CELL BIOLOGY LAB MODULE: SESSION 1

Location: 3110 Digital Computing Laboratory (DCL)

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Cell Biology Lab Exercise 1: Lifting and Seeding Cells

Supplies

70% ethanol in spray bottle

Kimwipes

Pipet aide in the biosafety hood

10 mL and 5 mL pipettes

Tube with cell media

Tube with PBS (phosphate buffered saline)

Tube with TRED (trypsin-EDTA)

Water bath set at 37^{o}C

Dish with cells

Glass Pasteur pipettes in sterile square metal box

Vacuum pump

Incubators with and without CO_2

Inverted phase microscope

New, sterile 10cm Petri dish

Procedure

1. Wash your hands and spray them with 70% ethanol.
2. Wipe down the surface of the biosafety hood with 70% ethanol and a large Kimwipe.
3. Take warmed media, PBS and thawed TRED out of the water bath. Spray (with 70% ethanol) and wipe the tubes you will be using. Place them in the biosafety hood, and loosen the caps before beginning. Get the pipettes you will be using out of the drawers. Spray, wipe and place these in the biosafety hood.
4. Remove your dish with cells from the incubator. View the cells under the inverted phase microscope to observe their shape and confluency. Take the dish to your biosafety hood and place in the hood.
5. Gently shake one glass Pasteur pipette from the metal container. Place the large end of the pipette into the vacuum tube, found on the right side of the biosafety hood. Remove the media from your dish with the vacuum pump (be careful not to scrape or touch the bottom of the dish with the pipette tip). Throw away the glass pipette in the sharps box.
6. Place a new 5 mL pipette on the pipet aide. Pipette 5 mL of PBS into your dish. Throw the pipette away. Rock the dish back and forth gently to spread the PBS across the surface of the cells. Try not to "spray" the PBS directly at the cells to prevent the cells from being washed away.
7. Using a new glass Pasteur pipette and the vacuum pump as before, remove the PBS from the dish. Throw away the Pasteur pipette into the sharps box.
8. Using a 1mL pipette and pipet aide, get 1 mL of TRED from the tube and dispense into the dish. Rock the dish for 5-10 seconds to distribute the TRED.
9. Place the dish in the "non CO_2" incubator for 1-5 minutes. During that time, check to see if your cells have detached.
10. When your cells detach, use a 5 mL pipette and pipet aide to obtain 4 mL of media and dispense into the dish. Gently pipette the liquid up and down.
11. Calculate how many cells are in your dish as you learned to do previously.
12. Write the volume of cells you need to seed in the new dish _____mL (1)
13. A dish holds 7-8 mL of solution. Calculate how much media you need to add to the dish. Write it here: _____mL (2)
14. Get a new 10 cm Petri dish. Spray, wipe and place it in the biosafety hood. Do not open the lid outside the hood.

15. Using a new pipette and the pipet aide, pipette the calculated media volume (2) out of the media tube and transfer into your new dish. Throw away the pipette.
16. Using a 5 mL pipette and the pipet aide, pipette the cell volume (1) out of your first dish and transfer into the dish with media. Gently swirl your dish in a "figure 8" to spread the cells around the dish. Throw away the pipette.
17. Replace the lid on all your tubes. Using a Sharpie, label your dish with your group member's initials around the top of the lid. Place your dish in the 5% CO_2 incubator.

Cell Biology Lab Exercise 1: Cell Handling

Information

Equipment in Cell Culture

There are several pieces of equipment and tools used commonly by those who practice cell culture.

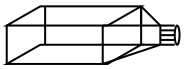
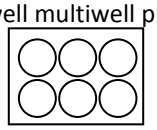
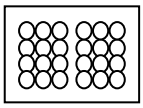
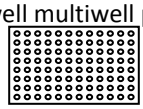
1. **Pipet aid or automatic pipette**: A tool used to aspirate and dispense volumes of fluid accurately and safely. Care must be taken to not pull fluid into the mouth of the pipet. Top button will draw fluid up into the pipet; bottom button will eject fluid from the pipet. Doing this either too fast or past the fluid level will result in the formation of bubbles that can disrupt your cells.

2. **Sterile serological pipets**: Pipets come in various sizes, wrapped individually with a cotton plug at the top. Carefully open the top of the package, insert end into mouth of pipet aid, turn the pipet so the numbers are facing you and then remove the rest of the package.

3. **Various serological or conical tubes and bottles:** Most commonly used are 4 mL and 15 mL round bottom tubes with a snap cap. The snap cap has two stops. To fully close the tube, press past the second stop. Conical tubes come in 15 mL or 50 mL size with a screw cap. Bottles have been pre-sterilized for this course and have a screw cap. To easily perform your techniques, it is best to loosen all lids in the hood before beginning. If you can, hold the lid in your hand when bottle or tube is open. If your dexterity does not allow for this, you may set the cap down either facing up or down (it is a point of argument as to which is more sterile)

4. **Micropipettes:** They come in various sizes and they are used for dispensing very small volumes of liquid.

5. **Culture dishes and dishes:** They come in various sizes (see table below). These are sterile packaged and the surfaces are usually treated in a way that the cells will adhere. Typical bacterial Petri dishes are not suitable for mammalian cell adhesion.

Tissue culture vessel	Surface growth area	Volume of media	Volume of trypsin
60mm (6cm) dish 	21.0cm^2	4.0mL	0.5-0.6mL
100mm (10cm) dish 	78.5cm^2	10.0-mL	1.0mL
T-25 dish 	25cm^2	7.0-8.0mL	0.5-0.8mL
6 well multiwell plate 	9.6cm^2 per well	2.0mL per well	0.2-0.3mL per well
24 well multiwell plate 	2.0cm^2 per well	0.8-1.0mL per well	0.08-0.10mL per well
96 well multiwell plate 	0.32cm^2 per well	0.1-0.2mL per well	0.01-0.02mL per well

Aseptic Technique

One of the most important concepts for performing tissue culture is the **aseptic technique**. The media the cells grow in is rich with very nutrients. Bacteria and fungi (present on every surface) as well as the cultured cells will proliferate in the media. The presence of bacteria and fungi will compete with the cultured cells for the nutrients and dissolved CO_2 and O_2. Even bacteria and fungi that would not harm humans can contaminate the culture.
The goal of aseptic technique is to avoid introducing bacteria and fungi into the culture. Doing so takes practice and attention to detail, but you should also use common sense. The following is a list of certain things to be aware of and guidelines to follow.

1. Work in a culture hood set-aside for tissue culture purposes. Most have filtered air that blows across the surface to keep microbes from settling in the hood.

2. Wear short sleeves or roll your sleeves up. Turn your baseball caps back if you MUST wear them, tie long hair back and remove rings and watches.

3. Wash hands with soap and water before beginning the procedure and rewash if you touch anything that is not sterile or within the hood.

4. Spray down your hands, work surface, and anything that will go into the hood with 70% ethanol. Rewipe at intervals if you are working for a long time in the hood. This will reduce the number of bacteria and mold considerably.

5. Do not breathe directly into your cultures, bottles of media, etc. This also means to keep talking to a minimum. No singing or chewing gum. No texting!

6. Work as quickly as you can within limits of your coordination. Also, keep bottles and dishes closed when you are not working with them. Avoid passing your arm or hand over an open bottle.

7. Use only sterilized pipettes, plates, dishes and bottles in the hood for your procedures.

8. Remove sterile pipettes from the package just before use. Set up the numbers on the pipet so that they face you. Never mouth-pipet, use the pipetting aid. Change pipets for each manipulation. If the tip of the pipet touches something outside of the dish or bottle, replace with a new one. Never use a pipet twice.

9. When handling dishes and plates, take care to not allow media to slosh near the rims or openings. This is one common way for microbial contamination to get into the dish.

Discard all used items immediately in the proper receptacle. Glass goes into a sharps box, plastic pipettes go into the trash bag, and excess cells are treated with a diluted bleach solution for a few minutes and then discarded down the drain.

Solutions Used in Media

1. DMEM (Dulbecco's Modified Eagle Media**)** - buffered (at pH 7.2) media used to grow cells *in vitro*. Contains glucose, glutamine, and essential salts, which are necessary as energy sources and for proper balance for cell growth. It also contains a pH indicator (phenol red) to indicate when the media is too acidic (orange) or too alkaline (purple).

2. Pen/Strep (penicillin/streptomycin) - two antibiotics added to the DMEM to keep the levels of bacterial contamination to a minimum. Usually added at a 1% of total solution.

3. FBS (Fetal Bovine Serum) or Newborn Calf serum (NCS) - serum added to the media as a nutrient source for the growing cells. Serum is the fluid obtained when blood is allowed to clot and the clot (containing cells and clotting factors) is discarded. The substances in FBS are undefined and vary from batch to batch so is not used by some researchers and is avoided for industry purposes. The components of serum are also the items that make the media "bubble". Care must be taken to avoid bubble formation in the culture as it will disrupt the cell layer or possibly introduce microbes. Added at 5-10% of total media solution.

4. Sodium Pyruvate - a component of the Kreb's cycle needed by most cells to grow in culture. Sometimes included in the DMEM. Added at 1% of total solution.

5. Sodium Bicarbonate - buffers the solution. Helpful for some cell lines grown at higher CO_2 concentrations.

Cell type classification

Cells come from many sources and can be classified depending upon that source. Below are a few common definitions.

1º Culture - cells taken directly from a tissue (i.e., fibroblast from an embryo chick tendon) and grown *in vitro*.

2º Culture - are cells taken from a primary culture and passed or divided *in vitro*. Therefore, once a primary culture has divided and proliferated it can be termed a secondary cell culture. These cells normally have a limited number of divisions or passages. Once they reach their limit they will undergo *apoptosis* (programmed cell death).

Cell Line - some cells in a passage may undergo a genetic mutation, thus altering them in such a way as making them immortal. They will grow beyond their limit: therefore, will not undergo apoptosis. Such cells will grow indefinitely. This is the definition of the cells you are using in lab today.

Transformed Cell Line - this is a cell line that has been transformed by a tumor-inducing virus or chemical. These cell lines often grown without attaching to a surface and will proliferate to a much higher density in a culture dish. Transformed cell lines have the ability to cause tumors (benign or cancerous) when injected into a susceptible animal.

Hybrid Cell Line (Hybridoma**)** - cell line where two different cell types have been "fused' together creating a single cell which often confers the ability of both individual cell's characteristics into one.

More cell culture terminology

Contact inhibition - A normal cell (primary, secondary or cell line) culture will exhibit this phenomenon, where the cells that come into contact with other cells will cease their growth and arrest in G_0 of the cell cycle. Transformed cell lines tend not to be constrained by this density-dependent inhibition of cell division, and will "pile up" in layer upon layer as they proliferate.

Anchorage dependence - The majority of cell cultures require that the cells bind to an extracellular surface (matrix) for growth. Transformed cells lack this dependence for anchorage and thus can grow freely in the culture/media solution. Cells that float freely *in vivo* such as blood cells also are anchorage independent (also known as suspension culture).

Passage number - The number of time the cells have been removed from the culture plate and then replated. Sometimes, the passage can refer to the completion of a cell cycle, but this definition is not pertinent to our lab today.

Additional Equipment used in Cell Culture

CO_2 incubator - humid chamber kept at 37° C and usually between 5% -10% CO_2 where cells can grow.

Non-CO_2 incubator **-** heated dry chamber without additional gas, used to expedite the trypsinization process.

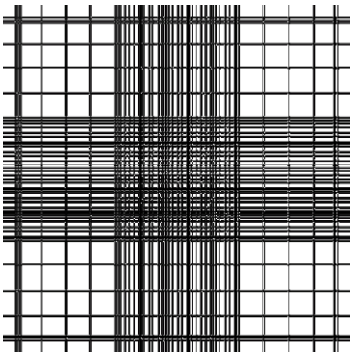
Vacuum aspirator – is placed near the hood, and used to permanently aspirate liquids from the plate or dish.

Inverted phase microscope - The primary obstacle of biological microscopy is that most objects have poor contrast (most everything is primarily water). Phase microscopy exploits differences in refractive indices in different areas of cells to create contrast, thus no stain is needed. The objective lenses are below the sample and the oculars are above, thus the term 'inverted'.

Hemacytometer - specially designed counting chamber with a precisely etched counting grid for counting cells. Each square of the hemacytometer represents a total volume of 0.1 mm^3 (10^{-4} cm^3) under the coverslip. Since one cubic cm is approximately 1 mL, you can determine the cell concentration and the total number of cells using the following calculations:

$$\text{cells/mL} = \frac{\text{total number of cell}}{\text{number of squares x dilution factor x } 10^4}$$

DIAGRAM OF ONE CHAMBER OF A HEMACYTOMETER



Other Useful Solutions

PBS-CMF (phosphate buffered saline-calcium magnesium free) - buffered solution used to "wash" or dilute cells. It is buffered at 7.2 so as not to disrupt the cells when rinsing. It is warmed to 37^{o} C before use.

TRED (trypsin-EDTA) - used to detach the cells from a culture dish. Trypsin cleaves a peptide bond (lysine or arginine) in the fibronectin (of the extracellular matrix) while EDTA chelates calcium ions (on which cell-cell adhesion depends) in the media. This is also a reason we use PBS that is free of calcium ions, since it is such a vital component in extracellular binding. Since trypsin is an enzyme, it works better at higher temperatures so the plate with a thin layer of TRED is set into a non-CO_2 incubator for a few minutes to increase the speed of enzyme function.

Extra cautions while using TRED:

- When warming to place on cells, don't leave it in water bath too long or it will self-digest.
- When initially adding TRED, remove the extra fairly quickly or you will aspirate all of your cells.
- Trypsinizing cells for too long (greater than 10-15 minutes) will reduce cell viability.

Trypan blue - an exclusion dye. Cells that are living have intact membranes and cannot take up the dye. These cells will appear bright and refractive under the phase microscope while dead or membrane damaged cells will take up the dye and appear dark. Useful in conjunction with the hemocytometer to count cells and determine their viability.

Bleach - used to kill any remaining living cells in the dishes and tubes before disposal. This is an acceptable method for disposing of Biosafety Level 1 materials.

Observing Cultured Cells

While maintaining your cultures, you will want to assess their health before you work with them. You also need to do this every day if possible in order to determine if your aseptic technique is good and your media is appropriate. This is easy to do by making the following observations:

Check the color of the media. It indicates the pH of the media and the health of the cells. After a day or two of growing, it is normal for the media to appear slightly orange (acidic) since the cells waste products will lower the pH. However, a yellow color may indicate microbial growth. If the media turns dark red or purple, this is a bad sign because there it indicates a problem with the CO_2 concentration (usually too low, or you forgot to loosen the cap of your dish). Your cells are most likely dead at a high pH. Cells can tolerate slightly acidic media.

Observe the cells under the inverted phase microscope. Healthy 3T3 fibroblast cells will be spread out and adhere to the surface of the plate. Dead or dying cells will be rounded and appear highly refractive (bright). Dividing cells will also appear round and refractive because they must detach from the dish in order to divide (look for mitotic figures). If most of the cells are floating or are in an uncrowded dish, your culture is most likely dead.

After passaging, healthy cells will adhere to the plate within an hour or so. Cells are susceptible to damage from rough treatment including the addition of cold media, over-trypsinization, foaming the media with the pipette, and exposing the cells to air for too long. Treat your cells gently.

Observe the *confluency* of your culture. A fully confluent plate has cells covering most of the surface with very little space between cells. If about 60% of the plate is covered with cells with 40% of the plate visible, the culture is considered 60% confluent. Best results are obtained with a plate of about 70-90% confluency (a 20% confluency will not give good cell counts and most of the cells may be lost during passaging).

Once anchorage dependent cells are growing, what can you do to keep them growing?

1. **"Feed them"**: Remove old media and replace with fresh warm media. Usually done when confluence is low and culture is less than 4-5 days old.

2. **Lift and replace on same plate**: Remove old media, rinse plate with warm PBS-CMF and using trypsin EDTA, lift the cells from the plate and then replace media. Again, this is for low confluence but when culture is more than 4 days old. Secreted factors remain on the plate to facilitate growth. Detaching the cells may keep them from spontaneous "transforming" into an unusual phenotype.

3. **Subculture, pass (passage) or split them**: Different terms for the same process. Remove all media, rinse plate with warm PBS-CMF, trypsinize to remove cells from plate, resuspend cells in warm media and transfer some cells to a new plate (or plates) with fresh warm media.

CELL BIOLOGY LAB MODULE: SESSION 2

Location: 3110 Digital Computing Laboratory (DCL)

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Adjunct Associate Professor, Department of Bioengineering
Lab Assistant: Joanne Li, Department of Bioengineering

Cell Biology Lab Exercise 2: Staining of Actin and Nuclei

Supplies

Box of Kimwipes

1 piece of parafilm

1 Petri dish (6 cm) seeded with fixed fibroblasts

Foil box

10 ml PBS

1 ml glycine in tube

1 ml triton-X in tube

Liquid waste container

Micropipettor (1000µl) with tips

Procedure

1. Look at your cells using a microscope. Do they look healthy? Using a micropipette, gently remove the PBS from the dish. Place the tip of the pipette on one edge and tilt the fluid towards the pipette tip to minimize disruption of the fixed cells. Avoid scraping/touching the bottom of the dish with the pipette tip. Discard into the appropriate waste beaker.
2. Using a micropipette, gently add 1 mL of glycine and tilt the dish to cover the surface. Allow solution to sit for 5 minutes.
3. Gently remove the glycine and discard into the waste beaker.
4. Using a new tip, gently add another 1 mL of glycine and allow to sit for 5 minutes.
5. Gently remove the glycine and discard into the waste beaker.
6. Gently rinse five times with 1 mL of PBS five times. The same tip can be used assuming minimal contamination of the stock PBS.
7. Gently remove the last PBS wash and using a new tip add 1 mL of 0.4% Triton X-100 (detergent). Let it stand for 15 minutes in order to permeabilize the cell membranes.
8. Gently remove the Triton X-100 and rinse 5 times with 1 mL PBS. Discard all fluid into the waste beaker.
9. Use aluminum foil to cover and keep your cells out of the light as much as possible. Using a new tip, gently add 500 µL of rhodamine phalloidin. Put the aluminum foil on to hide the dish from light. Swirl to ensure that the dye covers the dish. Allow it to sit for 30 minutes.
10. Gently rinse with PBS 3 times and discard all solutions into the waste beaker.
11. Using a new tip, gently add 500 µL of DAPI and again cover it and protect it from light for 10 minutes.
12. Gently remove the DAPI and rinse with PBS 3 times. Discard all solutions into the waste beaker.
13. Gently add 1 mL of PBS and wrap the lid onto the dish with parafilm.
14. Store in the dark until your instructor is ready to help you view your cells on a fluorescent microscope.

Information

The cytoskeleton

Virtually all eukaryotic cells contain an intracellular support system known as the cytoskeleton. Microtubules (approximately 20-25 nm in diameter), intermediate filaments (7-11 nm in diameter) and microfilaments (3-7 nm in diameter) are the three components of this web-like mesh that inhabits the cytoplasm and gives dimension and shape to the cell. Microfilaments, named because of their relatively small diameter, will be the focus of this exercise. The primary component of a microfilament is filamentous actin (F-actin). These actin filaments consist primarily of globular subunits (G-actin) which are actually single polypeptides about 375 amino acids long. When cultured cells are faced with a novel

environment such as new ECM proteins or artificial scaffolding made of metal, plastics or ceramics, the receptors on the cells sense this and send the information into the cell via integrins to the cytoskeletal components (particularly actin). Current research investigating cellular response to artificial surfaces has begun to discover that the cytoskeletal response to topography is transmitted to the nucleus and may determine the upregulating of genes that will eventually to choose whether the cell will adhere, migrate, proliferate, differentiate or undergo apoptosis.

Fluorescence as a research tool

Fluorescence microscopy is used to detect structures, molecules or proteins within the cell. Fluorescent dye can be a molecule that is a direct stain or probe for specific structures or it can be bound to a non-fluorescent probe (an antibody or other molecule) that recognizes specific structure.

The fluorescent microscope makes use of two highly specialized filters called **barrier filters**. These filters assure that only light of specific wavelengths reach the observer's eye. In the case of rhodamine, "red" light is emitted by the dye when it is excited by "green" light. Essentially, the first barrier filter only lets light with wavelengths of 520-560 nm (green light) pass through and this light is cast upon the stained object which then emits light at a much longer wavelength. This emitted light then passes to the second barrier filter, which allows only the red light (650-720 nm) to pass through to the viewer's eye. Thus, through the fluorescent microscope, we see rhodamine stained F-actin as red fibers against a black background.

This exercise highlights a prominent technique that is commonly used to visualize F-actin before and after specific manipulation. This technique involves the use of the fluorescent stain **rhodamine phalloidin** in conjunction with a fluorescent microscope. Phalloidin is a small bicyclic peptide of seven amino acids derived from the deadly mushroom *Amanita phalloides*. Rhodamine phalloidin is a fluorescent conjugate that readily binds to F-actin filaments, but unlike antibodies to actin, is unable to bind to monomeric G-actin. This provides a high level of staining precision resulting in an extremely large contrast between stained F-actin and non-stained background.