BE 167L - Bioengineering Laboratory

Lab 5: Mammalian Cell Culture

Prelab reading

Mammalian cell culture is one of the most essential tools in biotechnology and cell biology. In the following set of labs, you will learn how to culture a widely used cell line, 3T3 fibroblasts.

In general, mammalian cells are cultured on plastic surfaces often called “tissue culture plastic” or “tissue culture polystyrene”. You may see petri dishes or multi-well dishes made of polystyrene that are not intended for tissue culture, such as those used in earlier labs for PDMS stamping or other storage and uses. If they are intended for tissue culture, they will be marked as “tissue culture treated” and they will be pre-sterilized. Be aware of the difference when gathering or ordering your supplies. Cells will not adhere to non-treated polystyrene.

Cells are cultured, or expanded (grown to increase their numbers for use in experiments) most commonly in tissue culture flasks with canted (bent) necks. You will see them in different sizes and referred to as, for example “T75” or “T25” flasks. The number indicates the surface area available for cells to adhere. T75 flasks have 75 cm^2 of culturing surface area while T25 flasks have 25cm^2. It is useful to know what the surface area is, so that when you count your cells, you know what the density of the cells is. The density allows you to make comparisons between culture conditions that may have been different scales. For example, seeding a T75 flask with 1 million cells in it means a cell density of 13,333 cells/cm^2. What is the equivalent number of cells you can put into a T25 flask so that the cells feel the same cell density condition? A 6-well plate has 6 wells, each with a surface area of 9.6 cm^2. How many cells would you put into each of these wells for a similar cell density?

Different types of cells expand or proliferate at different rates. Many stem cells may take several days to double in number and may change their doubling time depending on how densely they are spaced. For instance, when they become too dense, a phenomenon called *contact inhibition* may cause them to stop proliferating. Also, if they are particularly sensitive, a very sparse seeding density may prevent them from receiving the right intercellular signals to proliferate at the standard rate. You will calculate the exact doubling time for your conditions and cell type by counting them at different time points. Knowing the doubling time and appropriate seeding densities (density of cells when replating after passaging) can help you to plan and prepare for experiments appropriately and efficiently by knowing how many cells you will have by a certain time and how many you need to start with.

Many mammalian cells are “adhesion dependent”, meaning they need to be attached to a substrate and spread out using focal adhesions and other ways of attachment. The hydrophilic surface treatment of polystyrene is often adequate for their attachment. Some mammalian cells are not adhesion dependent and may be cultured in flasks or wells that are not treated for adhesion, like hematopoietic stem cells or B-cells of the immune system that naturally remain detached. They remain suspended in the medium and must be collected by centrifugation. Other cell types are adhesion dependent, but require more than the typical tissue culture treatment of polystyrene. For example, some neuronal cell types are typically cultured on surfaces that are coated with proteins or amino-acids like gelatin or collagen, or lysine to improve their adhesion to the surface, otherwise they are easily detached just by bumping or shaking. You may notice many options for tissue culture treatments when looking at flasks and plates, and this is why. Be aware of your cell type and what it prefers.

The medium that cells are cultured in is specially formulated for each cell type. In general, it starts with a basic nutrient medium with a pH indicator and buffering salts. You may add serum, which contains a wide range of proteins and enzymes to help the cells adhere and proliferate, as well as antibiotics to keep the medium bacteria-free while culturing. This is also where researchers may modify culture conditions, by changing the contents or ratios of contents in the medium (adding growth factors, changing the pH, adding small molecule drugs to the medium) to change the behavior.

Refer to your lecture notes on the specific contents of your 3T3 cell medium for basic growth conditions and maintenance of the cell line.

Oxygen is important for keeping the cells alive. They get this from dissolved oxygen in the medium, which diffuses into the medium from the air. There is a rough volume of medium recommended for the size of your culturing container so that the thickness of the medium layer above the cells allows adequate oxygen diffusion to the cells. Too much medium may reduce the oxygen levels where the cells are at the bottom, suffocating them. Too little medium may allow too much oxygen or quick evaporation and dry patches where cells may die. Always make sure your cells have the right amount of medium for the vessel size.

The medium must be replenished on a regular basis. You will take out the old medium and put in fresh medium. Think of it as their food, and once they use up the nutrients in the medium, they need to be fed again. You are also removing accumulated waste products. Many components in the medium, such as components of the serum, degrade quickly under physiological conditions like those in the incubator. This is why serum is stored in the freezer, and the complete medium is stored in the fridge and lasts only a month in the fridge. Once it is warmed up and stored in the incubator with cells, components begin to degrade even quicker, so you must replenish those by changing the medium. It is also a good idea to warm up only the amount of complete medium that you plan on using on a given day, to avoid degrading the whole bottle.

Your medium contains a buffer to maintain pH 7.4. This buffer relies on CO_2 to keep it from releasing into the atmosphere and becoming more basic (turning purple). The incubator has a CO_2 sensor and injects CO_2 from a tank to keep the level at 5%, which is the level of CO_2 in your body and what is needed to maintain the pH of the medium. The purpose of this tray is to keep the humidity in the incubator at ~100%. This keeps your medium from evaporating during the culture period. If you notice that you have less media in your flask than you put in, check the water pan, it is probably empty. The humidity is also important for the type of CO_2 sensor in the incubators. If the humidity is not high enough, the sensors become inaccurate and will not keep the correct CO_2 levels, which will negatively affect the pH of your medium. Every time you open the incubator, the humidity goes down and the CO_2 level drops. The longer you leave it open and the wider you open the door and the more turbulence you create when you open and close it, the closer it gets to the room air. An incubator that starts with the low humidity of the room can take 3-4 hours to reach full humidity, which means that it can take 3–4 hours for the CO_2 sensor to work properly. Keep the doors closed. Open and close them gently, and only open them as wide as you need.

Cells are living things and can be shocked by quick changes in their environment. When changing the medium or passaging, try to warm up their new environment before putting them in it. Often you will see researchers put the fresh medium in the new flask and put it in the incubator to adjust the CO_2 levels and temperature of the medium while trypsinizing and counting the cells. If they have to keep the cells out of the incubator, they might keep them in a capped tube so that the atmospheric air won't continuously change the pH of the medium they're in, or keep them in PBS and reduce their temperature by placing them on ice so that their metabolic needs are slowed. It is important to know what conditions affect the components of your medium and your cells so that you can take the proper precautions when working with them. Setting up experiments can take longer than you think! Don't let your cells wither away in the biosafety hood while you do calculations and pipette solutions. It can take as little as 30 minutes for the pH of your cell culture medium to increase to toxic levels sitting on the counter or under the microscope without the CO_2 environment of the incubator.

This is the first in a series of cell culture labs in which you will learn many ways to quantify cellular behavior and growth kinetics. An essential laboratory skill to cultivate is the ability to perform experiments in parallel and plan ahead. Each lab period you should be prepared to passage your cells to keep them alive and proliferating for use in the next lab, while also performing the experiments of the current lab. In this first lab you will learn how to prepare your cell culture reagents, how to work in a Biological Safety Cabinet with cells, and how to count and passage your cells for continuous proliferation. You will prepare flasks and plates of cells for the next lab in appropriate quantities for estimating confluency and basic hemocytometer cell counting.

Watch the lab primer and cell passaging videos.

Mammalian cell culture

Refer to the online videos for instruction on how to passage cells. Come to section with questions about cell culture for your TA to assist you. You will be tested on aspects of cell culture on your lab practical exam, so be sure to use your time in section to learn these techniques properly.

In the teaching lab you will be using NIH 3T3 mouse embryonic fibroblasts. Fibroblasts are the predominant cell in your connective tissue and actively secretes collagen to maintain these tissues. In addition, fibroblasts are a major player in wound healing and for these reasons are actively studied.

> The cells you will be using in this lab come from a cell line isolated and initiated in 1962 at the New York University School of Medicine Department of Pathology. 3T3 refers to the cell transfer and inoculation protocol for the line, and means "3-day transfer, inoculum 3 x 10^5 cells." Using this protocol, the immortal cell line begins to thrive and stabilize in cell culture after about 20-30 generations of in vitro growth. George Todaro and Howard Green, the scientists who first cultured this cell line, obtained the cells from desegregated NIH Swiss mouse embryo fibroblasts. The cell line has since become a standard fibroblast cell line."

Taken from http://www.nih3t3.com/; visit this website to learn more about this cell line.

Preparation

Reagents

- High Glucose, DMEM
- DPBS, with calcium and magnesium
- Trypsin
- Fetal Bovine Serum
- Penicillin-Streptomycin

Supplies

- Micropipette tips
- 5 mL serological pipette
- 2 mL serological pipette
- 15 mL centrifuge tube
- 50 mL centrifuge tube
- Microcentrifuge tubes
- 1 T25 and 1 6 well plate

Equipment

- Biological safety cabinet (BSC)
- Pipet-aid — When using these, avoid aspirating liquid through the pipet and into the attachment head. If this does happen, **please inform your TA immediately.** It can be easily fixed if dealt with at the time.
- Phase contrast microscope
- Centrifuge
- CO_2 cell culture incubator

Biological Agent Safety

In addition to your usual protective equipment, (lab coat, gloves safety glasses), you should be aware of these things. All disposable items that come in contact with them should be put into solid biohazard waste containers, this includes gloves, pipettes, tips, flasks, paper towels, tubes… All liquid solutions that come into contact with them or contain them should be bleached for 30 minutes and then flushed down the drain with copious amounts of water (bleach will corrode the sewer pipes if you don't rinse with lots of water). Though you should be careful, don't put items into the biohazard bin if they are *not* actually contaminated with a biohazard. Serological pipettes, bottles, tips, and tubes that only contact sterile liquids that have not contacted human cells are *not* hazardous and can go in the regular trash. The university pays a vendor to haul away and incinerate all the biohazard waste. Don't put things in that don't need special treatment, it's wasteful. The water in the water baths contain a chemical that keeps bacteria and mold from growing in the body-temperature bath, so don't get it on your skin or splash it around too much. Wash your hands after contact.

Please refer to section IV of the CDC guidelines for Biosafety in Microbiological and Biomedical Laboratories, 5th edition (posted on CCLE) and the lab-specific Biosafety Manual for more information.

Procedure

Preparing Reagents

In this course, you will culture your cells in high glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. In addition to using media, you will also use DPBS, and trypsin in the first part of the lab you will aliquot your own stock of media, DPBS and trypsin to use for the rest of the quarter. In addition, you will 'complete' your media by adding the supplements described above. **All reagents to be used for cell culture must be aliquoted in the hood for sterility.**

Preparing Complete DMEM (10% FBS + 1% Pen/Strep)

1. Aliquot 44.5 mL of DMEM into a 50 mL tube.
2. Aseptically add 5 mL of FBS and 500 µL of Pen/Strep
3. Label your tube with Contents (DMEM Complete), Name, Section, and Date. Complete media can be kept in the fridge for approximately a month before it will no longer sustain cells. Other media formulations may have different shelf lives depending on their contents. See the reference sheet below for an example of how to label properly.

Passaging cells and counting

Summary of steps

- There will be a flask ready for each group.
- Students will lift cells from their flasks using trypsin and use a hemocytometer to determine the cell density in the original flask.
- Students will passage cells to different split ratios (seeding densities). Each group of students will seed into 3 wells of a 6 well plate. Each well of a 6 well plate has an area of ~9 cm^2 and you will be seeding at 500, 4,000, and 10,000 cells/cm^2. Your remaining cells will go into a new T-25.
- The TA will organize your clean-up and show you how to deal with the biohazard waste. Liquids must be aspirated into the flasks.
- Place solids in appropriate biohazard bins according to type, TAs will take the full bags to the waste collection site.
- In the next lab you will count the cells in each of your flasks to determine the growth kinetics (doubling time), and re-plate cells for the protein assay in the next lab.

Passaging Cells (Volumes are given for a T25 flask)

1. Spray down gloves with 70% ethanol before entering hood.
2. Spray down and wipe hood surface and sash with ethanol.
3. Warm up PBS, medium, and trypsin to 37°C in water bath. Spray PBS, media, and trypsin bottles with ethanol and place in hood.
4. Tighten the cap and remove a flask with cells from the incubator. View under the microscope to assess 80% confluency for cell passaging. Check confluency with your TA before bringing cells back into the biosafety cabinet. **Note**: Be sure not to tip flask toward the neck when transporting to avoid possible contamination. Always check you cells for confluence and contamination under the microscope before you do anything.
5. Aspirate media from cells using a new 2 mL serological pipette. **Note**: Instead of replacing the entire pipette in subsequent steps, you can instead place a 200 µL pipette tip on the end of the serological pipette, and swap that as needed.
6. Add 3 mL 1X PBS with a 5 mL serological pipette and pipette aid and gently rock the flask back and forth. **Note**: When pipetting, be sure to always hold the pipette upright to avoid fluid contamination into the pipette-aid.
7. Aspirate PBS with the 2 mL pipette into the vacuum flask.
8. Repeat steps 6 & 7. Use a *new* serological pipette to avoid contaminating the PBS. These PBS wash steps serve to ensure removal of *(i) ions that promote binding of cells to surfaces* and *(ii) trypsin inhibitors that are present in serum*.
9. Add 2 mL 0.25% EDTA-Trypsin to cleave receptors involved in cells adhering to the surface and to chelate the ions that promote binding. COAT EVENLY. If you are trypsinizing a larger flask, like a T75, you will need more trypsin to coat the bottom of the flask, 5 mL would be adequate for a T75.
10. Place flask in the incubator for 5 minutes.
11. View under the microscope to ensure cellular detachment. If there is still a small proportion of cells that remain attached, agitate the flask to detach the remaining cells by gently tapping the bottom of the flask. If the majority of the cells are attached, wait 2 more minutes and check again. **Note**: Horizontally maintain the flask (i.e. keep it on a flat plane) when agitating. See examples during the demos.
12. Pipette 4 mL of fresh media into flask with a 5mL serological pipette. Suck up the solution and rinse the attachment area several times to ensure that all the cells have been collected.
13. Pipette cell solution from the flask into a 15ml conical tube. Mark the tube so that you can identify it.
14. There should now be ~6 ml of cell solution in your tube. Gently flick the tube or agitate to resuspend the cells and quickly remove 200 µL to a sterilized microcentrifuge tube (this will be used for a hemocytometer count). Centrifuge the 15 mL conical tube at 150 x g (2500 rpm in our machine) for 5 min. **NOTE:** do *not* start the centrifuge without checking with the TA to make sure that it is properly balanced.
15. While your cells are centrifuging, count the cells in your microcentrifuge tube by resuspending them and transferring 10µL to each side of a hemocytometer. Calculate how many cells are spinning down in the centrifuge.
16. Aspirate supernatant from tube, bringing the aspirator down to the point of the curvature, then tilting the tube to the side to allow the remaining media to move to the aspirator, and being *very careful* not to touch the cell pellet with the aspirator. A slight amount of media will remain attached to the pellet. **Note**: A new tip on the aspirating pipette can be used here in case the previous was contaminated by being out in the open.
17. Knowing how many cells are in your large tube, resuspend the pellet in the appropriate volume of fresh complete media so that you have a final concentration of 1 million cells per mL. Ensure a homogeneous, even distribution of cells by pipetting up and down. It is important that you completely break up the pellet. Be careful not to overflow the media in the 15 mL tube when pipetting up and down. Ask your TA for help if you do not feel confident about this step.
18. Plate your cells at the specified concentrations with the provided well plate, be sure to label each well properly: 500, 4000, and 10000 cells/cm^2. For example, assuming your cells are at 1 million cells per mL, and that you need a total of ~100,000 cells for a well seeded at a density of 10000 cells/cm^2. You would take 100 uL of cells from your cell solution, and combine that with 2.9 mL of media (you want to have 3 mL of media in each well

plate).

19. Plate the rest of your cells into a new T-25 and properly label it.
20. Remove the aspirating pipette and place it in the biohazard sharps container.
21. Review cells under the microscope. You should see different densities of round cells floating in different planes for each of your wells. *Note*: The microscope should be ethanol sprayed prior to placing the flask on it.
22. Spray hands with ethanol.
23. Wipe flask with ethanol sprayed kimwipe prior to placement in the incubator.
24. Ethanol spray and wipe work area. Clean up for the next section!

Cell culture reference table

Plate	Surface Area (cm^2)	Trypsin (mL)	Growth Media (mL)
6 well plate	9.6	2	3
24 well plate	2	0.5	0.8
96 well plate	0.5	0.1	0.2
T-25	25	2	4
T-75	75	5	10

Proper Labelling

Cell Culture Flask: `3T3 P1, "Experiment", 9/30/18, AM`

Liquid reagent (ie. Media): `DMEM Complete, Section 1, 9/30/18, AM`