Introduction to Sterile Cell Culture

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INTRODUCTION TO CELL CULTURE

Tissue culture/cell culture is a technique in which cells are grown and cultured in a laboratory.

Cells

There are 2 main categories of cells that are cultured in a lab. Primary cells refer to cells which have been freshly isolated from organisms. Some examples of these are culture of immune cells derived from blood or bone marrow. Many tissues can also be disrupted to single cell suspensions, and cultured to promote growth of a specific cell type (e.g. culture of neurons or microglia from murine brain tissue). Usually primary cells require very specific growth factors to stimulate cell division, and these cells do not tend to grow in culture indefinitely. This is in contrast to cell lines. Cells lines are immortalized cells that have the capacity to grow indefinitely in an incubator given the right conditions. There are two types of mammalian cells that are typically cultured in labs. Suspension *cells*, such as lymphocytes, grow in a flask suspended in liquid media. *Adherent cells* such as fibroblasts and epithelial cells need strong attachment to extracellular matrix, and these cells grow on plastic plates that have been specially treated for tissue culture. There are many ways to obtain immortalized cells, one main way being isolation of cancer cells from patients or animals. An example of this is the HeLa cell line, which was isolated from a cervical tumor of a patient in the 1950s; He and La are the beginnings of the patient's first and last name (Henrietta Lacks). The Immortal Life of Henrietta Lacks is a book that describes the ethical issues of race and class in medical research and use of cell lines. Sometimes cells can be immortalized by infecting with viruses; human B cell lines can be made by infecting human B cells with EBV (Epstein-Barr virus).

Maintenance of cells

Cells are cultured in flasks and dishes. They become *confluent* when they grow to the point where they are about to run out of room and nutrients. Adherent cells reach confluence when they grow to cover most of the plate surface; suspension cells reach confluence when they reach $1-2x10^{6}$ /ml (this number depends on the cell type). A given cell line can be maintained in a laboratory indefinitely by placing some (an "aliquot") of the cells in fresh media in a new flask/plate before they become confluent; this is called "*passaging*" or "*splitting*" cells. Cells tend to lose their function when they have been in culture for a long time, and for this reason many labs keep track of the "passage number" for each cell split and when that number gets to be high, they throw away the cells and thaw out new cells from their frozen stock. When cells are not being grown in culture they can be put in a special media that contains "anti-freeze" materials such as DMSO (dimethyl sulfoxide) and stored in liquid nitrogen tanks (liquid phase of liquid nitrogen is -196°C, the vapor phase -156 °C).

Media

Mammalian cell culture media is a pH-balanced solution of salt, amino acids, vitamins and sugars. There are many different types of media, each with a slightly different set of ingredients. Commonly used media included *DMEM* (Dulbecco's modified Eagle's media) and *RPMI* (from Roswell Park Memorial Institute). Media is not used by itself; it

is often supplemented with animal serum, which contains nutrients such as growth factors that provide the signal cells need in order to grow and divide. *Fetal calf serum (FCS)*, also sometimes called fetal bovine serum (FBS), is preferably used in mammalian cell culture since it is readily available and since it is from a fetal source and therefore contains lower levels of serum antibodies, which may inhibit cell growth. FCS is first heat-inactivated via incubation at 56°C for 30 minutes, which destroys complement proteins that can have unwanted cytolytic, vasoactive, and chemokine effects. Often antibiotics such as penicillin and streptomycin are also added to media in order to prevent bacterial contamination. Finally L-glutamine, used for protein synthesis and nucleotide metabolism by cells, is sometimes added to media since it is unstable and is easily broken down and must be replenished. Media is often red in color due to the presence of pH indicator dyes. Red/orange signifies a neutral pH; yellow color indicates an acidic pH; and magenta/purple indicates an alkaline pH. When cells grow to confluence, media will turn yellow due to excess secretion of metabolic byproducts by overcrowded cells. Repeated overgrowth can result in compromised cellular function, so it is a good idea to split cells before they reach that point.

Plate/flask

Plastic is normally hydrophobic, but tissue culture plates/flasks have been treated with a special mix of gases so that it has negatively-charged chemical groups on surface, therefore making it hydrophilic. (Water will bead up on a bacterial culture plate, which is not tissue-culture treated. When you add water to a tissue culture plate, you will notice it spreading.) Tissue culture plates are also sometimes coated with extracellular matrix material such as collagen, polylysine, or fibronectin to aid the growth of certain more 'fussy' cells (e.g. neurons, endothelial cells). Tissue culture plates look a lot like bacterial plates, so be careful not to mix them up. When using a flask, remember that flasks rest on their sides in the incubator but once they are taken out they should be handled upright to avoid media splashing into the lid. Tissue culture flasks come in various sizes; standard sizes are the T-25, T-75 and T150 which have a culture surface are of 25 cm², 75 cm² and 150 cm² respectively. T-25 flasks hold 5- 10 ml of media, T-75 flasks hold 15-20 ml media, and T-150 flasks hold 40 ml media. TC plates also come in different sizes; the most commonly used ones are 60 mm or 100 mm plates (diameter).

CO₂ Incubator

Incubators provide an environment in which cells grow by providing optimal humidity, temperature, and pH. For mammalian cell culture we use a special incubator that maintains a set CO₂ gas level (5-10% for most mammalian cell lines). DMEM and RPMI media are buffered with sodium bicarbonate, and the physiological pH of the media is maintained around 7.2-7.5 via CO₂. The optimal temperature for almost all mammalian cells is 37°C. A tray of autoclaved water inside the incubator provides humidity via evaporation.

Laminar flow hood

There are many airborne microbes that can contaminate cell cultures during passaging, and therefore cell passaging is done in a special sterile environment inside a laminar flow hood. Air circulation inside a laminar flow hood is such that no outside air can get in, and the inside air is constantly filtered (HEPA filter), thereby creating a sterile environment. These hoods are often equipped with UV lights that can be turned on (when the hood is not in use) in order to keep contamination to minimum. It is important to note that these hoods are designed to maintain sterility of the air inside. You are still responsible for making sure surfaces and items brought into the hood (including your hands) are disinfected. Also, these biosafety cabinets are not suitable for protection from hazardous fumes or vapors. In general, a chemical fume hood will protect the outside world from its contents. A biosafety cabinet will protect its contents from the outside world.



Figure 1: Circulation of air in a laminar flow hood

What do I do with my cells? INTRODUCTION TO CELL ANALYTICAL TECHNIQUES

The ability to culture cells allows us to perform innumerable assays to determine cell specific responses within our system of interest. Limited only in our imagination and the availability of reagents to test our hypotheses. For example, these *in vitro* assays can be used to determine cell changes during differentiation, cell responses to changes in environment (e.g. pH, temperature, hypoxia) or to interactions with other reagents (e.g. toxins, chemicals, biological molecules) or interactions between different cell types.

To measure cell responses additional downstream assays are required. These will depend on what you are investigating, but can include assays to measure biochemical reactions, such as enzyme activity or cellular metabolism; changes of protein levels, such as receptor upregulation; changes in protein activity, such as protein phosphorylation; changes in protein location, such as nuclear translocation of a transcription factor and many others.

Some commonly used techniques to analyze cell responses are:

Western Blot: This technique is also known as immunoblotting because it uses an antibody to detect a specific protein (antigen). The specificity of an antibody-antigen interaction enables the identification of a target protein from a complex mix of proteins, such as a cell lysate. The proteins are first separated by size via SDS PAGE. After electrophoresis, the separated molecules are 'blotted' onto a membrane (nitrocellulose or PVDF). Any remaining protein binding sites on the membrane are then blocked by incubation of the membrane in a buffer containing non-specific proteins such as BSA or Milk. Usually the blot is then incubated with the antibody against the protein of interest which can be directly conjugated to an enzyme such as horse-radish peroxidase (HRP) or the blot can be subsequently washed and incubated with an enzyme-conjugated secondary antibody. Binding of the antibody to the protein of interest is detected using a substrate for the enzyme. For example, HRP can catalzye the conversion of a chromogenic substrate into a colored product, that produces a colored band on the membrane, or it can produce light when acting on chemiluminescent substrates such as ECL, which can be detected via film or specially adapted cameras.

ICC: Immunocytochemistry is a technique used to stain cells for a specific protein or antigen using an antibody that binds to it. Antibodies can be conjugated to enzymes, and staining detected chromogenically as described in the Western blot, or more usually they are conjugated to a fluorophore which can be detected via fluorescence microscopy or flow cytometry. Note: IHC, or immunohistochemistry, is a similar common technique used to identify a protein or proteins within a section of a tissue sample.

Fluorescence Microscopy: Fluorescence of cells can be directly imaged using a fluorescence microscope. The fluorophores that can be detected depend on the excitation and emission filters installed on the microscope.

Flow Cytometry: A flow cytometer is a high throughput device capable of measuring several parameters in 10s-100s of thousands of individual cells per sample. The parameters consist of FSC (forward scatter, equivalent to cell size), BSC or SSC (backscatter or side-scatter, equivalent to cell complexity/granularity) and FL (fluorescence). The fluorophores that can be detected by individual flow cytometers is dependent on the excitation and emission spectra of the lasers and filters installed on each machine. For example, we have a Sony SH800 flow cytometer which has 3 lasers, and 6 filters, which means a total of 6 different fluorescent 'channels' can be observed for each cell. Samples are aspirated automatically into the flow cytometer, in sheath fluid, and are channeled into the flow cell. Here they are aligned so that they travel through the light/laser beam one cell at a time (single file). The FSC, SSC and FL signals for each cell are detected and the data is stored ("Acquired") for each individual cell. Users can then analyze the data by looking at the parameter or parameters of interest. Analysis of data uses "dot plots" to look at 2 different parameters for individual cells on the same graph (e.g. FSC/SSC or FL1/FL2). Histograms are used to look at the distribution of one parameter over the entire population of cells (usually FL). Often people will compare the MFI, or Mean Fluorescence Intensity, from a histogram between one sample and another. The term 'flow cytometry' is often incorrectly used interchangeably with FACS, or 'fluorescence-activated cell sorting'. This is a specific type of flow cytometry in which a subpopulation of cells with specific parameters are "sorted" into a collection chamber after data acquisition. The Sony SH800 at CSULB is also a 'cell sorter' or FACS machine.

Protocols

Steríle Tíssue Culture (TC) Techníque

Handling of the hood

- The TC room should have all the windows closed to minimize aberrant airflow.
- The order of turning ON a laminar flow hood: open the hood sash (= window), and then turn on the blower. In some hoods, the blower comes on automatically.
- The order of turning OFF a laminar flow hood: turn off the blower, and then close the sash.
- Use of UV for the hood: when you turn off the hood for the day you can turn on the UV light for ~ 15 minutes to sterilize the inside. Do not leave the UV on all day/all night (bulb life), and be careful not to be exposed to UV yourself.
- It's best to allow the blower of the hood to run for a little while before you start working so that the inside air has a chance to filter through.
- The front sash should be opened at the right height (as indicated by a marking on the hood frame) when the blower is on. The outside air near the front of the hood circulates into the filter grid just inside of the hood front, allowing no dirty air to get to the inside area where tissue culture takes place. If the window-opening aperture is smaller, the airflow becomes accelerated and the outside air can land past the front air intake, introducing contaminants into the hood.
- It is a good idea not to put too many items inside the hood since they can disrupt smooth airflow.
- Make sure not to put anything on the front air intake.
- Do not put unnecessary/dirty items inside the hood. (i.e. notebooks, ice buckets, your head, etc.)

Ethanol

- 70% ethanol is used to sterilize certain items in tissue culture. You should know that ethanol will cut down on the amount of microbes but not completely eliminate them, and therefore you need additional sterile techniques. While the air is sterile, you should work under the assumption that surfaces are not 100% sterile.
- Wipe the inside working surface of the hood with 70% ethanol.
- Wipe items such as tube racks and pipet-aids with kimwipes sprayed with 70% ethanol.
- Make sure you do not heavily douse items with 70% ethanol; simple wiping will do. (You do not want liquid getting inside a pipet-aid, etc.)
- Your hands should be gloved and wiped with ethanol.
- What NOT to ethanol (yes, "ethanol" is a verb in TC!.)
 - There is no need to wipe down plastic disposables that are already sterile, such as tissue culture flasks, plates, and sterile disposable pipets.
 - If it is an item that "lives" inside the hood (tubing, racks, burner, etc), isn't touched often, and is repeatedly sterilized via UV, you do not have to use ethanol on it.
 - Sometimes there are items that shouldn't get wet and therefore cannot be wiped down, such as paper-wrapped pipets.

While you are carrying out cell culture

- Clothes
 - Your lab coat should be clean. (If not, it's better for your cells if you wear clean street clothes rather than a dirty lab coat.)
 - Watch out for your sleeves. It can drag onto/into sterile items/cells/plates, etc. Use rubber bands around your wrists if necessary.
- Posture
 - Use a chair and sit down at the hood. No standing while working at the hood it is not the right height.
 - It helps to rest your elbows on the hood so that your hands are more stable when pipetting.
 - Always be aware of the pipet and pipet-aid when working in the hood so that you know if the tip of the pipet touches anything non-sterile. Consider any surface non-sterile (even if it was wiped with ethanol).
- Handling of bottles/tubes
 - When you are in the hood and ready to pipet, it helps to loosen the bottle/tube caps (just a tiny bit) before you pick up your pipet otherwise they might be hard to open with one hand.
 - When you open a sterile bottle or a tube inside the hood, keep the caps away from you, with the inside facing up, in the back of the hood. The same goes for plate lids and flask caps. Make sure the caps are not in direct traffic of hands and other material (minimizes contamination). Also get into the habit of not passing your hands over sterile bottles and tubes that are open.
 - Do not graze your fingers on the bottle mouth/tube opening/plate rim when opening caps/lids.
 - The mouth of the media bottle, if it's made out of glass, can be flamed when first opened and when you are about to cap. This minimizes introduction of airborne contamination. Skip this step if the bottle/tube is plastic.
 - When you are dealing with sterile liquid in tubes or bottles, try not to get liquid into the cap (no tilting, shaking, sloshing, etc.). When you open a cap, whatever liquid was there will slide down the sides of the bottles/tubes sometimes on the outside, increasing your chances of contamination.
 - Serum (in complete media) will stain the stainless steel, so if you have any media dripping in the hood, please immediately clean it up. Thanks.
- Handling of flask, pipets & plates
 - How to open individually-wrapped sterile pipets: do this inside the hood (past the front air intake), and break the wrapper so that the tip end of the pipet does not touch anything non-sterile.
 - When you are opening a new sleeve of sterile TC plates, make sure that ALL the plates stay closed while you are getting yours out.
 - Make sure the pipet you choose is appropriate for the volume you are about to pipet.

- Pipet-aid: Pipet-aid is used to pipet volumes from 0.1 ml to 50 ml at a time. There are two buttons on the side of the pipet-aid. The upper button is to draw up the liquid and the lower one is to eject. A disposable pipet fits into a nosepiece of a pipet-aid. Do not (accidentally) draw up the liquid into the pipet-aid itself. For some models there is a self-locking mechanism that will stop the flow as soon as some liquid is introduced, so if your pipet-aid stops working, that's probably what happened. Do not tilt a liquid-filled pipet horizontally; this could cause contamination. Lastly, it is a good idea to let the pipet end touch the tube/flask/plate wall when pipetting out (minimizes splattering).
- Handling a vacuum aspirator
 - Gently shake the box of autoclaved sterile Pasteur pipets to remove a single Pasteur pipet carefully from its canister without touching the remaining pipets
 - Put the Pasteur pipet tip near the edge of a plate and not in the middle of the cells so that you don't aspirate off your cells.
 - After you are done aspirating, leave the glass pipet on the tubing for a few seconds longer to make sure that all of the liquid is gone (otherwise the liquid will drip right out and make a mess).
- Other important points
 - When pipetting out media (or any liquid), have the pipet tip touch the tube/flask/plate surface so that it gently slides down the side. Hovering/shaking pipet tips will introduce unnecessary contamination.
 - Once you expel media from the pipet, stop immediately. You will form lots of bubbles if you keep pipetting out.
 - Use the racks as much as possible inside the hood; don't keep holding onto tubes/bottles, etc., if you can let it safely sit.
 - Make sure you differentiate biohazard waste vs. normal waste. Anything that's been touched by FCS or cells should be considered biohazard.
- Finishing
 - Remove all items from the hood that you brought in.
 - Wipe the work area with 70% ethanol.
 - Make sure the pipet aid is off or plugged into the charger.
 - Make sure vacuum is off.
 - Make sure the aspirator flask is not full.
 - \circ Turn off the hood (order: blower off sash down UV on).

You will not remember everything the first time; with repeated practice you will hopefully remember most of them. Each time you do cell culture see how many of the above points you can remember without looking at your manual.

Preparíng TC Medía

There are two common types of media: DMEM and RPMI. DMEM is often used for adherent cells, whereas RPMI is for suspension cells (usually lymphoid in origin). The ATCC (ATCC.org) has a comprehensive database that describes media compositions for commercially available cell lines.

Recipe for DMEM or RPMI "complete" media

DMEM or RPMI mixed with; 1. heat inactivated FCS*, final concentration of 10% from 100% stock and

2. Penicillin/streptomycin/L-glutamine mix, 1X final, from 100X stock solution

Note: TC ingredients are not autoclaved; they are purchased filter-sterilized, or the whole bottle of media is filter-sterilized after preparation

Media is stored in the refrigerator at 4°C after preparation

Colordoffer Dreatics 1.
Calculation Practice 1:
You need to make 500 ml of <u>DMEM complete</u> or <u>RPMI complete</u> ,
How would you make the media (how many ml of each would you mix)?
ml of 100% FCS
ml of 100X Pen/Strep/Glu
mi of DMEM or RPMI

*To heat inactivate FCS, aliquot to 50ml or less and immerse in a waterbath at 55° C for 30 minutes

Detaching Adherent Cells

DETACHING ADHERENT CELLS FROM A PLATE/FLASK

In order to work with adherent cells, you must first detach the cells from the TC plates they are growing on. There are 3 approaches to this, mechanical, enzymatic, and non-enzymatic.

Mechanical detachment requires use of a cell-scraper to physically scrape the cells up from the bottom of the plate/flask.

- Advantages: fast, efficient
- Disadvantages: can cause cell disruption/death
- Uses: when splitting cells
- Protocol:
 - 1. Always check your cells under the microscope before you start
 - 2. Remove media from dish/flask to remove any dead cells/debris
 - 3. add fresh media
 - 4. gently scrape the surface with a sterile scraper

Enzymatic detachment uses proteases such as trypsin to lightly digest cell surface adhesion proteins responsible for attachment. It is important that you don't trypsin-treat cells for too long or they will start to aggregate into large clumps and it can be difficult to revert back to a single-cell solution

- Advantages: efficient
- Disadvantages: can cause clumping, may remove molecules from the surface of the cell that affect your analysis
- Uses: when splitting cells

Non-enzymatic detachment uses EDTA to chelate calcium and magnesium ions. Adhesion molecules require Ca/Mg to function, and by removing these you decrease attachment of the cell to the surface of the plate/flask

- Advantages: most gentle removal method, retains cell surface molecules on cells
- Disadvantages: slower and can be less efficient
- Uses: when splitting cells
- Protocol (for trypsin/EDTA):
 - 1. Always check your cells under the microscope before you start (make sure they are adherent!)
 - 2. Pipet or aspirate media from dish/flask to remove any dead cells/debris
 - 3. Wash surface with sterile PBS, swirl gently, and aspirate off the PBS. (This is a wash step to remove any residual serum). Serum contains Ca/Mg and trypsin inhibitors, so it's important to get rid of as much serum as possible before adding detachment media. Be gentle so that you do not shear off the cells. *Make sure the PBS is Ca/Mg free*.

- 4. Immediately add a volume of trypsin or non-enzymatic detachment solution and cover the plate. Incubate the plate at 37°C for around 5 minutes (do not over-trypsinize)
- 5. Pipet cells using a p1000 to increase force. Tilt plate towards you and pipet in a semi-circle (rainbow!) motion to detach cells
- 6. Observe the plate under a microscope to see if cells have detached; you will see them round up if trypsin is working. If not, return the plate to the incubator and observe a couple minutes later. If it's been a while and you don't think they are detaching, tapping the side of the plate gently against the side of the bench may help loosen them (but be careful not to splash the solution!)
- 7. Bring the plate to the hood and add some DMEM complete media and pipet up and down to disperse cells. Be careful not to splash while pipetting up and down, and try not to foam up the media too much. The detachment solution is now neutralized by the serum in the media.
- 8. Proceed with cell splitting and/or cell counting. If whatever you need to do will take a long time (i.e. hours), do not leave the trypsinized/resuspended cells on the plate as they will start to re-attach; move the cells to a 15 ml tube and keep the cells on ice.
- 9. Do not forget to dispose of the waste in appropriate bins. Whatever touched cells or FCS or cells should be considered biohazard and goes into biohazard bins.

Splitting Adherent Cells

HOW TO SPLIT ADHERENT CELLS

Splitting adherent cells involves taking a dense cell culture plate, detaching the cells so that they are released from the plate, and taking an aliquot of the cell suspension and transferring it onto a new plate with fresh media. We calculate how to split cells (= how much of the current culture is to be transferred to a new plate and how much new complete media to add) based on how confluent the cells are at the time of splitting, how many days the cells will be in culture, how fast your cells grow, and how many plates of cells you will need. We say cells are "x % confluent" based on the % of the plate surface area covered by cells. Be sure to look at various points of a plate to get a good feel for the overall/average confluence of a plate; Make sure that your cells do not go much past ~90% confluency, just to be safe; you do not want to overgrow your cells. Many cells double approximately once a day.

Example Calculation

If you have a 80% confluent plate of Raw264.7 mouse macrophage cells, and you need one plate of 80%-confluent cells 2 days from now, you need to split the cells so that the fresh plate today will have 20% confluency. (Tomorrow @ ~40%, the day after @ ~80%; Raw cells divide once/day) The first thing to do is to figure out the dilution factor for the cell split. Since the cells are at 80% before the split, and you need them to be at 20% after the split today, the dilution factor for today's split is 4X. After trypsinization, you should add 2mL DMEM complete to resuspend the cells, so the total volume is 4mL. Since you need to dilute the cells 1 to 4, what you'd need to do is to transfer to a new plate only ¹/₄ of the current trypsinized cell suspension, which would turn out to be 1 ml. Make up the rest of the volume in the new plate using fresh DMEM complete so that it will hold 5 ml; in this case you would add 4 ml of DMEM complete. *Note: You do not use C1V1=C2V2 for adherent cell split calculations*.

Before you start...

- The hood should be prepped before you start.
- Warm up the media and detachment solution in 37°C water bath so that the cells do not get shocked.
- When ready to work on cells, take the media bottle out and wipe the outside with alcohol.
- Place all necessary material (media, pipet, and new flask) inside/near the hood.
- Reminder: sterile technique!!

Protocol

- 1. Observe your cells and decide/calculate how you will split the cells.
- 2. Carry out detachment (described in previous section)
- 3. During the incubation, label new plate(s) appropriately.
- 4. Add the right amount of DMEM complete into the new plate(s). You can pick how much DMEM to add to make your dilution calculation easy!
- 5. Once cells have been resuspended at the end of detachment, add the right amount of cells to a new TC plate (that already has fresh media).
- 6. Rock gently a few times so that the cells are distributed evenly. Do not swirl in a circle as that concentrates the cells in the center.

Practice Calculation 2:

You want a confluency of 80% on Tuesday. Today is Friday. Assume you trypsinize with 2mL and neutralize with 4mL media.

Cells are 60 % Confluent today

I need them to be ______% confluent after I split them today

My dilution factor is

I will need to transfer _____ ml of trypsinized cell suspension to a new plate

that contains ml of complete media to make the total volume 5ml

Counting Cells

A hemacytometer is a specialized microscope slide used to count the cell number in a given volume (=cell concentration). A small amount of cell culture, often diluted, is pipetted between the hemacytometer and a cover slip via capillary action, and the hemacytometer is placed under a microscope. Hemacytometer has nine engraved counting areas (1 mm x 1 mm dimension each) that can be seen under a microscope, and since the cover slip sits 0.1 mm above the surface of the hemacytometer, the volume of cell culture placed in each counting area is 0.1 µl. People usually refer to a cell concentration as a number of cells per ml. Since 10^3 µl equals 1 ml, you should multiply the number of cells per counting area (which is the number of cells per 1 µl= 10^{-4} ml) by 10^4 in order to get the cell number per 1 ml. If the culture is too dense and therefore you diluted it before putting it onto the hemacytometer, do not forget to multiply the cell count by the dilution factor in order to determine the true cell count per ml.

It is really important to resuspend your cells thoroughly right before taking out an aliquot for counting. Cells start to settle to the bottom as soon as you stop resuspending. Therefore you should make sure that your cells are resuspended really well, and that you take out an aliquot immediately after you stop resuspension. Don't wait too long. Some suspension cells do stick to plastic (lightly) even if they are supposed to be suspension cells, so when you are resuspending cells be sure to "wash" the surface area where cells had been resting in the flask.

Cell culture sometimes has some dead cells, and it is important to count only the live cells in order for the cell count to be a true reflection of cell function. One can exclude dead cells by using a dye called trypan blue. Only dead cells absorb trypan blue (live cells have the capacity to pump out the dye), and therefore you should only count the non-blue cells.

Example Calculation:

If you counted 24 cells in one counting area and 22 cells in another, the average cell count per counting area is 23 cells. If you added equal volume of trypan blue, thereby diluting the cell culture 2-fold, you should multiply this cell number by 2 in order to get 46 cells per counting area. Since a counting area contains 10^{-4} ml, multiply this number by 10^4 in order to get cell concentration in ml. 46×10^4 =4.6 $\times 10^5$ cells/ml. You have 4.6×10^5 cells per 1 ml of culture. (Use scientific units: not 46×10^4 but 4.6×10^5)

Before you start...

- The hood should be prepped before you start.
- Reminder: sterile technique!!

Protocol

1. If using adherent cells, trypsinize them and resuspend them in DMEM complete. If using suspension cells, no need to worry about trypsinization. Using the same pipet you used for resuspension to take out just a little of the cell suspension into a microtube. Make sure that it is a well-resuspended sample.

- 2. At this point, you can bring the microtube to the bench since you will no longer be culturing this small sample of cells. Place 20 μ l of the cell suspension in a *new* microtube (Remember, good resuspension lightly vortex the tube before you take that 20 μ l out!). The aliquot to be counted does not have to be kept sterile.
- 3. Add 20 μ l of trypan blue to the microtube that already has 20 μ l of the cells. Mix thoroughly by pipetting up and down. Note this is a 1 in 2 dilution.
- 4. Fill a hemacytometer by taking approximately 10 μl of (cell + trypan blue) mix (use a p10 or p20 pipetman) and letting it slowly get soaked up between the hemacytometer and the cover slip through the groove. Again, make sure that the cells you put into the hemacytometer is a well-resuspended sample from the microtube.
- 5. *Immediately* observe under a microscope. If you see cells floating around, that means you've added too much volume (more than 0.1 µl/counting area). Clean the hemacytometer and cover slip with ethanol and repeat the previous step. Make sure to quickly vortex the tube before taking a new sample in case cells have settled.
- 6. For the most accurate count, you should count all nine counting areas. If the numbers per counting area are very different, re-do the count, taking care not to flood the hemacytometer. To calculate cell concentration you will need to determine the average of these counts. (Once you get good at counting , or if you have over 100 cells per counting square, you may be able to get away with just count two counting areas if cells are spread out evenly.) There will be some cells that are sitting on the borderlines of a counting area; count only two out of the four borders. Whatever the number of cells in a counting area should be multiplied by 2 since the counted culture is a 50:50 mixture with the trypan blue solution (2 is your dilution factor). This number x 10⁴ is your cell count/ml. Do not forget to always use the "/ml".
- 7. Clean the hemacytometer and cover slip with 70% ethanol and kimwipe.
- 8. Calculate the cell concentration as follows:

Average # cells per square x dilution factor = $x \ 10^4$ cells/ml



Photo credit: Melissa Rouge, Colorado State University

Figure 2: Hemacytometer with 9 individual counting areas/ counting squares (3x3).

Splitting Suspension Cells

Just like adherent cells, we calculate how to split suspension cells based on how concentrated the cells are at the time of splitting, how many days the cells will be in culture, how fast your cells grow, and how many flasks of cells you will need. Using a hemocytometer you can calculate the number of cells per ml in your flask. Make sure that your cells do not go much past $1-2 \times 10^6$ cells/ml, because you do not want to overgrow your cells. You also do not want to dilute them beyond 5×10^4 cells/ml. As an example, EL4 (mouse lymphoma) cells double approximately every 1.5 days.

Example Calculation:

You measure your cell concentration of EL4 to be 8×10^5 cells/ml. Today is Friday midday and you want a concentration of $\leq 1.5 \times 10^6$ cells/ml on Wednesday midday. This is just over 3 doublings (3.5). To be safe, you decide to split your cells to 1×10^5 cells/ml. This will double to 2, then 4, then 8×10^5 cells/ml then 12×10^5 cells/ml (= 1.2×10^6 cells/ml), by Wednesday.

Practice Calculation 3:

You want a concentration of $\sim 8 \times 10^5$ EL4 cells/ml on Friday midday. Today is Tuesday midday. EL4 double once every 1.5 days

Cells are at 0 x10 cells/illi today	Cells are at 6	x10 ⁵ cells/	ml today
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I need them to be ______ cells/ml after I split today

Using C1V1 = C2V2,

I will need to transfer _____ ml of cell suspension to a new flask

that contains _____ ml of complete media to make the total volume 5ml

Before you start...

- The hood should be prepped before you start.
- Warm up the media in 37°C water bath so that the cells do not get shocked when media is changed.
- When ready to split cells, take the media bottle out and wipe the outside with alcohol.
- Place all necessary material (media, pipet, and new flask) inside/near the hood.
- Reminder: sterile technique!!

Protocol

1. Remove the culture flasks from the incubator and examine your cells under a microscope. It is a good idea to do this every time you work with cells so that you can see their relative health and contamination status. Place the culture flask inside the hood (standing up).

- 2. Determine the cell concentration by counting
- 3. Calculate how you will split the cells. You can use C1V1=C2V2.
 - a. The total volume in a T25 is 5 ml.
 - b. The total volume in a T75 is 15ml
 - c. The total volume in a T150 is 40ml
- 4. Label a new flask with your name, date, and cell name.
- 5. Draw up appropriate ml of fresh complete media (it depends on your cell count and calculations) into the pipet and transfer to the flask. Make sure the pipet tip is well inside of the flask, touching the plastic surface; this minimizes unnecessary splashing around the neck of the flask.
- 6. Resuspend the cells in the original flask really well. Use a 5 or 10 ml pipet for this. Some cells stick to the plastic, so make sure you wash them off the flask wall (just the side they were resting on). Remove appropriate ml of cells from the old flask and transfer to the new flask. (If the volume to transfer to a new flask is less than 1 ml, switch to a 1 ml pipet at this point. Do not use a pipetman since it's not sterile. Be careful with 1 ml pipets they draw up really quickly and you might draw liquid into the pipet-aid body.)
- 7. Cap the new flask and transfer it back to the incubator while holding it vertically. The flasks should lie on its side once inside the incubator so that cells have as much surface area to settle as possible. Be careful with the orientation of the flask cap it should point upward. (If the cap does not have an air filter, loosen the cap 1/2 turn so that gas exchange can occur. Close the incubator door as soon as you are done since keeping the door open for a long period of time will make it difficult for CO₂ and humidity levels to re-equilibrate quickly.

Freezing Cells

Cells can be frozen for long term storage. This involves centrifuging cells, resuspending the cell pellet in freezing media, and freezing the cell mixture in sterile cryotubes.

Components of the "freezing media"

90% FCS

10% DMSO (dimethyl sulfoxide; prevents ice crystals from forming and shearing cell membrane)

Per cryotube: ½ of the content of a confluent 10-cm plate (adherent cells) or an entire confluent 5ml flask (suspension cells).

Protocol

- 1. Determine cell concentration/confluency and calculate how much cells you will freeze. I freeze around 10⁷ cells per vial.
- 2. Prep the laminar flow hood and have a bucket of ice handy. Ice buckets stay OUTSIDE the hood.
- 3. Trypsinize if your cells are the adherent type. With suspension cells go to the next step.
- 4. Once you have a cell suspension (with appropriate number of cells to freeze in one tube), transfer it to a sterile 15-ml tubes (in the hood).
- 5. Centrifuge the tube at 1200 rpm at 4°C for 10 min. (Make sure you balance the rotor.)
- 6. While the cells are spinning, label a sterile cryotube with a *cryomarker* (it won't come off in extreme cold or ethanol). Be sure to include the date, cell name and your name.
- 7. Once centrifuging is done, bring the cells into the hood. Aspirate off the media supernatant with a *sterile* glass Pasteur pipet, or p1000.
- 8. Flick the tube to loosen the cell pellet.
- 9. Add 1 ml of cold freezing media with a sterile 1 ml pipet. Pipet to resuspend the cells well. Watch the liquid level in the pipet if you are using a 1 ml pipet since it's easy to suck the liquid into the pipet-aid with 1 ml pipets. (Keep the cells on ice when not working with them.)
- 10. Transfer the cells to a cryovial (also inside the hood). Tighten the vial cap and put the vial on ice. The cells shouldn't warm up, so as soon as you put the cells in a cryovial, cap and put them on ice.
- 11. Bring the cells down to -80 °C freezer slowly. Insulated devices such as a "Mr Frosty" can be used to slow the freezing reaction, or you can start in the -20oC freezer overnight, then move to -80oC freezer next day if necessary.
- 12. For long-term storage the vials would be moved to a liquid nitrogen tank.

Thawing Frozen Cells

Cells can also be revived after being frozen. Cells need to be quickly thawed in a 37°C water bath and then cultured, following a wash. Cells are frozen in freezing media that contains DMSO, and it is important to remove DMSO before cell culture. Freezing media can also have high levels of FCS which has different osmolarity than media, so it is important to add media slowly as not to shock the cells.

Protocol

- 1. Get the hood ready. Set out and prep all the items you will be using. Remember your sterile techniques. You do not need to warm up the media for this procedure you want your complete media to be at room temperature.
- 2. Get ice. Do not start the rest of the protocol until you are ready. It's because once you thaw the cells you have to proceed to the end of the procedure without interruption or waiting.
- 3. Get your frozen vial and put it on ice.
- 4. Bring the vial to 37°C water bath and let it warm up. Don't immerse the whole vial into the water immerse just enough so that the frozen part can thaw. (If you dunk the whole thing in, or you let the water go above the cap level, then non-sterile water from the water bath can enter the vial and contaminate your cells.) Gently shake the tube so that the frozen cells thaw evenly. Do not just put the vial in the water bath and walk away you must stay with it and shake it so that you can take out the vial the moment it's thawed. This only takes a minute.
- 5. Keep the vial on ice. Bring the vial to the laminar flow hood. (The ice bucket stays outside the hood.)
- 6. Wipe the outside of the vial with ethanol-soaked kimwipe to minimize contamination from the outside.
- 7. Inside a hood, pipet the content of the vial into a 15 ml sterile tube (make sure you label your tube) with a sterile 1 ml pipet. Watch out for how fast you are pipetting with a 1 ml pipet; it goes fast.
- 8. Add 8 ml of sterile complete media (depending on which cell type) into the tube, *a few drops at a time*. After each addition (of drops of media) you should agitate the tube so that it is well mixed. Do not just squirt in the whole 8 ml the cells have to adjust to the osmolarity of the media and you must drip in 8 ml of the media into the tube slowly. Once you've added about 3-4 ml of media, you can start adding the media faster. Cap and gently tilt the tubes a couple of times to mix well.
- 9. Spin the tubes at 1200 rpm at 4°C for 10 minutes. Be sure to balance the rotor. Keep the tube on ice while you wait for your turn at the centrifuge.
- 10. Aspirate off the media supernatant with a sterile glass Pasteur pipet and a vacuum aspirator or pipette off.
- 11. Flick the tube to loosen the cell pellet.
- 12. Resuspend the cells (pipet up and down well) in sterile complete media (5 ml).
- 13. Put them into a plate (60mm) or flask (T25) and incubate.

CALCULATION ANSWERS!

Calculation Practice 1:

You need to make 500 ml of <u>DMEM complete</u> or <u>RPMI complete</u>, How would you make the media (how many ml of each would you mix)?

50 ml of 100% FCS (final concentration, 10%)
5 ml of 100X Pen/Strep/Glu (final concentration 1x)
445 ml of DMEM or RPMI

Practice Calculation 2:

You want a confluency of 80% on Tuesday. Today is Friday. Assume you trypsinize with 2mL and neutralize with 4mL media.

Cells are 60 % Confluent today

I need them to be <u>5 %</u> confluent after I split them today

My dilution factor is 12

I will need to transfer 0.5 ml of trypsinized cell suspension to a new plate

that contains 4.5 ml of complete media to make the total volume 5ml

Practice Calculation 3:

You want a concentration of $\sim 8 \times 10^5$ EL4 cells/ml on Friday midday. Today is Tuesday midday. EL4 double once every 1.5 days.

Cells are at 6 $x10^5$ cells/ml today

I need them to be 1.5×10^5 cells/ml after I split today

Using C1V1 = C2V2,

I will need to transfer 1.25 ml of cell suspension to a new flask

that contains 3.75 ml of complete media to make the total volume 5ml