Module 4 Primer: Cell Culture Equipment, Media Preparation, and Cell Counting

Learning Objectives:
Students will learn to count cells with a hemocytometer and learn to use a biological safety cabinet (BSC). The teaching assistant will demonstrate passaging cells.

Background:

Laboratory Equipment for Cell Culture
Biological Safety Cabinet (BSCs)
Also called a “hood” or, more properly, a “laminar flow hood”. BSCs are used to protect laboratory personnel and the environment. BSCs provide a ventilated workspace for culturing cells that may be carrying pathogens. Three different classes of BSCs exist. The classification is defined by the level of protection. In BIOE202 we will be using Class II BSCs.

A secondary purpose of the Class II BSC is to protect the experiment (for example, cultured mammalian cells) from contaminants. The air is filtered by a HEPA filter that removes bacteria, viruses, and other contaminants. Class II BSCs are required for BSL2 work and are required to be inspected regularly to ensure proper function. BSCs need to be cleaned regularly to minimize the risk of experiment contamination.

While BSCs are not required for BSL1 work, we will be using them to protect our experiments from potential contaminants.

Unlike a fume hood, which pulls air up and out of the work area to protect you, a BSC blows air down in a curtain. Be careful not to obstruct any airflow in the hood. Avoid inducing turbulent flow or creating stagnant spots by blocking the air between the vents and your cultures. Avoid any dirty air flowing across your cultures. Work carefully when moving cultures between the hood, microscope, and incubator. Never breathe on your plates, and avoid anything that would cause air to flow into the hood (like talking or whistling). Keep lids on cultures and media as much as possible. One of the hardest skills to develop is minimizing the amount of time that lids are open for plates and bottles. When you become comfortable with the procedures and techniques, try to hold an open bottle and cap in the same hand without ever allowing the cap to be set down.

BSCs should never be used in place of a fume hood.


Incubator
A humidified chamber kept at 37 deg C and usually supplied with 5-10% CO₂ for
cell growth. Assume the inside of the incubator is sterile. Use aseptic technique when adding or removing anything from the incubator. Although the instructor and TAs will ensure proper operation of the incubators in your laboratory, research labs must replace the water bath and CO₂ supplies roughly once per month, and decontaminate the entire incubator every 3-12 months. In the BIOE202 lab one incubator is kept heated without CO₂ to use during cell passaging.

Vacuum Aspirator
A vacuum pump or building vacuum supply connected to a liquid trap and a hose running into the hood. Aspirating pipettes fitted into the hose provide a convenient way to remove fluid that is no longer needed. The liquid trap contains bleach (10% of final container volume) for disinfection of aspirated liquids. Only use aspirating pipettes with the vacuum aspirator. The cotton filters in the serological pipettes can become dislodged and clog the vacuum tubing and/or vacuum and cause damage.

Inverted Phase Contrast Microscope
The most commonly-used microscope in the lab, phase contrast microscopes are used to check the morphology and density of cell cultures. It is also used for cell counting. The primary obstacle of biological microscopy is that most objects have poor contrast. Cells are almost universally transparent and filled with water. Phase microscopy exploits differences in refractive indices in different areas of cells to create contrast, so no stain is needed. The objectives are located below the sample, thus the term "inverted." Under phase contrast, the edges of the cells become more defined.

Water Bath
The bath typically contains a few inches of water kept at 37 deg C for heating bottles of media and other reagents or materials that are stored cold but must be warmed before using with cells. Apart from growth medium, these liquids include:

- Phosphate buffered saline (PBS): widely used to wash or dilute cells. PBS has a pH and ionic content identical to cell medium.
- Trypsin-EDTA (or TRED): an enzyme that cleaves peptide bonds in the extracellular matrix, releasing anchorage dependent cells from the plastic and allowing them to be passaged.

In this lab, you will be using Trypsin-EDTA (TRED for short). Ethylenediaminetetraacetic acid (EDTA) is a calcium chelator, and it disrupts the bonding between cell-cell adhesion proteins called cadherins. Without EDTA, many trypsinized cells would aggregate or lift in a sheet.

There are two important things to consider when using trypsin.

- If left in a water bath too long, trypsin will self-digest and be less effective. Use trypsin as soon as it reaches room temperature.
- Cells left in trypsin for too long will be damaged by it. As soon as all cells
are detached from the surface, they should be resuspended in medium. Any remaining and active trypsin will then be occupied by the vast quantities of protein in serum and will stop damaging the cells.

Aseptic Technique Concepts
Aseptic technique refers to a set of practices designed to prevent cell cultures from being contaminated by any other organisms. The media used in cell culture are very rich in nutrients, and many bacteria and fungi will outcompete mammalian cells for these nutrients if they get into culture. Contaminated cultures do not behave normally, and mammalian cells frequently die as a result.

Use Sterile Materials
Anything placed in the hood should be sterilized ahead of time. Many materials (including plates and serological pipettes) are sterile until opened, and should be opened inside the biosafety cabinet. Others (including micropipette tips and glass jars) are sterilized in an autoclave before use. Anything that can't be autoclaved (pipet aids, micropipettes, anything from the water bath) can be sprayed with 70% ethanol before use. Spray down the work surface in the biosafety cabinet with 70% ethanol before working. Before putting them in the hood, wash your hands and forearms with antibacterial soap. Pull on nitrile gloves, and liberally spray the gloves with 70% ethanol. Any time you pull your hands out of the biosafety cabinet, spray them again before working. Never touch the inside of a plate, medium bottle, or bottle cap with anything other than a pipette. Ideally, avoid touching these surfaces even with a pipette. If any pipette touches anything other than the inside of a plate, medium bottle, or bottle cap, throw it away. This seems like a waste of material, but the cost of a contaminated culture is significantly greater than a wasted pipette.

Plan Ahead and Work Carefully
The longer your cells are outside of the incubator, the higher risk of contamination. Ensure that you have all necessary materials ready before bringing your cells into the biosafety cabinet. Work quickly but within the limits of your coordination. When handling flasks 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 culture. Loosen the caps on your bottles after putting them in the hood so that you can open them with one hand.

Cell Culture Media
Cells are cultured in a carefully balanced solution designed to provide nutrients, buffer pH, and maintain osmotic pressure. This section summarizes some of the ingredients you will encounter this semester.

Dulbecco's Modified Eagle Medium (DMEM)
Buffered (pH 7.2) medium used to expand fibroblasts. DMEM contains the salts and nutrients necessary for cell growth. When prepared with serum, it is often
referred to as `growth medium.' Some important components of DMEM include:

- Glucose: a sugar, energy source.
- Sodium pyruvate: a component of the Krebs cycle, also added as an energy source.
- Sodium bicarbonate: a buffer, balances pH in conjunction with CO₂ in the incubator.
- Phenol red: a colorimetric pH indicator.
  - Red: normal pH.
  - Orange or Yellow: acidic, often indicative of bacterial contamination.
  - Purple: basic, often indicative of low CO₂.

There are many types of cell culture media beyond DMEM. The specific medium is typically chosen based on the cell line being used.

Antibiotics
Penicillin and streptomycin are often added to keep levels of bacterial contamination in control. They are typically added together at 1 vol.% of the final solution, or 100 U/mL. Some media will also contain fungicide.

Serum
Serum is typically added at 10 vol.% as a nutrient source for growing cells. Serum is the fluid obtained when blood is allowed to clot and the clot (containing cells and clotting factors) is discarded. It contains high levels of proteins, including growth factors and adhesion proteins. The richest and most-commonly used serum is fetal bovine serum (FBS). The substances in serum are undefined and vary from batch to batch. Many researchers carefully test serum from several batches before ordering a large amount to maintain consistency from experiment to experiment.

Differentiation Factors
Additional supplements are often added to induce differentiation or provide raw materials for extracellular matrix maturation. For example, dexamethasone, insulin, and isobutylmethylxanthine will be added to growth medium to induce the 3T3-L1 cells to become adipocytes (fat cells).

**Counting Cells with a Hemocytometer**
A hemocytometer is a specially designed chamber used for counting cells. The bottom plate is etched with a grid to aid in counting cells, while the top of the chamber is a precisely-weighted cover glass. When properly assembled, each square of the hemocytometer (denoted by the dashed line below) has an area of 1 mm² and the cell suspension has a thickness of 0.1 mm, resulting in a volume of 100 nL. By convention, we inject ~10 μL of cell suspension into each side (two sides) of the chamber and count 5 squares on each side.
Cells counted in a hemocytometer are often first mixed with a dye called Trypan blue. The stain does not penetrate the membranes of viable (healthy) cells, but does enhance the contrast making them easier to count. Trypan blue will accumulate in the cytosol of dead cells, so we can use it as a measure of culture viability.

When trypan blue is mixed with a cell suspension, we can use equation 1 to calculate the cell concentration (in cells/mL).

\[
C_s = \frac{N \times D}{S} \times 10^4
\]

where \( C_s \) is the number cells in solution [cells/mL], \( N \) is the total number of cells counted, \( D \) is the dilution factor (with trypan blue), \( S \) is the total number of squares counted, and \( 10^4 \) is a conversion factor to get from 100 nL to 1 mL. A typical count (with a dilution of 1:1) after lifting a T-25 flask might be 200 cells in 10 squares, giving us \( 4 \times 10^5 \) cells/mL.
Figure 3: Diagram of cells to be included and excluded in the cell count.

### Table 3: Common Trypan Blue Dilution Factors

<table>
<thead>
<tr>
<th>Cell Suspension : Trypan Blue Ratio</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 1</td>
<td>2</td>
</tr>
<tr>
<td>1 : 2</td>
<td>3</td>
</tr>
<tr>
<td>1 : 4</td>
<td>5</td>
</tr>
<tr>
<td>1 : 5</td>
<td>6</td>
</tr>
<tr>
<td>1 : 9</td>
<td>10</td>
</tr>
<tr>
<td>1 : n</td>
<td>n+1</td>
</tr>
</tbody>
</table>

We often need to know the total number of cells we have in suspension. To get that, simply multiply your cell count by the total volume shown in equation 2.

\[
\text{Volume of Cell Solution Needed: } V_{cs} [mL] = \frac{\rho_s \times SA}{C_s} \tag{2}
\]

where \(V_{cs}\) is the volume of cell solution needed [mL], \(\rho_s\) is the cell density [cells/cm\(^2\)], SA is the surface area of the culture dish or flask [cm\(^2\)], and \(C_s\) is the viable number of cells in solution [cells/mL].

We will often count the numbers of live and dead cells separately to get a viability using equation 3.

\[
\text{viability(%) } = \frac{V}{T} \times 100 \tag{3}
\]

where T is total cells and V is viable cells.

Most healthy cell cultures should range from 90 to 100% viability. Large numbers
of dead cells indicate unhealthy or contaminated cultures. Ensure accurate viability calculations by counting the cells immediately after adding Trypan blue.

NOTE: Any cells left in Trypan blue long enough will die.

The trickiest thing about using a hemocytometer is identifying what is truly a cell and what is not. Viable cells should appear pale and round. Nonviable cells should be roundish and dark blue. You may observe oblong or geometric shapes of a similar size as cells. These are often aggregates of protein, and they can show up in serum that hasn't been mixed very well. Fibers from Kimwipes will also frequently show up in the hemocytometer.

Clean hemocytometers with 70% ethanol and dry them with a Kimwipe before and after use. It's best to clean your hemocytometer before you even add trypsin to your cells (~10 min before you need it), because any residual ethanol will immediately kill cells and throw off your viability count.

**Safety Considerations:**
Trypan blue is a suspected carcinogen. Gloves and lab coats must be worn at all times when handling Trypan blue. If you get Trypan blue on your skin or in your eyes you must wash the area or flush your eyes for a minimum of 5 minutes and seek medical attention. The Safety Data Sheet is located in the ELN. Use extreme caution when handling Trypan blue. Excess/used Trypan blue solutions need to be collected for proper disposal by the Division of Research Safety.

**Schedule:**
This is a one-session (Return Lab only) primer activity to prepare for subsequent mammalian cell culture labs (Module 4). While there is no quiz during this lab, subsequent quizzes and labs will rely on the information and techniques learned in this lab.

**Lab Exercises:**
Counting Cells
Practice Counting
Your TA will lift and resuspend cells with Trypan blue. Closely observe how the hemocytometer is loaded-you will be performing this next week. Using one of the microscopes and viewing a single hemocytometer, determine a cell count and viability. Record your answer on the whiteboard. When each student has recorded their calculation on the board, compute the mean and standard deviation (a measure of variation). Record these values in your laboratory notebook.

Practice Calculations
In the lab, you will often have to quickly determine how many cells to seed in a new plate after counting your cells. Given the mean cell count you derived above, calculate the number of cells required to seed the following vessels. Record
these numbers in your lab notebook and note whether it is possible given the cell density. Refer to Table 1 for media volumes.

1 *10⁶ cells in a T-25 flask
1 *10⁶ cells in a 10 cm plate
5 *10³ cells in each well of a 6-well plate
100 cells in each well of a 96-well plate

Table 1: Common cell culture dishes, flasks, and plates with the optimal and recommended medium and TRED volumes.

<table>
<thead>
<tr>
<th>cell culture vessel</th>
<th>surface growth area</th>
<th>media volume</th>
<th>TRED volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mm (6 cm) dish</td>
<td>28.27 cm²</td>
<td>4 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>100 mm (10 cm) dish</td>
<td>78.5 cm²</td>
<td>10 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>T-25 flask</td>
<td>25 cm²</td>
<td>7 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>T-75 flask</td>
<td>75 cm²</td>
<td>15 mL</td>
<td>3 mL</td>
</tr>
<tr>
<td>6-well plate</td>
<td>9.6 cm²</td>
<td>2 mL/well</td>
<td>0.3 mL/well</td>
</tr>
<tr>
<td>96-well plate</td>
<td>0.32 cm²</td>
<td>0.2 mL/well</td>
<td>0.02 mL/well</td>
</tr>
<tr>
<td>LabTek chamber</td>
<td>1.8 cm²</td>
<td>0.5 mL/chamber</td>
<td>0.05 mL/well</td>
</tr>
</tbody>
</table>

Preparing Growth Medium
Each group will prepare a single bottle of medium today. Your group will use this bottle in lab in the coming weeks. Ensure excellent aseptic technique is used, as a contamination today gets carried over for many labs.

Materials Needed:
bottle of DMEM (89 mL)
calf serum (10 mL)
pen/strep (1 mL)

Protocol
1. Place the tubes of calf serum (CS) and pen/strep (P/S) in the water bath to thaw.
   • CS and P/S are stored frozen.
2. Spray your work surface with 70% ethanol. Always wipe the hood down from back to front.
   • 70% ethanol is sufficient to kill most contaminants.
3. Spray your bottle of DMEM and thawed tubes of CS and pen/strep and place them in the hood. Quickly dry these with a Kimwipe if necessary-you do not want any ethanol to contaminate your medium.
• We clean the outside of the bottles and tubes after they have been in the water bath to minimize possible contaminants.

4. Loosen the caps on your bottles and tubes.
   • By loosening the caps ahead of time this makes it easier to work in the BSC.

5. Transfer 10 mL CS to the bottle of DMEM using the pipet-aid. Remember to avoid pulling liquid all the way up to the cotton plug of the serological pipet.
   • CS provides 3T3-L1 cells with additional nutrients and growth factors.

6. Using either the pipet-aid or micropipette (whichever you are more comfortable with), transfer 1 mL of pen/strep to the DMEM bottle.
   • P/S helps prevent contamination of our cultures.

7. Close the bottle of growth medium and pull it out of the hood. Label it with your names and place it on the appropriate shelf in the 4 deg C refrigerator.

8. Remember to spray down your hands before you put them back in the hood.

9. Clean up: throw empty tubes and used pipets in the trash and spray down your work surface.

**Before You Leave**
Ensure that your medium bottle is labeled and stored on the appropriate shelf in the refrigerator. Clean any spills and throw away any trash.