. For neuron differentiation, add retinoic acid to the final concentration of 5 μM to the differentiation media.

Day 7:

17. Coat 24-well plates with 0.1% gelatin for 5 minutes. Remove gelatin from the wells.

18. Add 1.5 mls of differentiation media to each well of the 24-well plates. Add 5 μM retinoic acid to the media for neuron differentiation.

19. Using a wide-bore pipette tip, transfer the EBs from each well of the 96-well plate to each well of the 24-well plate. Be careful not to break the EBs. Some of the wells in the 96-well plate may have no EBs formed. (Alternatively, you can transfer the EBs to a low-attachment Petri dish first. This way you can see the EBs floating, and then you can transfer the EBs from the dish to the 24-well plate.)

Immunocytochemistry

To visualize the neurons, you may fix the differentiated cells and use an anti-Map2 antibody to bind to the Map2 protein in the neurons. Use a fluorescent-labeled secondary antibody to visualize the neurons with a fluorescent microscope.

Appendix A

List of required equipment and suggested vendors:

Equipment	Suggested vendor
Cell culture Incubator	Thermo Scientific
Clinical centrifuge	VWR
Freezer (-80^{o}C)	Thermo Scientific
Hemocytometer	Fisher Scientific
Inverted microscope and fluorescent microscope	Olympus or Zeiss
Laminar flow hood	Thermo Scientific
Liquid nitrogen tank	Thermo Scientific
Pipetmen (p20, p200 and p1000)	Gilson
Pipette pumps	Fisher Scientific
Sterilizing Oven or autoclave	Thermo Scientific
Vacuum pump	Thermo Scientific
Water bath	VWR or Fisher Scientific
Water purification system	Millipore

List of required supplies and suggested vendors:

Supplies	Suggested vendor
1, 5, 10 and 25 ml disposable sterile serological pipettes	Fisher Scientific or VWR
1.5 ml Microfuge tubes	Fisher Scientific
15-ml and 50-ml conical centrifuge tubes	BD Falcon
500 ml 0.2 μm low protein binding filters	Nalgene
Cell culture flasks	Sigma (Corning)
Cell culture multi-well plates	Sigma or BD Falcon
Cryo-vials	Nunc or Sigma
Glass Pasteur pipettes	Fisher Scientific
Micropipette tips (preferably filtered)	Gilson
Petri dish	Fisher Scientific or VWR
Wipes	VWR

List of required reagents and suggested vendors:

Reagent	Suggested vendor
4% Paraformaldehyde	USB Corporation
Calcein AM	Invitrogen
Crystal Violet	Sigma
Dimethyl Sulfoxide	Sigma
DPBS	Sigma
Fetal bovine serum	Gibco or Cellgro
Fugene® HD	Roche
Gelatin	Sigma
Leukemia Inhibitory Factor (LIF)	Sigma, Invitrogen
L-Glutamine	Sigma
MEM	Sigma or Cellgro
Methanol	Sigma
Mitomycin C	Sigma
Non-essential amino acids	Gibco or Cellgro
Penicillin-Streptomycin	Sigma
Plasmids	Clontech
Propidium Iodide	Invitrogen
Retinoic acid	Sigma
Sodium Pyruvate	Gibco or Cellgro
Trypan Blue	Gibco or Sigma
Trypsin	Gibco or Sigma
β- Mercaptoethanol	Sigma

Appendix B

Practice problem solutions:

The following are solutions to the problems in Appendix B of the student manual.

1. Take out 0.1 mls of the culture and mix it with 0.9 mls of PBS for 10X dilution. Next take out 100 µls of the diluted sample and mix it with 9.9 mls of media for another 100X dilution. The new cell suspension is 1000X less concentrated than the original culture. For dilutions it is recommended to use the less expensive PBS rather than media. For cells that will be plated remember to dilute into media.

2. First figure out how much volume she needs by using the C1V1=C2V2 formula, where C1 is the starting concentration, in this case 10^5 cells/ml. V1 is the starting volume which is unknown. C2, the final concentration in this example, is 5000 cells/ml and V2, the final volume, is 5 mls. Solving for V1, she gets 0.25 mls. So, she needs to mix 0.25 mls of her cells with 4.75 mls of media for total of 5 mls in a T25.

3. Using the C1V1=C2V2 formula
C1= 10^5 cells/ml
V1= ?
C2= 100 cells/ml
V2= 5 mls
Solving for V1= 5 µls.
This volume is too small for accurate measurements. So first she dilutes her cells 20X by mixing 100 µls of her cells with 1.9 mls of PBS. The diluted sample is 5 x 10^3 cells/ml (20X less concentrated than the original concentration). Using 5 x 10^3 cells/ml as C1, she now calculates 100 µls for V1. So she takes out 100 µls of the diluted cells and mixes it with 4.9 mls of media in a T25.

4. Concentration=150/10 x 10^4 x 1 (dilution factor) = 1.5 x 10^5 cells/ml

Total number of cells =
5mls x 1.5 x 10^5 cells/ml = 7.5 x 10^5 cells

5. Concentration of dead cells =
(60/5) x (10^4) x (2, dilution factor) =
2.4 x 10^5 cells/ml

Concentration of live cells =
(300/5) x (10^4) x (2) = 1.2 x 10^6 cells/ml

6. Total dilution factor=
10 (dilution in PBS) x 2 (dilution in Trypan blue)= 20

Cell concentration = 60/10 x 10^4 x 20 =1.2 x 10^6 cells/ml.

7. C1= 200/10 x 10^4 =2 x 10^5cells/ml
C2 =1000 cells/ml.
V2 = 24 wells x 1ml/well= 24 mls
However, always make some extra for the master-mix to count for pipetting errors. So V2=26 mls (2mls extra).
V1 = (1000) (26) / 2 x 10^5 = 0.13 mls
He prepares a master-mix by mixing 0.13 mls of his cells with 25.87 mls of media for total of 26 mls. He then aliquots 1 ml of the mix into each well.

8. C1= 400/10 x 10^4 = 4 x 10^5 cells/ml.
C2= 100 cells/ 2 mls for each well =50 cells/ml
V2 = 2mls/well x 6 wells = 12 mls
However she needs to make extra to count for pipetting errors, so V2= 14 mls
Solving for V1 using C1V1=C2V2
V1 = 50 x 14 / 4 x 10^5 = 0.00175 mls =1.75 µl
This volume is too small to represent the whole culture. You should try to measure volumes larger than 50 µls for more accuracy. Therefore, she dilutes her cells 100 times first by mixing 100 µls of her cells with 9.9 mls of PBS. The concentration of the diluted sample is 100 times less than the original culture, so she needs to take out 100 times more

volume for the same number of cells. So, she takes out 175 µls of the diluted sample and mixes it with 13.825 mls of media to make the master-mix. She then aliquots 2 mls from the master-mix into each well.

9. C1 = 240/5 x 10^4 = 4.8 x 10^5 cells/ml
She then calculates the final concentration (C2). Each T25 flask is 25 cm^2, so she needs to seed 25 x 1000 = 2.5 x 10^4 cells total. She is going to use 5 mls for the T25.
C2 = (2.5 x 10^4) cells /(5) mls = 0.5 x 10^4 cells/ml
V2= 5 mls for the T25
Solving for V1 using C1V1=C2V2 formula.
V1 = 0.05 mls= 50 µls
She mixes 50 µls of cells with 4.95 mls of media in a T25.

10. C1 = 60/10 x 10^4 = 6 x 10^4 cells/ml
For each well C2=3 cm^2 x 2000 cells/ cm^2 x 1 ml= 6000 cells/ml
V2 is 12 mls for the 12-well plate plus 2mls extra to count for pipetting errors. So V2 = 14 mls.
Using C1V1 =C2V2 solve for VI.
V1 = 1.4 mls
He mixes 1.4 mls of his cells with 12.6 mls of media. He then aliquots the master-mix by pipetting 1 ml into each well.

11. C1=315/10 x 10^4 = 3.15 x 10^5 cells/ml
Total number of cells=
(3.15 x 10^5 cells/ml) (5 mls) = 1.57 x 10^6 cells.
He transfers all of his cells to a centrifuge tube and spins the cells for 3 minutes. He then removes the supernatant and resuspends the cells in <u>1.57 mls</u> of freezing media in order to get 1 x 10^6 cells/ml. He then transfers the resuspended cells to cryovials and freezes them.

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