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Plant and Animal Cell and Tissue Culture Lab Manual

BIOL 451

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Qatar University

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General Information

Welcome to the Cell and Tissue Culture Laboratory.

This course is unusual in that it presents two completely separate fields of research in one semester. As such, this manual has been divided into two main parts – an Animal Cell Culture part and a Plant Tissue Culture part – and each part has lab numbering starting from "1". This is because, depending on the availability of the lecture instructors, the course may be presented in two different orders: either the Animal part first, or the Plant part first. The lab portion will follow the order set by the lecture instructors.

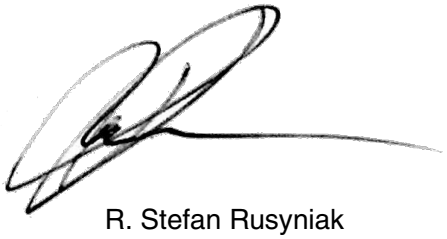
Please note that the practical part of this course is still a work in progress – I may have to update some of the lab materials from week to week. It is also very dependent on the resources that are available to us from week to week, and as such, it may deviate a bit from the schedule listed in your syllabus.

This manual and any handouts that might be posted online for you will form the laboratory textbook for the course. They will contain the protocols for your exercises for each lab, as well as any relevant theoretical information, and definitions that you will need to perform your lab work effectively. Please be sure to bring this manual and any posted handouts to every lab.

It is important that you read through each laboratory exercise and theory before coming to the lab. This will help you understand why you will be doing certain things and will make it more likely that you will successfully complete the exercises in the allotted time, it also helps to ensure that the labs are conducted safely. Prior preparation and understanding are important and will be periodically tested using quizzes (these are likely to occur near the start of the lab).

I hope that you enjoy the labs and learn a lot in the process. I would appreciate any comments or suggestions that you have for future improvements (although, please keep in mind that I have limited resources and time).

I wish you much success in this semester,



R. Stefan Rusyniak

Lab 1: Introduction to the Lab

In this lab, we will be discussing some of the administrative details relevant to the lab part of the course. Please be sure you've looked at the syllabus and read through this section of this manual **before** start of the lab.

Learning Objectives:

Students will:

- Demonstrate their knowledge of Lab Safety Rules by coming in dressed appropriately.
- Identify all pieces of safety equipment in their lab.
-

Pre-Lab Questions:

Read through the syllabus for the lab and answer the following:

- Is there a midterm in this course?
- How much are quizzes worth?
- Should you have a lab notebook?
- What are your TA's office hours?

Use any resources you think will be helpful to answer the following:

- What is an MSDS?
- Which MSDS section contains information on the toxicity of a chemical?
- Is your skin hydrophobic or hydrophilic?
- What does "aqueous" mean?
- What does the word "biohazard" mean?

Lab Safety

Your concern for safety should begin even before starting your lab activity. Please make sure you **always read your lab manual / handouts**, and think about each laboratory assignment **before starting**. Doing so, will alert you to any chemicals and other potential hazards that you might encounter in the lab that day. It will also ensure that you've had a chance to think about how you will perform certain activities and why they must be performed in certain locations or in a certain order.

Always remember that you are not the only person working in a lab and your actions and knowledge (*or lack of knowledge*) can affect the safety of others. For this reason please familiarize yourself with the locations of safety equipment (safety shower, eye wash station, first-aid kit, fire extinguisher, and blanket) in the lab, as well as the location of the medical clinic office in case medical assistance is needed. Also, please make sure your books, bags and clothing are placed out of the way where they are unlikely to cause problems.

In order to protect yourself and others follow basic safety procedures in the labs. Some of our classes involve the use of harmful chemicals so please know and follow the following rules:

1. You **must wear a laboratory coat** for all laboratory work. You will not be allowed to work in the lab without one (and will be marked as absent).
2. **Eating, drinking, and chewing gum are prohibited** in the laboratory at all times.
3. Bulky clothes, abaya, long scarf, dangling jewelry and short skirts can be a hazard in lab. Please do not wear them to the lab or make sure they are secure and unlikely to cause problems.
4. **Confine long hair and scarf** when in the laboratory so that it will not catch on fire or come into contact with chemicals.
5. Wear shoes rather than sandals at all times in the laboratory. **Wearing open-toed shoes is hazardous** in a lab environment (you will be asked to leave the lab and marked as absent).
6. Special care is needed if you wear contact lenses since chemicals splashed in the eye may get under the lens and therefore be difficult to rinse.
7. Mascara has harmful effect on ocular lens (eyepiece) of microscope, and therefore should be avoided.
8. Do not use flammable liquids near open flames. Most organic liquids are flammable.

9. Smell chemicals carefully and only when instructed to do so. Waft odors towards your nose rather than sniffing directly.

10. **Wash hands thoroughly** with soap and water immediately after a spill and before leaving the lab.

11. Any broken glass should be removed from a work area and placed in the glass waste container. Biological waste should be disposed of in biohazardous waste containers.

12. Notify your lab instructor immediately if you are injured in any way.

Notebooks

A laboratory notebook contains an accurate and detailed account of experimental procedures and results. It needs to be neat, well-organized and as complete as possible. The level of detail should allow another researcher to repeat your experiment and replicate your results.

It is important to keep an accurate and detailed laboratory notebook. Sometimes, a small detail may not seem important when you first see it, but it might be useful when you're trying to analyze the results – especially when they are not what you expected. Being able to go back through your procedures and observations in your notes can help you explain why your results are different from what you would expect. It might turn out that you skipped a step in the protocol, or that you made a mistake somewhere along the way (sometimes mistakes are good things – they lead to new discoveries).

The lab notebook is the property of the lab and not of the researcher who is writing in it. In a research setting, a laboratory notebook never leaves the lab. This is important, because many researchers working in that lab may need to refer to that notebook in order to perform their work.

For example, you might be a graduate student who is studying a particular gene. When you finish your project, you will need to be able to refer to your notebook to write your thesis. However, others may need to be able to use your notebook as well.

- Perhaps you made some modifications to a procedure that gave improved results – other people in your lab would benefit from having access to your notes on this procedure.

- Maybe one of your friends in the lab asked you to complete some part of an experiment for them, you would put your notes and observations in the notebook and your friend would later need to access them.

- Also, after you have completed your studies and left the lab, other graduate students might be asked to continue your research to find out more – these new grad students will benefit from having access to your notes (so they don't have to start from the beginning).

Advice on keeping a "proper laboratory notebook" varies depending on who you ask in a teaching lab, but there are a few things that are commonly done. Because some research can lead to financially rewarding discoveries and a laboratory notebook can be used as evidence of prior discovery in cases of disputes over intellectual property / patents, there are a few commonly agreed guidelines for keeping a notebook. Much of it has to do with ensuring that the contents of the book cannot be easily altered:

1. Notebooks should be permanently bound. This means that it should not be possible to remove (or add) pages from them without it being easily noticed.

2. All pages should be numbered. Again this will make it easier to notice if a page has been removed.

3. You should have a date on every page. This allows the identification of when an experiment was first performed or when a discovery was made.

4. You should write in pen and never use white-out or corrective tape. If you need to make corrections then just neatly cross out the incorrect material. A notebook that shows evidence of erasing or removal of data (use of corrective tape, etc.) is not trusted.

5. A laboratory notebook should have your name in it, and a contact number in case the book is found by someone who wishes to return it.

Because a laboratory notebook tends to contain the details of many experiments, it is also a good lab practice to include a Table of Contents on the first 1-2 pages. In the Table of Contents you should list the important experiments and items, and indicate on which pages those items can be found.

Things your TA would like to see in your notebooks:

The page numbers and dates should be in there as mentioned above. For each lab, you should have a brief introduction – just a couple of sentences to summarize the purpose of the lab and the techniques used. This should be followed by a flowchart for that lab and any calculations that might be needed (get these done before the lab, so you don't have to spend time on them during the lab).

You should write your procedure and observations as you do the experiment or very shortly after you've done it – it should be completed before you leave the lab each day. It is much better to have an accurate but somewhat messy description, than it is to have a

less messy and less accurate one, written based on your memory a day or two after the lab was completed.

Lastly, any results/data that are posted for you after the lab should be added to the notebook with a brief explanation of what the result shows. You don't need to make any major conclusions about the results, but should write down information about the picture or graph that might be useful to understanding it later.

Part 1: Animal Cell Culture

Lab 2: Practical Aspects of Work in the BSC

In this lab, we will get an idea of what you already know about asceptic technique. Due to the way this lab is organized, we will not have much time for this, so it is important that you come to the lab prepared and on time. Please read the lab handout, and watch all assigned videos linked in this module - they should prepare you for this week's exercise.

In the lab, you will be demonstrating proper asceptic technique to be used at the biosafety cabinet (BSC). This is not a test - you are not being evaluated on this - but it will give your TA some idea of what you already know and what needs improvement in the class as a whole.

Learning Objectives:

Students will:

- Demonstrate awareness of and compliance with lab safety rules
- Explain the importance asceptic technique in a cell culture lab
- Describe the features of a biosafety cabinet and how it works
- Identify problematic behaviours, which might adversely affect the results
- Demonstrate good asceptic technique
- Properly dispose of wastes generated during the lab

Pre-Lab Questions:

Try to answer these in as much detail as possible before completing this module. Once you finish the readings and videos in this module, come back to this activity and think about how you could improve your initial answers.

- Is it okay to have two people working in a BSC at the same time? Explain.
- You are working in a BSC and there are people standing behind you, talking and moving around. Is this okay? Explain.
- You are trying to demonstrate a procedure for your friend at the BSC, should you be talking and describing what you're doing, or only demonstrating? Why?

Introduction to the BSC

A biological safety cabinet (BSC) looks a lot like a fumehood, but is much more specialized. The main focus of the BSC is the provision of a well-ventilated and clean laboratory workspace. This allows us to work with many types of potentially hazardous materials in a safe way, it also allows us to minimize the chances of contaminating our biological samples while working on them.

There are several different types of BSCs, but Class II BSCs are typically used for cell culture work (you should never perform any cell culture work in a Class I cabinet).

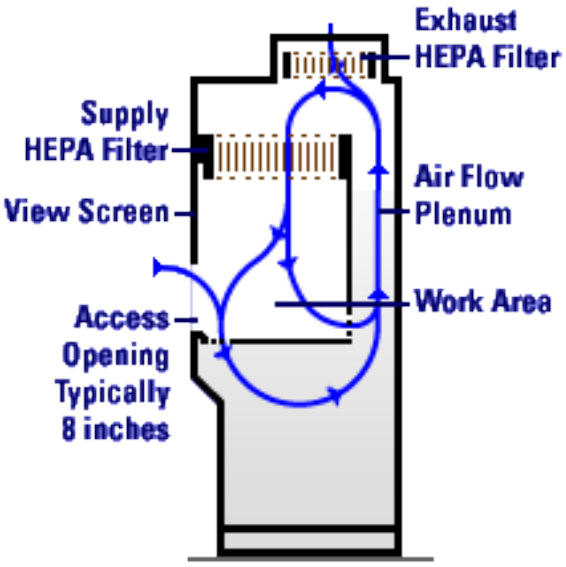


Illustration 1: Airflow in a typical Class II Biosafety Cabinet.

Source: http://www.ucdenver.edu

The linked video (https://youtu.be/oIuWQqzw324) shows the airflow in a typical BSC. Please note how much focus is on ensuring that only clean air is coming into contact with the sample inside the BSC and on the fact that it is again filtered after coming into contact with the sample. Air is drawn in from the lab and circulated in the BSC to provide protection for the sample and for the researcher.

As you watch this video, notice that air is being drawn in from the outside through the air-vent at the front of the BSC, just at the point where the glass sash comes down and is sent for filtration through a HEPA filter. This ensures that there is ample air available to generate the necessary air currents inside the BSC.

At the same time, there is also a curtain of air being generated just behind the glass sash. This "curtain" acts as a primary protective barrier between the air inside the BSC and the air in the lab. It is very important to ensure that there is minimal disruption to this air barrier. Researchers minimize that disruption by moving slowly and avoiding movements of their arms from side-to-side as much as possible.

The air in the BSC is passed through a HEPA filter, which is able to remove harmful bacteria and viruses. As a result, these filters need to be periodically replaced and the BSCs need to be re-certified on a regular basis to ensure proper functioning.

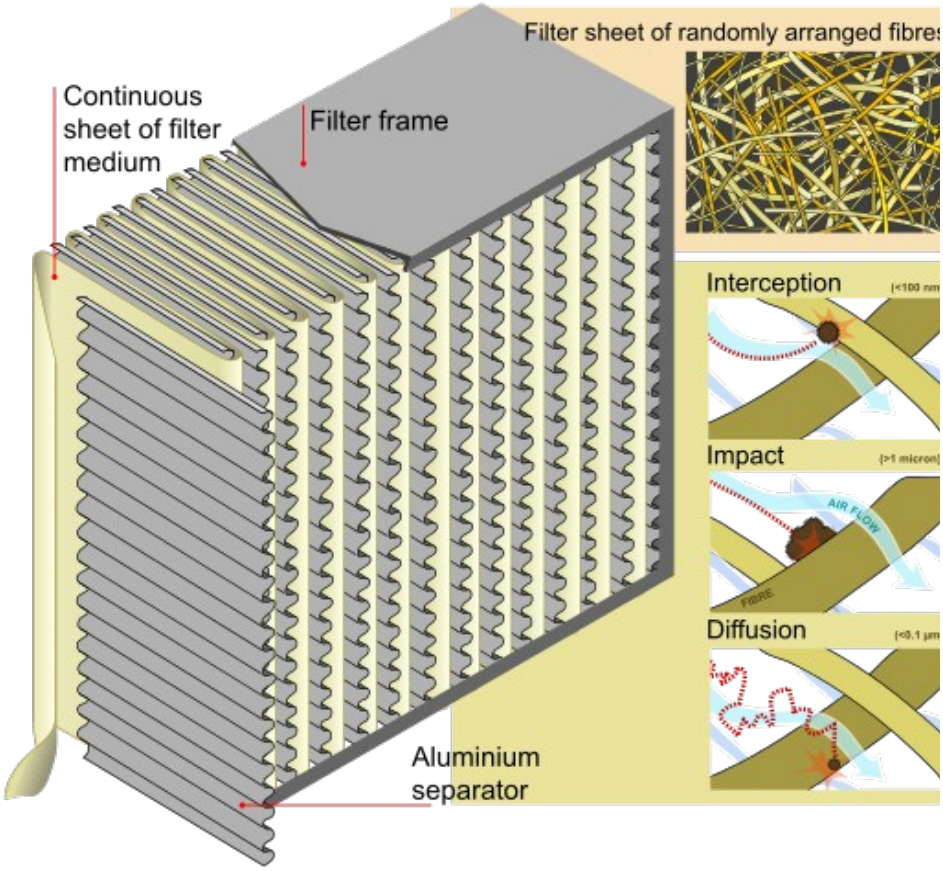


Illustration 2: Anatomy of a HEPA filter.

Source: https://commons.wikimedia.org/

Asceptic Technique in Animal Cell Culture

Good asceptic technique is critical to successful cell culture experiments. While you have already learned about asceptic technique in your Microbiology course, it is very likely that you will need to spend some more time learning about it for this course, and that you will need to be even more careful in our labs. This is because of the differences between the cells you were working with in that course and the cells we will be using. In Microbiology labs, you were dealing with bacterial cells - these were unicellular organisms, selected for their ability to grow well under lab conditions. They were used to growing on their own, even under less than ideal conditions, and had some ability to fight off pathogens.

In this course, you will be dealing with human cells - cells very similar to your own. These cells would normally grow inside the body, where they would be provided with the appropriate conditions (ie. the right temperature, the right oxygen concentration, the right pH, the right levels of ions and minerals, the right signaling molecules and hormones, etc.), and where they would be protected from pathogens by the body's defenses. Growing these cells outside the body means you have to provide all these things and need to ensure that these cells are not exposed to pathogens. This is where good asceptic technique will help you.

Please watch the following videos describing typical technique used in the BSC before coming to class.

- Video 1: https://youtu.be/dSxH9xNmhWg
- Video 2: https://youtu.be/9CN4-AlZPLc

As you will discover, the asceptic technique being demonstrated is quite different from what you have already learned in your other courses. Please pay close attention to the skills being demonstrated, you will be expected to demonstrate many of them as you carry out your exercise on your own in the BSC in this week's lab.

Practical Lab Activity: Asceptic Technique

In this week's exercise, you will be demonstrating your asceptic technique. It is important to understand that **this is not a graded test** – I simply want to see what each of you knows about this important set of methods, and the level of practical skill that you have developed in other labs. It will give me an idea of what needs more attention in this semester.

Just to give you a quick overview: you will prepare one plate and one culture tube. While the exercise is very simple - 15min should be more than enough time – it will allow me to observe several important skills. Once everyone has completed it, we will all return to the lab and I will provide you with general feedback (don't worry, I will not be pointing out individual student mistakes).

Since we only have 2 BSCs in the lab, I can only have two of you performing the lab exercise at a time. At the start of the week, I will post a schedule to let you know at what time you should attend this first lab (keep an eye on the announcements section on Blackboard).

In this lab, **you will be given 15min** to complete the exercise below. Please **make sure you are on time** - if you show up late, it could affect everyone else coming after you.

Materials:

- 70% Ethanol in spraybottles
- Phosphate Buffered Saline (PBS)
- A 15ml tube of Cell Culture
- A 50ml tube of Media
- Pipettes (5ml, 10ml, 25ml)
- Petri plates
- Capped tubes
- Incubator
- Biosafety cabinet

Procedure:

1. Prepare your work area.
    - Spray ethanol on your gloved hands to sterilize them
    - Clean the cabinet with 70% Ethanol
    - Asceptically add the necessary pipettes, tubes and plates
    - Asceptically add the necessary reagents
    - Asceptically add the cells (do this last)
2. Label your plate with your name and today's date
3. Add 8ml of the Media to your plate
4. Transfer 2ml of the Cell Culture (the smaller tube) onto your plate.
5. Close the lid and swirl the plate gently to distribute the liquid evenly over the surface

6. Label a capped tube with your name and today's date
7. Transfer 2ml of the Cell Culture from the small tube into a clean tube
8. Add 5ml of Media from the large tube into the tube you just prepared
9. Cap your tube and gently mix the contents
10. Dispose of your tools and place your tube and petri plate in the incubator
11. Clean up the cabinet and place all the tools back where you initially found them for the next student to do this procedure.

Post-Lab Questions:

Based on your and the class performance in today's lab:

How do you think you did in today's lab – was your asceptic technique perfect?

What do you think was the most common problem among all the students in the lab?

What are some of the details that you didn't think about, but which could result in contamination of your cells?

What skills do you think you will need to focus on and practice as you go through this semester?

Lab 3: Culturing Animal Cells

In this week's lab, we will be discussing the maintenance of cell cultures. In the practical part, you will be learning some of the most basic and most commonly used techniques in animal cell culture. Since we will be working with adherent cultures, you will learn how to detach the cells from their plates (this is trypsinization) and how to count cells.

Learning Objectives:

Students will:

- Describe the composition of media used in animal cell culture and understand its importance.
- Explain how cells attach to the culture vessels.
- Explain why Trypsin is needed and why it's only used for short periods of time
- Explain how and why trypan blue is used in determining cell viability
- Perform an accurate cell count and calculate cell concentration

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Pre-Lab Questions:

Read the linked webpage to learn more about the use of Trypan Blue staining for determination of cell viability:

- http://bitesizebio.com/13687/cell-counting-with-a-hemocytometer-easy-as-1-2-3/
    - Why does Trypan Blue staining allow you to differentiate between living and dead cells?
    - Complete the online exercises in the “Practical Aspects of Counting Cells” Section

Read the “Background” information at the link below and answer the following questions:

- https://www.sigmaaldrich.com/technical-documents/articles/biology/cell-dissociation-with-trypsin.html
    - What is Trypsin and how does it work?
    - Should Trypsin be used with all cell lines? Why or why not?

Please complete the Online Discussion Task for this week.

Creating Conditions for *in-vitro* Animal Cell Growth

In order to grow animal cells *in-vitro*, a hospitable environment must be provided. In most cases, this includes a surface for the cells to attach to, nutritive culture media, and appropriate physical conditions.

Solid Support

In their natural environment, most cells tend to be attached to something - ie. matrix material. Many cell cultures used in research originate from epithelial cells, which are normally attached to a matrix material known as a basement membrane and/or to each other. So, in most cases, animal cell culture involves providing a solid support for the cells to attach to - cells grown in this manner are known as **adherent cells**.

There are a few cell types (ie. lymphocytes - these are used for antibody production), which do not require attachment - these types of cultures are known as **suspension cultures**.

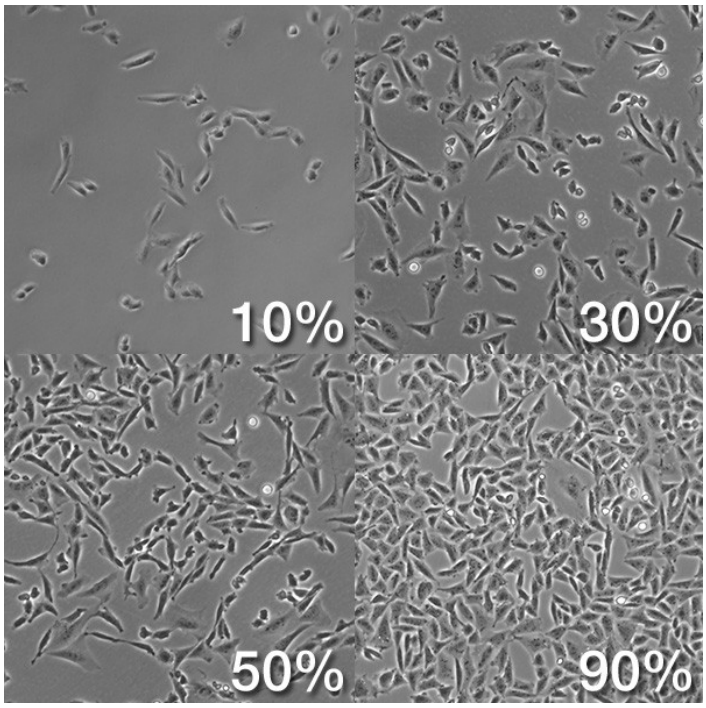


Illustration 3: Cultured cells showing different levels of confluency. Cells in the upper-left quadrant cover ~10% of the available space (10% confluent), while the cells in the lower-right quadrant are occupying ~90% of the growth surface (90% confluency).

Source: biology.stackexchange.com

Usually, the solid support requirement is met simply by providing a treated polystyrene (plastic) petri plate. The plastic plates are "treated" because typical plastics are hydrophobic (they're petroleum products) and thus cells would not be able to attach to them. So the treatment generally involves making the plastic negatively charged. In some cases, the culture vessels can also be coated with typical extracellular matrix materials like collagen, laminin or fibronectin.

In general, adherent cells are grown in monolayers, and thus culture growth is limited by the surface area. Because normal cells tend to respond to environmental cues like crowding, by stopping proliferation (contact inhibition), this means that most cultured cells will stop dividing when there is no more space (ie. the cells have reached 100% **confluency**).

Since, there are space limitations, it means that cells will need to be removed from the surface in some way in order to keep the culture growing - this can be done by enzymatic methods or physical methods.

Nutritional Support

In the natural environment, cells have access to nutrients, hormones, etc. In-vitro, this needs to be simulated by providing an artificial media solution. This media must take the various requirements of the cells into account, so its composition may differ depending on the types of cells that are being cultured or on the type of experiment that is being conducted.

Typical media for animal cell culture is actually a very complex mixture. You can learn about the basic requirements for a cultured human cell here (www.biology-pages.info). The page compares it to the requirements of a bacterial cell, but you can also see the all the components under "Ham's Medium".

In general, the culture media, will contain **glucose, lipids and fatty acids, ATP and amino acids, vitamins and minerals**. **L-Glutamine** is often added separately, just before the media is about to be used, because it is degraded in the presence of normal media and serum over time.

Glutamine is an essential amino acid - cells can't synthesize it, so it needs to be provided in the media. However, once added, it degrades relatively rapidly to ammonia (especially at 37°C), which is toxic to cells. Newer forms of commercially prapared media often use a more stable form of glutamine called GlutaMAX.

New, more stable, forms of Glutamine have been developed, but media containing glutamine probably shouldn't be used after about 3 months - it's unlikely that there will be enough Glutamine in the media at that point to support proper cell growth.

Another important component of complete media is **serum**. Serum (sera - plural) is the fluid component of blood (no cells or clotting proteins), and is thus a complex mixture of **trace elements, electrolytes, proteins, vitamins, growth factors and hormones**. Serum seems to help simulate the animal cell's natural extracellular environment by providing our synthetic media with the necessary factors to promote cell growth.

There are three main problems with the use of serum in experimental work:

- Ethical problem: Serum is extracted from the blood of animals - it's usually calf serum. Thus animals are harmed in the large-scale production of this resource.
- Reproducibility problem: Serum composition is variable and undefined - its composition varies with each animal, so experiments done with cultures grown in media that had different sera added experienced different growth conditions. Thus some variables can't be absolutely controlled.

- Potential contamination: Since serum is collected from the blood of a living animal, there is always the possibility that it may contain various pathogens. Since cultured cells are being grown outside the body, they do not have the benefit of protection provided by an immune system. They are thus very vulnerable to infection.

  Additionally, the discovery of Bovine Spongiform Encephalopathy ("mad cow disease") has resulted in worries over the presence of prions in the serum and has limited the potential suppliers of calf serum to places that are very isolated and have never shown any evidence of the presence of the disease (like Australia and New Zealand).

For these reasons, many researchers try to find ways of avoiding the use of serum. There are some serum substitutes that can be used with some cell lines, there are also some cells that are able to grow in serum-free media. But for many cells, serum is needed.

Physical Conditions

Besides simulating the tissue fluids surrounding the cultured cells, the physical conditions of the body must also be replicated to allow optimal growth of cultured cells. This includes the appropriate **temperature** (37°C) as well as **pH** (7.2 - 7.4). The pH is usually maintained through the use of a bicarbonate buffer in the media. This requires that the incubator provides a constant supply of CO_2 (usually 5% CO_2) to ensure that the pH is maintained.

The media also usually contains a pH indicator called Phenol Red (that's what gives the media its colour), which can alert the researcher to sudden changes to the pH of the media (as happens in cases of microbial contamination).

The Growth Curve

Given the appropriate conditions, cultured animal cells follow growth patterns that may be familiar to you from Microbiology class.

When introduced into a new environment, animal cells will experience a brief period of slowed

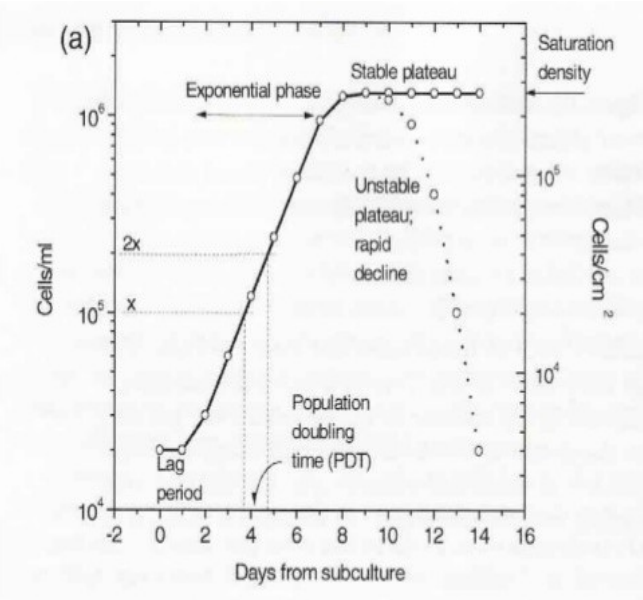


Illustration 4: Growth curve of cultured cells showing the different stages of growth.

Source: www.slideshare.net

proliferation (lag phase) before entering a prolonged phase of optimal growth (exponential growth phase / log phase).

Eventually, wastes like lactic acid from glucose metabolism, and ammonia from Glutamine metabolism will build up to toxic levels, causing the cell proliferation to slow down and cell death to increase. The build up of lactate is likely to be visible in the colour of the media - the Phenol Red will turn more orange or yellow to indicate the acidification.

Additionally, at some point, space is also likely to become limited and will cause a slowdown in proliferation. Thus the cells will enter the plateau phase.

Since the purpose of growing cells in culture is to study their responses to different conditions, it is best to use cells that are growing optimally. Thus log-phase cells are generally collected for experimental work. In practical terms, it means that researchers generally wait until their cell cultures reach 70% - 90% confluency before using the cells for their experiments or subculturing them.

In either case, the adherent cells will first need to be detached from their physical support.

Cell Detachment

In this course, we are using plastic (polystyrene) petri plates which have a negatively charged surface. The reason this charge is important is that when complete cell culture media is added to the plate, some of the extracellular adhesion proteins contained in serum (like fibronectin and vitronectin) will bind to the negatively charged surface by charge-charge interactions, and then stimulate cell attachment.

This attachment of the cells will involve the interaction of the cells' own transmembrane proteins with the proteins (ie. fribronectin) bound to the plate.

Later, when subculturing the cells, we will need to be able to detach them. For this, we will be using an enzyme called **Trypsin** - a serine protease - which cleaves peptide bonds in other proteins. When a solution containing this enzyme is added to a plate with attached cells, the enzyme starts to break down the proteins on the surfaces of these cells.

Some of these proteins will be the ones used by the cell to attach themselves to the plate, thus the enzyme will cause a weakening of the attachment and will allow us to remove the cells from the culture plates more easily.

It is important to note, however, that the trypsin enzyme is not specifically targeting the attachment proteins. It non-specifically cleaves any proteins on the cell's surfaces, therefore a treatment with trypsin should be relatively short to minimize the damage to any other cell surface proteins; especially ones that our cells might need, or ones that we may want to study in the experiment.

When trypsinizing cells, it is good practice to periodically check for "rounding" of cells using a microscope. If the cells look like they are still attached to the plate but look round, they are ready to be detached.

If most of the cells are detached and floating in the media, then the incubation with trypsin has been too long (they are "over-trypsinized"). You should try to avoid over-trypsinizing your cells. Once you observe that the cells are rounded, just give the plate a couple of sharp taps from the side. The physical stress produced by this tapping should help to detach the cells.

Practical Aspects of Trypsinization

The serum in the media contains **protease inhibitors like α1-antitrypsin**, which will prevent the trypsin enzyme from working. For this reason, the media (serum) must be completely removed from the cells before trypsin is added. This is done by first aspirating (pipetting up) the media, and then by washing the cells with an isotonic buffer like PBS (Phosphate Buffered Saline). The PBS is used to help wash off any remaining serum proteins from the cells, this way we minimize the chances that α1-antitrypsin is still present when the Trypsin enzyme is added.

Once the enzyme is added to the cells, the culture dishes are placed in an incubator to allow the enzyme to work optimally - trypsin will work best at 37°C.

At the end of the trypsinization, the reaction is stopped by the simple addition of more complete media (media that contains Serum).

Cell Counting

When setting up an experiment with cultured cells, it is useful to know the starting number of cells. This is especially useful when setting an experiment with multiple samples that will be compared to one another at the end of the experiment - you would want to start with the same number of cells in all the samples. For this reason one of the first things to be done at the start of any cell culture experiment is a cell count.

The Haemacytometer

Cell counting is done in a Haemacytometer, which is basically a specialized microscope slide that has raised edges (“shoulders”) to support a coverslip, and a set of lines (“Grid”) etched into it to divide up the sample and make it easier to determine the cell concentration.

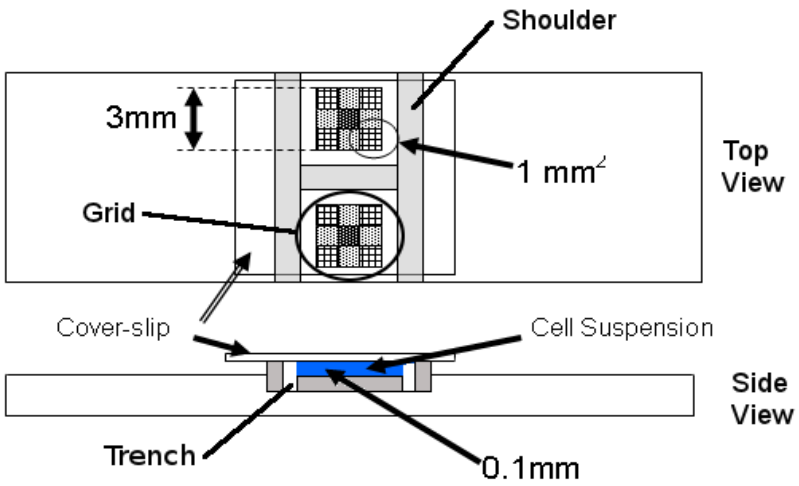


Illustration 5: Diagram of a typical Haemacytometer, shown from the top and from the side. The dimensions of the counting chamber allow us to accurately determine the cell concentration of the applied sample.

Source: https://slideplayer.com/slide/275078/

Because the hemacytometer has an exact volume under the coverslip, one can determine the concentration (cells/ml). In general, cells in one of the large corner squares (like the one labeled as "1 mm^2" in the above diagram) of the chamber are counted. Because the dimensions of that square are 0.1cm x 0.1cm (equivalent to $1mm^2$) and the depth of the chamber is 0.01cm, the volume of liquid under that one large square is $0.0001cm^3$ or 0.0001ml (because $1cm^3$ = 1ml). Thus, once the cells in the above square have been counted, the concentration is easily determined by dividing the number of cells by the volume of that area of the chamber. The cell concentration of the original cell suspension will be the same as that of the chamber – except for any dilutions made.

Because the cells in the counted sample are randomly distributed, it is important to ensure that the result of the count is confirmed by counts of other areas of the counting chamber. For this reason, all four of the corner squares are counted. This helps to ensure that any bias in terms of the distribution of the cells in the counting chamber is removed from the final number.

Viability

One of the challenges faced by cell biologists is that cells are generally very small and it is very difficult (especially in cell culture) to visually differentiate between living and dead cells. However, the success of many experimental manipulations depends on starting with a healthy and abundant cell population. For this reason, biologists will often perform viable cell counts on their cell samples before continuing with their experiments.

Since we know that cells use their membranes to control the flow of molecules into and out of the cell, we can use this ability to help us make that distinction. Dead cells do not control the flow of molecules across their membrane and will allow the accumulation of a variety of dyes like Trypan Blue inside their cytoplasm (and appear blue under the microscope). This is in contrast to living cells , which will exert control over what will enter and will exclude such dyes or actively pump them out if they do manage to enter (and appear white). Thus, living cells can be distinguished from dead cells and a percentage of viable cells in a sample can be calculated.

Practical Aspects of Counting Cells

Counting the cells in a total of 10 of the squares will give the number of cells in a combined volume of 1ul, thus it's a simple way of getting a cell concentration that doesn't require any calculations.

- Check the video on this website to get a better idea of how it's done: http://www.sigmaaldrich.com/video/life-science/counting-of-cells.html
- You can practice performing cell counts using this link: http://cbii-au.vlabs.ac.in/Hemocytometer/experiment.html
    - Change to "Binocular View"
    - Change the "No: of squares counted" to 5

Practical Lab Activities: Cell Culture Maintenance Skills

In this lab, you will be performing three very common tasks in cell culture. You will be using trypsin to detach your cells from their solid support, you will then perform a cell count and determine the viability of your culture. Once that's completed, you will add your cells to fresh media on new plates (subculturing). The procedures you will perform are used 2-3 times per week in a typical cell culture lab, and learning to do them efficiently and cleanly is critical to the success of your experiments in this course.

Exercise 1: Trypsinization

Materials

- 70% Ethanol in spraybottles
- Phosphate Buffered Saline (PBS)
- A 15ml tube of Cell Culture
- A 50ml tube of Media
- Pipettes (5ml, 10ml, 25ml)
- Petri plates
- Capped tubes
- Incubator
- Biosafety cabinet

Procedure

You need to make sure you're starting with warm growth media and warm trypsin. Keep in mind that large solutions need more time to warm up to 37°C, so factor that into your preparation (have a member of your group come in about 30min before the lab to place your solutions into the water bath to warm up). The PBS can be at room temperature.

1. Prepare your work area.
   - Spray ethanol on your gloved hands to sterilize them
   - Clean the cabinet with 70% Ethanol
   - Asceptically add the necessary pipettes, tubes and plates
   - Asceptically add the media
   - Asceptically add the PBS
   - Asceptically add the cells (do this last)
2. Aspirate the media and discard it.
3. Wash cells with 5ml of PBS, aspirate, and discard.
4. Add 1.5ml of warmed Trypsin solution and rock the dish 4–5 times to coat the cells.
5. Place the dish in the CO_2 incubator at 37°C for ~3 min.
6. Remove dish from the incubator
7. View the cells under a microscope to confirm rounding/detachment. Tap the side of the to assist detachment if necessary
8. Add 5ml of growth medium to the plate. The serum in the growth medium inactivates trypsin activity.
9. Gently pipet up some of the media from the plate and wash the plate with it 2 times. This will help collect as many cells as possible from the plate and to break up clumps of cells.

   Please note: If pipetted too vigorously, the cells will become damaged. Ensure that pipetting does not create foam.

10. Transfer the cell suspension into a blue-capped tube.

Exercise 2: Determining the Cell Count

Materials (per group)

- Eppendorf / Microfuge tubes
- Micropipettor (P200) and tips
- Cell Suspension
- 0.4% Trypan Blue Stain
- Microscope

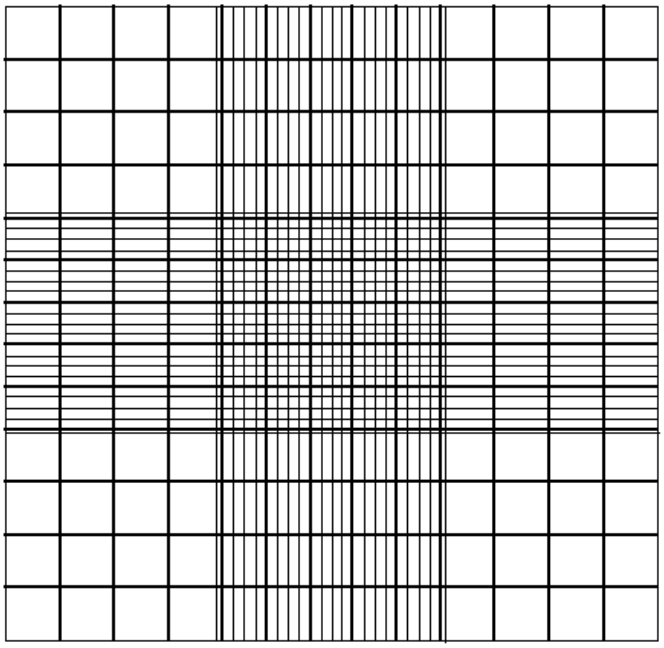
Procedure

1. Clean the haemacytometer and cover slip with 70% Ethanol
2. Obtain the cell suspension and mix gently before taking a sample for analysis.
3. Transfer 40µl of cells into an Eppendorf tube
4. Add 40µl of 0.4% Trypan Blue stain

You've just made 2-fold dilution

5. Mix gently again and wait for about 2min.
6. Take 10µl of the stained cells (don't forget to make sure they're well mixed)
7. Apply a small amount of the sample at the edge of the coverslip of the counting chamber and allow the liquid to be drawn under the coverslip by capillary action.

8. Do the same to the second counting chamber
9. Place the Haemacytometer under a compound microscope and view under 10x objective
10. Wait for the cells to settle and stop moving.
11. Count the cells in 5 of the **large** squares in the counting chamber.



12. Count cells in 5 of the large squares in the second counting chamber.
13. Since the volume under one of the large squares is 0.1µl, then adding the cells from all 10 large squares will give you the number of cells in 1µl.

This gives you a concentration in cells/µl of the diluted solution.

14. To obtain the concentration in the original cell culture, you still need to convert it to cells/ml and multiply by the dilution factor.

(total live cell # in 10 counting areas) x (1000µl in 1ml**) x (dilution factor)**

15. Calculate the viability of the cell culture sample.

Live cell number / Total cell number (living and dead) x 100

Exercise 3: Seeding Plates

After determining a cell count, you must determine how much of the cell suspension on a plate. This week, you will be plating part of your culture on some 96-well plates for your next experiment, and the remainder will be added to a fresh petri plate to ensure that you have a supply of cells for later experiments.

In order to be able to obtain reliable results from next week's experiment, we will need to load 20,000 cells per well (final volume 100µl) on the 96-well plate (we will only be loading a total of 16 wells). Based on your cell counts, calculate the volume of your culture that you will need to add to each well to have 20,000 cells/well.

A) I need ____20,000_____ cells per well.

B) Cell count results show that my cell suspension has _____ cells/ml

C) Therefore, I need $\frac{\text{(A)}}{\text{(B)}}$ _____ ml of the cell suspension per well.

Depending on the cell count you obtained in the previous exercise, there is a possibility that the answer you get will be higher than 100µl (meaning your cell sample is too dilute). This poses a problem. Discuss this problem with your group and come up with a short procedure you could perform to make your cell sample more concentrated.

Materials

- 70% Ethanol in spraybottles
- Complete DMEM media
- Pipettes (5ml, 10ml, 25ml)
- Petri plate and 96-well plate
- CO_2 incubator
- Biosafety cabinet

Procedure:

1. Make sure you're working in a clean work area.
   - Make sure you have the necessary pipettes and tubes
   - Aseptically add the 96-well plate to the BSC
   - Aseptically add the cell suspension (the culture you counted)
2. Obtain the cell suspension and mix gently before taking a sample.
   When working with a 96-well plate, do not use the outer rows and columns of the plate. Do not put samples into Rows A and H and Columns 1 and 12. Samples put into these outer wells tend to evaporate more than the other samples and will therefore have different volumes from the other samples (this may affect the results).

3. Add the appropriate amount of cell suspension into each of the wells needed for the experiment.
    - See your calculations above for the appropriate volume.
    - Make sure you gently mix your cell suspension tube frequently (every 3rd well) to ensure that your cells are evenly distributed in the tube
4. Add the appropriate amount of DMEM media to bring the volume up to 100µl

When doing this procedure, you may notice that adding a small volume of cell suspension to each of 16 wells and then adding another small volume of DMEM to the same wells seems a bit inefficient. Discuss this problem with your group.

5. Incubate the plate overnight in the CO_2 incubator at 37°C to allow the cells to attach to the bottom.
6. The next day, view the 96-well plate under the microscope to confirm that the cells have attached and that they have similar numbers in all wells.

The plate can now be used for experiments.

Lab 4: Cytotoxicity Assays

In this week's lab, you will be performing a cytotoxicity assay. This is a test which helps you determine if a particular treatment condition (drug, chemical, etc.) has had a negative impact on the growth of your cultured cells. There are several types of cytotoxicity assays and none of them are "perfect", so this module will give you an overview of the types of cytotoxicity assays that are commonly performed in cell culture labs. In the actual lab, we will be performing one of those assays and discussing the interpretation of the results from this assay.

Learning Objectives:

Students will:

- Describe the reasoning behind viability testing.
- Explain how different properties of living cells can be used to test for evidence of viability.
- Explain the difference between cytotoxic and cytostatic effects.
- Describe some of the limitations the methods available for cytoxicity testing.
- Explain how the MTT assay works and what is accomplished in each of the steps in the procedure.
- Interpret the results of an MTT assay.

Pre-Lab Questions:

Please complete the exercise at the link below, then go to the Blackboard Discussion Board for this lab and participate in the Online Discussion Task for this week.

- https://h5p.org/node/438867

Much cancer research focuses on determining if a particular compound/chemical will cause the death of cancer cells (ie. toxic to cancer cells). Since things that can kill cancer cells, can usually also kill normal cells, it can also be useful to determine the lowest effective concentration of that compound. Thus we need a way of finding out how many cells are alive or dead.

You have already learned one way of testing cell viability in the previous lab, but you also know that can be very time-consuming. It would also be impractical if testing several samples. Thus, a quick method, which will allow us to test cell viability in multiple samples simultaneously is needed. Such methods are called “cytotoxicity assays” and they fall into a few general categories.

Cell Membrane Integrity

Loss of cell membrane integrity is one of the signs that a cell is no longer functioning/living. The inability of a dead cell to control flow across its membrane can be seen as a general "leakiness". Molecules that are normally intracellular will tend to flow out of the cell and molecules that would normally be kept out are able to enter and stay in the cytoplasm. Below are a few ways that loss of membrane integrity can be detected:

Lactose Dehydrogenase Assay

- Tests for the release of Lactose Dehydrogenase (LDH) from the cytoplasm into the media. LDH is used to convert lactate to pyruvate, and NAD+ to NADH in the process. We can test for the presence of NADH by adding an electron acceptor like the tetrazolium salt INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride). NADH reduces INT to formazan (a coloured product).

- Here's the logic behind this test: Presence of formazan in media = NADH is in the media = LDH is in the media = loss of membrane integrity. Therefore dead cells.

Glucose-6-Phosphate Dehydrogenase Assay

- Tests for the release of Glucose-6-Phosphate Dehydrogenase (G6PD), a cytosolic enzyme used in Glycolysis. In this reaction, G6PD which has leaked out of the cells, is used to generate NADPH. The NADPH is then used as a reducing agent to convert our indicator molecule - resazurin - to fluorescent resorfurin. The ability to use fluorescence makes this a very sensitive assay and allows us to detect death even in a small number of cells.

Glyceraldehyde-3-Phosphate Dehydrogenase Assay

- Tests for presence of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) in the extracellular fraction. When present in solution, GAPDH can be used in conjunction with with 3-Phosphoglyceric Phosphokinase to produce ATP. ATP can

then be detected using a bioluminescence assay called a luciferase assay (again, very sensitive).

Neutral Red Assay

- Neutral Red is a "vital dye" - it enters living cells and accumulates in the lysosomes. It can be used to determine the amount of living cells because the dye leaks out of the lysosomes of dead cells (due to membrane leakiness) and does not permanently stain them. In this way it seems to do the opposite of what you saw with the Trypan Blue dye in the previous lab.

- The reason we can use this dye in a cytotoxicity assay is that multiple samples can be treated with Neutral Red at the same time and any cells that are still alive will hold onto the dye, but the permeability of the dead cells' membranes will not improve over time, so it's a more stable indicator. In contrast, with Trypan Blue, over time the living cells will not be able to keep all the dye out and will eventually look blue.

So, why do we bother doing other assays? Sometimes, cell death processes start within the cell long before membrane integrity is lost. Thus, early stages of cell death will be missed using these methods, and a chemical's effect may be underestimated.

Another complication that is more relevant to the Neutral Red Assay (but also to any assay targeting a specific organelle), is that not all cells will have the same number of lysosomes. Thus two cell samples with the same number of cells but different number of lysosomes per cell would give different results with neutral red, indicating that one of them had more cell death than the other.

Cell Division

One of the defining characteristics of life, that we all learn in elementary school or high school, is the ability of living things to reproduce. So, it might seem logical to try to use that ability as an indicator of living cells in your sample. The way we can test for this on a large scale is to look for things that change significantly during the cell cycle in a mitotically-active cell population. The most obvious choice here would be DNA - the amount of DNA doubles in an actively cycling cell just before mitosis.

DNA Synthesis Assay

- One of the most common ways of testing for DNA synthesis is to add a radioactive (radiolabeled) nucleotide to the cell samples and then measure the level of incorporation of that "tagged" nucleotide into the DNA of the cells in the samples over time. At the end, the more radioactive the cells are, the more of the nucleotide was used in DNA synthesis and therefore there was more cell division in that

sample. The obvious drawback is the use of radioactivity in the experiment and efforts are constantly being made to develop safer alternatives to radiolabeling.

One of the problems with testing for mitosis is that it won't always differentiate between cytotoxic and cytostatic effects of the chemical being tested. Cytostatic agents are analogous to bacteriostatic agents you learned about in Microbiology class - they inhibit cell proliferation but don't actually kill the cell (not cytotoxic). Distinguishing between them may require a visual inspection (ie. counting of cells in a cell colony)

Another problem is that not all cell types are mitotically active. The cells we grow in culture were taken out of a living organism (which does reproduce), but they themselves do not constitute an organism - they are highly specialized and often limited in their ability to reproduce. In some of the most active tissues and organs in your body, only about 5% of the cells may be undergoing mitosis, while some of the cells in your body never undergo cell division. So depending on the types of cells being used in your experiment, these assays may not produce any useful data.

Metabolism

Since all living cells carry out some very basic functions related to energy, it is possible to use that to test for cytotoxicity. Most such assays target molecules or reactions specific to the mitochondria and mostly differ in which enzyme or metabolite they target. Here are the most commonly used assays for active metabolism:

MTT Assay

- Probably the most commonly used cytoxicity assay. Seems to mostly detect mitochondrial dehydrogenase activity (although there is some evidence that it's not just specific to mitochondria). MTT is able to cross the cell membrane, it's converted to formazan inside the cell. Formazan is not soluble and thus can't exit the cell.
- Needs to be solubilized in order to quantify it spectrophotometrically (this adds an extra step to the protocol as well as increasing the volume of the sample).
- Logic: More blue = more metabolism = more living cells.

Alamar Blue Assay

- This one is becoming more popular because it's non-toxic and non-destructive. Cells are not killed in the process and Alamar Blue can be used to study the same cells over time. Uses both a colourimetric indicator and a fluorescent indicator.
- Growth of cells causes reduction of the dye (more colour/fluorescence), while growth inhibition causes dye oxidation (less colour/fluorescence)

- Logic: More colour/fluorescence = more metabolism = more living cells

The limitation of these types of assays is related to the fact that we're relying on a specific organelle as an indicator of cell death. The assumption made by these assays is that active metabolism means that a cell is alive, and that a lowered levels of metabolism in a sample (remember we're looking at many cells all at once) indicates cell death. But lowered metabolism could just mean that the chemical being tested simply affects metabolism in some way, but doesn't actually cause cell death. Less metabolism does not necessarily mean more dead cells.

Practical Activity: MTT Assay

The MTT assay (Methyl-thiazolyl-tetrazolium assay) is a colourimetric test for cell viability. In this assay, a yellow solution of MTT is added to a cell sample, and the reduction of MTT by dehydrogenases and reducing agents (like NADH) in the cells leads to a conversion of MTT into a water-insoluble, blue-purple product called formazan. Since reducing agents like NADH are products of actively respiring cells, and it has been shown that dead cells do not convert MTT into formazan, this colour conversion is used as an indication of cell viability.

Additionally, since the amount of purple colour produced is proportional to number of living cells (we are making some assumptions here), spectrophotometry can be used to quantify and directly compare samples treated with different amounts of a compound/chemical of interest.

In this exercise, you will be performing the MTT assay on the cells seeded in 96-well plates. Your cells have been treated, for a period of 24hrs, with several different concentrations of a chemical which might be toxic.

Exercise: MTT Assay

The purpose of this assay is to measure the number of metabolically active cells. It does this by exposing all the cells in a sample to a chemical called MTT (a yellow tetrazolium salt). This chemical will be reduced to a water-insoluble purple product (formazan) in the presence of metabolically active (ie. living) cells.

Materials

- 70% Ethanol in spraybottles
- Complete DMEM (containing FBS and Antibiotic mixture)
- DMSO
- Pipettes (5ml, 10ml, 25ml)
- 96-well plates
- Capped tubes
- CO_2 incubator
- Biosafety cabinet

Procedure:

1. Prepare your work area.
    - Clean the cabinet
    - Add the necessary pipettes and tubes
    - Make sure all this is done in a sterile way

2. Remove one of the 96-well plates from the incubator and place it in the cabinet
3. Add enough MTT reagent to give a final concentration of 0.5mg/ml (stock is at 5mg/ml)
    - If you have 100ul of cell culture in each well, add 10ul of MTT
    - If you have 200ul of cell culture in each well, add 20ul of MTT
4. Incubate for 3 hours in the CO_2 incubator at 37°C.
5. Aspirate the media (leaving only cells on the plates).
6. Add 100ul of DMSO
    - This helps to solubilize the formazan crystals to make the colour of the sample in the well more uniform.
7. Cover your plate with aluminum foil and leave it on the bench (not incubator) until it can be processed on a plate reader.
8. Use a 96-well plate reader to read absorbance at 570 nm
    - This piece of equipment is in the research complex, so leave your samples for me to scan. I will post the results as soon as I have them.

Any differences in average absorbance readings between the different treatment conditions means that there are different numbers of metabolically active cells in the wells.

Lab 5: Cell Migration and Invasion

In this week's lab, we will be looking at ways of studying cell migration and cell invasion. The protocols we will be using in the lab are some of the most commonly used methods for studying these two related processes.

Learning Objectives:

Students will:

- Explain the principle of cell migration testing
- Explain how cell invasion occurs and how it can be studied in a laboratory
- Describe what a basement membrane is
- Interpret the results of these assays

Pre-Lab Questions:

Please take a look at the video below. As you watch the cells move, please notice the extension of the cell membranes and think about what has to happen inside the cell to allow this to happen.

- https://youtu.be/ZUUfdP87Ssg
  - What is chemotaxis?
  - What role do cell receptors play in this video?
  - What role does the cytoskeleton play in this video?

Cell Attachment

As discussed earlier in the course, one of the requirements for culturing cells is providing them with a physical support - the cells of most cell lines need to attach to a surface before they can start functioning normally. In fact, many cells initiate apoptosis if they do not find a suitable surface to bind to.

An interesting overview of the history of Cell Culture Dishes can be found here (www.sigmaaldrich.com).

While there are some cell lines that can be grown in suspension culture, most of the cell lines we work on in the labs are epithelial in origin and thus need to be attached to something.

Plastics are Hydrophobic

Modern labs tend to use plastic dishes for culturing their cells (many decades ago, cells were cultured in glass dishes), but plastics are a petroleum product, and are thus hydrophobic. Cells would normally have great difficulty interacting with such surfaces, which is why the plastic vessels produced for cell culture are specifically treated to make the plastic surfaces hydrophilic.

This means that you can't just use any plastic petri dish for cell culture - the plates have to be specifically produced for cell culture use.

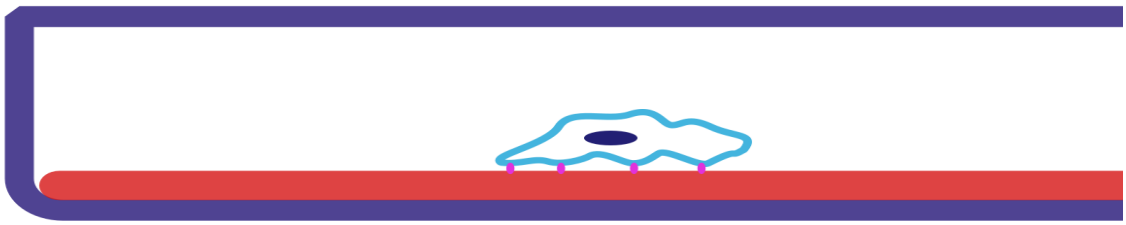


Illustration 6: Cell using its cell surface receptor proteins (pink) to attach to a peri dish coated with adhesion molecules (red).

While cells are still not able to easily bind to the treated plastic polymers, they are able to bind to any adhesive proteins that bind to the treated plastic surface first. For example, the treated surfaces allow for the binding of proteins like vitronectin and fibronectin (through charge-charge interactions). These proteins are commonly found in serum, and provide a target for cell attachment through cell surface receptors like integrins.

Cell Movement

Cell migration is a naturally occurring and important process in the development, maintenance and repair of normal tissues. All of these require the coordinated movement of cells into particular locations, often in response to specific external stimuli (chemotactic agents or physical forces).

The process of cell movement is very complex and still not fully understood. It can be accomplished by a few methods, but in general, it involves cytoskeletal changes which affect the cell shape (**Extension**), production of new attachments to the extracellular matrix (**Adhesion**) along with more cytoskeletal changes (**Translocation**), and removal of unneeded attachments (**Adhesion Disassembly**).

This movement can be complicated further by the fact that, in their natural environment, cells are embedded in a complex and dense 3D mesh of proteins and proteoglycans.

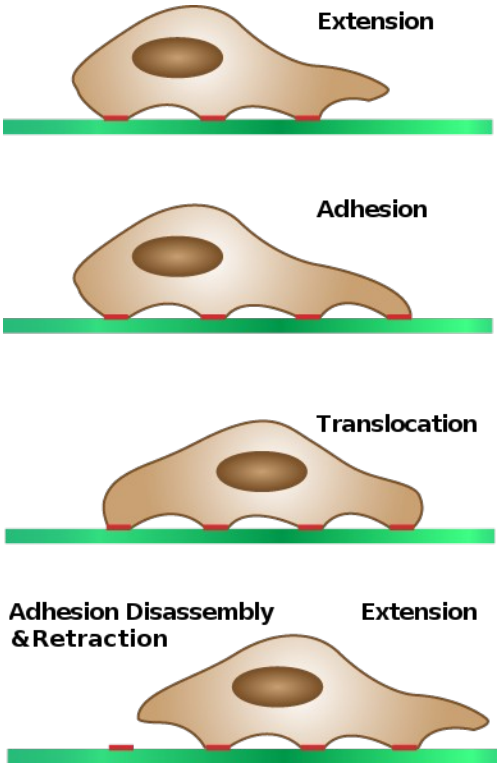


Illustration 7: Summary of cell migration steps.

Source: https://commons.wikimedia.org

While, *in-vitro* studies of cells are often done on 2D surfaces such as plastic petri dishes, in their natural environment cells deal with a 3D matrix (see below).

This, complex matrix tends to put more limitations on cell migration. In order for cells to move through the extracellular matrix *in-vivo*, they need to have the ability to degrade the matrix to create space through which to move. This is achieved by the expression and export of proteases such as the matrix metalloproteinases (MMPs). The expression of

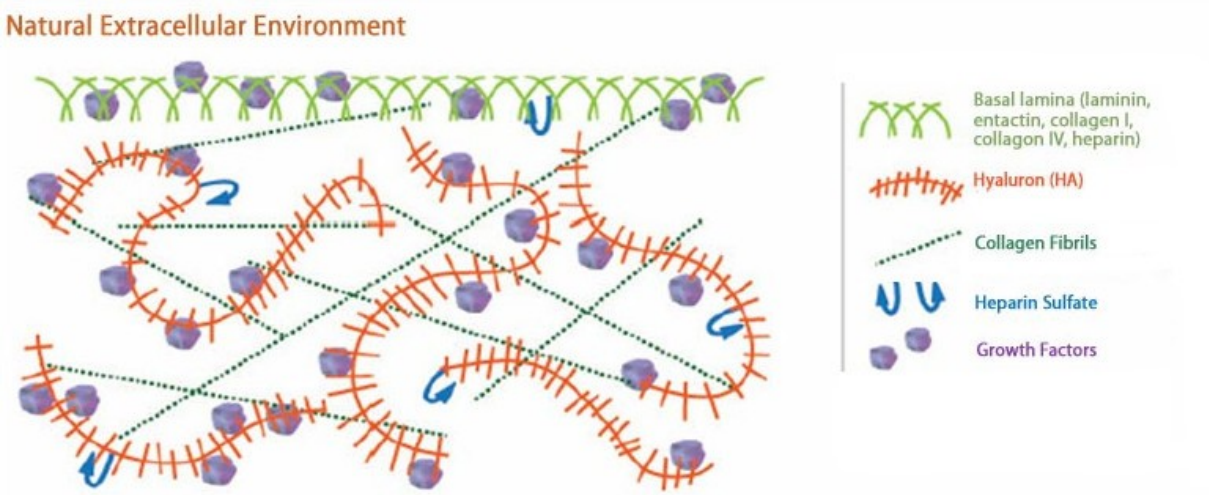


Illustration 8: Visual representation of the composition of the Extracellular Matrix (ECM) in the body.

Source: http://www.labcluster.com/

some specific MMPs will also allow cells to break through basement membranes - this is called "**invasion**" and is often associated with malignant tumors.

In order to simulate this environment, various manufacturers have developed a variety of 3D matrix materials that can be used in research. One of the most commonly used is Matrigel (by Corning), which is a gelatinous mixture composed of structural proteins such as laminin, entactin, collagen and heparan sulfate proteoglycans, as well as a variety of growth factors and signaling molecules. This composition is very similar to the natural environment of the cells and can thus elicit much more complex and realistic behaviours compared to typical cell culture conditions.

The reason we would want to study all this is that cell migration and invasion are crucial to the metastasis and spread of cancer. Cancer cells can migrate as individual cells or in groups, and this starts when they are able to detach from their neighbours and acquire increased cell motility. Part of this will involve the ability to use MMPs to break down the surrounding matrix and enter blood or lymph vessels, which will lead to the spread of these cells and the formation of new tumors in other parts of the body.

Practical Activites: Migration and Invasion Assays

While there are several ways to test a cell's ability to migrate or to invade. One of the most commonly used tools is the "Boyden Chamber". The Boyden Chamber is composed of two compartments separated by a "porous membrane". Cells are seeded in the upper chamber and provided with two types of media – the upper chamber contains basic media, while the lower chamber contains complete media (with FBS).

The FBS contains molecules which are capable of inducing cell migration by chemotaxis. These chemotactic factors diffuse across the porous membrane, establish a concentration gradient, and bind to the relevant cell surface receptors. Activated cells will then **migrate** through the pores (holes) in the membrane separating the two chambers in order to get to the higher concentration of the chemotactic agent. Cell migration can then be measured by counting how many cells are found on the underside of the porous membrane.

The same system can be used to detect cell **invasion** if a dense matrix is placed between the cells and the porous membrane. The cells then have to degrade the matrix before they can migrate into the lower chamber.

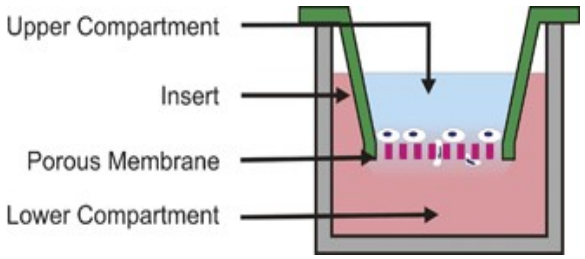


Illustration 9: Organization of a Boyden Chamber.

Exercise 1: Invasion Assay

In this protocol, we will be using a Boyden chamber to determine if our cancer cells are able to migrate across a membrane (matrigel) as a result of chemotaxis. We will be adding our cells suspended in DMEM (no FBS) into a transwell insert suspended over a well. The well itself (the "lower compartment") will contain normal cell culture media (with FBS) which contains chemotactic agents that will attract the cells. We will measure how many of the cells migrate into the lower chamber after 24hrs, and determine if our treatment has any effect on the migration across this membrane (invasion) of these cells.

Materials

- 70% Ethanol in spraybottles
- Phosphate Buffered Saline (PBS)
- Complete DMEM (containing FBS and Antibiotic mixture)
- Trypsin
- Pipettes (5ml, 10ml, 25ml)
- 24-well plates
- Capped tubes
- CO_2 incubator
- Biosafety cabinet

Procedure:

- **Seeding *(day 1)***

1. Make sure you're working in a clean work area.
    1. make sure you have the necessary pipettes and tubes
    2. add the 24-well plate
    3. add the inserts
    4. add the cell culture plate (do this last)
2. Trypsinize the cells.
    1. Aspirate the media
    2. Wash the cells twice with PBS
    3. Spin down the cells at 700g for 10min and aspirate the trypsin solution
    4. Wash with PBS and spin down again
    5. Resuspend cells in 2.2ml of DMEM (without FBS)
3. Transfer 200ul of cell suspension to an epitube for cell counting.
4. Perform a cell count. (use 20ul of cell suspension and 20ul of Trypan Blue)
    - We would like 100 000 cells per each insert on the plate (~$2x10^5$cells/ml).
    - Result of the cell count: _____ cells/ml

5. Perform the necessary dilution to be able to plate 0.5ml of cell culture per insert
    - Prepare 3 test tubes of 2ml of basic growth media with different concentrations of the treatment
        1. for 0.4mg/ml, add 4ul of 100mg/ml stock for every 1ml of total DMEM
        2. for 0.8mg/ml, add 8ul of 100mg/ml stock for every 1ml of total DMEM
        3. for 0.0mg/ml, add 8ul of 70% ethanol for every 1ml of total DMEM
6. Add 1ml of Complete Culture Media (DMEM with FBS) to the bottom chambers
7. Incubate the plate overnight in the CO_2 incubator at 37°C to allow the cells to migrate.

- **Detection of Migration *(day 2)***

8. Make sure you're working in a clean work area.
    1. make sure you have the necessary pipettes and tubes
    2. add the 24-well plate

make sure all this is done in a sterile way

9. Carefully aspirate the media from the lower and upper chamber
10. Wash 2x with PBS
11. Add 0.5ml of fixative to the upper and to the lower chamber
12. Leave the cells in the fixative for 2min
13. Remove the fixative
14. Wash 2x with PBS
15. Add 0.5ml of methanol to the upper and to the lower chamber
16. Leave the cells in the methanol for 10min
17. Remove the methanol
18. Wash 2x with PBS
19. Incubate the cells (in the lower chamber) with 0.5ml of Cresyl Violet for 10min
20. Use a cotton swab to wipe the inside of the insert to remove unmigrated cells
21. View inserts under the microscope to count the number of cells that migrated

Exercise 2: Migration Assay

This assay is also sometimes referred to as the “wound-healing assay”. The purpose of this assay is to determine whether your cells are able to move across a space and to measure how quickly this happens. We can do this by taking a confluent cell layer and introducing a scratch into it, we then apply our treatment to the cells and take pictures of the scratched area every few hours to see if the cells migrate to cover up the scratch.

Materials

- 70% Ethanol in spraybottles
- Phosphate Buffered Saline (PBS)
- Complete DMEM (containing FBS and Antibiotic mixture)
- Trypsin
- Pipettes (5ml, 10ml, 25ml)
- 6-well plates
- Capped tubes
- CO_2 incubator
- Biosafety cabinet

Procedure:

1. Trypsinize cells and perform cell counts
2. Plate out ~300,000 cells per well in a 6-well plate
3. Incubate the plate at 37C until the cells are almost confluent. Check on this daily.

The Migration Assay

4. Prepare your work area.

   Make sure all this is done in a sterile way
5. Remove the 6 well plate from the incubator and view the wells under a microscope.
6. While the plate is on the microscope stage, draw a straight line with a sharpie across the wells on the underside of the plate.
7. Place the plate in the cabinet.
8. Using a sterile yellow micropipette tip, introduce a scratch (perpendicular to the line you drew) into each well. Use the lid of the plate to help you make sure it's straight and uniform (use it like a ruler).
9. Carefully aspirate the media and wash each well with 2ml of PBS
   - Be very gentle, you do not want to disturb the cells that remain on the plates. Sheets of the cells may detach and lift off if you are not careful
10. Carefully remove the PBS and replace it with fresh media or media supplemented with our plant extract.
    - Do 2 wells with complete media

- Do 2 wells with 70% ethanol (10ul of 70% ethanol per 2ml of complete media)
- Do 2 wells with 0.5mg/ml of plant extract (10ul of extract per 2ml of complete media)

11. Take pictures of each scratch in each well under the microscope as soon as possible
    - this is time 0
    - use the sharpie line to help you find the same spot each time after this.
12. Incubate the plate in the CO_2 incubator at 37°C.
13. Take pictures of the scratches every 2 hours for the first day and then at 24 hours

Any differences in the size of the scratch between the time points can be measured using specialized software. We will then be able to compare those to the negative control to determine if our plant extract has any effect on cell migration.

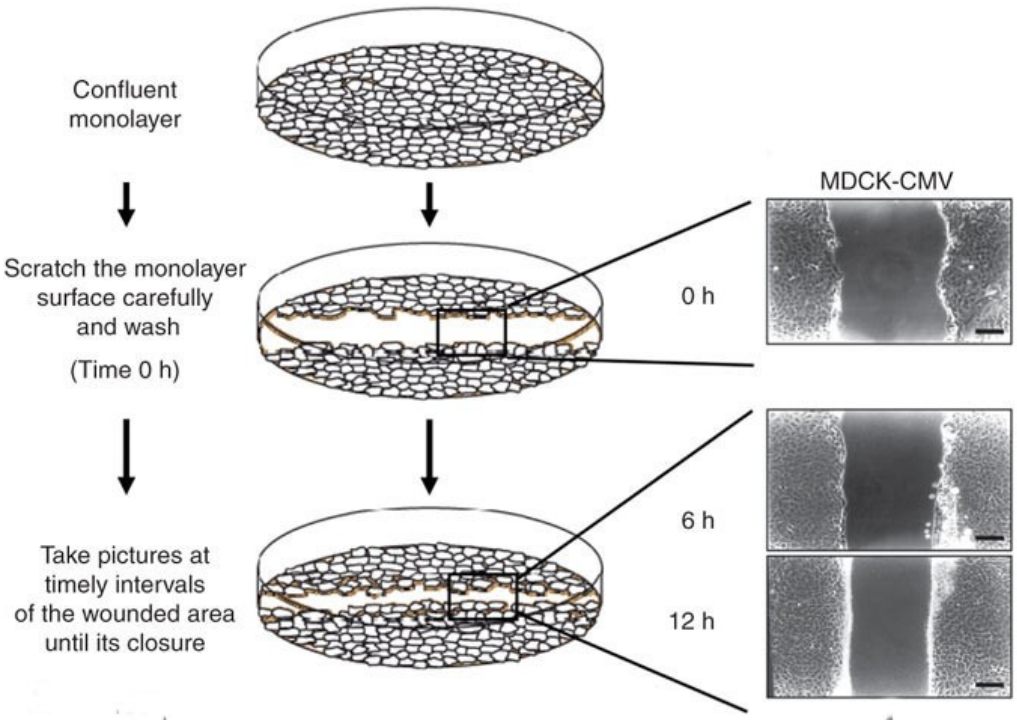


Illustration 10: Visual overview of the scratch assay

Lab 6: Fluorescence Microscopy

In this week's lab, we will be looking at how fluorescence microscopy works and how it can be used to detect things like the presence of apoptotic cells in a culture sample.

Learning Objectives:

Students will:

- Explain what a fluorophore is
- Explain the principle of fluorescence microscopy
- Interpret fluorescence microscopy images
- Explain how apoptosis can be detected

Pre-Lab Questions:

Please view at least the first 7min of the linked video (view the whole thing if you have the time). It will help you to understand fluorescence and flourophores.

- https://youtu.be/AhzhOzgYoqw
  - What are some of the advantages of fluorescence microscopy?
  - What is a fluorophore?
  - What is the difference between "excitation wavelength" and "emission wavelength"?

Basic Principles Behind Fluorescence

Fluorescence microscopy has become a very common tool in the study of cells. It allows us to specifically, and easily, localize specific proteins/enzymes or organelles inside single cells, and thus allows us to gain a better understanding of a cell's ultrastructure as well as the movement of molecules and organelles within a cell.

This is accomplished through the use of special molecules which can be induced to produce light (glow) when exposed to high energy wavelengths of light. These molecules are called fluorophores, and they can be attached in various ways to our cellular components of interest.

In order to understand how this works, it is useful to understand the difference between fluorescence and phosphorescence.

Fluorescence is a property of some substances, where they absorb electromagnetic radiation – at some specific wavelength of light – and nearly simultaneously emit light at a longer wavelength (thus lower energy). You may have seen this in action when viewing an agarose gel in molecular biology lab. The gel is lit with ultraviolet light (which your eyes can't detect) and the Ethidium Bromide, which has become associated with the DNA on the gel, fluoresces at a lower wavelength - one that is easily visible to the human eye. Once the UV light is turned off, the "DNA" stops glowing.

Phosphorescence differs from this only in that the substance would continue to glow for some time after the UV source had been turned off - many analog watches have phosphorescent materials on their dials to make them easier to read in the dark. They absorb light energy while exposed to daylight, and later emit it through phosphorescence while in the dark.

The British scientist Sir George G. Stokes first described fluorescence in 1852. He noticed that in fluorescence, the emitted light always occurred at a longer wavelength than the excitation light. This can be explained by the basic principle of chemistry which states that atoms can absorb energy by sending their electrons to higher-energy orbitals. The subsequent return of these electrons to their normal, lower-energy orbitals results in the release of energy (often in the form of light – fluorescence or phosphorescence).

In the process of this conversion, some of the initial energy is lost (due to entropy) and thus the light energy emitted in the process is at a lower energy level (longer wavelength) than the initial excitatory energy (shorter wavelength). This is now called the **Stokes' Shift** and is very useful to us in fluorescence microscopy.

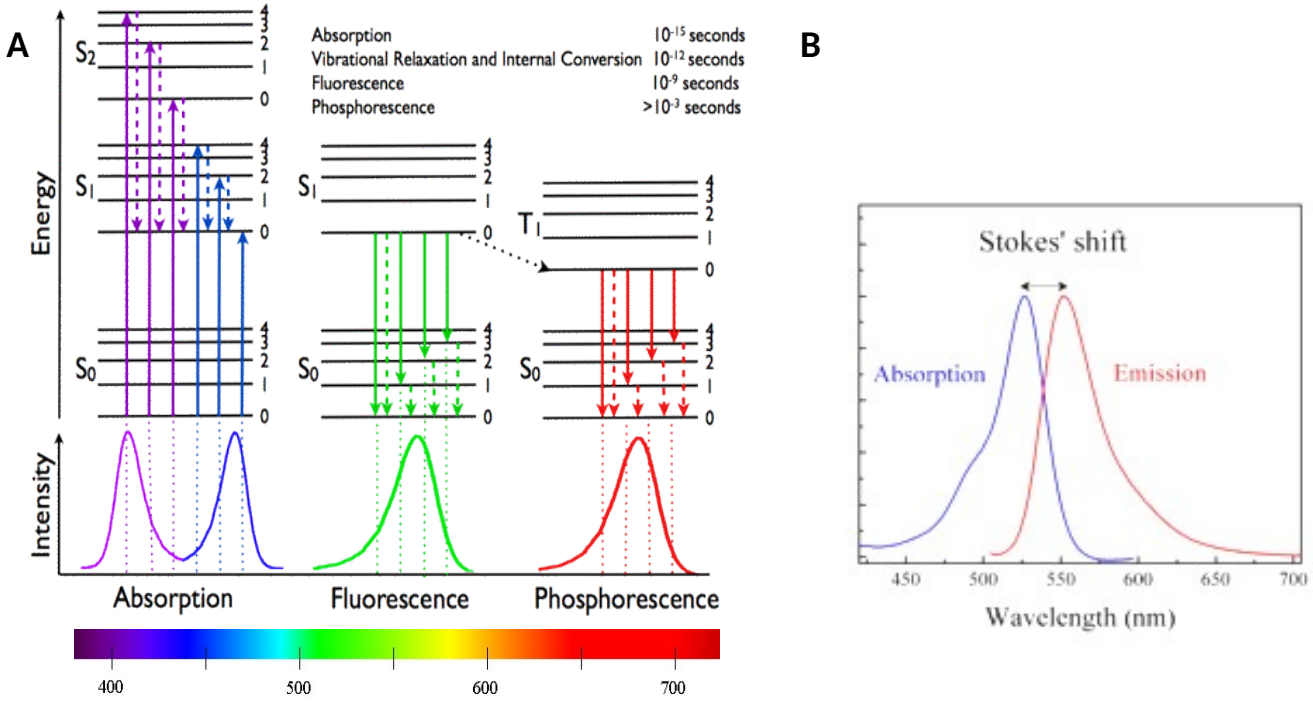


Illustration 11: A visual representation of the changes in the energy state of electrons in response to the absorption of light energy (A). High energy (low wavelength) light excites electrons, causing them to move to higher energy orbitals. The return to their normal orbital results in the release of energy as light at lower energy (higher wavelength). The difference between the excitatory wavelength and the emitted wavelength is known as the Stokes' Shift (B).

Fluorophores

Most **fluorophores** are small organic molecules, which are capable of absorbing light energy at a specific wavelength and emitting light at a longer wavelength.

Some of these molecules can often be used to directly stain biological macromolecules - for example DAPI stain binds to AT-rich regions of DNA and thus stains nuclei, while Phalloidin directly binds to actin and can thus be used to help visualize cytoskeletal structures.

Other fluorophores are chemically attached to antibodies, which can then be used to identify just about any molecule of interest inside a cell.

Additionally, some naturally occurring proteins have fluorescent properties (ie. GFP - Green Fluorescent Protein). The genes encoding these proteins are often inserted into expression vectors, which are then used for cloning and expressing a particular protein of interest (ie. tubulin). Due to the presence of the fluorescent protein sequence in the expression vector, the gene you clone into that vector will be attached to the fluorescent protein gene and result in the expression of a recombinant protein – this is your protein of interest (ie. tubulin) with the fluorescent protein (ie. GFP) covalently attached to it. This is called a fluorescent "tag".

These fluorophores can then be used in combination to highlight several specific cell components on the same slide. In such cases, fluorophores with different emission wavelengths are used to help ensure that each fluorophore is detected individually. Once pictures are taken at each emission wavelength, the images can be merged to get a more complete picture of the location of various components inside a cell.

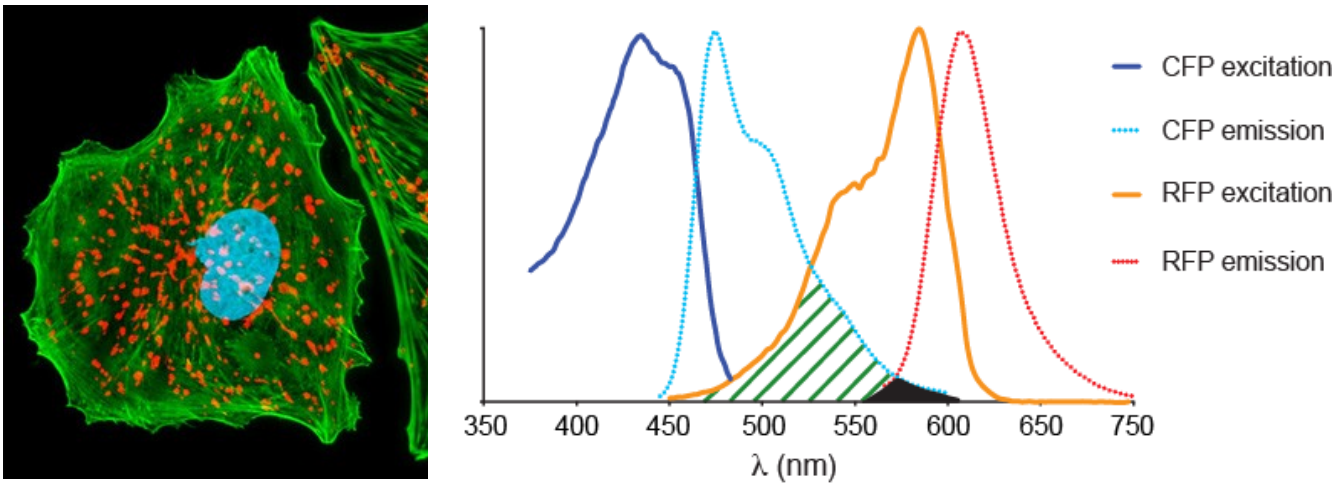


Illustration 12: [Left] A typical composite image composed of 3 separate fluorescence microscope images, each detecting a different cellular component - Nucleus is in blue, Mitochondria in red, and the Cytoskeleton in green. Each of these was detected individually, then the three images were merged to produce the image seen here. [Right] Excitation and emission spectra of two fluorescent molecules (CFP – Cyan Fluorescent Protein, RFP – Red Fluorescent Protein) showing that they have different excitation and emission peaks. The "peak" of the excitation spectrum is the wavelength used to activate the fluorescent molecule and the microscope is set to detect the "peak" emission wavelength, in this way, each cell component is detected separately.

Fluorescence Microscopy

Fluorescence microscopes utilize high-energy wavelengths of light (usually in the UV range) to activate the fluorophores on the slides. The fluorescent stains then emit light at a lower wavelength which can be viewed/detected by the observer. Because different fluorophores are activated by different excitatory wavelengths, the fluorescent microscope must have a way to specifically select them. This is accomplished by the use of different **excitation filters**, which block all other light and only permit the desired wavelength to pass through them.

When the excitation wavelength of light strikes the sample and activates the fluorescent molecules, a longer wavelength of light is emitted into the objective lens. Before this emitted light can be detected, however, it passes through an **emission filter** in order to block any other light (ie. any reflected “excitation” light).

This ensures that the resulting image will only show the location of the structures stained by the fluorophore being activated at that time. Because all other light is being blocked by the excitation and emission filters, the areas of the slide surrounding the highlighted structures will be black; this results in excellent contrast (a clear delineation of the structure of interest from everything else).

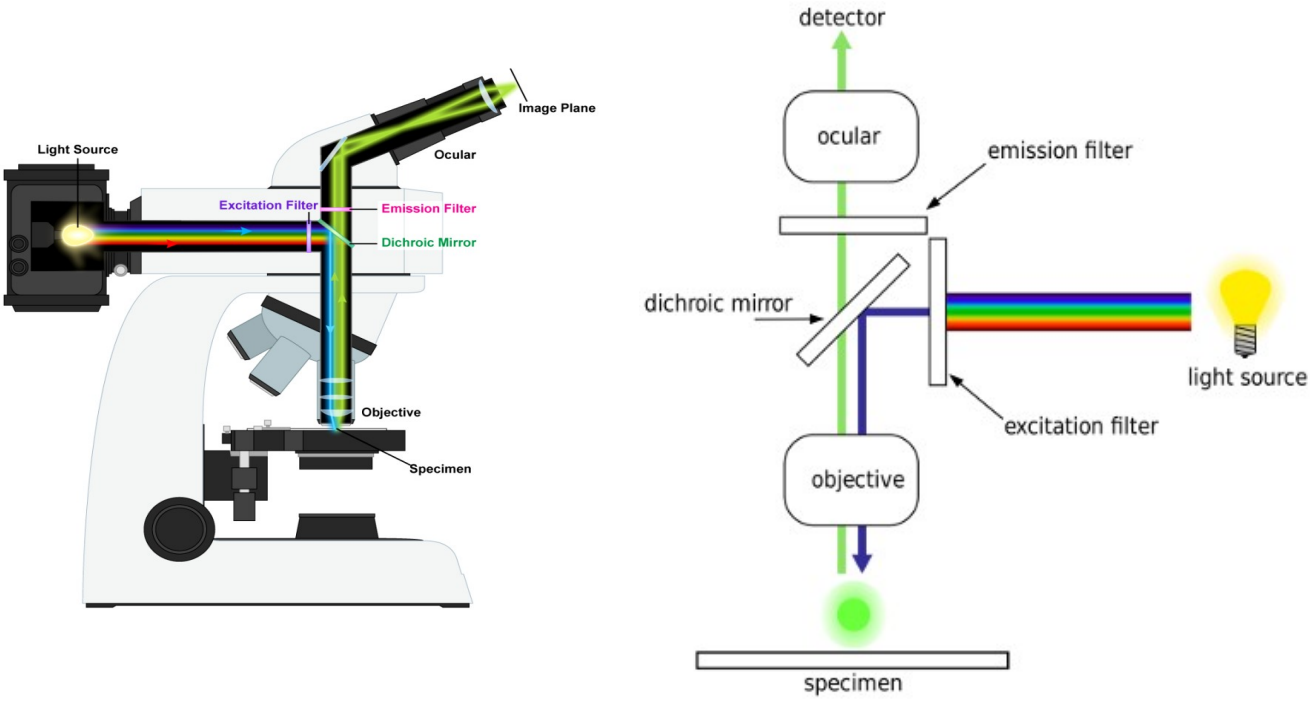


Illustration 13: The basic structure of a typical fluorescence microscope [Left]. A schematic diagram of the filter cube showing how it functions [right].

Source: www.netclipart.com

Since the background colour is black and only the tagged components are visible in fluorescence microscopy, it is important to be able to show the presence of cells on a slide (in case the molecule or organelle of interest is absent). For this reason, we try to highlight something that is likely to be present in all cells – ie. nuclei.

Practical Activity: Apoptosis and Fluorescence

There is a variety of ways in which apoptosis can be detected by fluorescence microscopy – there are several apoptosis-specific molecules that could be tagged or detected with fluorescent antibodies. One of the simplest ways, however, is to simply look at the nuclei of the cells.

DNA fragmentation is a hallmark of the later stages of apoptosis. At this time, DNA is being degraded and the nucleus starts to undergo some very characteristic changes. Thus detecting this change in the appearance of the nuclei can be relatively simple even with a fluorophore that is not specific to apoptosis.

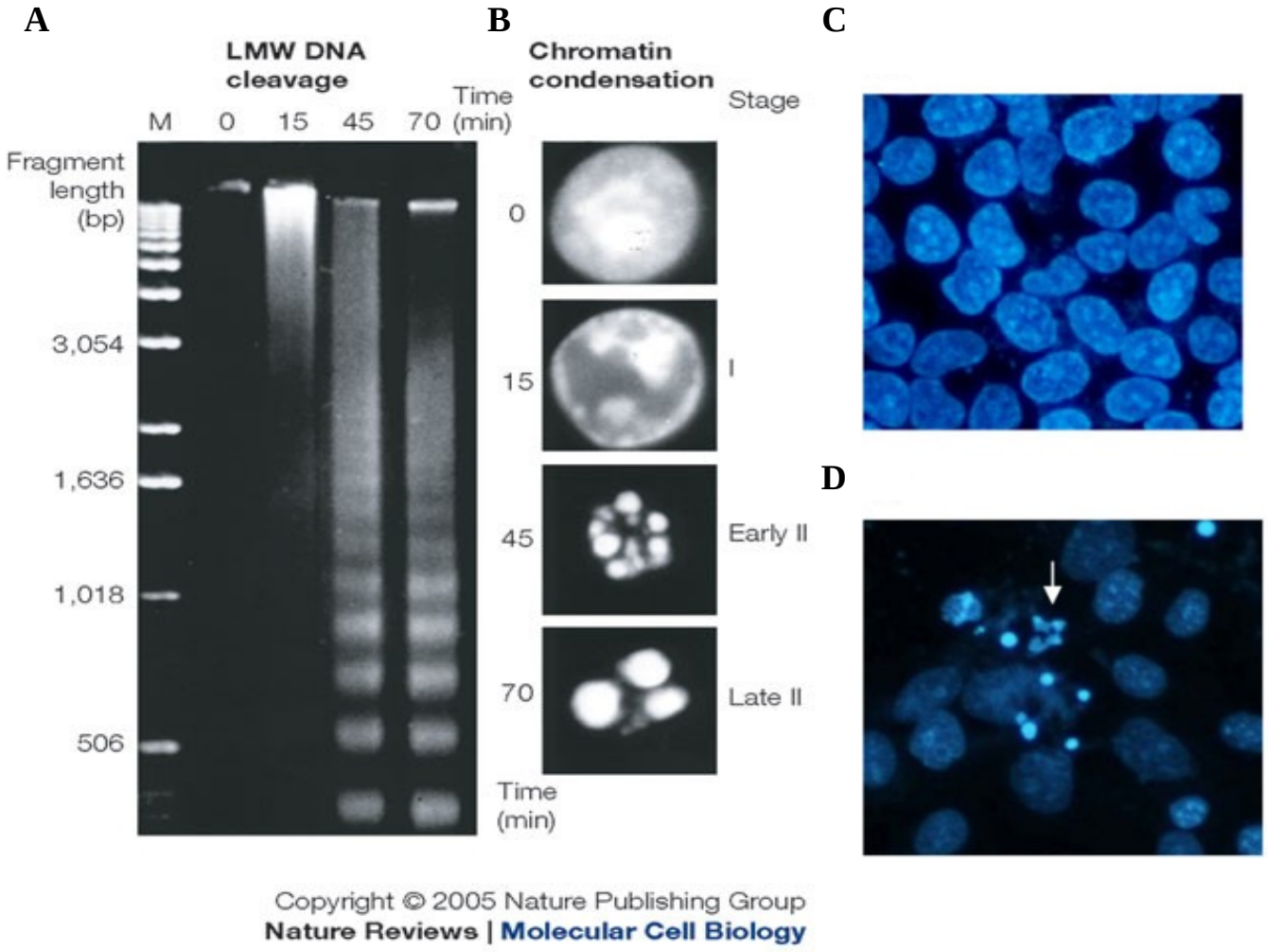


Illustration 14: Agarose gel showing the characteristic DNA degradation pattern seen in apoptotic cells (A), and accompanying images of the appearance of the nuclei at these stages (B). DAPI-stained images of non-apoptotic cells (C) and apoptotic cells (D, arrow).

Exercise: Fluorescent Staining of Nuclei

In this experiment, we will be using a kit containing the Hoechst 33342 dye to stain cell cultures treated with 0.5mg/ml of our treatment condition.

Materials

- 70% Ethanol in spraybottles
- Phosphate Buffered Saline (PBS)
- Complete DMEM (containing FBS and Antibiotic mixture)
- Trypsin
- NucBlue fluorescent staining kit
- Pipettes (5ml, 10ml, 25ml)
- Treated and untreated cells
- Capped tubes
- CO_2 incubator
- Biosafety cabinet

Procedure

1. Trypsinize the cells (0.5ml of trypsin per well)
2. Add 2ml of growth media
3. Transfer each culture into separate test tube
4. Centrifuge tubes at 700g for 10min
5. Remove media
6. Resuspend cells 1ml of PBS
7. Take out a sample to do a cell count and a viable cell count
8. Add 2 drops of NucBlue stain to each tube
9. Incubate for 20min
10. Mix cells and pipette a 10ul sample onto a slide and coverslip it
11. View slide under a fluorescence microscope
    - Excitation wavelength => 360nm
    - Emission Wavelength => 460nm

Part 2: Plant Tissue Culture

Lab 1: Asceptic Technique in Plant Tissue Culture

In this lab, we will be discussing the basics of plant tissue culture. We will also be initiating some sterile plant tissues for use in later labs, by sterilizing seeds and preparing them for germination at different conditions.

Learning Objectives:

Students will:

- Know the basic requirements for working with plant tissue culture
- Understand the need for aseptic conditions and surface sterilization of plant materials.
- Prepare seeds for plating and growth on sterile media.

Pre-Lab Questions:

Please watch the linked video and answer the following questions:

- https://youtu.be/zd0iVJrQwyY
  - List one likely source of bacteria mentioned in the video
  - Based on what you saw, in what way is working with plant tissue culture similar to animal cell culture?
  - Based on what you saw, in what way is working with plant tissue culture different from animal cell culture?
- How is a Laminar Flow Hood different from a Biosafety Cabinet (BSC)?

Introduction

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under **sterile conditions** on a nutrient culture medium of known composition. In order to achieve the aseptic conditions media are generally sterilized by autoclaving (except the thermolabile components – refer to the lecture notes). Surface sterilization of living plant materials (**explants**) is required because these are naturally contaminated on their surfaces with microorganisms. Surface sterilization of living plant materials is done with chemical solutions. Most commonly used are alcohol and sodium hypochlorite, or mercuric chloride. Mercuric chloride has limited use due to its toxicity to animal tissues (ie. it's not safe for you to use).

Explants are then cultured on solid or liquid media, which support the growth of these isolated tissues. These media are generally composed of inorganic salts with a few organic nutrients (refer to the lecture notes), vitamins and plant hormones. Media are solidified with the addition of a gelling agent, usually purified Phytagar or Gelrite.

Since these media are full of nutrients (ie. sugars), they will allow for the growth of contaminants just as easily as the growth of your plant cells. In fact, due to the faster rate of division of the bacteria and/or fungi that could contaminate your media, they are likely to outcompete your plant samples for the resources in the media and cause problems with the culture of your plants of interest. Thus, the sterilization of your plant materials and a good asceptic technique are essential to successful plant tissue culture.

Sample Sterilization Methods

Practical Activity: Seed Sterilization and Culturing

Seeds of tomato or mustard are treated using different dilutions of Clorox solutions and different times, according to the matrix below.

		Clorox Concentration		
		0% Clorox	10% Clorox	20% Clorox
Length of Incubation	5min	1 tube	1 tube	1 tube
	10min	1 tube	1 tube	1 tube
	20min	1 tube	1 tube	1 tube

You will then "plate" them onto plates containing MS media as well as plates

containing filter paper and sterile water. You should end up with a total of 18 plates for analysis next week.

Sterilization of equipment and other materials

Autoclave 1 L of distilled water used for washing seeds or explant.

Forceps, scalpels and glassware (like beakers, glass petri plates etc.) are wrapped in aluminum foil or paper before putting in autoclave. After autoclaving these should be placed at 60^{o}C in oven for drying.

Preparation of sterile work area:

Prepare the BSC or Laminar Flow Hood for work

- Spray ethanol on your gloved hands to sterilize them
- Clean the cabinet with 70% Ethanol
- Asceptically add the needed materials (ie. pipettes, tubes, plates)

Seed sterilization, and culturing on filter paper and MS media:

1. Obtain and label 9 capped tubes (Falcon tubes)
2. Transfer 30-40 seeds into each tube
3. Add 5ml of each Clorox solution to 3 of those tubes and start your timers
    - 3 tubes of 0% Clorox (sterile water)
    - 3 tubes of 10% Clorox
    - 3 tubes of 20% Clorox
4. Incubate the tubes at RT for either 5, 10, or 20min
5. Mix the contents of the tubes frequently
6. After 5min, move your first set of tubes (0%, 10% and 20% Clorox) to the BSC
    - Be sure to spray your tubes with some 70% Ethanol
    - Start the next part with the 20% Clorox sample, then process the 10% Clorox sample, and do the 0% Clorox sample last.

For each tube:

7. Pour the contents of the tube, through a sieve, into the waste beaker
8. Pour about 10ml of sterile water back into the falcon tube to wash out any remaining seeds and pour that back through the sieve
9. Repeat the above step 3 more times (each time, you're also rinsing the seeds in the sieve)
10. Invert the sieve over a piece of filter paper to remove the seeds from the sieve
    - Tap the seeds out and make sure there aren't any seeds left on the sieve – you will be using it for the next set of seeds
11. Carefully pick 10 seeds (using forceps) and place them onto a round, wet filter paper inside a petri dish.
    - Try to separate the seeds so that each is about 1cm away from its neighbour
12. Transfer 10 more seeds, in a similar way, onto a plate with MS media.
13. Repeat these steps for the remaining tubes

14. After the 10min incubation is completed, transfer the 3 tubes (0%, 10% and 20% Clorox) to the BSC and restart the procedure at step 7 above
15. After the 20min incubation is completed, transfer the 3 tubes (0%, 10% and 20% Clorox) to the BSC and restart the procedure at step 7 above
16. Once you have all your plates (18 plates) completed, seal them off with parafilm (your TA will demonstrate) and place them in a growth chamber.
    - **Your results will be ready for analysis in the next lab**

Results

Effect of Sodium hypochlorite on contamination rate and germination percentage of different seeds.

1. Effect of (Clorox) concentration on contamination (CT%) and germination rate (GR%) in seeds of _____.

Seeds plated on wet filter paper		Sterilization time (min)					
		5min		**10min**		**20min**	
Clorox concentration % [V/V]		CT %	GR %	CT %	GR %	CT %	GR %
	0%						
	10 %						
	20 %						

1. Effect of (Clorox) concentration on contamination rate (CR%) and germination rate (GR%) in seeds of _____.

Seeds plated on MS media		Sterilization time (min)					
		5min		**10min**		**20min**	
Clorox concentration % [V/V]		CT %	GR %	CT %	GR %	CT %	GR %
	0%						
	10 %						
	20 %						

Name: _____ Student's ID: _____

Homework 01

Media preparation and seed surface sterilization

Please submit an electronic copy of this assignment in the online dropbox on Blackboard.

Experimental Design

1. Why were you asked to process your tubes starting with the 20% Clorox sample in 'Step 6'?

Plant Growth

2. Did the seeds grown on water germinate? Why?

3. Present the data from this experiment in the form of a bar chart.

4. Based on what you see in your graph, discuss your results.

Lab 2: Plant Media

In this lab, we will be preparing some MS media for use in later labs. We will also be looking at our results from the previous lab and discussing them.

Please be sure you've read through all of this handout **before** the start of the lab.

Learning Objectives:

Students will:

- Understand the need for a defined Nutrient Medium.
- Prepare some MS media plates for use in future labs.
- Understand the roles of growth regulators in plant growth and development, the need for the addition of these to the growth medium.
- Analyze the data from the previous lab

Pre-Lab Questions:

Go to the website below and read through the descriptions of the main Phytohormone categories:

- http://passel.unl.edu/pages/informationmodule.php?idinformationmodule=956783940&topicorder=10&maxto=10&minto=1
- http://www.biologydiscussion.com/plants/hormones-plants/role-of-auxins-and-cytokinins-in-culture-medium-plants/71833

Answer the following questions:

- Why would we want to add auxins to our growth media?
- What do you think would happen if you plated your seeds on media that contained some gibberellins?
- Based on the graph at the bottom of the second article (second link), what can you conclude about when you would want to use the different plant growth regulators?

In order to perform any controlled studies, one must ensure consistent growth conditions for the organism under study. One of the most complex conditions that needs to be controlled is the composition of the growth media.

In order to survive and perform their functions, cells need energy (usually in the form of carbohyrates), they need to be able to produce proteins and enzymes (made up of amino acids), they also need to be able to synthesize DNA and RNA (made up of nucleotides), and need to extend and produce new membranes (made up of lipids).

Thus, the growth media needs to provide some basic "building blocks." In addition to this, it also need to contain various minerals, vitamins and hormones, without which the cells will not be able to survive.

Here are some of the typical components found in media used in plant tissue culture:

- **Inorganic minerals** - N. P. K, Ca, Mg, B, Mo, Cu, Zn, Mn, cl.
- **Organic nutrients** - sucrose, glucose, peptones, glycerol, coconut water etc.
- **Vitamins** - Thiamine (B_1), Niacin/Nicotinic Acid (B_3), Pyridoxine (B_6), Biotin (B_7), Folic Acid (B_9), Ascorbic Acid (C), Myo-inositol
- **Amino acids** – Asparagine and Glutamine (good sources of Nitrogen), Glycine, Arginine, and Cysteine.

This media is often supplemented with Agar (Agar [Phytagar] 0.6%, Gelrite 0.1 – 0.2 %) in order to provide firmness to the media.

Since not all plants require the same growing conditions, there is more than just one type of growth media. Different researchers, working on different plants, have developed different mixtures of the above components to meet the requirements of their chosen plants (see Table 43.1 below). But the media developed by Murashige & Skoog is currently the most commonly used one.

Aside for the basic components, some media plates/tubes will also be supplemented with Growth Regulators (plant hormones / phytohormones). These are signalling molecules, naturally found in plants in very low concentrations, which have the ability to induce changes in the cultured plant tissue to make certain cells start to proliferate and differentiate to form specific organs/structures (ie. roots).

Most of these signalling molecules are generally divided into five main groups:

- **Auxins** – Indole-3-acetic acid Free acid **(IAA)**, 2,4-Dichlorophenoxyacetic acid (2,4 – D), Indole-3-butyric acid (**IBA**), alpha-Naphthaleneacetic acid Free acid (**NAA**)
- **Cytokinins** – 6-Benzylaminopurine (**BA**), Kinetin, Zeatin, 1,3-Diphenylurea (**DPU**), 6-(gamma,gamma-Dimethylallylamino)purine (**2iP**)
- **Gibberellins** – Gibberellic acid (**GA3**), Gibberellin A4 Free acid (**GA4**)
- Abscisic acid (**ABA**)
- Ethylene

There are a few other categories (Brassinosteroids, Salicylic acid, Jasmonates, Plant peptide hormones, Polyamines, Nitric oxide (NO), Strigolactones, Karrikins), but the above are the main ones.

These molecules help plants to coordinating the growth and development of cells and tissues in events like the formation of flowers, stems, and leaves, senescence (the shedding of leaves), and the development and ripening of fruit.

In our labs, you will mostly be dealing with the first three of the main types of Phytohormones – Auxins, Gibberellins, and Cytokinins.

TABLE 43.1 Composition of commonly used plant tissue culture media

Components	*Amount (mg l^{-1})*				
	White's	*Murashige and Skoog (MS)*	*Gamborg (B5)*	*Chu(N6)*	*Nitsch's*
Macronutrients					
$MgSO_4.7H_2O$	750	370	250	185	185
KH_2PO_4	—	170	—	400	68
$NaH_2PO_4.H_2O$	19	—	150	—	—
KNO_3	80	1900	2500	2830	950
NH_4NO_3	—	1650	—	—	720
$CaCl_2.2H_2O$	—	440	150	166	—
$(NH_4)_2.SO_4$	—	—	134	463	—
Micronutrients					
H_3BO_3	1.5	6.2	3	1.6	—
$MnSO_4.4H_2O$	5	22.3	—	4.4	25
$MnSO_4.H_2O$	—	—	10	3.3	—
$ZnSO_4.7H_2O$	3	8.6	2	1.5	10
$Na_2MoO_4.2H_2O$	—	0.25	0.25	—	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025	—	0.025
$CoCl_2.6H_2O$	—	0.025	0.025	—	0.025
KI	0.75	0.83	0.75	0.8	—
$FeSO_4.7H_2O$	—	27.8	—	27.8	27.8
$Na_2EDTA.2H_2O$	—	37.3	—	37.3	37.3
Sucrose (g)	20	30	20	50	20
Organic supplements					
Vitamins					
Thlamine HCl	0.01	0.5	10	1	0.5
Pyridoxine (HCl)	0.01	0.5	1	0.5	0.5
Nicotinic acid	0.05	0.5	1	0.5	5
Myoinositol	—	100	100	—	100
Others					
Glycine	3	2	—	—	2
Folic acid	—	—	—	—	0.5
Biotin	—	—	—	—	0.05
pH	5.8	5.8	5.5	5.8	5.8

Practical Activities: Plant Tissue Culture Media Preparation

Exercise 1: Preparation of hormone stock solutions

Plant growth regulators are available in powdered form. These are dissolved in proper solvents [see the table 1 below] and diluted with water to prepare the required stock solutions. The stock solutions are usually 1000X – this means that they are 1000 times more concentrated than concentration that you will be actually applying to your plant cells (ie. the "working concentration). Doing it this way makes the math easier later on.

Usually a 1g/L stock solution is prepared. To prepare this stock:

1. weigh out 0.2g of powder of one of the growth regulators
2. dissolve it in the solvent as given in table 1 below
3. dilute with distilled water to a final volume of 200ml.

Some general guidelines are:

- **Auxins** are soluble in few drops of ethanol 70% or 1 N NaOH [may be heated if required], then diluted in required amount of distilled water.
- **Cytokinins** are soluble in few drops of ethanol 70% or 1 N HCl, then diluted in required amount of distilled water.

- Natural hormones are thermo-labile (will be destroyed when heated), so they should be filter sterilized and added to media **after** it is autoclaved.
- Synthetic growth regulators are thermo-stable (will not be destroyed by heat) and are added to media before autoclaving.

Table 1. PGR preparation and storage requirements.

Class	Name	Solvent	Diluent	Storage	Sterilization
Auxins	2,4 –D	1 M NaOH	H_2O	-20°C	Co-autoclave
	NAA	1 M NaOH	H_2O	-20°C	Co-autoclave
	IAA	1 M NaOH	H_2O	-20°C	Filter
Cytokinins	BA	1 M HCl	H_2O	-20°C	Co-autoclave
	Kinetin	1 M HCl	H_2O	-20°C	Co-autoclave
	2iP	1 M NaOH	H_2O	-20°C	Filter
	Zeatin	1 M HCl	H_2O	-20°C	Filter
Gibberellins	GA3	Alcohol	H_2O	-20°C	Filter

Exercise 2: Media preparation

Basal media is prepared from the premixed basal media [basal salts and vitamins] obtained from Duchefa Biochemie (Netherlands).

- Add 800 ml of distilled water to a 2 liter Erlenmeyer flask.
- Weigh 4.5 grams of premixed powdered medium and dissolve in the distilled water.
- Weigh 30 grams of sucrose and dissolve in the solution in flask.
- Adjust the pH to 5.7 ± 0.1 with 1 M KOH solution.
- Bring the volume up to 1 L with distilled water.
- Add 6 grams of Phytagar.
    - If you're adding a synthetic PGR, you would add 1ml of it at this point.
    - Send media for autoclaving (the agar will dissolve there).

Media sterilization (autoclaving):

Put the media in Erlenmeyer flask inside autoclave and set the temperature to 121°C for 15 min. For operation of autoclave follow the instructions carefully.

After autoclaving the media is cooled down to 40°C [or hot enough to be touch with hand comfortably]. Media is poured in sterilized petri dishes [culture plates], test tubes, culture flask or bottles and allowed to solidify at room temperature.

In our case, you will make some plates.

Results from the Previous Experiment

Obtain your group's plates from last week's experiment and check to see how many seeds on each plate have germinated. Remember that in each case you plated 10 seeds, so if 9 or them germinated, then that's a 90% germination rate; if 6 of them germinated, then that's a 60% germination rate.

You will also be looking at evidence of contamination on your plates. The plates that were treated with water are your negative controls for this, so your 10% and 20% plates will be compared to those.

You can use the tables below to collect your data for analysis

Seeds plated on wet filter paper		Germination		Contaminants	
		Number	Percent	Number	Percent
5min Incubation	0%				100%
	10%				
	20%				
5min Incubation	0%				100%
	10%				
	20%				
5min Incubation	0%				100%
	10%				
	20%				

Seeds plated on wet filter paper		Germination		Contaminants	
		Number	Percent	Number	Percent
5min Incubation	0%				100%
	10%				
	20%				
5min Incubation	0%				100%
	10%				
	20%				
5min	0%				100%

Incubation	10%				
	20%				

Lab 3: Callus Initiation

In this lab, we will be inoculating some MS media with some explants. We will be using the seedlings generated in the previous labs for part of the procedure, but we will also be using a non-sterilized "mother plant" to produce some explants, in order to practice our sterilization technique.

Please be sure you've read through all of this handout **before** the start of the lab.

Learning Objectives:

Students will:

- Identify and select the appropriate parts of a seedling for use in the protocol
- Inoculate explants at an appropriate density
- Describe the three main stages of plant callus development
- Explain the importance of dedifferentiation

Pre-Lab Questions:

Watch the following video:

- https://youtu.be/11QOg4dLa3U

Answer the following questions:

- What is a meristem / meristematic tissue?
- Where are meristems found?

Complete the matching exercise at the link below:
(feel free to use any online resources to help you):

- https://h5p.org/node/421434
- Take a **screenshot** of your result and **upload it** through the "Prelab 3" Dropbox under "Assignments"

Introduction

In order to obtain explants, we generally start with whole plants, whose tissues are differentiated, mature, and often non-dividing. After the transfer of freshly cut explants into growth-promoting conditions, cell division is initiated on the cut surfaces, and as a form of wound healing. This growth is fairly unorganized and is referred to as a **callus**.

A typical plant callus undergoes three main stages of development. Initially, there is a period of preparation for and initiation of cell division. Then the callus enters a period of abundant cell division, before resources in the culture media become limiting and the division slows down. This description may sound familiar – you've probably discussed a very similar growth pattern in your microbiology class.

During that second stage, the normal/differentiated plant tissues in the explant, undergo modifications to become meristematic. This phenomenon of mature cells reverting back to meristematic state to form undifferentiated callus tissue is called **dedifferentiation**.

Callus cells continue to proliferate without differentiating leading to the establishment of **primary culture**. Eventually (in the third stage) differentiation occurs within the tissue mass with the help of growth regulators leading to the formation of complete plants. The subculturing of that primary callus on fresh media results in the production of **secondary callus cultures**.

Inoculation of cultures

The placement of explants onto the growth media is known as **inoculation**. It should be done in a way that ensures that the explants have good contact with the media (especially the cut sides), so the explants are gently pressed down into the media.

It is also important to note that explants tend to release some essential substances once inoculated, and these chemicals diffuse into the media near the explant. These substances can be quite important for growth, it is therefore useful to ensure that the size of the explant is large enough to be able to produce enogh of these substances, and that the explants are close enough together to ensure that they can have access to the chemical produced by their neighbours. Thus, the size of the explant should be at least 1cm in length, and the explants are often inoculated about 1-1.5cm apart.

Practical Activity: Preparation of explants for culturing on solid media

Exercise 1: Preparation of explants from sterile seedlings

Explants from your aseptically grown seedlings from the previous labs will be used for part of this lab. You will also prepare explants from provided non-sterilized plants. You will use both to try to generate some calluses on media supplemented with two different Auxins.

1. Place your seedling plates and media plates in the BSC
2. Cut 5–10mm pieces of shoot, hypocotyl, root or cotyledons from the seedlings, and place them on the media plates.
    - Because they were grown under asceptic conditions, they shouldn't need surface sterilization (but please make sure they're taken from plates that are not contaminated).
3. Place explants on MS medium containing either 2 mg/L 2, 4 – D or 1 mg/L NAA.
    - Place the explants on the media in a way that brings the cut ends close to the media (ie. stems should be lying on their sides so both cut ends are in contact with the media), and gently press them down to ensure good contact

Please be sure to use sterilized scalpels and forceps for preparing these explants. Scalpels and forceps must be kept ethanol and flamed before each transfer.

[Caution: ethanol should not be placed near the flame. Don't put hot (flamed) forceps or scalpel in ethanol].

Exercise 2: Preparation of explants from non-sterile plants

Selection of the explants (Outside the BSC)

1. The explants should be collected from plants neither too young nor too old.
2. Select healthy-looking plants and discard any damaged leaves/stems.
3. Wash the explants with tap water followed by distilled water.
4. Cut the stems if they are too long and collect them in a beaker.
5. Detach the leaves from the stem with petiole intact and collect it in a beaker.

Sterilization of the explants (Inside the BSC)

6. Place the pre treated explants in a sterilized beaker inside the hood.
7. Rinse leaves/stem with Clorox 15% for 15 minutes.
8. Discard Clorox.
9. Thoroughly rinse three times with autoclaved distilled water
10. Discard water.
11. Rinse leaves/stem with 70% EtOH for 30 sec.
12. Discard EtOH.
13. Thoroughly rinse three times with autoclaved distilled water

Exercise 3: Inoculation of the explants

This part must be done Inside the BSC

14. Cut the petiolar end and apex of the leaf with the help of forceps, resulting into approx. 1cm sized leaf explants.
15. Similarly cut the lower and top portion of the stem with the help of forceps, resulting into stem sections of 1cm size.
16. While cutting the stem sections remove the nodal portion of the stem and select the internode section of the appropriate size.
17. Inoculate the leaf/stem with the help of the forceps placing them horizontally in separate media plates.
18. Place explants on MS medium containing either 2 mg/L 2, 4 – D or 1 mg/L NAA.
19. Press the explants gently against the surface of the agar to make good contact.
20. Label the test tubes with name and type of the explant, date of inoculation and the type of media.

Lab 4: Shoot Culture

In this lab, we will be inoculating some MS media with some shoot meristems collected from a plant called *Syngonium*. There are two parts to today's lab. In the first part, you will be practicing dissecting out nodal meristematic tissues. In the second part of the lab, you will be preparing nodal explants and inoculating them into some media.

Please be sure you've read through all of this handout **before** the start of the lab.

Learning Objectives:

Students will:

- Identify the locations of meristematic tissue
- Dissect apical and axillary buds to expose the meristems
- Explain the importance of culturing plants from meristem tissues

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Pre-Lab Questions:

Watch the following video:

- https://youtu.be/cD9CFtpLL2s

Answer the following questions:

- Why did the researchers need to use shoot tip meristem culture on their *Arabidopsis halleri* plants?
- What kinds of tools do you need in order to obtain a meristem for culturing?

Introduction

Please watch the linked video in the pre-lab section. That's your introduction.

Practical Activities: Culturing Meristematic Tissues

Exercise 1: Dissection and isolation of a shoot meristem

In the first part of the lab, we will be practicing the art of exposing/dissecting meristematic tissues. Since there is not enough room for all of you to do this in the hoods, we will be doing this under non-sterile conditions for practice.

1. Identify a part of the plant that is likely to have a meristem. Please note:
    - once you see a flower bud on a shoot, it will not have a shoot apical meristem – meristematic tissues are VERY small, so look for small buds.
    - auxilary nodes are often hidden beneath the petiole of a leaf at the node – you will have to pull away the leaf's petiole completely from the stem to see the node.

2. Cut a piece of the plant that will be large enough to be easy for you to handle under a dissecting microscope, and remove any large leaves covering the region you want to dissect.
3. Place the plant sample under the dissecting microscope at low magnification and use forceps to "clean up" the area (ie. carefully remove any remaining parts of the leaves that you removed earlier).
4. Identify the bud that contains meristematic tissue
5. Increase the magnification and notice that the bud is covered by very small leaves.
6. Carefully remove the small rudimentary leaves, and the leaves of the leaf primordium. (see below)

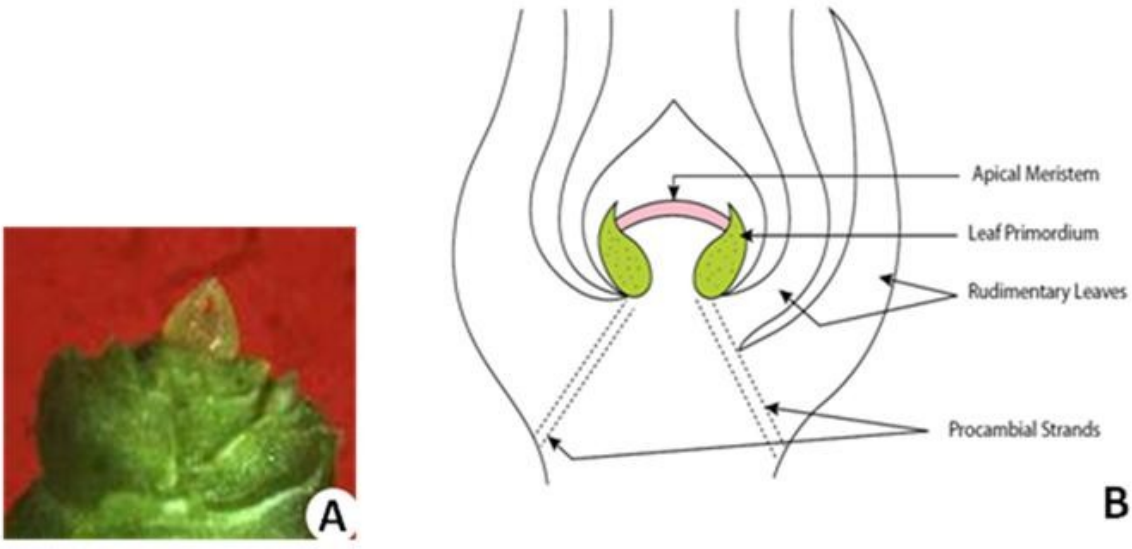

Illustration 15: Structure of a typical apical meristem. (A) Exposed leaf primordium and meristem after dissection. (B) Organization of tissues surrounding the apical meristem. (Source: nptel.ac.in)

Once you've successfully dissected a few meristems, you will go to the Tissue Culture Lab and work on Part 2 of the lab.

Part 2: Plant Tissue Culture

Exercise 2: Preparation and culturing of nodal explants on solid media

Explants from *Syngonium* plants will be prepared by cutting 1-2cm pieces of shoot and nodal tissues. These will be partially dissected to expose the meristematic tissues before being inoculated individually into tubes, jars or magenta boxes containing specially prepared media.

We will not be fully isolating the meristem tissue for this part of the lab – we will simply be using explants which contain meristematic tissues and removing most other plant organs to ensure that the meristems are the most likely to be responsible for any plant regeneration that we observe later.

Composition of different media for *Syngonium* propagation

	SEM*	SMM*	MS
Basal Media	MS	MS	MS
PGR 1	2iP @ 3mg/L	2iP @ 20mg/L	
PGR 2	IAA @ 1mg/L		
pH	5.7	5.7	5.7
Gelling Agent	Phytagel	Phytagel	Phytagel

* SEM = *Syngonium* Establishment Medium; SMM = *Syngonium* Multiplication Medium

Today's lab would be the start of a much longer protocol (carried out over many weeks) that is summarized in the figure below.

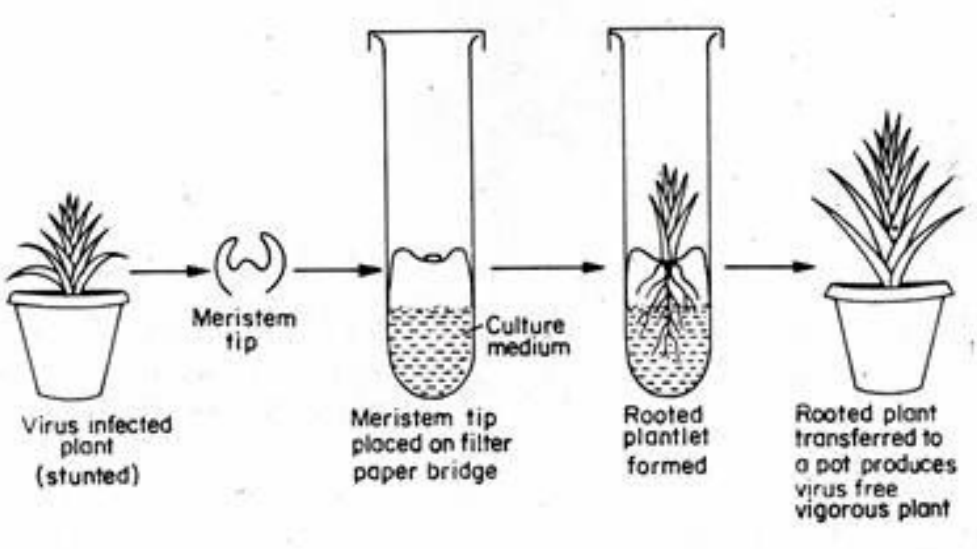

Illustration 16: Overview of meristem tip culture to produce a virus-free plant. (source: biologydiscussion.com)

Initial Cleaning and Explant Praparation:

1. Select a *Syngonium* plant and cut off a regions containing meristematic tissues.
2. Wash the explants with tap water followed by distilled water.
3. Remove leaves from the stem/branches to obtain small explants containing shoot buds (apical, axillary), approximately 1-2cm in size.
    - **For Apical Shoots**: Cut the shoot to be about 1cm long, and remove all the surrounding leaves to expose the apical meristem (as much as possible).
    - **For Nodal Explants**: Remove the leaf/leaf base that covers the bud at the node, and also remove any adventitious roots (if present). Cut the stem to leave approx. 1cm on either side of the bud.
    - Keep the apical explants and the nodal explants in separate beakers

Sterilization of the explants (Inside the BSC)

1. Place the pre treated explants in a sterilized beaker inside the hood.
2. Wash the explants with 10% Clorox for **2-3min**.
    - Please note that the time of disinfection is much shorter than in the previous labs, because we're dealing with a partially dissected and very tender tissue. Incubating too long could kill the cells we're trying to culture.
3. Discard Clorox.
4. Thoroughly rinse three times with autoclaved distilled water
5. Discard water.
6. Place one explant per tube of solid medium (you should inoculate tubes with apical and with nodal explants to compare their growth characteristics).
7. Observe shoot growth and proliferation over the next few weeks.

Lab 6: Anther Culture

In this lab, we will be inoculating media with some wheat (*Triticum turgidum*) anthers. There are two parts to today's lab. In the first part, you will be viewing and dissecting wheat spikelets under a dissecting microscope to familiarize yourself with the structure of the florets found inside. In the second part of the lab, you will be isolating wheat anthers, under asceptic conditions, and using them to inoculate some media.

Please be sure you've read through all of this handout **before** the start of the lab.

Learning Objectives:

Students will:

- Identify reproductive parts of a plant
- Dissect spikelets to expose the anthers and stigma
- Explain the usefulness of culturing plants from anthers
- Describe factors that increase the success rate of anther culture

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Pre-Lab Questions:

Use this handout and any online resources that you think might be helpful to complete the exercise found in the link below:

- https://h5p.org/node/437108

Submit a screenshot of your results from the final summary slide through the dropbox on Blackboard.

Introduction

Whether you're a scientist working on increasing crop yields, or on genetically engineering a plant to give it some useful properties (ie. drought-resistance, or ability to grow on polluted soil), you will find anther culture to be a very helpful tool.

Anther culture allows scientists to generate **haploid** embryos and plants. These haploid plants can then be treated with a chemical called **Colchicine**, to double the chromosome number and generate what is commonly known as a **Double Haploid (DH)** plant – a plant with two copies of the same chromosomes. These plants are **homozygous** for all of their traits, making it much easier to use them in genetic crosses to generate new plant varieties with useful traits. This form of plant breeding significantly speeds up efforts in crop improvement around the world.

Haploids are useful because:

- They carry only one allele of each gene, thus any recessive mutation or characteristic are seen in the phenoype.
- Lethal genes can be eliminated from the gene pool.
- Can be used to produce homozygous diploid or polyploid plants for plant breeding.
- Shortens the time the production of superior hybrid genotypes.

Additionally, haploids can be very useful when trying to insert a particular gene into the genome. It means that you need to insert that gene into only one chromosome. A double haploid plant can then be generated which will carry two copies of your gene of interest (it will be homozygous), thus ensuring that your gene will be expressed even if it encodes a recessive trait.

Besides anther culture, there are several other methods of producing haploids. Sometimes, it occurs naturally – this is extremely rare. And sometimes, **inter-species hybrids** are generated (ie wheat is crossed with corn), which produces haploids through the process of **chromosome elimination**. This works well for some species, but not for others. Induction by physical and/or chemical treatment can also result in chromosome elimination and yield haploids, but it is difficult to get predictable results with this method.

Anther Culture

Anther culture is one of the most common *in vitro* methods to obtain haploid plants, because it is the easiest to perform. This method relies on haploid cells called **microsporocytes**, which would normally develop into the male gametes – pollen grains. These microsporocytes develop inside a plant organ called the anther, and this is the part of the plant that is removed from a flower and used to inoculate media in order to produce a callus.

The callus that is produced will likely result in a mixture of haploid and diploid plants (because the walls of the anther are composed of diploid cells), but haploid plants are quite common and this is a much quicker and simpler method than **pollen culture** or **ovule culture**, which involve separating the haploid cells from their organs (see Fig 1).

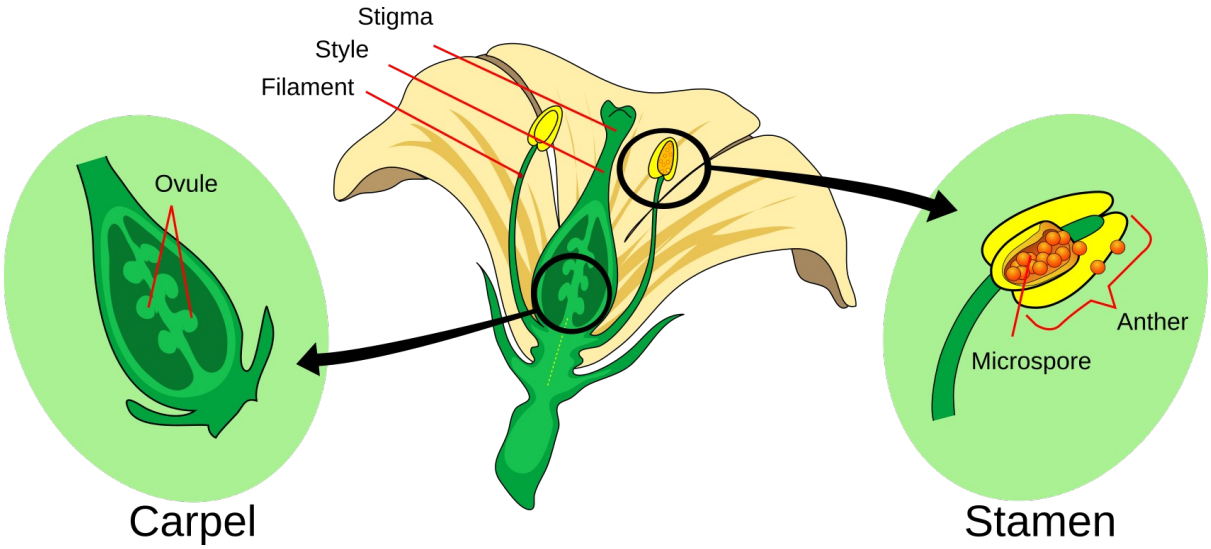

Illustration 17: Basic anatomy of the reproductive organs of a typical flower.Image modified from: https://commons.wikimedia.org/wiki/File:Mature_flower_diagram.svg

One of the things that needs to be understood about producing plants directly from microsporocytes, pollen cells or ovules, is that this is **not** something that normally occurs in nature. By utilizing these methods, scientists are redirecting these cells away from their normal development program (ie. the production of gametes), and forcing them into a very different pathway. Thus researchers need to be aware of several factors that can improve their chances of success.

The starting plant material is one important factor – some individual plants are more suitable than others. This is partially due to genetic factors, but also due to the health and age of the plant – younger plants, just starting to flower, tend to make better anther donors.

The **stage of development** of the microsporocytes / pollen grains is also important. Cells that are still early in development, or very late in their development are less likely to produce haploid plants. For this reason, you will be using spikelets (Fig 2B & C) from the middle portion of the provided wheat spikes (Fig 2A) – they tend to contain cells close to the optimal stages of development.

Illustration 18: Wheat Nomenclature. (A) Wheat Spike, composed of numerous spikelets. (B) A single spikelet, with the 'glumes' removed, showing anthers emerging from behind the 'lemma' of a few florets. (C) Organization of the florets in a spikelet.

Lastly, the cells need to be induced to follow an abnormal path of development (ie. produce a plant instead of becoming gametes). This requires some sort of treatment to stress the cells and "knock" them out of their normal activity. Depending on the plant species, this may be a chemical treatment, exposure to low or high temperature, or the absence of light.

Practical Activities:

In the case of today's protocol, wheat tillers (stems) were harvested when the majority of anthers in the spikes contained mostly mid- to late-uninucleate stage microsporocytes. Tillers with spikes at this stage were clipped off at ground level and tagged. Then, they were put in water and maintained for 6–8 days at 4°C in the dark.

Exercise 1: Dissection of wheat spikelets

In the first part of the lab, we will be practicing the art of dissecting the spikelets and opening up the florets to expose the anthers. Since there is not enough room for all of you to do this in the hoods, we will be doing this under non-sterile conditions for practice.

1. Obtain a wheat spike (one per group should be enough)
2. Use your fingers or forceps to remove the outer/lower glume – the part of the spikelet with the actual long spike on it (the awn) – from one of the spikelets.
3. Inside a spikelet are several florets. Use your forceps and a dissecting microscope to help you open up one of the florets and identify and remove the anthers – there should be 3 anthers per floret.
4. Try to remove the anthers from another floret <u>without the use of a microscope</u>.

    - Use the images below and the ones on the previous page to help you understand what you're looking at.

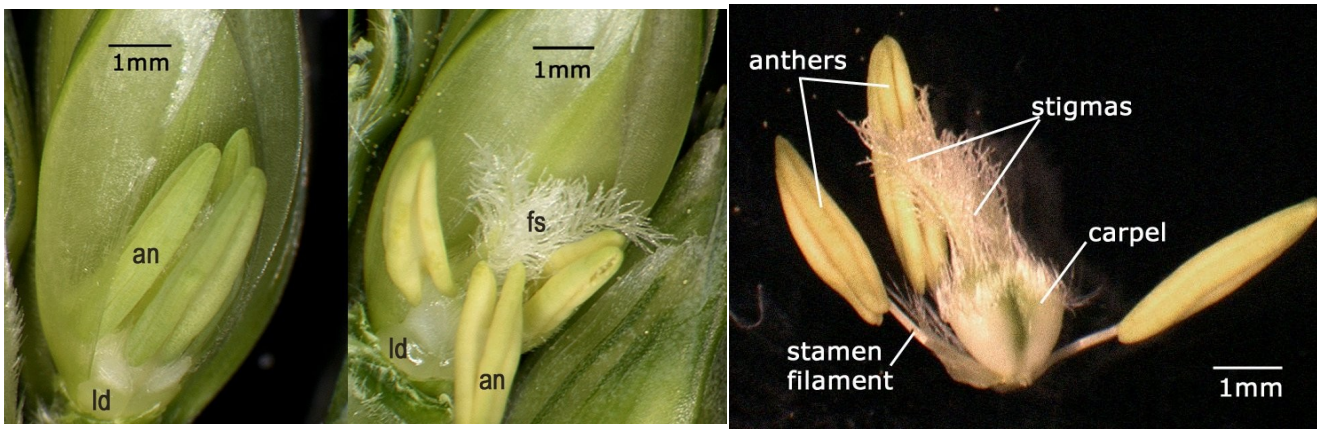

Illustration 19: Dissection of a wheat floret. (A) Interior of a young floret - the anthers [an] are still green. (B) Interior of a more mature floret – the anthers [an] are filled with yellow mature pollen, and the stigmas [fs] are more developed, and the lodicule [ld] is more swollen. (C) Reproductive parts of a wheat floret, with all the surrounding tissue dissected away. Image Source: http://bio-gromit.bio.bris.ac.uk/cerealgenomics/

Exercise 2: Preparation of anthers for culturing on solid media

Once you feel confident in your ability to find and pull out anthers. Go to D103 and isolate some anthers for inoculation into some media. This will have to be done in the BSC, so you won't be using a dissecting microscope for this part – this is why you were asked to practice the dissection without a microscope in Part 1 as well.

1. Obtain a wheat spike and remove enough of the tiller (stem) to ensure that the whole explant can be covered by liquid in your beaker when sterilizing.
2. Place the spike in a sterile beaker and cover completely with 70% Ethanol for 1-2 min.
3. Remove the ethanol and cover the spike with 20% Clorox solution for 7-10 min.
4. Rinse the spike five times with sterile deionized water.
5. After the last rinse, leave the spike to drain properly for about 10min
6. Place the spike in a sterile Petri dish and dissect out the anthers.
7. Inoculate 10 anthers onto a plate with one of the media listed below.

Table 1. Callus initiation from Wheat anthers on MS medium supplemented with either 2 mg/L 2, 4 – D or 1 mg/L NAA.

Explants	Callus initiation percentage	
	2 mg/L 2, 4–D	1 mg/L NAA
Week-2		
Week-3		
Week-4		