Cell Culture Experiments

Cell cultures are another way to observe and analyze biological systems. These in vitro (*in glass; outside the living body and in an artificial environment*) models allow experimental manipulation and direct observation of living and developing cells. Cell culture provides an opportunity to study aspects of cellular development and basic biochemical mechanisms in a controlled setting.

A major advantage of cell culture systems is that aspects of metabolic, chemical and morphological changes induced in the cultures by experimental variables can be assessed in a relatively straightforward fashion. Morphological changes in cells can be examined easily and photographed in living cultures using only a light microscope. Cultures can be fixed and stained for more in-depth morphological examination at any time throughout the culture period.

Cell cultures offer a novel approach for the examination of the direct effect of exogenous (*factors outside of the organism*) agents including cellular toxins, pharmacological substances such as drugs, etc., on specific groups of cells. Variables such as tissue distribution and metabolism of the experimental chemical used in whole animal studies can be avoided using cell cultures.

There are basically two types of cell cultures, primary and secondary. Cells obtained from a living animal and maintained in vitro are called primary cultures. Primary cultures can be further categorized into dissociated cultures or organotypic (*typical of the organism*) cultures. Dissociation or breaking the connection between cells either mechanically and/or chemically yields cultures with individual cells that can be plated into a culture dish. Organotypic cultures are not dissociated and whole pieces of tissue are placed in a culture vessel. Cells grown as organotypic cultures, also called explant cultures, maintain their relationships to surrounding cells, unlike dissociated cells that lose cell-to-cell interactions. Organotypic cultures are more appropriately called tissue cultures since the tissue relationships present in vivo (*in the body*) are maintained. All primary cell cultures have a limited life span and can be maintained for only a short period of time.

Secondary cultures are cell lines that have been derived from a single cell. Since these cell lines have been propagated for years they tend to be less differentiated than cells in vivo, and some of these cell lines have been obtained from transformed or cancer cells. Therefore, caution must be used in extrapolating information gained from the use of secondary cell cultures back to in vivo situations. However, they are a rich source of homogenous cells.

**PC6 Cells:**

You will be using a rat secondary cell line, PC6 (Figure 1.), derived from a pheochromocytoma, (from Greek phaios "dark", chroma "color", kytos "cell", -oma "tumor") a tumor of the adrenal glands that developed in an irradiated rat.

These are living cells. All of the metabolic processes that we will discuss in this course are happening in these cells, except, of course, photosynthesis! In addition, they are a very specialized type of cell called adrenal medulla cells.
Figure 1. Photomicrographs of PC6 cells at low and high density. Density refers to the number of cells per cm² of the culture vessel. The cells on the left at low density have just been placed into the culture vessel. The cells on the right have been allowed to grow in the culture vessel for several days, and the culture is said to be “near confluence” or close to covering the entire surface of the culture vessel.

You will design an experiment to examine the effects of a substance of your choice using these cells. Use your imagination! Are you curious about how a particular substance affects cells? Will the cells respond by increasing their number (cell division), or will the cells show an increase in the number of neurites, or will the cells die? Does the substance affect the cell cycle, growth potential, or is it cytotoxic? Unfortunately, there are some constraints on your choice of substance. NO ALCOHOL! Alcohol is a fixative and will simply kill your cells. Your substance must be soluble in water; in other words, it must dissolve in water. The medium used to keep your cells alive is water-based, and although lipid-soluble substances are often tested on cell cultures, this just adds one more level of complication. (Can you think why?) You may select a substance that is not “pure,” but you should realize that any of the other components in the compound might affect the cells in addition to the substance you selected. For example, if you want to test the effect of aspirin on your cells and want to use a tablet of aspirin that you purchased over the counter, realize that you are really testing the effects of acetylsalicylic acid and everything else in the tablet that may include polyethylene glycol, calcium phosphate and/or lactose.

Besides choosing your substance, you must determine the concentration of the substance to use. You will have to conduct a literature search to determine if your substance has been used in in vitro preparations, what concentration was used, and how cells responded to the substance. You may choose a substance that has not been evaluated in vitro; in this case, you will have to decide on your own concentration.
Making Solutions:

Knowing the final concentration of your substance in the medium surrounding your cells is crucial to your experiment; therefore, spend some time getting used to the nomenclature used to describe and to make-up solutions. You may make solutions depending on percentage of substance in solution (w/v) or based on molarity. There are also formulas to convert between percent and molarity.

Molar Solutions: Moles of compound in 1 liter of solution

Molarity is defined as the number of moles of a chemical or substance in one liter (L) of a solution. One mole is equal to one gram molecular weight of a substance, so 1 mole/L is equal to the molecular weight (or formula weight, FW=grams/mole) of the substance in grams dissolved in 1L and expressed as a 1molar solution or 1M. For example, the molecular weight of table salt or sodium chloride (NaCl) is 58.44g. A 1M solution of NaCl is made by dissolving 58.44g of NaCl into a final volume of 1L of water. Notice that you do not add 1L of water to 58.44g of NaCl but you dissolve 58.44g of NaCl in a smaller volume, then you add enough solvent (usually water) to a total volume of 1L. A 0.1M solution, or 1x 10^{-1}M, would have 5.84g of NaCl dissolved to a final volume of 1L. A 1mM (1 x 10^{-3}M) would have 0.0584g or 58.4mg of NaCl dissolved to a final volume of 1L.

Percent Solutions (w/v): Grams of compound in 100ml of solution

A percent solution (w/v) is the number of grams of the compound in 100ml of solvent. A 1% solution of any compound would have 1g of the compound dissolved in 100ml of solvent. For a 1% solution of NaCl, you would add 1g NaCl to a smaller volume of solvent until the NaCl dissolved, then add more solvent to a total volume of 100ml. A 3% solution would have 3g of NaCl to a total volume of 100ml, a 10% solution would have 10g to 100 ml.

Stock Solutions and Final Concentrations:

If you add several solutions of a particular concentration together, each of the solutions will be diluted according to the final volume of the combined solutions. If you add 1ml of a 1M solution of NaCl to a final volume of 10ml, you have just diluted your 1M solution 10 fold or 0.1M. 1ml of 1M to a final volume of 100ml would be 0.01M. Another way to consider this is in terms of the dilution of a stock solution. If you wanted a solution containing 0.01M (or 1 x 10^{-2}M) in 100ml, you might start with a stock solution of 1M which would be 100 times (100X) the final concentration you wanted, add 1ml of 1M and dilute it 100X or 1ml of 1M plus 99ml of solvent. A stock solution that is 5X would be 5 times the concentration you want; a 1X solution would be 1 part in 5 or 1ml of 5X and 4ml of solvent.

Requirements for Cells in Culture:

Cells grown in vitro require a culture medium, or “feeding medium” (FEED), that contains various factors necessary for cell attachment, growth, and maintenance of the cellular environment. Culture media contains a basal media formulation, various supplements depending on the cell type and, occasionally, antibiotics. A basal medium such as Minimal Essential
Medium (MEM) or RPMI 1640 includes vitamins and amino acids and is usually supplemented with various necessary components such as horse serum and/or fetal calf serum and glucose. The inclusion of animal sera to the feeding medium provides various factors; some of these factors are unknown or uncharacterized. FEED with serum supplementation are called, undefined media. Buffers, such as sodium bicarbonate, maintain the cellular and extracellular pH. Cells are usually grown in a CO$_2$ incubator. Sodium bicarbonate in the media equilibrates with the CO$_2$ atmosphere and maintains a pH of about 7.4 in the culture media. FEED is usually a “pink” color due to the addition of an indicator that measures pH of a solution. Media with a pH of 7.4 is orange-red; a yellow color implies an acidic condition (pH 6.5). A yellow color can be seen in cultures that are very active metabolically, sometimes due to a high number of cells in the flask, or in flasks that are contaminated with bacteria. Bacteria consume the nutrients producing acids. Cell cultures contaminated with bacteria die from starvation. Antibiotics may be added to the FEED to help prevent contamination.

The FEED needs to be changed every 2 to 3 days. Work out the feeding schedule for your cells; if you change the media on Monday, you need to change it again on Thursday. Changing the media on Tuesday requires another change on Friday. Keep an eye on the color of the FEED. Orange is best; if the FEED is yellow, there may be too many cells in the culture or the culture is contaminated. If your culture media is yellow and cloudy, then your cells are contaminated; do not refeed them! Contact an instructor!

Cells are grown at the body temperature of the original animal; for mammalian cells, this is 37°C. The CO$_2$ incubator in Dr. Barbara Christie-Pope’s lab (West Science 204) is set at 37° for mammalian cells.

Cultures can also be described as contact-dependent or contact-independent. All of the cells that we will be using are contact dependent meaning that they attach to a solid surface and do not grow suspended in the culture media. Cells that are dividing quickly may detach from the surface and then reattach after dividing. Occasionally cells may need to be subcultured or removed from their current flask, divided and placed into new flasks with fresh medium. This “splitting” of cultures requires removing the cells from the substrate and can sometimes be done by simply scraping the cells off the flask surface or by striking the flask. The cells are then collected by drawing them into a sterile, glass pipette, a procedure called aspiration. Repeatedly drawing the cells in and out of the pipette will break up any cell clusters. The cells are then divided into new flasks.

Some cells may require treatment with a proteolytic enzyme (this is an enzyme that digests proteins that adhere the cells to the flask) to release them from the culture vessel. Trypsin is usually used and the procedure is called trypsinization. Trypsinization requires a chelator like EDTA to tie up divalent cations like calcium and magnesium. Enzyme activity is inhibited by Ca$^{2+}$ and Mg$^{2+}$ ions. EDTA binds (chelates) these ions so trypsin can work. If you need to use trypsin, wash your cells with a Ca$^{2+}$, Mg$^{2+}$ free balanced salt solution prior to trypsinization. Adding these cations back along with medium containing serum stops the action of the trypsin. However, proteins are also found in the cell membrane. Exposing cells to excess trypsin or prolonged exposure will destroy the cells.

**Observation of Cells in Culture:**
Although one of the benefits of using cell cultures is observation of the cells in the living state, the microscopic examination of living cells requires special equipment. A special microscope, called an inverted scope allows a good visual image of cells in culture vessels.

Microscopic examination of cells requires that the cells be stained. You can’t stain living cells, not if you want them to continue living. Living cells look like bags of water and appear only slightly darker than the background. Using special optics called phase contrast on the inverted scope can enhance the contrast between the background and a living cell.

Aseptic Techniques:

One of the biggest requirements of growing and maintaining cells in vitro is aseptic or sterile techniques. Contamination can easily happen; the slightest touch of the tip of a glass pipette (Pasteur pipette) to an unsterile beaker can destroy your cultures and your project. Your cultures are not protected from contamination by the antibiotics in your FEED. Here are a few simple rules to follow to help avoid contamination.

1. Cell culture work is done under a laminar flow hood. You will be using the laminar flow hood in West Science 204.

2. Wash your hands with soap and water before working with your cells. Rinse your hands with 70% ethanol (EtOH). EtOH will dry your hands out; use hand cream after you are finished with your cultures.

3. Have everything you need within reach. Do not get up and leave your work area, then come back to it and resume working. Once you start, finish.

4. Loosen the lids/caps of all flasks, bottles, test tubes, etc. but do not remove lids until you are ready. You may have to remove a lid with one hand, loosening the lid will make this much easier. Immediately replace a lid when you through. If you need to put a cap down on the hood surface, place the inner surface down on top a paper towel soaked with 70% EtOH.

5. NEVER, NEVER POUR ANYTHING! Pipette all liquids. NEVER, NEVER MOUTH PIPET! Never let the pipette touch an unsterile surface. Even touching the tip of the pipette to an unsterile surface means contamination. Never lay a pipette down. If you must put down a pipette and intend on using it again, place it sterile tip down into a sterile test tube in a rack.

6. If a pipette has touched cells or the sides of a culture vessel, never place it back into a stock solution such as your FEED; this could contaminate your stock solution. You can use the same pipette to feed all your cultures, but be sure not to touch the tip of the pipette to the inside of the vessel; allow the FEED to run down the surface opposite to your cells. Do this gently; the flow of feed directly on top of your cells could dislodge them.

7. Discard old FEED into a sterile beaker. Splashing from an unsterile beaker back onto the pipette that you will use to remove FEED from the next culture will contaminate that
culture. After you are done feeding your cells, (replacing feeding medium) add a small amount of bleach to the beaker filled with old FEED and discard contents down the sink.

8. When you are finished working under the hood, remove all your materials, dispose of them properly and wipe down the workspace with EtOH.

**Counting Cells:**

Cell growth can be monitored easily by counting the numbers of cells contained within a culture. The density of cells in culture can affect cell proliferation and responsiveness to stimuli; therefore, it is important that each experimental condition be tested on cultures of cells plated at the same density. Both the number of cells plated into each well at the beginning of the experiment and the number in each well at the end of the experiment must be determined.

You will count your cells using an automated cell counter called a ViCell. The ViCell automatically mixes your cells with a viability dye called trypan blue. Trypan blue will only stain dead cells since living cells exclude the dye from passing the cell membrane. The ViCell provides the following information:

**Cell Count:** This is the total number of cells present in the sample. It is the sum of all of the cells counted by the ViCell. It has no relevance until it can be indexed to the number of cells present in a standard volume. In other words, you cannot compare cell counts between samples. This number is used by the ViCell program to calculate the total cells/ml (see below).

**Viable Cells:** This is the total number of living or viable cells in the sample. This is used to calculate the percentage of viable cells or what % of the cell count are viable cells. Remember that this is determined by the ability of living cells to exclude the dye trypan blue. Trypan blue cannot cross the outer cell membrane of living cells but can cross the cell membrane of dead cells since the membrane has been disrupted. Dead cells stain blue; viable cells are clear.

*Viability %:* is the calculation of the percentage of cells in the sample that are living. This is extremely useful in cytotoxicity studies, i.e. studies involving drugs or toxins.

**Total cells/ml:** This is the number of cells present in 1 milliliter of cell medium (“feed”). This is always represented as no. of cells x 10^6/ml. For example, a cell count of 0.5 x 10^6 cells/ml is 0.5 times 1,000,000 or 500,000 cells/ml. You should consider this measurement when comparing experimental groups such as controls vs. drug concentration.

*Viable cells/ml:* Total cells/ml includes all cells, viable or dead. This measurement only considers the total number of living cells. This is also a useful measurement to examine between experimental groups.

**Avg. diam (microns):** All of the cells in your cultures should be about the same diameter. However, if the cells have shrunken or expanded due to a substance or drug, this measurement will be smaller or larger, respectively, than that of control cells. Consider this in your analysis.

**Avg. circularity:** Cells that are dead may no longer be circular. This measurement may be in indirect way of determining if there is any debris in the sample.
Avg. background density: An increase in background density may indicate contamination (the media will be cloudy) or may indicate cellular debris from cells rupturing.

Note: You may want to analyze the 3 measurements indicated with an asterisk.