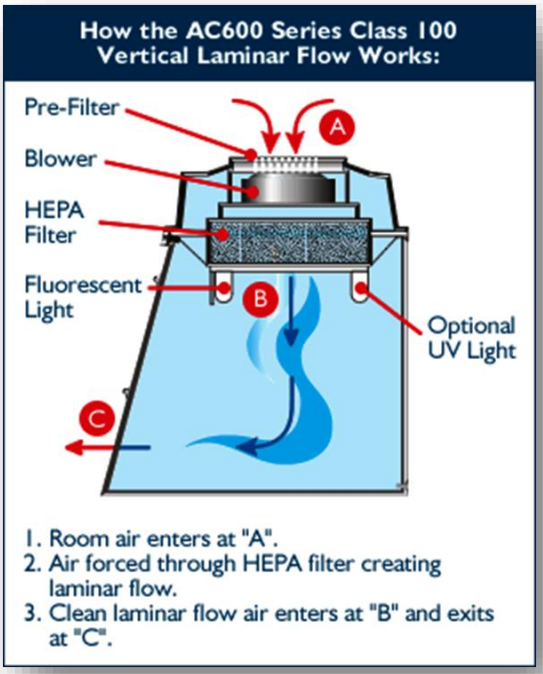
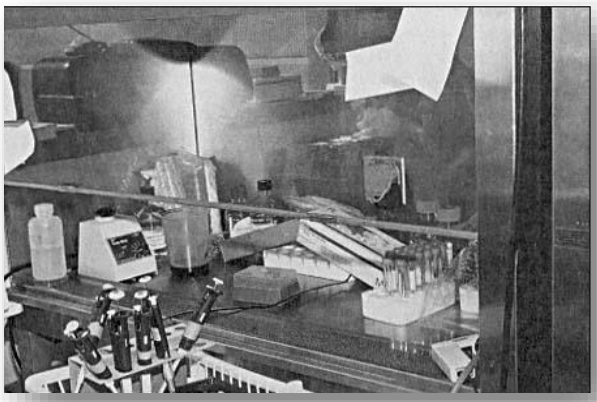
Setting Up a Cell Culture Laboratory
Basic equipments used in cell culture

General Equipments

- The tissue culture hood can be as simple as an open, laminar flow unit, with air passing initially through a HEPA filter and moving parallel to the work surface, exiting at the front of the hood.
- These hoods are generally available in 4-ft and 6-ft lengths, the latter being somewhat more convenient in terms of work space.
- Two people can also work side by side in a 6-ft hood. This is convenient if the experimental protocol requires two people to work together.



- Regardless of the size being used, it is important that the interior of the hood be as free of obstruction as is practical to optimize airflow.
- Keep only the minimum necessary equipment in each hood.
- Currently available biosafety cabinets have duplex electrical outlets, convenient for plugging in pipetting aids and gas and vacuum valves, and are equipped with UV lamp.
- There is little need for gas to supply a Bunsen burner in this type of hood. In fact, an open flame interrupts the airflow pattern in the hood and decreases barrier efficiency.
- In addition, an open flame creates a hazardous condition when working with flammable reagents in the hood. Thus, a flame should only be used when essential.



A dangerously overcrowded biosafety cabinet. The equipment stored in the hood and on the air intake grid will effectively break the sterile barrier for this hood.

Incubator

- It is important to know if the displays on the incubator control panel are reflecting in fact the actual conditions inside the chamber. For accurate temperature determination, a portable thermometer is recommended.
- The tissue culture laboratory needs a supply of CO_2 and any other gas that will be delivered to the chamber.

- Silicon tubing that can be sterilized by autoclaving should be used to connect the gas source to the incubator.
- Insulation is maintained by either a water jacket or an air jacket, with corresponding advantages and disadvantages.

- The critical point about incubator being to maintain an uninterrupted flow of gas.
- An in-line 0.2-μm filter to prevent introduction of mold and other potential contaminants.
- The water-jacketed incubator can maintain temperature over a longer period of time should there be a power outage, and this can be a critical feature for some installations.

- It is much heavier when filled, however the level must be maintained by periodic and the jacket drained when the incubator has to be moved.
- The air-jacketed incubator is lighter, has more moving parts to fail, comes up to temperature faster, but will lose heat much faster when the fan goes off (e.g., in case of an electricity).
- More importantly, the interior design and construction, materials used, and ease of assembly and disassembly can determine in part how well the cultures can be maintained free of contamination.
- Contaminating mold will grow on stainless steel, labeling tape, and even plastic, so it must be easy to remove shelves and clean when necessary.
- Copper shelving and interior walls can inhibit the growth of such organisms but it is expensive, and unless all hardware components are of copper construction, one cannot completely inhibit the growth of mold on interior surfaces.
- Routine cleaning of stainless steel or aluminum shelves with a disinfectant and ethanol rinse will help to reduce these risks.

- Be careful to use a disinfectant recommended by the incubator supplier. Many excellent disinfectants are volatile and will kill cultured cells as well as contaminants.
- The chamber should be allowed to equilibrate overnight after a thorough cleaning prior to returning the cultures.

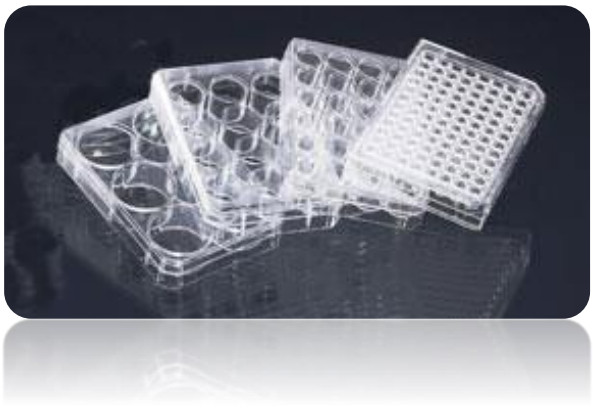
- The incubators run at 37C and 5% Carbon Dioxide to keep the medium at the correct pH.
- They all have meters on them to register temperature and gas level.
- There are alarms to indicate when these deviate from set parameters.

Keep the door open for as short a time as possible

Standard Microscopes

- The ability to capture an image directly from the microscope is becoming increasingly important.

Plasticware and Glassware



Tissue culture plasticware is available in a variety of formats, ranging from routine plates to larger multiwell plates in 96-, 48-, 24-, 12-, and 6-well configurations and flasks.

- Plasticware, although expensive, is now preferred over glass for pipettes, tissue culture dishes, and so forth.
- Glassware used for tissue culture should be autoclaved with moist heat for 30 min at a pressure of 15 lb/in^2.
- Empty bottles should have their screw caps loosely on and covered with foil. Partly filled bottles should have their caps tightened.

Freezers

- Liquid nitrogen freezers for long-term storage of cell cultures. These can be "portable" units, which can be moved when needed and hold up to 2,500 vials. This type of unit is filled with NO_2 manually.

- It is absolutely necessary to regularly monitor the liquid nitrogen levels.

- Cells may be stored frozen at -80C for a few months but lose viability rapidly at this temperature.

An example of a water purification system that produced water suitable for medium preparation

Water purification system

- The standard tissue culture laboratory should have a reliable source of water for preparing medium.
- There are some cell types that will not grow if water quality is poor.
- Do not use tap water or water straight from a deionizing column.
- The water source should be tested, as some bottled waters are better than others for making media.
- Some cell types being far more sensitive to water quality than others.
- Nonetheless, all cells respond to water quality and it is important to be able to control this as much as possible.

- Choice of media depends on the type of cell being cultured.
- Media is supplemented with antibiotics e.g. penicillin, streptomycin etc.
- Prepared media is filtered and incubated at 4 C.

Figure 4. Media sterilization through a 0.22 µm membrane filter assembled in a filter holder.

Vaccum counter

Particle counter

- An electronic particle counter, is extremely useful when conducting experiments that require numerous and regular cell counts.

General Equipments

Cell Culture Media

Introduction

- Nutrient mixtures that used for culture the cells are usually called *media.*
- Usually, these mixtures will almost always be supplemented with serum or another complex biological fluid (milk and plasma), or with a defined mixture of hormones and growth factors.

- The ongoing experimental works of understanding the medium with defined components, largely has been responsible for our increased technical ability to maintain a broad range of functional cells *in vitro.*

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All media are composed primarily of water. If media are prepared in the laboratory, the water source can make a critical difference in the quality of the media prepared.

This is especially important for serum-free media where trace metals and organic compounds present in some distilled or deionized water can cause severe toxicity problems for some types of cells.

What Does the Medium Do?

The nutrient mixture is the cornerstone of cell culture. Having the correct nutrient mixture can often be the determining factor in failure or success in growing a cell in vitro.

The nutrient mixtures can be substituted only by using even more complex fluids, which sometimes fail to support cell growth.

The medium provides essential nutrients that are incorporated into dividing cells, such as amino acids, fatty acids, sugars, ions, trace elements, vitamins, cofactors and ions, and molecules necessary to maintain the proper chemical environment for the cell.

Some components may perform both roles; for example, the sodium bicarbonate may be used as a carbonate source but also may play an important role in maintaining the appropriate pH and osmolality.

Media containing both "essential" and "nonessential" amino acids and also contain lipids; most contain a mixture of fatty acids, and some contain more complex lipids (e.g., cholesterol).

Some media contain macromolecules such as thymidine, adenosine, and hypoxanthine that can be synthesized by cells in vitro. Adding more of these to the medium may nonetheless improve the growth of some cells by maintaining an appropriate pool size of precursors in the cells.

Many media contain the common vitamins such as niacin, folic acid, riboflavin, inositol, thiamine, and so forth. While these vitamins are essential to continued cell replication, a detrimental effect may not be seen until several cell doublings after their removal from the medium.

Other vitamins such as vitamins D (1,25-dihydroxycholecalciferol), C (ascorbic acid), E (a-tocopherol), and A (retinol, retinoic acid) are not commonly added to media formulations because they are unstable in solution.

However, these may prove beneficial or even essential for some cell types and should be added separately. They may also be involved in maintaining the differentiated state of the cell, in regulating cell functions, or acting as antioxidants.

All media contain some energy source, usually glucose, although their levels can vary widely (0.8-5 g/liter).

Amino acids and glucose, as well as ions such as NaCl, having a nutritional role, as well as contribute to the osmolality of the medium.

In addition to the bicarbonate buffering system, the medium may also contain some phosphate buffer and perhaps complex organic buffers.

Most media contain phenol red as a pH indicator. This is very helpful in rapidly assessing the pH of the medium of all the cultures in an incubator. Phenol red can be added to media if it is not part of the medium powder or if a more obvious color is desired.

It should be noted than phenol red has weak estrogenic activity, which may be a consideration with some cells.

Medium may also contain antioxidants or reducing agents (or these might be added separately).

Types of media

Recently, several media commonly used.

Some media [e.g., minimal essential (ME) medium, Dulbecco's Modified Eagle's (DME) medium] were developed specifically for use with serum supplementation and high density growth of cells (Dulbecco and Freeman, 1959; Eagle, 1955).

In contrast, others such as Ham's nutrient mixtures F12, F10, and the Molecular Cellular Developmental Biology (MCDB) series of media were tailored specifically for growing a given cell type at low density (Ham, 1965; Ham and McKeehan, 1979).

Leibovitz (L-15) medium is designed to grow cells in air rather than CO2 and is useful when CO2 incubators are not available (e.g., the teaching laboratory), or when cells are shipped or handled extensively outside the incubator (for example, during a long tissue dissociation protocol).

Usually the media for growing mammalian cells have similar components, while the insect cell culture media (e.g., Grace's medium) is quite different, reflecting the different metabolic needs of insect cells

Note that composition and concentrations of components of several media differ qualitatively and quantitatively.

Commonly Used Media

Media	Applicable to
Basal media Eagle (BME)	Growing cells with serum
Minimal essential media (MEM)	Growing cells with dialysed serum
Dulbecco's Modified Eagle's media (DMEM)	Many virus transfected cells, growth with serum, high-density growth
Ham's F10 media Ham (1963)	CHO cells, low density, low serum protein
Ham's F12 nutrient mixture (F12)	Serum growth, many cells, serum fre
Media	**Applicable to**
William's media E	Rat liver epithelial cells
RPMI 1630	Mouse leukemia cells, cells in suspension
RPMI 1640	Human leukemic (and other) cells
Leibovitz L-15 medium	Buffered for air, human tumors
McCoy's 5A media	Human lymphocytes

Media	Applicable to
MCDB 131	Human endothelial cells
Media 199	Chick embryo fibroblasts
Neurobasal medium	CNS neurons
BGJb medium	Fetal rat long bones
Fischer's media	leukemia cells

- The CO2 setting on incubators should be chosen to match the medium to be used.
- Each medium has been formulated with components designed to work with a specified CO2 concentration (most ranging from 0 to 10% CO2-air mixtures) to provide a bicarbonate buffering system giving a pH of 7.0-7.4.

- Mismatch of medium bicarbonate levels and CO2 incubator levels will result in the medium pH being out of the optimal range for cell growth, resulting in slower growth or cell death.
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If media designed for use with different CO2 levels are to be used in the same incubator, the bicarbonate levels should be adjusted so that they all buffer correctly at the CO2 level to which the incubator is set.

- The ability to alter the bicarbonate concentration when the medium is made is clearly one advantage of preparing liquid medium in the laboratory from commercial powders.
- Considerations that should be kept in mind when altering the bicarbonate levels are; that the bicarbonate concentration itself is important for some cell types; that altering the

bicarbonate changes the total buffering capacity of the medium; and that altering the bicarbonate changes the osmolarity of the medium.

- If a new cell line is brought into the laboratory, determine what medium is recommended for its growth.
- This information can be obtained from the same source as the cells.
- If the recommended medium is incompatible with the CO2 settings on the incubator used for other cells grown in the laboratory, or is not commonly prepared in the laboratory, one might wish to change the growth medium.
- Keep in mind, when trying to repeat published data, that cells grown in a different medium may respond differently in some parameters measured.
- For example, estrogen-requiring cells previously grown in F12 medium and then switched to DME medium may have a diminished response to exogenously added estrogens in this medium because of the weak estrogenic activity of the phenol red, which is present at a much higher concentration in the DME medium.
- If one wishes to grow a primary cell in culture and no published data exist on growing that cell type *in vitro,* or one wishes to grow the cells in a different manner (e.g., with defined supplements rather than serum), it is best to screen several of the commercially available media before deciding on the one that is best for that particular use.
- This can be done by obtaining five to ten candidate media powders from a supplier, preparing them all in the laboratory using the same water and supplementary components, and doing a direct comparison of cell growth.
- If endpoints other than cell growth are important, measure these too in each of the media.

Media Preparation

- Media can be purchased as prepared liquid media, made up in the laboratory from dried powders containing most of the components of the nutrient mixtures, or prepared in the laboratory from individual stocks of the individual components or groups of the components.
- We do not recommend purchasing liquid medium, especially for serum-free culture work. Medium components deteriorate with time, and do so faster in solution. Some necessary components break down and are lost, others create toxic breakdown products or oxidize to toxic components.

- Some prepared liquid media can be frozen. Those that form a precipitate when thawed should not be frozen.
    - While it varies from cell to cell and with serum-supplemented or serum-free media, we have found that 2 weeks is a safe storage time for serum-free media, or longer if serum is added when the medium is prepared.
    - Outdated medium can be used for washing cells or preparing tissues for primary culture.
- In any case, it is always safe to store the prepared powdered medium and make liquid medium in the laboratory on a regular basis.
- Powdered nutrient mixtures generally have a shelf life of a year or more if stored in moisture-proof, airtight containers in the dark.
- If the laboratory does not use large volumes of media, the 1-liter packages are convenient.
- Preparing medium in the laboratory from components immediately before use is obviously the best way to insure that the medium contains the desired components in the desired form. This is essential if the investigator wishes to study the role of the nutrients themselves or to optimize the nutrient portion of the medium.
- However, most laboratories will find that preparation of medium from commercial powdered nutrient mixtures and a limited storage of the prepared media in a light-tight refrigerator will be adequate for their needs.
- All antibiotic agents have some toxicity, so any antibiotic to be used should be tested over a defined concentration range on the cells of interest.
- Serum can be added to medium when it is made up and the mixture filtered.

- However, since the serum has a longer shelf life than the medium and different cells may require different levels or kinds of serum, we find it more convenient to make up the medium without serum, store the sterile serum separately, and add it to the medium as needed for each experiment.
- When using disposable filters, the first 30 to 100 ml of medium through the filter should be discarded to avoid contaminating the medium with chemicals washed out of the filter.
- The media bottles should be labeled, dated, and stored at 4C°.

Serum Treatment

- Cell culture media can be supplemented with sera from any species of animal; bovine (fetal, newborn, or adult), horse, or human sera are the most frequently used.
- Additionally, serum varies from animal to animal, with changes in diet, and seasonally. Therefore, there is considerable variability from lot to lot of the commercially available sera.
- In addition to whole sera, which is allowed to clot and the clot removed, the blood can be collected with an anticlotting agent and the cellular portion spun out, resulting in plasma.
- Serum and plasma, even from the same animal, are quite different in composition and their effect on cells.
- Sera can be treated before use in one or more ways: filtration, heat treatment, or fractionation.
- These treatments can act as an added insurance against contamination, can remove or inactivate toxic components of the serum, can remove or inactivate growth-promoting or differentiating components of the serum, and specifically can remove low- or high-molecular-weight components of the serum or particular serum fractions.
- It is recommended that the only way to insure good results is to thoroughly test several lots of serum for their ability to support the desired cell characteristics (e.g., growth, differentiation, or lack of differentiation; specific biochemical markers; protein production, etc.) and then buy a large quantity of the best lot, store it at -20 to -80C°, and use it for the next several years.

- Most commercial sera come sterilely packaged. It is best to purchase serum that has been sterilely collected as well, as an added insurance against viruses or mycoplasma, which can go through some filters.
- Human sera should be collected from known donors or blood banks that test for the common viruses such as human immunodeficiency virus (HIV) and hepatitis.

Testing Media and Components-Quality Control

- We have stated that it is best to prepare media from commercially available powdered nutrient mixtures.
- It is important to keep good records and do quality control testing of reagents used in making the medium.
- We generally keep one set of glassware exclusively for medium making. This is rinsed well with distilled water but not washed with detergent between each use. This avoids the possibility of any detergent residue getting into the medium.
- When weighing out these reagents, a disposable tool should be used. This avoids contaminating these reagents with other, potentially toxic chemicals that may be in use in the laboratory.
- The major component of the medium is water.
- Water purity is very important for good-quality medium.
- Water quality can be more critical when cells are grown in serum-free medium.
- However, some cell types can be extremely sensitive to poor medium quality even when serum is used.
- We like to test media powders, critical hormones, and sera and buy enough of the best lot to last a year.

- Testing should include daily growth curves, from which one can calculate population doubling time and saturation density .

Medium Optimization

- There is still a need for more experimentation to derive the optimal media for other cell types (e.g., newly derived cell lines, human cell lines, etc.) or other culture needs (e.g., very-high-density culture, controlling differentiation through culture conditions).
- Optimizing the medium in which a primary culture or cell line is grown can lead to increased growth, increased protein secretion, increased viability, increased phenotypic stability, and better control of differentiation.
- Optimizing the nutrient mixture is an important part of this process.
- The best way to optimize the nutrient mixture is to sequentially perform dose-response curves on each component, select the optimal range for each, and retest each component.
- Note that the cells will tolerate a broad range of concentrations for some components, but will have a very narrow optimal concentration range for others.

Primary Culture

Types of Cultures

Primary Culture

Cell Line Culture

- **Primary culture** refers to the cells that are placed in culture directly from the tissue of origin.
- These are called *primary cultures* until the first subculture.
- After the first subculture, they may be called *secondary cultures,* and thereafter, if continued passage is possible, a *cell line.*
- Cells when removed from an organism and placed in suitable culture environment will attach and grow are called as primary culture.
- Primary cells have a finite life span.
- Primary culture contains a very heterogeneous population of cells.
- Sub culturing of primary cells leads to the generation of cell lines.
- Cells such as myocytes, macrophages and neurons do not divide in vitro so can be used as primary cultures
- . While the majority of cell culture studies use established cell lines, there are some instances in which primary culture is preferred.

- ❖ The desire to study normal terminally differentiated cell types, such as neurons, myocytes, or T cells *in vitro,* obviously would require primary culture, since these cell types do not divide *in vivo.*
- ❖ In addition, it has proved that some dividing cells don't maintain their differentiated function *in vitro* through multiple passages.
- In any case, there will always be a need to perform primary culture in order to study the properties of cells that are only recently removed from the *in vivo,* in order to learn more about their functions *in vivo.*
- These cultures can contain mixed cell types or consist predominantly of a single cell type.

However, primary cultures seldom consist exclusively of a single cell type

- During primary cell culture, attempts may be made to mechanically or enzymatically purify the cell type of interest during the tissue dissociation.
- In the next step, primary cultures can be maintained in conditions chosen to positively select for the survival of only one cell type (established cell line).

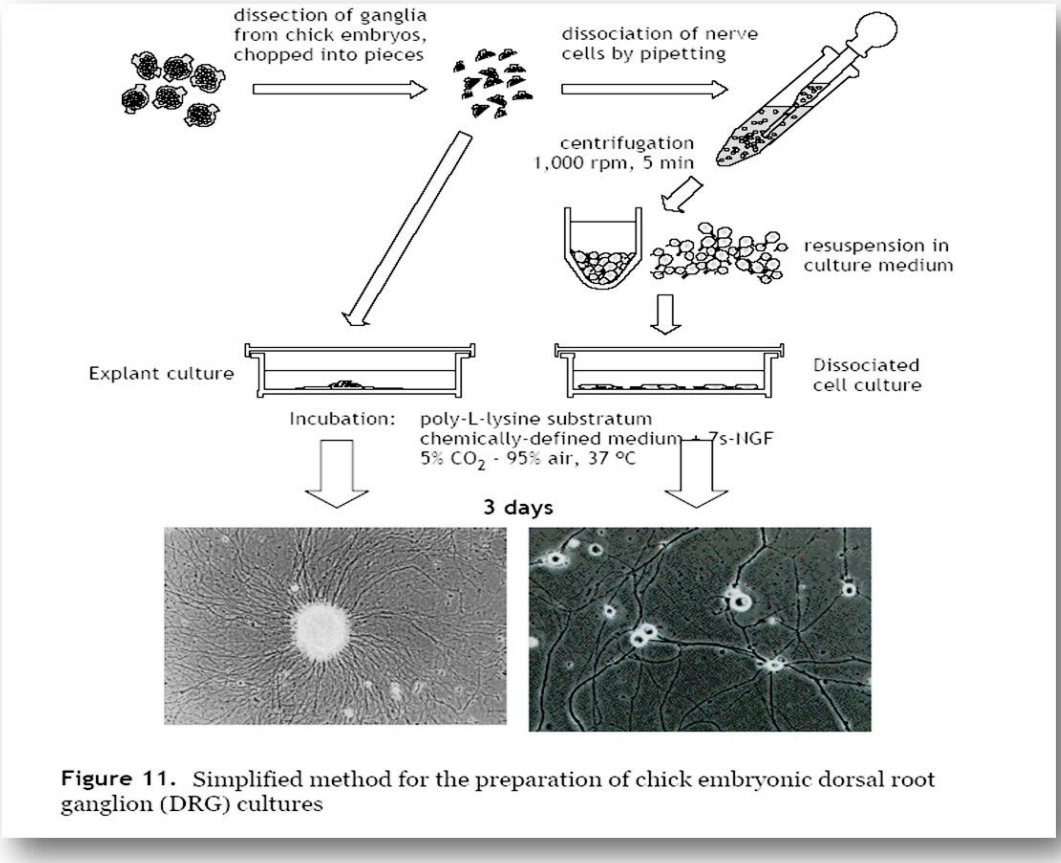


Figure 11. Simplified method for the preparation of chick embryonic dorsal root ganglion (DRG) cultures

- The drawbacks of primary culture are that;

1. They are frequently time consuming to prepare and require the use of live animals or fresh tissue.
2. There can be considerable variation from one preparation to another, particularly if prepared by different people.
3. Finally, the cultures are continuously changing from the time the cells are removed from the body until they die or adjust to the culture conditions used.

4. This can include changes in the mix of cell types in the culture, changes in cell shape, changes in cell-cell associations, and changes in the factors secreted from the cells and the receptors and other cell surface proteins present on the cells.
5. Primary cultures from rodent tissues can routinely be obtained from adult, neonatal and fetal animals.
6. The advantage in using adult tissue is usually the volume of tissue available for harvest.

But where the investigator is interested in establishing a cell line, long-term culture of a specific cell type, neonatal or fetal tissues have the advantage of providing a rapidly dividing population of cells whose long-term proliferative capacity is great and can frequently form immortal cell

❖ The general points in trying to obtain a primary culture is outlined below:

1. The preliminary experiments trying different factors and can often be performed in 24- or 48-well tissue culture dishes to obtain the maximal data with minimal tissue.
2. It is important to be able to see the cells in the dish and inspect them visually frequently.
3. Cell purity, cell yield or minimization of cell damage should take priority in designing your protocol.
4. Determine what markers will be used to follow the cell type of interest and to determine cell purity and function throughout the culture. It is important to have more than one marker if the cells are to be studied in long-term primaries or a cell line is to be obtained.

After dissociation check for viability. If the cells of interest are not released from the tissue, try another dissociation method.

- If the cells have been released from the tissue but are dead, try a milder dissociation.
- If the tissue is still undigested, try different dissociation enzymes, more enzymes, and/or longer treatment times.
- . Inoculate plates at high and low density. If live cells of the type desired are seen, continue incubating the cultures.
- 7. Try several different media and different supplements, including serum and serum-free, hormone-supplemented medium and conditioned medium.
- Determine the percentage of the cell type of interest in the culture and how this changes over the duration of the life of the cultures.
- 9. If none of the above has worked, try a different species (or strain) of animal, a different age of animal, or co-culture the cell type of interest with nearby cells or related (or not so related) feeder cell lines.

10. If you have had success, publish your achievements.

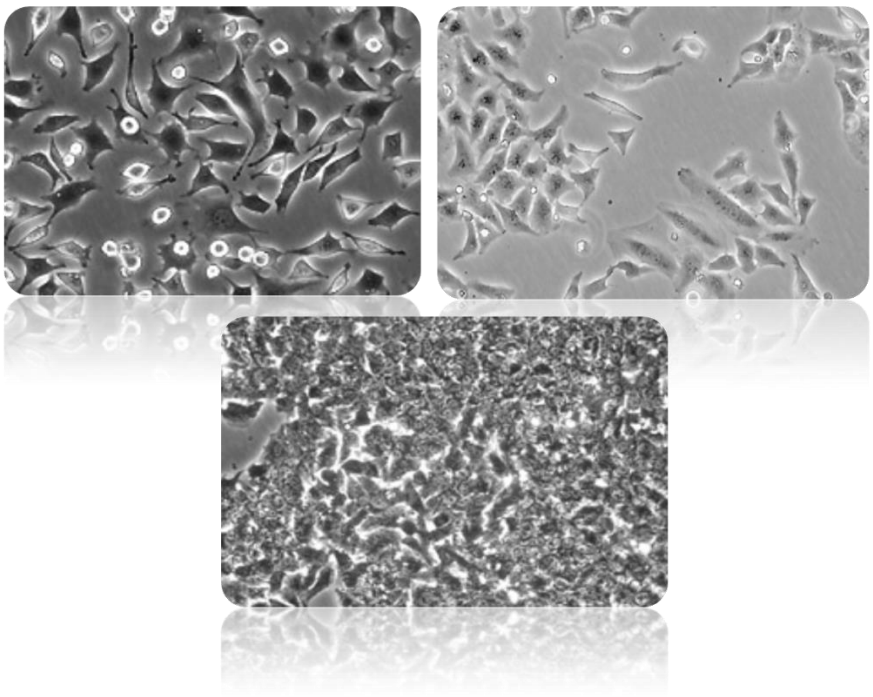
- Publishing a complete description of the method used to derive primary cell culture is important for all those investigators who may later use these cells.
- This initial publication should always be cited in any later publications using the cells.

Subculturing

- Subculturing is a process to provide periodically fresh nutrients and growing space for continuously growing cell lines.
- The frequency of subculture and split ratio, or the density of cells plated, will depend on the characteristics of each cell line being carried.
- If cells are split too frequently or at too low a density, the line may be lost.
- If cells are not split frequently enough, the cells may exhaust the medium and die, or a different type of cell may be selected for in mixed cell cultures.
- In general, once the correct timing and split ratio are found for a particular cell line, it should be used consistently for that line, with only minor variations when absolutely essential.
- In practice, subculture involves removing the growth media, washing the plate, disassociating the adhered cells, usually enzymatically (e.g. with trypsine), although some cells may be removed by repeated pipetting or gentle scraping, and diluting the cell suspension into fresh media.
- If the culture is maintained in serum-free media, however, it is necessary to neutralize the enzyme by using an appropriate protease inhibitor, such as soybean trypsine inhibitor.
- If this involves the use of serum and the split ratio is low (e.g., 1:100), it is usually not necessary to remove the residual enzyme.
- But, if the cell line is carried at a very high split ratio (e.g., 1:2-1:5), it is advisable to wash the cells after enzyme treatment even if serum is present.

Subculturing Adherent Cells

Subculturing Adherent Cells



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Materials/Solutions

- All materials/solutions are sterile:

1. Growth medium

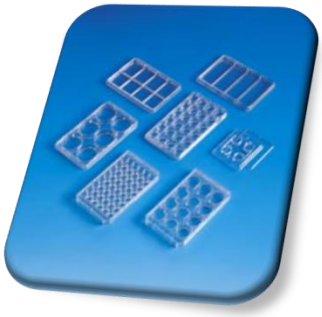
2. Phosphate-buffered saline (PBS)

3.Trypsine solution

4. Culture dishes

5. Hemacytometer or electronic particle counter

Subculturing Adherent Cells



Procedure

1. Remove medium from the plate.

- If the cell line adheres tightly, the medium may be discarded. 9
- If many cells in the plate are floating or only loosely attached, medium wont be discarded, the plate should be gently shaken or washed with a pipette and the loose cells saved and recombined with the trypsine-dispersed cells before replating.
- If this is not done, the overall phenotype of the culture will change with time, as each passage will preferentially select for the more tightly adherent cells.
- 2. Wash 1´ with 5 ml of PBS.
- 3. Add 2-3 ml trypsine. Allow the trypsine to cover the plate.

4. Incubate at 37C°. The time will vary depending on the cell type and whether it is a primary culture or an established cell line, but generally it will take 2-3 min.

- It is a good idea to remove the plate from the incubator and look to see if the cells have rounded up after about 2 min and check every minute thereafter.
- If the cells slough off when its side is gently tapped against the bench, they are ready.

Do not trypsinize beyond the time required to detach cells, since this will damage the cells and may reduce plating efficiency

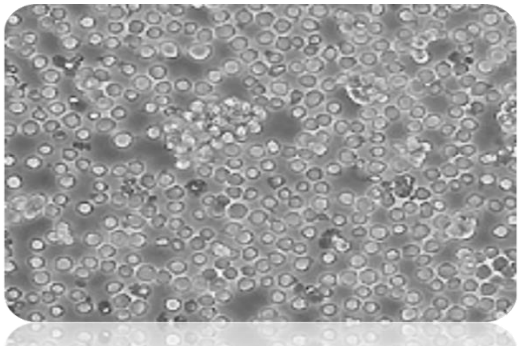
5. When the cells have rounded up and slough off, re-suspend in 5 ml of serum containing medium and wash cells by centrifugation at 800 rpm.

- Re-suspend in 5 ml medium. [This wash step may be omitted if the cells are to be split at a low split ratio (>1:50) in serum containing medium].

- If using serum-free media, the trypsine should be neutralized with 1 ml of a 1 mg/ml solution of soybean trypsine inhibitor (STI), diluted to 10 ml with medium, and wash by centrifugation for 3-4 min at 900 rpm.
- The supernatant is then aspirated and the pellet re-suspended by repeated pipetting in 5 ml of growth medium.
- 6. If the cells are primary or secondary cultures or the cell line is one with which you have had no experience, a high seeding density is recommended, i.e., 2.5 ml of cell suspension to 7.5 ml of growth medium (a 1:4 split ratio).
- If precise cell counts are needed, an aliquot of the cell suspension should be counted at this time.
- It is a good idea to seed stock plates at several densities, for example, 1:4, 1:8, 1:20, and 1:40 split ratio.

Subculturing Suspension Cultures

❖ Cultures of cells that grow in suspension in flasks can be maintained by diluting an aliquot of the suspension into fresh growth medium.



- All materials/solutions are sterile:

1. Flasks
2. Growth medium
3. Hemacytometer or electronic particle counter

Procedure

1. Hold the flask upright and pipette the cell suspension up and down two or three times to disperse any clumps.

Transfer 200 µl to 1 ml of the suspension to a fresh flask containing 10 ml of growth medium.

- If a split ratio is less than 1:10, the cell suspension should be placed in a conical tube, diluted to 10 ml with medium and centrifuged at 900 rpm for 3-4 min.
- Re-suspend the resulting cell pellet into fresh growth medium and aliquot the appropriate number of cells into the number of flasks needed.
- Note, while many published methods suggest washing or diluting cells in PBS, we prefer to use serum-free medium (outdated medium can be used here).
- This seems to improve viability, especially for handling delicate cells.
- Using serum to wash cells also prevents any rapid changes in osmolarity that may occur when switching from medium to PBS and back and maintains an energy source for the cells.
- Basically, with using serum the cells are less stressed during an difficult period.

Cell Viability

- Total cell number in a culture dish *in vitro* is a balance between the rates of cell growth, or mitosis, and cell death. So, the ability to differentiate between live and dead cells is therefore important.
- Cells can be counted before, during, and after setting up an experiment to accurately and directly monitor and standardize experimental conditions.
- For this purpose usually two methods can be used:

1. **Trypan blue method**
2. **Acridine Orange-Ethidium Bromide method**

- # Trypan blue method

- This method is a differential count of the cells that exclude trypan blue (viable) and those that take up the dye (dead).

- In this method live and death cell were counted by using a hemacytometer or an electronic particle counter

- The hemacytometer is undoubtedly the cheapest and most labor intensive method for counting cells, but it can be used to provide data as accurate as that obtained by any other method and to provide an assessment of both total and viable cell counts.

Materials

1. **Trypsinized cell cultures**

2. Improved Neubauer hemacytometer with coverslip

3. Counter

4. 0.4% trypan blue in PBS

5. Pasteur pipettes

6. Microscope

Procedure

1. Make a 1:1 dilution of cell suspension with 0.4% trypan blue. This can be further diluted with PBS if necessary.

2. Carefully re-suspend with a Pasteur pipette.

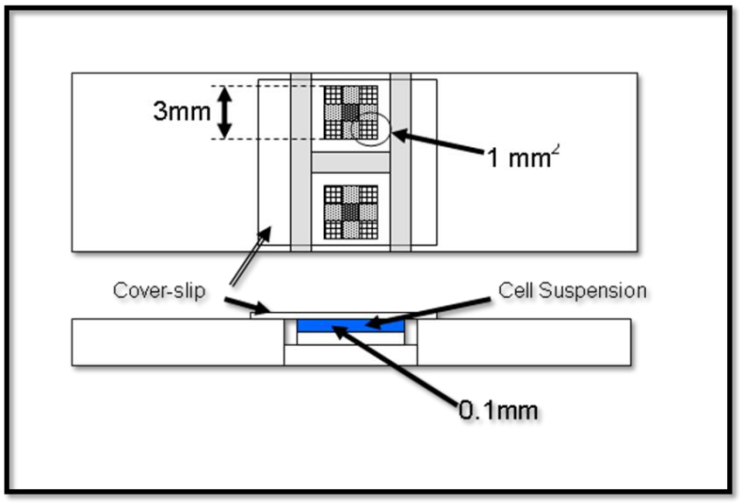
3. Cover the hemacytometer chamber with the coverslip and place a drop of the suspension from the Pasteur pipette at the edge of the "V" shape on the chamber.

- **Repeat for the other side of the chamber. It is important not to overfill or underfill, but rather to allow the drop to be drawn over the surface by capillary action.**

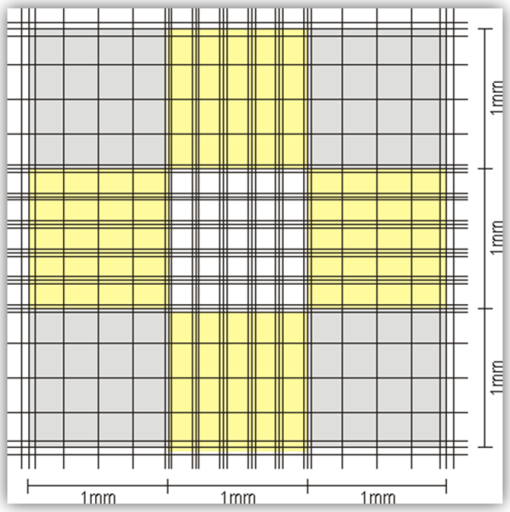
- **4. Place the chamber on the stage of the microscope.**

- **5. Initially focus on the etched lines of the chamber with low (4×) power.**

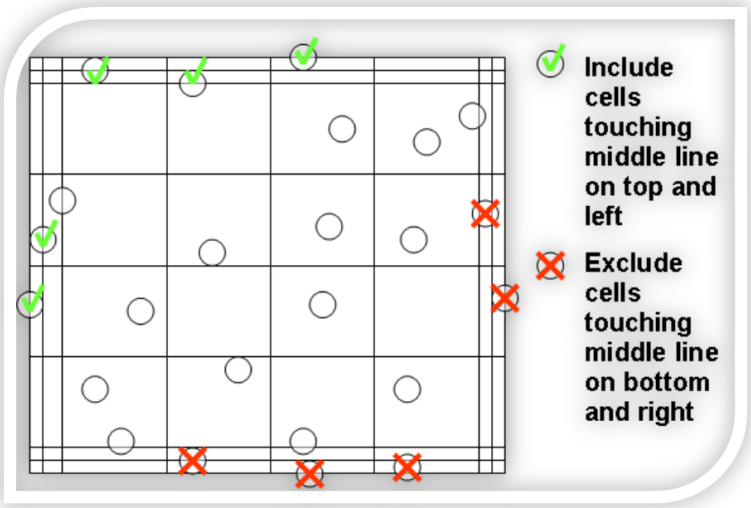
- **6. The hemacytometer consists of nine 1mm^2 squares that are divided into 25 smaller squares. The volume of one 1mm^2 square is 0.1 mm^3 or 10^{-4} ml(1mm^3=0.001ml). Using a 10× objective, focus on one of the 25 smaller squares bounded on all sides by three parallel lines.**



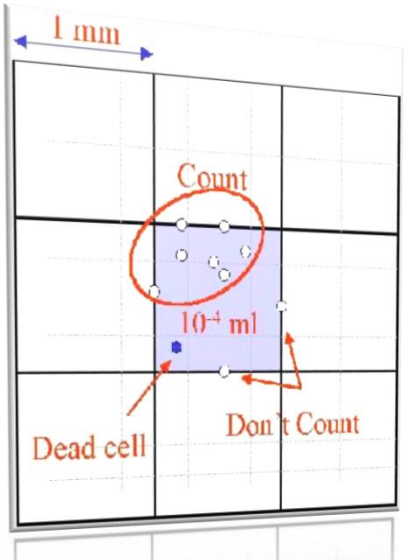
The hemacytometer shown can be used as an inexpensive method to determine viable cell number.

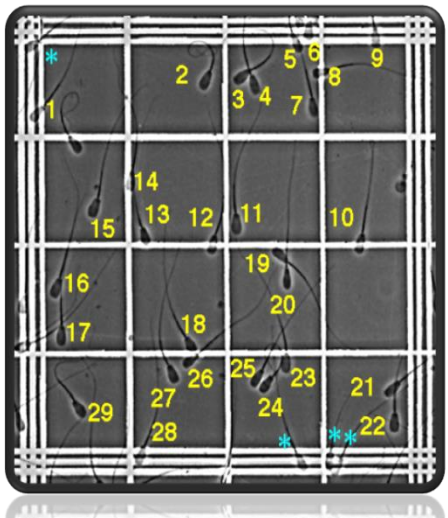


- For a viable cell number, count all the cells that exclude the dye. For estimation of percent viability separately count the blue cells and those that exclude the dye.
- A multichannel push-button counter is very useful here.
- In order to count cells that lie on the border, counting those cells that lie on middle line in the top and the right and not on middle line in the bottom and left depends on your orientation.



1. **Trypan blue method**





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7. Count at least 100 cells/mm^2 for calculation of percent of cell viability. If you count fewer than 100 cells in the square, count one or more additional squares.

8. Repeat for the second side of the chamber.

9. For calculation the number of cells/ml, multiplying the number of cells counted in a $1mm^2$ square (or the average of however many squares you counted) by 10^{-4}.

2. Acridine Orange-Ethidium Bromide method

- This method is quite easy to see the cells and do differential counts .
- A fluorescent microscope with an appropriate filter, however, is required.
- Both acridine orange and ethidium bromide are DNA intercalating dyes and therefore mutagens. Handle and dispose with care.

❖ Ethidium bromide is a dye that is only able to pass through the membrane of a dead cell. Acridine orange is a membrane-permeable dye that will stain all cells in the sample.

❖ EB also dominates over AO.

Materials

1. Trypsinized and prepare cell suspension.

2. Ethidium bromide-acridine orange (EB-AC) stock solution: ethidium bromide, 50 mg; acridine orange, 15 mg. Dissolve in 1 ml 95% ethanol and rise to a total volume of 45 ml in purified water. Store in 1ml aliquots at -20C°.

3. PBS

Procedure

1. Make a working solution of EB-AC by diluting a 1ml aliquot of stock solution into 100 ml PBS. This can be stored, light tight, at 4C° for up to 1 month.

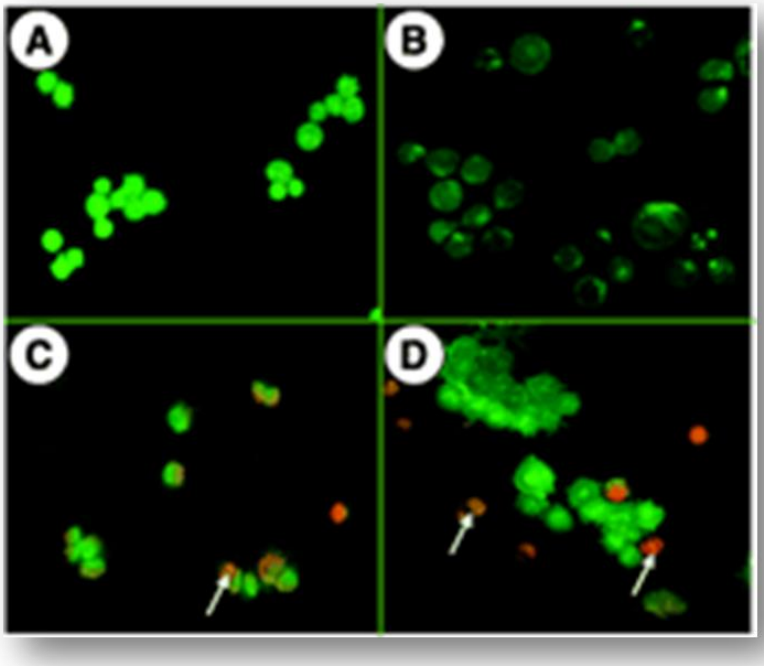
2. Mix equal volumes of cell suspension: EB-AC.

3. With a Pasteur pipette add a drop (100 µl) to a slide and cover with a coverslip.

4.Observe with an fluorescent microscope.

5.Count the live/dead cells with a counter. The live cells will fluoresce green and the dead cells will fluoresce orange.

6.Determine percent of viability by calculating: (Live cells/Total cells counted) × 100.



Freezing

Materials

1. Freezing medium. This should be 90% normal growth medium and 10% glycerol or 10% dimethyl sulfoxide as a cryoprotective agents.

2. Freezing vials, 2 ml or 5 ml.

3. Freezing container.

Procedure

1. Remove the cells from the plate using trypsin or whatever agent is generally used to subculture the cells.

2. Wash the cells once in fresh medium by gentle centrifugation.

3. Re-suspend in the freezing medium at the desired cell density.

4. Aseptically pipette the cell suspension into freezing vials.

Freezing vials can be polypropylene or glass.

Glass vials must be flame sealed and then leak tested by immersion in a dye bath before freezing.

Leaky vials not only might lead to cross-contaminated cultures, they might explode on thawing, as the nitrogen that entered during storage rapidly becomes a gas and expands.

However, cells frozen in glass vials will not leak unexpectedly and therefore cross-contaminate the culture, this is a more laborious and time-consuming method.

Most small laboratories now use polypropylene freezing vials.

Do not freeze or store cells in liquid nitrogen in any kind of container other than those designed for frozen cell storage.

Many plastics will become brittle at liquid nitrogen temperatures and explode when removed, possibly injuring laboratory personnel.

5. Label each vial individually with the designation and passage number of the cells, the date of freezing, and the initials of the person freezing the cells.

It is important that each vial be labeled, not just one label placed on the box or cane.

Boxes and vials can be dropped, and more often than not, only those individually labeled will be salvageable.

Many freezing vials have an area of the surface suitable for writing on with an indelible (make sure it is indelible in organic solvents and liquid nitrogen) ink pen.

If labels are to be taped or glued on, make sure the adhesive will withstand liquid nitrogen temperatures.

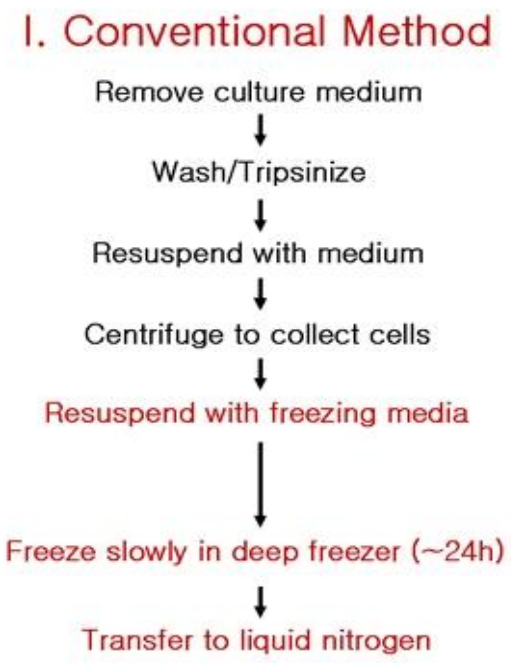
There is nothing quite so frustrating as a freezer full of vials of your precious collection of frozen cells, all of whose labels are at the bottom of the tank.

6. Cells should be frozen slowly.

A steady decrease of one degree per minute is ideal.

It should take a minimum of 4 hours to reach liquid nitrogen temperatures.

There are machines that will freeze vials at this exact rate. However, this is clearly not a required expense for the average cell culture laboratory.



Vials of cells should be placed in a freezing rack or a polystyrene plastic tube rack.

The rack or container should be placed in the refrigerator for 30 min, then moved to a -80C° freezer, where it should remain for at least 60 min and no longer than overnight.

The vials may be transferred from the freezing container to the liquid nitrogen freezer.

Freezing container (rack

Alternatively, nitrogen tank manufacturers make inexpensive freezing necks that fit their standard tanks. This replaces the insulating cap and holds the vials high in the neck of the tank, allowing them to be frozen slowly by the nitrogen vapors.

Cells may be stored for a short time at -80C°, but they will continue to degenerate rapidly at this temperature and so should not be stored at -80C° for more than a few weeks.

Equipment for freezing and storing cells at liquid nitrogen temperatures. There are three different-sized tanks

Frozen cells should be stored at -180C°. This is liquid nitrogen temperature.

Cell storage tanks, made by several manufacturers, are specially designed to handle these low temperatures.

A good tank should have a long "holding time" (the amount of time it takes all the liquid nitrogen to evaporate if the tank is not refilled), hold a reasonably large number of vials, and have a good inventory system for storing and finding the vials.

Some type of alarm or an automatic filling system is very helpful.

It is wise to store frozen vials of these cell lines in at least two separate freezers so that everything is not lost if one goes dry because of carelessness or a defect in the tank.

Remember that this equipment, though usually stuck away in a corner, contains the heart of a cell culture laboratory and may contain irreplaceable cell lines and cultures that are stored nowhere else.

Frozen vials of cells frequently stay in a laboratory long after the student, fellow, or technician who froze them and placed them in the tank is gone. Therefore, a good record-keeping system is an essential part of cell storage.

Each vial of cells should be labeled with the complete name of the cells, the passage number, the date, and the name or initials of the person growing and freezing the cells.

A laboratory-wide system should be established to maintain records of what cell lines are frozen, how many vials are frozen, who froze them, why they were frozen at that time, and where they are stored.

If there is a laboratory computer conveniently available, the records can be kept in a database on the computer.

One laboratory member can be responsible for placing cells in the freezer and removing cells to be thawed.