College of the Canyons Introduction to Biotechnology: Custom Lab Exercises

Sterile Techniques, Tissue Culture and Cell Counting

Version 8-18-12

- **Tissue culture is a technique used by scientists to grow cells from various tissues in a laboratory setting.**
- **In this experiment you will propagate your own cell line for a number of weeks, conducting weekly propagation as well as quantifying and characterizing your cell culture.**
- **The type of cells you will be working with are insect cells. They are easy to obtain and can grow under a variety of conditions (unlike their mammalian counterparts).**
- **Cell cultivation requires stringent protocols and sterile technique. Therefore, in addition to the propagation of your cell line you will become familiar with these misc, sterile techniques and protocols.**
- **You will also record and graph the health of your culture by using an instrument called a hemocytometer to count both living and dead cells in your cell line.**
- **Growth optimization and prediction will be attempted as a prelude to a cell cryopreservation (freezing for long term storage) exercise.**
- **In future labs you will determine the average protein concentration of the cells in your culture (using previously prepared protein standard curves), as well as assess the success of your cryopreservation technique.**
- **A series of educational videos will be viewed and should be reflected upon to clarify key steps. Notes for the videos are included with this lab on the last few pages.** For more information on the College of the Canyons Introduction to Biotechnology Course, contact Jim Wolf, Professor of Biology/Biotechnology at (661)362-3092

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I: Objectives

1. To become proficient at sterile technique and basic cell culture.
2. To understand the importance of tissue culture in the field of molecular biology.
3. To successfully propagate your own insect cell line for a number of weeks.
4. To become familiar with a hemocytometer (and related calculations) and be able to use it to quantify living and dead cells in your cell line.
5. In later labs, assess protein concentration per cell and determine effectiveness of cell culturing cryopreservation techniques.

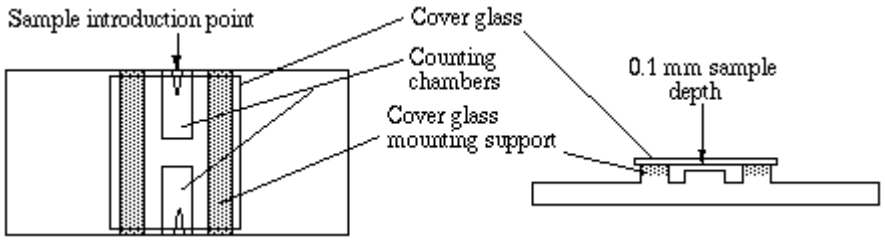
II. Background:

Over the next few weeks we will be learning the art (oops, I meant science) of tissue culture. Many of the most significant advances in the field of molecular biology have been made via tissue culture techniques. I say art of tissue culture because a lot of what has been learned is through trial and error and serendipity. Each organism has unique tissues, and so the tissue culturing techniques are both organism and tissue specific.

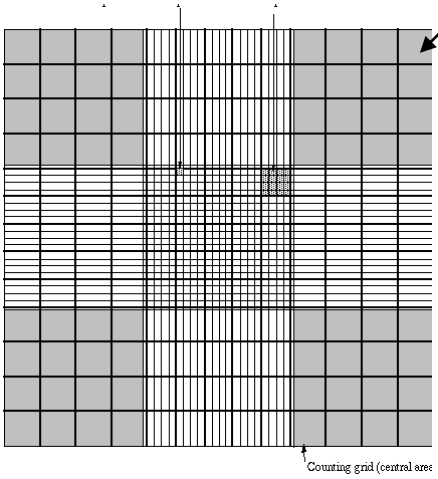
Once a tissue culture protocol has been worked out it is rarely changed. Some tissue culture techniques and actual cell lines date back over sixty years! There are three broad categories of tissue culture: plant, mammalian and other. Mammalian tissues are arguably the most difficult, for these cells require a constant stream of CO_2 gas in addition to the normal cell requirements. Plant tissue culture requires that you work around the cell wall.

The groups of cells we will be working with are insect cells (i.e. the "other" group from the above list). They are easy to obtain and can grow under a variety of conditions. The first few steps in becoming competent in working with cell cultures is to practice sterile technique, propagate a cell line and inspect the line for health and growth. Your sterile techniques should be better developed by now, as you have just completed a media preparation lab, where sterile technique is essential to proper media preparation. To practice sterile technique more, each of you will propagate your own cell line for 4-5 weeks, as well as quantify and characterize your cell culture via a graph. Finally, you will quantify, split, feed, cryopreserve and validate your cells towards the end of this lab. In a concurrent lab, you will assay the protein concentration per cell.

To count cells, a hemocytometer is used. The hemocytometer is a glass slide with grids inscribed on it. These grids are a set distance apart and create many small squares. If we count the number of cells in a particular square, and know the volume of the square, it will give us the number of cells per unit volume (diagram next page).



Ultimately you want to know the number of cells/ml. You will be counting cells in any of the larger corner grids, labeled gray in the illustration.



Each small square is 0.25mm wide with a depth of 0.1mm. Thus, the volume of each square is:

0.25mm x 0.25 mm x 0.1 mm = 0.00625 mm^3

At least ten squares should be counted. For example, if you count 700 cells in ten squares, then the average is 70 cells per square:

$$\frac{700 cells}{10 squares} = 70 \frac{cells}{square}$$

Now simply use dimensional analysis to determine the number of cells per mL:

$$\frac{70 \text{ cells}}{1 \text{ square}} \quad \frac{1 \text{ square}}{0.00625 \text{ mm3}} \quad \frac{1000 \text{ mm}^3}{1 \text{ cm3}} \quad \frac{1 \text{ cm}^3}{1 \text{ ml}} = 1.12 \times 10^7 \frac{\text{cells}}{\text{ml}}$$

The above calculation can be simplified by generating a simple multiplication per cell count. This number may also change if you use a substantial dilution factor (ours is very small, so we will ignore the dilution factor for our study), or if you use another set of squares.

To check for cell viability, the dye Trypan Blue is used. A healthy cell will have a vital cell membrane capable of keeping out many organic macromolecules. Healthy cells **exclude** Trypan Blue, but once the cell dies, the membrane is no longer capable of keeping the dye out, making it appear blue or dark (although the cell may otherwise appear fine).

Note: Light intensity is very critical when assessing cells with Trypan Blue. Every effort should be made to optimize the lighting in the microscope to maximize contrast. It is very rare that a sample will not have any dead cells in it, so double check your light environment to ensure that the blue dyed cells are indeed visible. Conversely, light can kill cells. So keep the light at a minimum and count quickly as time under the scope it limited!

Practice Exercise: Working from provided stock, count cells as described in steps 8.31 - 8.34 (few pages ahead). For comparison, look at provided dead cells to ensure you can see blue coloration under the microscope. To make dead cells, simply take a small sample (100 ul) of cells and add 20 ul of

isopropanol. Also: which squares to use, techniques to use to avoid counting cells twice, time limits and other considerations will be covered in supplemental reading materials, in the lecture and in educational video notes attached at the end. Suffice to say, this topic is very rich in detail!

Remember: the videos covering elements of this lab (at different times) have lecture notes included at the end of the SOP, FYI…

Sterile technique issues: While done at different times during the lab, the following sterile technique checks should be executed during EVERY lab that the student attempts (unless specifically noted otherwise). They are included below, but may actually be done before, after or even during the cell culturing procedure outlined in the steps under "III: SOP /Lab Activities.

III: SOP for sterile technique assay:

Required Materials for Sterile Technique Assay:
From your sterile media prepared earlier in the semester, get the following:
1, 65 mm SDA plate
1, TSA/Tween contact plate
2, 65 mm LB plates
set of sterile Q tips
aliquot of sterile water (in 1.5 ml micro-fuge tubes)
gloves and alcohol sprayer
labeling pen (sharp point, Sharpie or Lab-Line).

The following steps should be completed in concert with the lab exercise on passing cells. Review procedure completely and remember to do the exercises in a timely manner.

1. For one LB plate, using a LIGHTLY wetted Q-tip, gently swab the surface directly in front of and behind the intake screen on the bottom of the tissue culture cabinet. This should be done before the cell culture line is passed and *before any equipment / alcohol spray down has started.* Remember to use sterile water aliquots, and be careful to not contaminate other swabs when removing a swab from test tube they were sterilized in. Discard microfuge tube with water and Q-tip in bio-hazard (dispose only the used Q-tip). Label bottom of LB plate with date, surface checked, initials and and words "pre-culture".

2. For SECOND LB plate, using a wetted Q-tip, gently swab the surface directly in front of and behind the intake screen on the bottom of the tissue culture cabinet. *This should be done **after** the cell culture line is passed*. Remember to use sterile water aliquots, and be careful to not contaminate other swabs when removing swabs from test tube they were sterilized with. Discard microfuge tube with water and Q-tip in bio-hazard (dispose only the used Q-tip). Label bottom of LB plate with date, surface checked, initials and words "POST-culture".

3. For SDA plate, label bottom : cell culture, settling plate, date, initials. Once inside the hood, bring in the plate (sterilize outside with alcohol), and then open the plate in some location that is out of the way, but not directly next to the edges of the hood. This

plate should remain open for the **duration** of the lab exercise. Once you have finished passing all of your cells line (s), place the cover on the plate.

4. **For the TSA with Tween plate,** at the **END** of the procedure, place the tips of all five fingers, together, onto the surface of a the contact plate. Be gentle and remember to record: what hand, date, initials, etc.

All fours plates should be stacked, taped and then placed UPSIDE down in the 37 ^{o}C bacterial culture hood. Be sure to use hood marked for bacteria (obviously, not for hood marked for tissue culture). In the following lab, assess the plates for any colony forming units (CFUs).

After assessing plates and recording information, discard contaminated plates in the bio-hazard receptacle. Leave any apparently uncontaminated plates at the front with the professor.

III. SOP/Lab Activities: The below template is derived from actual industy protocols FYI…

Important: Always note any addenda that are posted by the instructor.

1. **Scope**: Maintain continuous culture for tissue culture experiments.
2. **Definitions**: Inoculum: Portion of old culture that is added to new media to encourage growth.
3. **References**: SOP Manual, Introduction to Biotechnology 230, College of the Canyons
4. **Reagents/ Supplies: NOTE ###**
    - 4.1 Stock tissues culture (*Drosophila sp*. cell suspension, stock culture)
    - 4.2 50 ml suspension culture flask
    - 4.3 1 ml disposable transfer pipette (sterile, individually wrapped)
    - 4.4 10 ml transfer pipet
    - 4.5 pipette pump (hand or electronic)
    - 4.6 isopropyl alcohol in spray bottle
    - 4.7 tissue culture hood
    - 4.8 Stock Hyband tissue culture broth
    - 4.9 2, 1.5 mL microfuge tubes (sterile)
    - 4.10 20-200 ul micropipette and tips (sterile)

NOTE ###: The above list should modified for days when you are the first to use the tissue culture hood (get enough supplies for about 12 students) and if you are doing cryopreservation and/or later labs with cell feeding (growth optimization). See that lab for additional details.

5. **Responsibility**:
    - 5.1 Transfer and propagation of cell line is the responsibility of students in Biotechnology 230.

6. **Hazard Communication**
    - 6.1 **Danger**: Isopropyl Alcohol: Flammable and irritant if inhaled.
    - 6.2 **Danger**: UV Light (avoid prolonged exposure to skin and eyes).

7. **Attachments**: Verification ledger: SOP as needed.

8. **Procedure**: The first person to set up the lab should get enough supplies for 12 persons (or about ½ of the class).

8.1 <u>Sterile field preparation</u>: ***Note: The first person to propagate should set out enough equipment for the entire class. The last person should clean out the hood and remove any unused equipment and other supplies still left in hood, restock, dispose, replace as needed. Also, remember to do your plate sampling at the appropriate times during this procedure (see previous).***

8.11 Place clean, new tissue culture flask, a disposable transfer pipettes, 10 ml transfer pipettes and pipette pump into the culture hood. As you are placing them in, spray with alcohol. Do not get alcohol into insect cell culture flask (cap is absorbent FYI and this step is notes twice as it is important). Make sure at address every item in the list in step 4. Spray down with isopropyl alcohol and illuminate hood with UV light source. Let dry.

8.12 You will be assigned a stock cell culture and vial of insect cell media <u>for your use only</u>. The insect cell media is in a 50 ml Falcon tube (blue cap, tapered bottom, volume demarcations on the side FYI). Label them clearly and keep track of them.

8.2 <u>Cell line propagation</u>: Check lab syllabus and remember your scheduled day of cell transfer (CT, Tuesday or Thursday).

8.21 Remove tissue culture media from refrigerator. Get stock cell culture and **inspect stock culture under tissue culture microscope to ensure no contamination**. If contaminated, discard in biohazard, and contact the professor for another sample If OK, while covering cap (with glover finger) so no alcohol gets in, spray down with isopropyl alcohol. Put into culture hood, turn **off** UV light and let dry.

8.22 Transfer 5 ml aliquot of stock culture broth (in 50 ml Falcon tube) into new tissue culture flask using 10 ml transfer pipette.

8.23 Inoculate tissue flasks (using only uncontaminated samples) with or 1.0 ml of cell culture. Gently agitate the flask to help dislodge the cells. Use a new transfer pipette for inoculation.

8.24 Agitate the **<u>old</u>** cell flask (again) and then aliquot a 100 µL portion of the cell culture into a microfuge tube. *Note: ensure that your sample is taken from your old culture, not the new one you just made! This is the culture you will count.*

8.25 Label flask appropriately and clean out your debris from hood.

8.26 Place new cultures into incubation chamber @ 25^{o} C and return tissue culture media to rack. Save the original cultures until told to dispose of them, or if they become contaminated.

8.27 If you are the last to use hood, clean it out entirely, spray with alcohol, turn on U.V. light for 10 minutes. Make sure glass panel is pulled down all of the way and wipe down with extra alcohol as needed.

8.28 Remember to use plates to assess technique as noted in plate protocol. This process will occur for 3-4 weeks. At the end of the cycle, a modified procedure (designed to help cells become more viable for cryopreservation.) will be conducted (*see cell feeding and freezing protocol*).

8.3 <u>Cell Counting</u>:

8.31 Obtain a microscope and hemocytometer. Clean the hemocytometer with EtOH (in spray bottle) and dry with lint free cloth (chem. wipe is good, in green and shite boxes, look like Kleenexs).

8.32 Agitate microfuge sample of cells and add Trypan Blue dye at a 1:10 dilution (10 µL of Trypan Blue to 100 µL of cell culture). Vortex sample.

8.33 Place a hemocytometer cover slip on the hemocytometer (vortex sample again immediately prior to adding cells) and use a micropipette to transfer a 25 ul sample of the cell culture sample (too little or too much can skew the counts FYI) with dye to the hemocytometer (see hemocytometer diagram on page 2). Note that the sample is added in the groove at the edge of the device, and that the cover slip is on the device at this time.

8.34 Use the grids identified in the illustration and count all viable and non-viable cells (separately) in each square within that grid.

NOTE: The size of the square chosen and counted is a function of the number of cells in a sample. Ideally, the cell counts should be between 20 and 50 per cell (depending on size, health, etc.). If the larger cells are yielding 100's of cells, use the smaller squares. If the cell count is still really high, you might consider a dilution. For example, blood samples are typically diluted 200:1 as the number of cells in a small square can easily exceed 1000's! If you elect to use another square, be sure to adjust your calculations.

8.35 **Reproduce table (on next page) in lab notebook and enter data as needed**. Be sure you can identify the correct grid lines and that the light environment is adjusted so that you can see Trypan Blue infused cells.

8.36 Refer to hemocytometer section of this lab to determine the number of viable *and* non-viable cells per mL.

8.37 Repeat counting process each time you propagate your cell line.

8.38 Clean the hemocytometer and cover slip using ethyl alcohol and be careful with them as they are **<u>fragile and very expensive</u>**.

8.39 Return the hemocytometer and cover-slips to the appropriate container, drawer, etc.

8.40 If you culture is contaminated, or is two or more weeks old, dispose of the vial in the biohazard container.

DO NOT FILL OUT THIS FORM! *(Reconstruct in lab manual)*:

Date		Number of cells per square										AVG	Cells/mL
		1	2	3	4	5	6	7	8	9	10		
	Viable												
	Non-viable												
	Viable												
	Non-viable												
	Viable												
	Non-viable												
	Viable												
	Non-viable												

DO NOT FILL OUT THIS FORM! *(Reconstruct in lab manual)*:

Plate Data "Example": Recreate the below table in your lab notebook. Remember, you will need room for at LEAST 4 lines for 4 weeks of data

Date:

	LB plate (before)	LB plate (after)	SDA plate	TSA/Tween plate
# of CFU's	_____	_____	_____	_____

Date:

	LB plate (before)	LB plate (after)	SDA plate	TSA/Tween plate
# of CFU's	_____	_____	_____	_____

Date:

Cell feeding and freezing protocol:

During the last week of cell propagation, the entire class will conduct this lab on a Tuesday. The idea is to feed your cells and to split them in such a way so that on Thursday, they will be at an ideal density of 5 million cells per ml. This will involve counting the cells, splitting to a density of around 1.25 million per ml and then feeding them. Ideally, the cells will divide twice between Tuesday and Thursday (as they usually divide every 24 hours, and the extra food will help ensure this). If things go as planned, the cells will be at the ideal concentration and in good health for the cryo-preservation protocol. After the freeze down, we will wait a few weeks and try again to grow out some of the newly frozen cells. You will freeze two lines of cells, this first one will be the control (the one we thaw and try to grow out), and the other vial will serve as "stock". Obviously, only if your cells grow out and survive, will we keep the cells in long term storage. Depending on technique, cell health, density, etc, your success, chances are about 25 %. Anyways….you will get some experience with this technique and we get some cell lines to keep as stock for future labs!!

Tuesday: Cell Feeding: Elements of this process will be demonstrated a few times at the front of the class. You then can try it with your own cell line. NOTE: During this lab, you do NOT need to assess the technique using the various culture plates (LB, SDA, etc.).

Stock Supplies (from earlier list of items).

4. **Reagents/ Supplies:**
   4.1 Stock tissue culture (*Drosophila sp*. Cell suspension.)
   4.2 50 ml suspension culture flask
   4.3 1 ml disposable transfer pipette
   4.4 10 ml transfer pipet
   4.5 pipette pump
   4.6 isopropyl alcohol in spray bottle
   4.7 tissue culture hood
   4.8 Stock Hyband tissue culture broth
   4.9 2 - 1.5 mL labeled micro-fuge tubes
   4.10 0.1-1 ml micropipet and tips (sterile) optional
   4.11 15 ml falcon tubes (sterile) optional

Also needed:
15 ml falcon tubes (sterile).
Pasteur Pipet tips and vacuum trap (sterile)
Feeding media (Minimal insect cell media enriched with FBS (Fetal Bovie Serum).
Steps:
5.1 Check sample under tissue culture scope for viability.
5.2 Gently agitate the sample and take a 100 ul sample from culture for counting.
NOTE: Your cell count may affect the following steps, so depending on cell #, you may or may not need to concentrate the cells. Your cells can stay in the Falcon tube for a little while, but do the counts and figure out what steps you need to take quickly.
5.3 Pour the remaining cell culture sample into a sterile Falcon Tube and label clearly. If your cells are more than 2.5 million per ml, than you will do a straight dilution. You do not need to spin the cells. If it is less than 2.5 million, you will need to concentrate the cells. So step 5.4 depends on your cell count. If you have lots of cells, then do the math and calculate the amount of cells you need to transfer to bring the original cell count to 1.25 million per ml. For example:

Lets say your cell counts are 4.5 million per ml. You need to split them to get to 1.25 million per ml. To do this, you need a C1V1 = C2V2 calculation.
1.25 million (5 mls) = 4.5 million (V2)
Solve for V2 = 6.25 million V2 = 1.39 mls
4.5 million
So…. Use 1.39 mls of the original culture (in the falcon tube), and add 3.61 mls of "new" feeding media. <u>Remember to double-check your calculation by asking "Does this make sense?"</u> The original culture is about 4 times more dense, so by adding 1.39 mls into 3.61 mls of media, you will dilute it roughly 4 fold, so, yeah, it makes sense.

If your cells are dilute (less than 2.5 million per ml) you will need to <u>*concentrate*</u> the cells…so follow the below steps.

5.4 Note the volume of cells on the side of the centrifuge tube. Spin at low RMPS 1,500 for 3 minutes. Remember to balance the centrifuge in the swinging bucket and that all vials have equal volume. *Remember to use centrifuge etiquette ! Ask if others need to use centrifuge, watch centrifuge while running, label all tubes, etc..*

5.5 (part one) Return to the tissue culture cabinet. Using a micro-pipet, aspirate and save 1 ml of the old media and place temporarily into a micro-fuge tube. Now aspirate all of the remaining old media, except for 1 ml (use volume demarcations on side of the Falcon tube as a guide). Note the below calculations to help you with your next steps. For example, your cells have a concentration of 1.4 million per ml (too dilute to doa simple dilution / transfer). After taking the cells to the centrifuge and concentrate ing the cells in 1 ml., this one ml now has all of the cells in it. So its NEW concentration is 8 million per ml (1.4 million per ml x 5 mls = 8 million). With a concentration of 8 million, use C1V1 =C2V2 to figure out the amount of cells required to give you 1.25 million in you 5 ml sample.

8 million V1 = 1.25 million (5 mls) V1 = 6.25 million V1 = 0.78 ml of cell / media.
8 million

Remember again to see if this makes sense….The cells are about 7 times more conc. Than you need and 0.78 is about 1/7 of 5 mls…so, yeah, it makes sense. Finally, using a new tissue culture vial, aspirate 0.78 mls (us a micro-pipet and sterile tips) of the cells in the Falcon tube. See below for details and remember to mix cells gently before transferring.

This said… Return to SOP.
5.5 (part two, largely a repeat of above, but know your calculations) In tissue culture hood, take out 1 ml of media and transfer into a sterile microfuge tube. Aspirate the rest of the media from the vial down to the 1 ml demarcation (use Falcon to clarify what the volume is). Do not disturb the pellet! Take the fluid level down to 1 ml (the extra "old: media is good for the cells, so always keep some old media around when doing transfers).
5.6 Now, gently agitate the cells. Tap it a few times and "BE GENTLE". If needed, use a sterile transfer pipet and aspirate / expel a few times to help "push" the cells off of the side of the vial.
5.7. Using above noted calculations, aspirate (using micro-pipet), enough of the original cells culture (from step 5.6) and transfer to a new culture vial.
5.8. Now add 1 ml of the old media you saved (in microfuge tube, step 5.5). Record the amount by noting volume on pipet.
5.9 Using sterile pipet…add enough of the enriched, **new media** to the vial to bring final total volume up to 5 mls total. You may need to make multiple transfers FYI. So using above example. 0.78 mls of cells are transferred, 1.0 mls of "old media" is also transferred, giving a final volume of 1.78 mls. To bring to 5 mls, add 3.22 mls of the **new, enriched media**.
5.10 Label culture vial accordingly and place into the tissue culture incubator.

Thursday: Cryopreservation:
Using the ideas covered in previous cell culture labs, you will want to set up and sterilize the hood. Each student will set up 2 vials. These vials are special vials that can withstand the extreme temperatures associated with freezing, hence the term cryo-vials is used. Essentially, each student will set up two of these vials, do a cell count, and few weeks later, check to see if the process worked. Here is a brief overview of the process:

SOP: Cryopreservation:
Set up the hood with the standard supplies. See previous list. You will also need 2 cryovials, DMSO (in a small beaker to keep vial upright), 20-200 micropipet and above noted equipment.
1.1 Assess tissue culture vial using tissue culture scope. If healthy, proceed, if not, dispose of cells and use a colleagues' culture.
1.2 Under tissue culture hood, transfer 900 ul of cell culture to each of the two cryo-vials (use a micropipet).
1.3 To each of the cryovials, add 100 ul of DMSO. *Again this substance should ALWAYS be left in the beaker to avoid tipping over the vial!*
1.4 Close the cryo-vial and invert a few times to mix.
1.5 Remove 100 ul of the cells from your culture vial to do a count. Record the cell count (viable and non viable) in your lab notebook. Be sure to be clear that these counts are for the "cryo-preserved cell line".
1.6 Label both vials VERY CLEARLY and place into cryo-preservation chamber.

Technical Note: You cell lines will be placed in a cryo-preservation chamber. It has room temperature ethanol in it. The chamber's size, amount of alcohol, etc have been worked out as that when the chamber is placed into the minus 80 Celsius freezer, the temperature will drop at a rate of about one degree Celsius per minute. This is important, as the slow drop helps to prevent ice crystals from forming. These crystals are further deterred by the DMSO (Di-Methyl-Sulpha-Oxide). This process takes about 2-3 hours. After this period of time has passed, the vials can be taken out of the chamber and stored in a more typical rack. After a week or two in the minus 80 freezer, the cells are checked. A vial is taken out of the freezer and QUICKLY thawed to room temperature and efforts taken to grow out the cells (media added, incubated, etc.). Only after a few weeks of cell passing, growth, can one confidently say the cryopreservation worked. This said, once the cells have "graduated" from this "test", they are placed in liquid nitrogen for longer term storage. The cells then form the basis of a cell bank which has an indefinite lifespan.

Grow-Out: After a week in the cryopreservation chamber, one of the two vials you prepared will be taken out of the minus 80 freezer and attempts taken to see if they are viable. This process will be under taken for a select number of cell lines. Depending on the success or failure of these cells lines, we will decide to either keep or throw out the other cell cultures! There will be a few comments in lab on how to do this, but this process will NOT part of your lab notebook.

IV. Post-Lab Questions/Activities:

The following post lab questions are for your benefit. The questions will help you to address a range of topics relating to the lab activity. Along with the post lab handouts, these questions will help to ensure that you have both correct information regarding the lab data and crucial lab processes. Complete the post lab questions at the end of the lab and post lab handouts (answers for both of these are available from your instructor, so ask) before making any lab-notebook entries.

1) Why is Trypan Blue used and how does it help with quantifying your cell culture?

2) Is there any correlation between the number of viable and non-viable cells as related to cell numbers? To put this another way, does the percentage of dead cells change with the overall number of cells?
3) Was there any significant change in your cell count from week to week? If so, what may have caused it? One or two ideas are fine.
4) Did your plates reveal any contamination? If so, what was it? Likely causes?

V. <u>Notebook Entries:</u> Data from the lab should be the focus of this section and if there are any incorrect results, you should discuss this as well as expected results. The questions in this section will help you address both your results and discussion. Your data should drive the discussion. An informed discussion is dependent on understanding the lab questions/ activities.

Your intro should be: 2 paragraphs, 4-5 sentences per paragraphs, maximum!

- Mention role of cell culture in modern biotech (1-2 sentences).
- Type of cell being transferred.
- Frequency of transfer and assaying using Trypan blue (viable verses non viable).
- Assessment of sterile technique using plates.
- Efforts to cryopreserve and validate process (very brief).

Results should be:

- One histograms showing viable and non-viable cell numbers.
- Daily count tally and final numbers should also be in this section.
- Table of "plate results" showing plate type, date, and number of CFU's per week.

Discussion should consider the following:

- Look closely at the data and see if you can note any trends.
- What are total cell numbers? Do they change in a significant manner?
- Express the viable and non-viable cells as a percentage of total cells. Are there relationships here?
- Was contaminate rare? Common? Any links with the health or viability of your cell line?
- **<u>Recall that all data is suspect as you are now refining /learning techniques. Many of your observations, ideas, etc., may be due to your technique and not really have a robust science idea behind them. In a nutshell, do not over analyze your data. Remember to not include any information on cell feeding and/or cryopreservation</u>**.

Microscopically Characterizing Cells

Video Adjunct

Artifacts: Not a cell, but other object(s) that can be possibly confused for a cell. Sources include: 1. Cell culture plastic (especially if dirty) 2. Debris (bigger or s
cells.) Note that Brownian movement can make particle appear to be alive as it is moving.

Morphology: Learn to anticipate the shape of the given cell line and look for things out of the ordinary.

Fibroblastic cells: (Used to propagate viruses.) Long cells that are attached to substrate.

Epitheloid cells: Used is tissue culture (cultured skin cells for grafting.) Polygonal and attached to substrate.

Lymphoid cells: Used for monoclonal antibody production: Round, in suspension, and highly refractive (appear to shimmer or glow.)

Cells that form a sheet will eventually abut against one another in a condition known as confluence. Cells like this are in a resting state, and can survive for some time, but will need to be propagated soon.

All cells are best viewed with phase contrast tissue microscope. Phase contrast permits best image, and the tissue scope configuration is one with the objective below the specimen and the light source above.

Morphology is somewhat predictable, but be aware that this can change significantly between individual cells, cell lines, growth phase and other factors.

Multinucleated cells: Occurs upon fusion of two cells. May be perfectly normal, or sign of viral infection (function of cell line.)

Single cell embryo: (biggest of the cells, up to 1-2 mm.) Can be individually Manipulated, and are often used in genetic studies (e.g. genotyping of developing embryo.)

Size: Can be determined by mixing in spheres of latex of known size...but may be problematic.) Preferred method is to use micrometer in the ocular lens and know the field of view of the magnification.

Growth pattern: Can be suspension, matrix, uniform, sheets or colonies.

Cellular Differentiation: Can be seen via diffraction or +/- of organelles.

Confluence (growth rate factor in attached cells): Determined by the percent coverage of the cell-growing surface. Takes some time to learn, and must understand idiosyncrasies of given cell line. Note percentage confluence determination techniques....

Quantifying Viable Cells

Video Adjunct

Note grid pattern and keep your bearing and scale clearly in mind (i.e. what are the exact dimensions of the square you are looking at?)

Clean hemocytometer with EtOH and dry with lint free cloth. Helps to ensure minimum artifacts.

Load Hemocytometer:

Trypan blue is a vitality stain, and is excluded, so blue cells are dying or dead.

Add trypan blue solution (0.25 % (is this correct?) is physiological saline) into cells at a 1:10 dilution. Place pipette at edge of hemocytometer at groove near interface of hemocytometer and cover slip.

Techniques note: If cells are loaded onto hemocytometer, and not immediately counted, placed into petri dish with moist towel in petri dish.

Techniques note: After thawing, wait for 10-15 minutes, as cells immediately out of freezing may take up trypan and still be vital. MTT is a vital stain, and if mitochondria are healthy, color will increase.

Count cells microscopically and identify viable cells.

Identify optimum grid size and assess cell numbers (viable/not viable) Count only cells along two sides of square (be consistent) and count at least 4 squares (preferably 10) to get average and then calculate # of cells/ml.

Alternative counting methods.

1. Coultier Counting: As cells flow by an electrode, the voltage changes (as a result of the cell's conductivity) and registers on the counter.

Good for large number of samples/cells. Requires standards and calibration and cannot always discern between live and dead cells. Also, apparatus is comparatively expensive.

2. Flow Cytometer: Apparatus is similar to Coultier Counter in that voltage registers cell passage. Additional channels allow for flow to be diverted. As a result, cells can be both counted and separated. Cost big bucks and lots of experience to calibrate and resolve different cell features. Sophisticated models can actually check chromosomes (can actually separate x and y chromosome sperm!)

Basic Sterile Cell Culture

Video Adjunct

Work Area: Cell culture cabinet. Do not use to store items, and position reagents so

that they do not block vents or laminar flow around individual reagents.

Supplies are nearby, so that person doing culturing does not need to move. Lighting kept to minimum as to avoid free radical oxidation of regents (unless sterilizing with UV prior to passing cells.)

Passing suspension cells: Sterilizing done with Et-OH and iodine solution. Scrubbed with aseptic foam onto glove (optional)

Sterile techniques: Sources of contamination: Technician, water bath, excessive supplies in cabinet, length of time of procedure.

Passing Cells: Cloudy sample implies need to transfer.

Note technician's pipetting technique. All reagents and supp li
taken to avoid crossing over, and cap is closely monitored. Pipette don'ts: do n
shaft of pipette, open only in hood, close when not in use, and use cotton plugs when possible.

Cell Culture Plastic: Quite variable: FYI....Culture wells, culture bottles and culture flasks.

Equipment: Keep sterile, especially water baths, cell culture hood and incubators.
Microbes Types: Virus, mycoplasm, bacteria (first three, not easily visible, but

can often see effects.) Fungal and molds often grossly visible.

Brownian movement: Random oscillation due to molecular bombardment. Can make inert particles move and thus appear living.

Chief sources of microbial contamination: poor sterilization, room air, dust, aerosols (from talking and sneezing), malfunctioning hood, chalk dust.