ZOO505

CELL AND MOLECULAR BIOLOGY

LABORATORY MANUAL

Contents:

Practical 1

Preparation of different stock solutions used in molecular biology (solution used in PCR, electrophoresis, DNA isolation, RNA isolation and Protein isolation)

A solution is a homogeneous mixture of one or more solutes dissolved in a solvent.

- ***solvent***: the substance in which a solute dissolve to produce a homogeneous mixture
- ***solute***: the substance that dissolves in a solvent to produce a homogeneous mixture

Note that ***solvent*** is the substance that is present in the greatest amount.

Stock Solution:
Stock solutions can best be described as solutions of known, accurate concentrations that will be diluted for future laboratory use.
Working Solution:
Working solutions are any dilutions that are made using the stock, generally into aqueous solution.

The following general instructions are applicable in the preparation of all reagents.
Use narrow neck apparatus such as graduated cylinders or pipettes to make accurate solutions closest to the volume being measured for preparing liquid reagents.
Store all reagents in sterile containers unless otherwise noted. Label all reagents with name, date prepared, initials of scientist who prepared the solution, lot number, and expiry date. Record each preparation in the lab's reagent logbook.

1M Tris-HCl [Tris (Hydroxymethyl aminomethane)] hydrochloride pH 8.0

Tris base	121.1g
H_2O	800ml

Adjust to desired pH with concentrated HCl.
Mix and add distilled water (H_2O) to make 1 Liter solution.
Store at room temperature

0.5 M EDTA (Ethylenediamine Tetra acetic Acid) pH 8.0

$Na_2EDTA.2H_2O$	186.1g
H_2O	700ml

Adjust pH to 8.0 with 10M NaOH (almost 50ml)
186.1g to 700ml
Raise the volume to 1 L using distilled water. Store at room temperature.

10 M NaOH

NaOH	400 g
H_2O	1 Liter

Store at room temperature.

10 mg/ml Ethidium Bromide

Ethidium Bromide	0.2g
H_2O	15ml

Raise the volume to 20mL using distilled water. Mix well and store at 4°C in dark.

TE (Tris 10 mM-EDTA 2mM) pH 8.0 (Lysis Buffer)

1M Tris-HCl pH 8.0	10 ml
0.5 M EDTA pH 8.0	4 ml
H_2O	800ml

Raise the volume to 1 L using distilled water.
Store at room temperature

Low TE (Tris 10 mM-EDTA 0.2 mM) pH 8.0 for DNA storage

1M Tris-HCl pH 8.0	10 ml
0.5 M EDTA pH 8.0	0.4 ml
H_2O	800ml

Raise the volume to 1 L using distilled water.
Store at room temperature

Proteinase K (20mg/ml)

Proteinase K	100 mg lyophilized powder
Ultra-pure H_2O	5ml

Aliquot and store at approximately -20°C.
CAUTION: Powder and solution of Proteinase K can be irritating to mucous membranes.

SDS 10% w/v

Sodium dodecyl sulfate	100g
H_2O	700ml

Heat at approximately 65ºC to dissolve.
Bring to a final volume of 1.0 L with ultra-pure water.
Store at room temperature.
CAUTION: SDS can be irritating to mucous membranes. Wear safety glasses, mask and gloves when handling.

TEN buffer (10mM Tris, 2mM EDTA, 400 mM NaCl)

1 M Tris-HCl pH 8.0	10 ml
5M NaCl	80 ml
0.5M EDTA	4 ml
H_2O	800ml

Raise the volume to 1 L using distilled water.
Store at room temperature.

50x TAE (Tris-Acetate-EDTA) Electrophoresis Stock buffer

Tris base	242g
Glaciall acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100ml
H_2O	800ml

Raise the volume to 1 L using distilled water
Store at room temperature.

1x TAE (Tris 40mM, Acetate 20mM, EDTA 2mM) Electrophoresis working buffer

50x TAE	10 ml
H_2O	to raise volume to 500 ml

The pH of diluted buffer is 8.3.
Store at room temperature.

10x TBE (Tris 90mM-Borate 90mM-EDTA 2mM) Electrophoresis buffer

Tris base	108g
Boric Acid	55g
0.5M EDTA pH 8.0	40 ml
H_2O	800ml

Raise the volume to 1 L using distilled water

Store at room temperature

2x Gel Loading Dye

2% Bromophenol blue	0.25 ml
2% Xylene cyanol	0.25 ml
Glycol	7ml
H_2O	10ml

Store at room temperature

5M Sodium Chloride

Sodium Chloride	292.2 g
H_2O	700 ml

Raise the volume to 1 L using distilled water.

Store at room temperature.

6M Sodium Chloride

Sodium Chloride	351 g
H_2O	600 ml

Raise the volume to 1 L using distilled water.

Store at room temperature.

References and further reading:

https://www.chem.purdue.edu/gchelp/solutions/whatis.html

http://ask.trilinkbiotech.com/q-what-is-the-difference-between-stock-and-working-solutions/

Practical 2

Isolation of DNA from human blood

DNA isolation is an essential technique in molecular biology. Extraction and purification are essential to determine the unique characteristics of DNA, including its size, shape and function. Isolation of high-molecular weight DNA has become very important with the increasing demand for DNA fingerprinting, restriction fragment length polymorphism (RFLP), construction of genomic or sequencing libraries and PCR analysis in research laboratories and industry. Also, DNA isolation is the first step in the study of specific DNA sequences within a complex DNA population, and in the analysis of genome structure and gene expression. The quantity, quality and integrity of DNA will directly affect these results. Scientists can buy ready-to-use DNA extraction kits. These kits help extract DNA from particular cell types or sample types. However, they can be expensive for routine use, so many labs have developed chemical methods for DNA extraction.

Principle:

The extraction of DNA involves three main steps, namely, cell lysis, protein separation, and DNA purification. Cell lysis is usually performed by incubation of cells in buffer containing detergent and protease. Cellular proteins are salted out or phase separated using organic solvents. Finally, DNA is isolated and purified either by alcohol precipitation or adsorption with silica and elution.

Reagents required:

- TE buffer (10mM Tris, 2mM EDTA, pH 8.0)
- TEN buffer (10 mM Tris, 2mM EDTA, 400mM NaCl)
- 10% SDS
- Proteinase-K solution 20mg/ml
- 6M NaCl
- Phenol-Choloroform-Isoamylalcohol (PCI) (25:24:1)
- Absolute Ethanol or Isopropanol
- 75% Ethanol
- Low TE buffer (10mM Tris, 0.2mM EDTA)

Consumables required:

- Filter barrier tips 200 µl
- Filter barrier tips 1000 µl

- Wide bore tips 1000 µl
- Falcon tubes 15 ml
- Microcentrifuge tubes 1.5 ml

Equipment required:

- Centrifuge for 15 ml falcon tubes
- Microcentrifuge for 1.5 ml tubes
- Adjustable micropipettes 1 ml and 200 µl

Procedure:

1. Add 1 ml chilled TE buffer to 200 µl blood. Mix by inverting the tube several times.
2. Centrifuge at 4000 rpm for 15 min at room temperature.
3. Discard the supernatant and add 900 µl chilled TE buffer. Re-suspend the pellet by vigorous shaking.
4. Centrifuge at 4000 rpm for 15 min at room temperature.
5. Discard the supernatant and add 800 µl TE buffer. Re-suspend the pellet by vigorous shaking.
6. Centrifuge at 4000 rpm for 15 min at room temperature.
7. Discard the supernatant and add 200 µl TEN/A1 buffer, 20 µl SDS (10% solution) and 10 µl Proteinase-K solution. Re-suspend the pellet by shaking and vortex mixing.
8. Incubate the mixture at 56°C overnight.
9. Next day, place the tubes on ice and add 50 µl of 6M NaCl. Shake the tube vigorously and place on ice again for 15 min.
10. Centrifuge at 4000 rpm for 15 min to pellet down the salts and proteins.
11. Transfer the supernatant in a fresh properly labeled 1.5-ml centrifuge tube.
12. Add equal volume of chilled isopropanol and invert the tubes gently till threads of DNA are visible.
13. Centrifuge at 8000 rpm for 1 min at room temperature. Discard supernatant.
14. Add 200 ul absolute ethanol and vortex for 15 sec.
15. Centrifuge at 8000 rpm for 1 min at room temperature.
16. Add 200 ul 75% ethanol and vortex for 15 sec.
17. Centrifuge at 8000 rpm for 1 min at room temperature.
18. Discard the supernatant and add 100 µl low TE buffer or sterile distilled water to dissolve the DNA pellet.
19. Store DNA at -20°C.

Precautions to be taken during DNA isolation:

1. Make sure that the vials and tips are DNase free.
2. Avoid vortexing during DNA extraction.
3. Make sure that the extracted DNA is treated with RNase to remove RNA.
4. It is good to use columns to purify the DNA. The columns will remove the salts presents in the DNA.
5. Ship the DNA samples on sufficient Dry ice.

References and further reading:

General aspects of DNA isolation and purification, in Basic Techniques in Molecular Biology. 2000, Springer. p. 1-32.

https://www.whatisbiotechnology.org/index.php/science/summary/extraction/dna-extraction-isolates-dna-from-biological-material.

https://www.sciencelearn.org.nz/resources/2036-dna-extraction.

Practical 3

Quantification of DNA and RNA through spectrophotometer

The spectrophotometer is an instrument which measures the amount of light that a sample absorbs. It works by passing a light beam through a sample to measure the light intensity of a sample. These instruments are used in the process of measuring color and used for monitoring color accuracy throughout production which ultimately is used to check product quality. A spectrophotometer covers ultraviolet (UV) region range (200–400 nm) in addition to visible range of electromagnetic spectrum.

A spectrophotometer is made up of two instruments: a spectrometer and a photometer. The spectrometer is used to produce light of any wavelength, while the photometer is to measure the intensity of light. The spectrophotometer is designed in a way that the liquid or a sample is placed between spectrometer and photometer. The photometer measures the amount of light that passes through the sample and delivers a voltage signal to the display. If the absorbing of light change, the voltage signal also changes.

Principle:

Nucleic Acids (nucleotides, RNA, ssDNA, and dsDNA) all absorb light at 260 nm wavelength; In aqueous solution, double-stranded DNA has a maximal absorbance near 260 nm.
Readings should be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentrate on of nucleic acid in the sample.

1 O.D. at 260 nm for double-stranded DNA = 50 ng/µl of dsDNA
1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA
1 O.D. at 260 nm for RNA molecules = 40 ng/µl of RNA

The reading at 280 nm gives the amount of protein in the sample.
Pure preparations of DNA and RNA have OD260/OD280 values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be

possible.

So typically, dilute sample 1 µl in 100 µl so the dilution factor is 100. Put whole 100 µl in spectrophotometer cuvette. The DNA concentration read will then be:

OD260 X 50 ng/ul x dilution factor

For example, if have OD260 = 1.6. Then the concentration is:

1.6 x 50 ng/ul x 100 = 8000 ng/ul or 8 ug/ul.

Equipment Required

- UV/VIS Spectrophotometer
- 1 mL quartz cuvette
- DNA/RNA samples
- TE Buffer
- Disposable 1 mL polyethylene Transfer Pipettes (Berol) [2 per group]
- Eppendorf tubes (1.5 mL) [2 per group]
- Ruler
- Kimwipes

Procedure

I. Setting up the Spectrophotometer (Beckman DU64)

1. Turn on the spectrophotometer at the power strip. Check that the printer is on line and ready.
2. Turn on the UV lamp source and allow it to warm up for 5 minutes.
3. Select the absorbance reading mode (ABS key).
4. Press the SCAN key "Edit" will be displayed.
5. Enter the starting wavelength as 280 nanometers (nm) and press enter.
6. Enter the ending wavelength as 260 nm and press enter.
7. The speed for the scan of the sample will be displayed. It should read 750 nm/min. If it does not, press the STEP key and scroll through the options until 750 nm/min is displayed. Press enter.
8. Upper limit will be displayed. Set the upper limit at 2,000 absorbance. Press enter.
9. Lower limit will be displayed. Set the lower limit at 0.000 absorbance. Press enter. The starting wavelength will reappear.
10. The instrument is now ready to be calibrated against a control solution. The purpose

of the calibration is to measure and then subtract from the samples absorbance any absorbance from the buffer solution.

11. Place 200 microliters (μL) of the TE Buffer into the quartz cuvette. This is the solution you will use to calibrate the instrument.
12. Open the sample compartment lid on the instrument.
13. Carefully wipe the cuvette with a Kimwipe and be careful not to get fingerprints on the quartz panels. Place the cuvette into the cuvette holder so that the quartz sides are in the path of the light source (left to right).
14. Close the sample compartment lid.
15. Press CALB. The absorbance of the TE Buffer solution will now be recorded in memory as the "background" and "Bkg" will be displayed.
16. Press READ, Calibration is complete when "Scan" is displayed. The instrument is now ready to measure DNA/RNA samples.
17. Open the sample compartment lid and remove cuvette.
18. Discard the 200 μL of TE Buffer.
19. Rinse the cuvette twice with the TE Buffer solution and drain the cuvette onto a Kimwipe.

II. Sample Preparation

20. Place 200 μL of the DNA/RNA sample in the cuvette and place the cuvette in the sample holder.
21. Press READ. The absorbance of the sample between 260 and 280 nm will be measured and
plotted as a graph on the printer.
22. Repeat steps 20 and 21 for any other DNA samples that you have been assigned.

Advantages & Disadvantages

It is an easier and cost-effective method with reliable results if performed adequately. The protein contamination cannot be reliably assessed with a 260:280ratio; this also means that it contributes little error to DNA quantity estimation.

References and further reading:

https://byjus.com/chemistry/spectrophotometer-principle/
https://www.ncbi.nlm.nih.gov/pubmed/18201813

Practical 4

DNA amplification through polymerase chain reaction

Polymerase chain reaction (PCR) is the in vitro amplification of specific sequences of nucleic acid. The processes of PCR and the enzyme DNA polymerase were named by Science magazine as the 1989 "Molecule of the Year" because they were likely to have the greatest influence on history.

Polymerase chain reaction (PCR), invented by scientist Kary Mullis of Cetus Corporation in 1983, for which he won a Nobel Prize in 1993, allows researchers to amplify fragments of DNA by several orders of magnitude without the need to use bacterial cells. It is a method for amplifying small samples of DNA or RNA, creating millions of copies in just a few hours, and has been described as 'molecular photocopying'. This makes it possible to analyze DNA when only very small amounts are available. Before PCR was widely available, the DNA had to be cloned into a vector and transferred to bacteria, and this could take weeks – PCR now takes up to a few hours for the same result. PCR is usually used to amplify stretches of DNA of up to 10 kb (kilo base pairs), though some techniques can work with DNA of up to 40 kb.

This technique has revolutionized many aspects of current research, including DNA cloning and sequencing, functional analysis of genes, the diagnosis of hereditary or infectious diseases, the identification of genetic fingerprints, and so on. The basic components of a PCR reaction include a DNA template, primers, nucleotides, DNA polymerase, and a buffer.

Principle:

Polymerase Chain Reaction is an in-vitro method for exponential amplification of a target portion of template DNA, which involves incorporation of nucleotides by DNA polymerase during thermal cycling. The basic components of a PCR reaction include a DNA template, primers, nucleotides, DNA polymerase, and a buffer. The process involves three major steps which are denaturation, annealing and extension/elongation.

Reagents Required:

- PCR master mix including Taq polymerase, dNTPs, $MgCl_2$ and buffer
  OR
- Taq DNA Polymerase, dNTPs, $MgCl_2$ and PCR buffer separately.
- PCR primers (Forward and Reverse)
- PCR grade water
- Negative and Positive Controls

Equipment Required:

- Thermal Cycler with analysis software
- Vortex Mixture
- Microcentrifuge
- Pipettes
- PCR safety cabinet

Consumables:

- PCR tubes/strips/plates according to equipment compatibility and requirement
- Filtered pipette tips
- 1.5 ml centrifuge tubes

Procedure:

1) Label the PCR tubes for samples and controls. In case of quantification experiments, tube will also be labeled for standards.
2) Thaw the PCR reagents and prepare PCR reaction mix. A generalized recipe of PCR is given in the following table. The amount of chemicals used may vary according to the desired protocol and manufacturer's instructions. Calculate the volume of total reaction mix required for the whole batch including samples, controls and standards.

Table 4.1: Preparation of PCR Reaction Mix

Reagent	**Stock Conc. (M1)**	**Final Conc. In Reaction Mix (M2)**	**Volume per Reaction (V1)**
PCR buffer	10x	1x	5 µl
Forward Primer (dilution)	10 µM	0.5 µM	2.5 µl
Reverse Primer (10 µM dilution)	10 µM	0.5 µM	2.5 µl
dNTP mix	10 mM each	0.2 mM each	1 µl
$MgCl_2$	25 mM	2 mM	4 µl
Taq DNA Polymerase	5 U/ µl	1.25 U	0.25 µl
Sterile distilled H_2O	To make up volume		31.75 µl
Total			47 µl

Or

Table 4.2 PCR using prepared 2x Master Mix

Reagent	Volume per Reaction
2x PCR Master Mix	25 µl
Forward Primer (10 µM dilution)	2.5 µl
Reverse Primer (10 µM dilution)	2.5 µl
Sterile distilled H_2O	17 µl
Total	47 µl

Mix the reagent by gentle vortex followed by short spin.

Aliquot the reaction mix in the individual PCR reaction tubes/well.

4) Add the template i.e; sample/ control in the appropriate labeled tube.
5) The volume of template varies according to the protocol in use. In the above example, 3 µl templates will be added to each tube so that 50 µl total reaction volume (V2) is achieved.
6) The DNA concentration of the template should be known so that the optimum input quantity of the template DNA can be used for PCR reaction. Optimal amounts of template DNA in the 50 µl reaction volume are 0.01-1 ng for both plasmid and phage DNA, and 0.1-1 µg for genomic DNA. High amounts of template increases the risk of generation of non-specific PCR products. Low amounts of template reduces the accuracy of the amplification.
7) Open the PCR machine's software and edit run parameters e.g. run ID, user ID, sample IDs, sample volume and cycling conditions according to desired protocol. A generalized 3 step cycling protocol for PCR is given.

Table 4.3 PCR cycling conditions

Step	Temperature	Duration	No. of cycles
Initial Denaturation	95^{o}C	3 min (in case of Hot Startitmay be prolonged upto 10 min)	1
Denaturation	94^{o}C	10 sec	35
Annealing	50-60^{o}C	20 sec	
Extension	72 ^{o}C	30 sec	
Final Extension	72 ^{o}C	5 min	1
Final hold	25 ^{o}C	Hold	1

1) Place the sample tubes in the thermal cycler, close the lid and Run the program.
2) After the completion of the PCR, remove the tubes from the thermal cycler and proceed for agarose gel electrophoresis or other downstream application. Otherwise store the PCR products at -20 ^{o}C.

References and further reading:

Guyer, R.L. and D.E. Koshland, The molecule of the year. Science, 1989. 246(4937): p. 1543-1546.

Gerald Karp, Janet Iwasa, Wallace Marshall - Karp's Cell and Molecular Biology. Concepts and Experiments-Wiley (2015)

https://www.xxpresspcr.com/all-news/pcr-101-an-introduction-to-pcr/

Delong, R.K. and Q. Zhou, Introductory experiments on biomolecules and their interactions. 2015: Academic Press.

Practical 5

Separation of different sized DNA fragments on agarose gel

Gel electrophoresis is the standard lab procedure for separating DNA by size (e.g., length in base pairs) for visualization and purification. Electrophoresis uses an electrical field to move the negatively charged DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones. Thus, you can determine the approximate length of a DNA fragment by running it on an agarose gel alongside a DNA ladder (a collection of DNA fragments of known lengths).

Principle

Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones. In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest.

Equipment Required

- An electrophoresis chamber and power supply
- Gel casting trays, which are available in a variety of sizes and composed of UV transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- Sample combs, around which molten agarose is poured to form sample wells in the gel.
- Transilluminator (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels.
- NOTE: Always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.
- Pipettes covering 1 to 100 ul range.

Reagent Required

- Electrophoresis buffer, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).

- DNA sizing standard/ladder.
- Loading buffer, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- Ethidium bromide, a fluorescent dye used for staining nucleic acids. *NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical – wear gloves while handling.*
-

Procedure:

To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

After the gel has solidified, the comb is removed, carefully so as to not to rip the bottom of the wells. The gel is inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. You can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.

The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively. When adequate migration has occurred, DNA fragments are visualized on the basis of Ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on an ultraviolet trans-illuminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

Migration of DNA Fragments in Agarose

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the $\log_{10}$ of either
their molecular weights or number of base pairs, a roughly straight line will appear.
Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles.

Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments. Chief among these factors are:
Agarose Concentration: By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilitate separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs.

Voltage: As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than small fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

Electrophoresis Buffer: Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel! Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.

Ethidium Bromide: Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels. As described above, it can be incorporated into agarose gels, or added to samples of DNA before loading to

enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore, its mobility.

Caution: Ethidium bromide is a mutagen and should be handled as a hazardous chemical (so wear gloves while handling).

Precautions:

1) Make sure that the Agarose is fully dissolved in the buffer.
2) Before casting the gel, the tray and comb should be wiped with ethanol.
3) Make sure that the gel in the Chamber is immersed in TAE Buffer.
4) Labeling should be proper.
5) Ensure that the connections are proper.

References and further reading:

Sambrook, J. and D. Russell, Gel electrophoresis of DNA and pulsed-field agarose gel electrophoresis. Molecular cloning: a laboratory manual, 2001. 1(3).
https://vlab.amrita.edu/?sub=3&brch=77&sim=1375&cnt=2.

Practical 6

Microscopy

Cells – the living unit of life; are so small that they cannot be seen by naked eye. The optical instrument that magnifies the image of these cells and enables us to view their morphological features is a microscope. Antony van Leeuwenhoek is often considered as the father of microscopy.

Among microorganisms, most bacteria measure in the range of 0.5 to 4 µm. Mycoplasma and Coxiella are the shortest among bacteria and Spirochetes the longest. Viruses are ultramicroscopic structures; they can't be seen by compound microscopes. They can be visualized by electron microscopes and their measurements are in nm (nanometer).

Various types of microscopes are:

- Simple microscope
- Compound microscope
- Phase contrast microscope
- Fluorescent microscope
- Interference microscope
- Electron microscope
- Atomic Force microscope
- Polarization microscope

A microscope is commonly used in a microbiological study of organisms. The basic modern microscope found in schools, hospitals, and research centers is a compound microscope which has a series of lenses to collect and focus the light transmitted through the specimen. The various parts of a microscope with their associated function are mentioned below.

Tube:

Tube or the body tube, connects the eyepiece to the objective lenses.

Eyepiece or ocular lens:

Eyepiece is the lens, present at the top and is used to see the objects under study. Eyepiece lens contains a magnification of 10X or 15X.

Resolving nosepiece:

It is also known as the Turret. Resolving nosepiece has holders for the different objective lenses. It allows the rotation of the lenses while viewing.

Objective lenses:

Generally, three or four objective lenses are found on a microscope, with ranges of 10X, 40X, 100X powers. Lenses are color coded, the shortest lens is of the lowest power, and the longest lens is high power lens.

Diaphragm:

Diaphragm helps in controlling the amount of light that is passing through the opening of the stage. It is helpful in the adjustment of the control of light that enters.

Coarse adjustment knob:

Used for focus on scanning. Usually the low power lens is used enabling the movement of the tube.

Fine adjustment knob:

Used for focus on oil. It moves the body tube for focusing the high power lens.

Arm:

It supports the tube of the microscope and connects to the base of the microscope.

Stage:

A flat platform used for placing the slides under observation.

Stage clip:

Stage clips hold the slides in proper place.

Condenser:

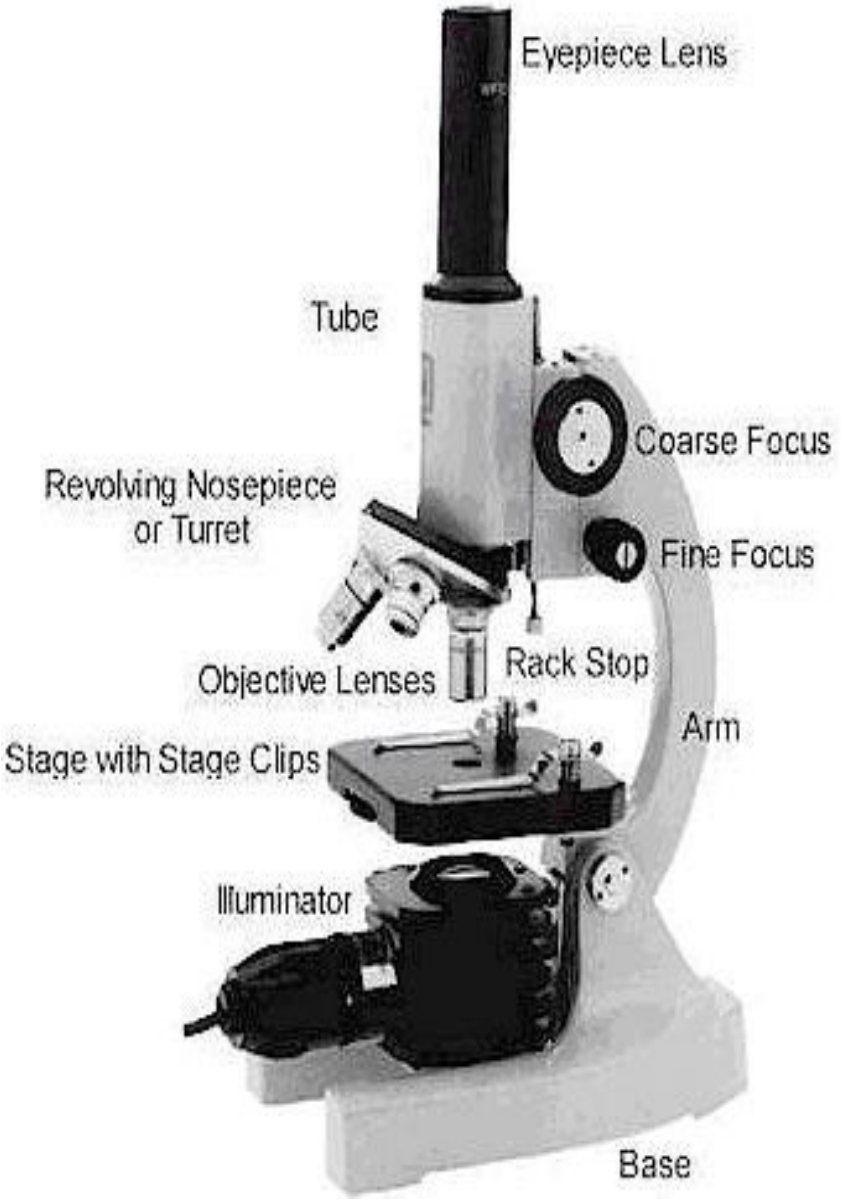
The main function of condenser lens is focusing the light on the specimen under observation. It gives a sharp image.

Base:

It provides basal support for the microscope.

Power switch:

It turns the illumination on or off.



A compound microscope

Proper usage and handling the microscope:

Microscope is a delicate instrument that must be handled with care. All kinds of mechanical

shocks must be avoided. The microscope must be lifted by holding its arm in one hard and supporting the base of the microscope with the palm of the other hand. The microscope must be kept in dust free environment. The oil must be wiped clean using a soft tissue paper. After usage the slide must be removed and cleaned before returning to its original place.

Principle of a Microscope:

A simple microscope works on the principle that when a tiny object is placed within its focus, a virtual, erect and magnified image of the object is formed at the least distance of distinct vision from the eye held close to the lens.

Procedure of properly using a microscope:

1) Set your microscope on a tabletop or other flat, sturdy surface where you will have plenty of room to work. Plug the microscope's power cord into an outlet. (Note: some compound microscopes don't use electric lighting, but have a mirror to focus natural light instead.)
2) Switch on your microscope's light source and then adjust the diaphragm to the largest hole diameter, allowing the greatest amount of light through. If you have an iris diaphragm, slide the lever till the most light comes through.. Rotate the nosepiece to the lowest-power objective usually 4x for 40x. It is easiest to scan a slide at a low setting, since you have a wider field of view at low power.
3) Place a microscope slide on the stage, either under the stage clips or clipped onto the mechanical stage if your microscope has one. A prepared slide works best when you do this for the first time. (If you do not have a prepared slide, place a strand of colored yarn or thread on a blank slide and place a coverslip over it.) Move the slide until the specimen is under the objective lens.
4) Adjust the large coarse focus knob until the specimen is in focus. Slowly move the slide to center the specimen under the lens, if necessary. Do this by nudging it gently with your fingers or by turning the slide control knobs if you have a mechanical stage.
5) Adjust the small fine focus knob until the specimen is clearly in focus. Then adjust the diaphragm to get the best lighting. Start with the most light and gradually lessen it until the specimen image has clear, sharp contrast.
6) Scan the slide (right to left and top to bottom) at low power to get an overview of the specimen. Then center the part of the specimen you want to view at higher power.
7) Rotate the nosepiece to the 10x objective for 100x magnification. Refocus and view your specimen carefully. Adjust the lighting again until the image is most clear (you will need more light for higher power). Repeat with the 40x objective for 400x magnification, which will enable you to see

all of the specimen detail that's necessary.

Applications of microscope:

1) It is usually used for the study of microscopic algae, fungi, and biological specimens.
2) It is commonly used by watchmakers to see the magnified view of small parts of a watch.
3) It is also used by the jewelers to see the magnified view of the fine parts of jewelry.
4) It is used to see the enlarged image of letters of a book, textures of fibers or threads of a cloth.
5) It is used to see the magnified view of different particles of different types of soils.
6) It is used by palmists to see an enlarged view of the lines of our hand.
7) It is used by skin specialists to find out various diseases of the skin.
8) It is also used to see the details of stamp and engravings.

Precautions and safety tips for using microscope:

1) Clean the microscope after each use.
2) Clean smudged lenses with lens paper. Avoid applying pressure with a cloth as the lenses are very fragile.
3) Wipe the stage (the platform that holds the slides) down thoroughly and disinfect the eyepiece with an alcohol-based wipe.
4) Handle glass slides carefully. If a slide breaks, ensure that the contents are properly disposed and report the incident in PeopleSoft if an injury occurs.
5) Turn off the light source when the microscope is not in use. This will improve lamp longevity and save energy.
6) Be aware if your microscope has a mercury lamp. A broken mercury lamp may release toxic mercury vapors.
7) When carrying the microscope, always use two hands with one hand supporting the base and theother hand holding the arm.
8) Properly store the microscope by lowering the nosepiece, turning off the light source, and placing the objective lenses on the lowest setting. Cover with a dust jacket.
9) Ensure your microscope is scheduled for preventative maintenance and keep the area around the microscope clean. For more information, consult the manufacturer's manual for your equipment.

References and further reading:

https://microbenotes.com/simple-microscope-principle-instrumentation-and-applications/
https://learning-center.homesciencetools.com/article/how-to-use-a-microscope-science-lesson/

Practical 7

Staining techniques (Gram staining)

Staining

It is a technique that is used to define and examine different types of microbes.

Types of staining techniques,

1. Simple staining techniques. Simple staining is performed with basic dyes such as crystal violet or methylene blue.

2. Differential staining techniques. Differential staining technique distinguishes two kinds of organisms. The technique is of two types; Gram staining and acid fast staining technique.

Gram stain technique is the most commonly used technique for study of microbes like bacteria.

Gram stain or Gram staining, also called Gram's method, is a method of staining used to distinguish and classify bacterial species into two large groups (gram-positive and gram-negative). The name comes from the Danish bacteriologist Hans Christian Gram, who developed the technique.

Materials required:

Clean glass slides, Inoculating loop, Bunsen burner, Bibulous paper, Microscope, Lens paper and lens cleaner, Immersion oil, Distilled water, 18 to 24-hour cultures of organisms.

Reagents: Primary Stain - Crystal Violet Mordant - Grams Iodine Decolourizer - Ethyl Alcohol Secondary Stain - Safranin

Procedure:

1. Smear preparation A small sample of microorganisms is placed on a slide and permitted to air dry. The smear is heat fixed by quickly passing it over a flame. Heat fixing kills the organisms, makes them adhere to the slide, and permits them to accept.
2. Flood the slide with crystal violet. Leave for 30 seconds and wash with distilled water.
3. Use iodine as a mordant by flooding the slide with iodine solution. Leave for 30 seconds and wash with distilled water.

4. Wash the slide with ethyl alcohol that acts as a decolorizing agent. Gram positive bacteria retain the crystal-violet-iodine stain; however, the Gram negative bacteria lose the stain.
5. Flood the slide with the secondary stain or safranin. Let it stay for 30 seconds and wash gently with distilled water. All the slide to dry in air.
6. Observe under the microscope, first at low magnification and then at higher magnification. Gram negative bacteria appear red while Gram positive bacteria appear blue or purple.

Results:

Summary of gram stain results

Application of	Reagent	Cell colour	
		Gram-positive	Gram-negative
Primary dye	crystal violet	purple	purple
Trapping agent	iodine	purple	purple
Decolorizer	alcohol/acetone	purple	colorless
Counter stain	safranin/carbol fuchsin	purple	pink

1. Gram-positive bacteria (thick layer of peptidoglycan-90% of cell wall)- stains purple.
2. Gram-negative bacteria (thin layer of peptidoglycan-10% of cell wall and high lipid content) –stains red/pink.

References and further reading:

Moyes, R. B., Reynolds, J., & Breakwell, D. P. (2009). Differential staining of bacteria: gram stain. *Current Protocols in Microbiology*, *15*(1), A-3C.

Practical 8

Preparation of cell organelles (prepared slides)

Materials required: prepared slides, light microscope, tissue papers to clean slides and microscope lens.

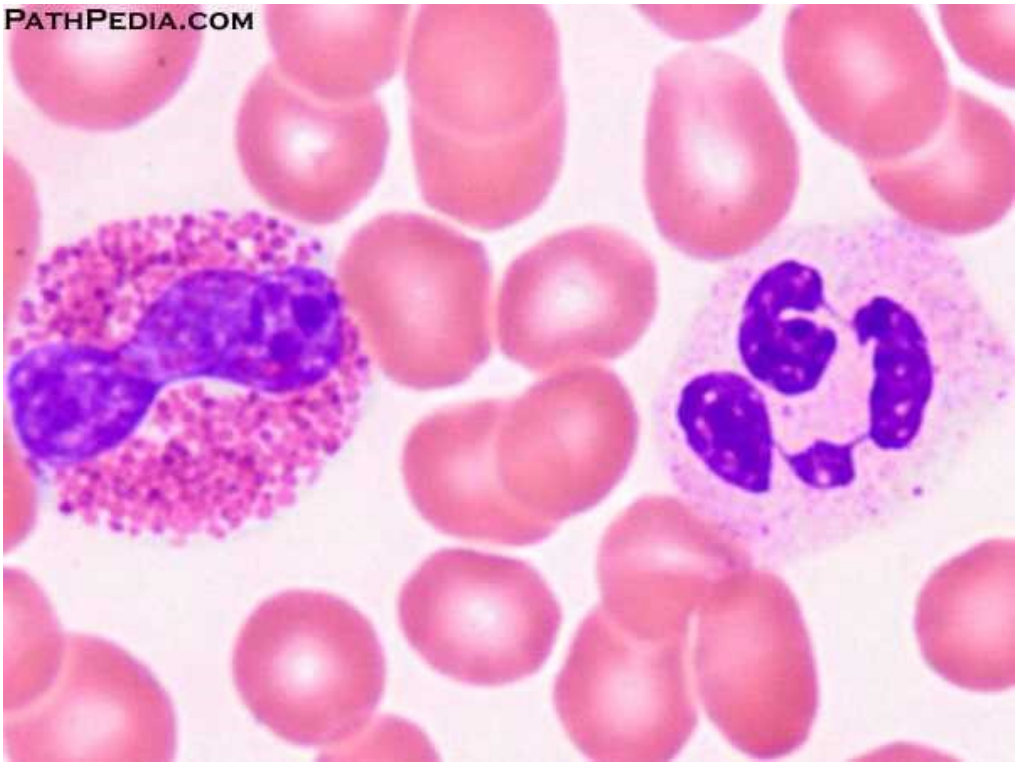
Procedure:

Focus all the available prepared slides of cells to examine different organelles present in them and record your observation in practical notebook.

You will be provided with the slides of animal and plant cells.

Results:

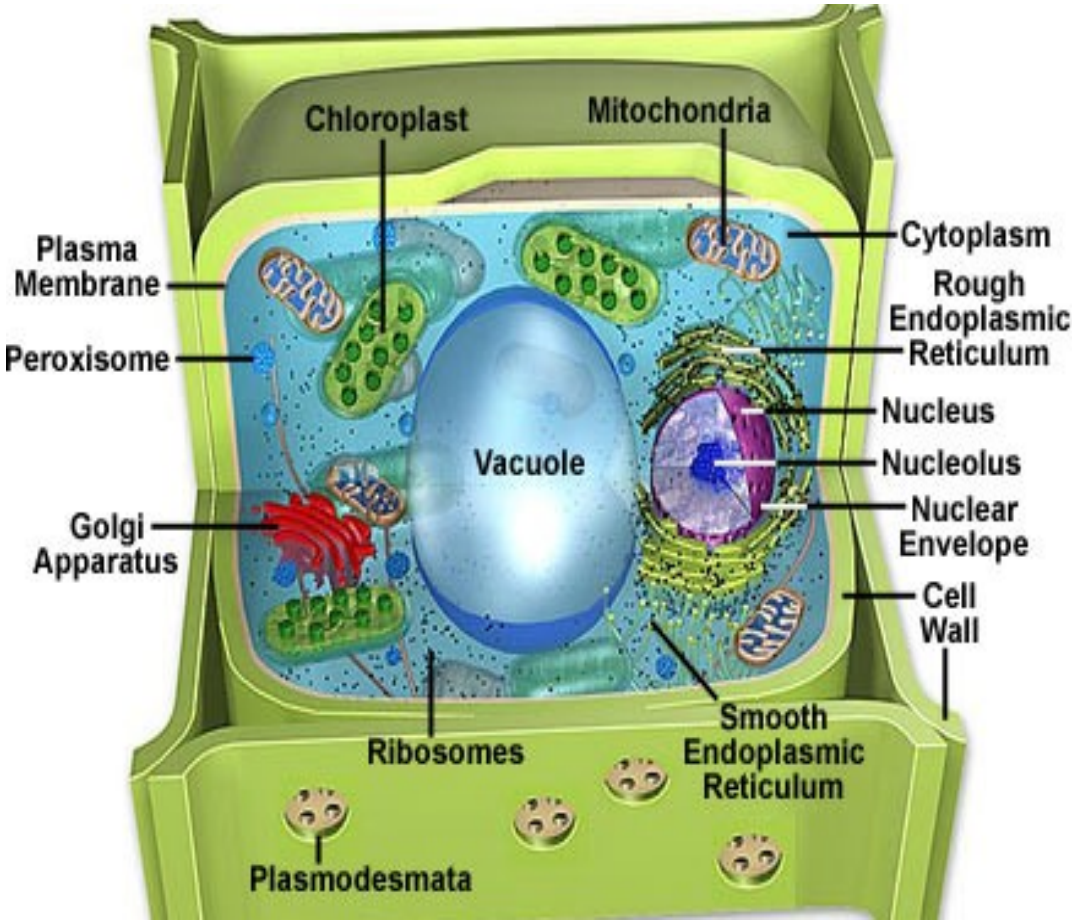
Internal structure of an onion epidermal cell



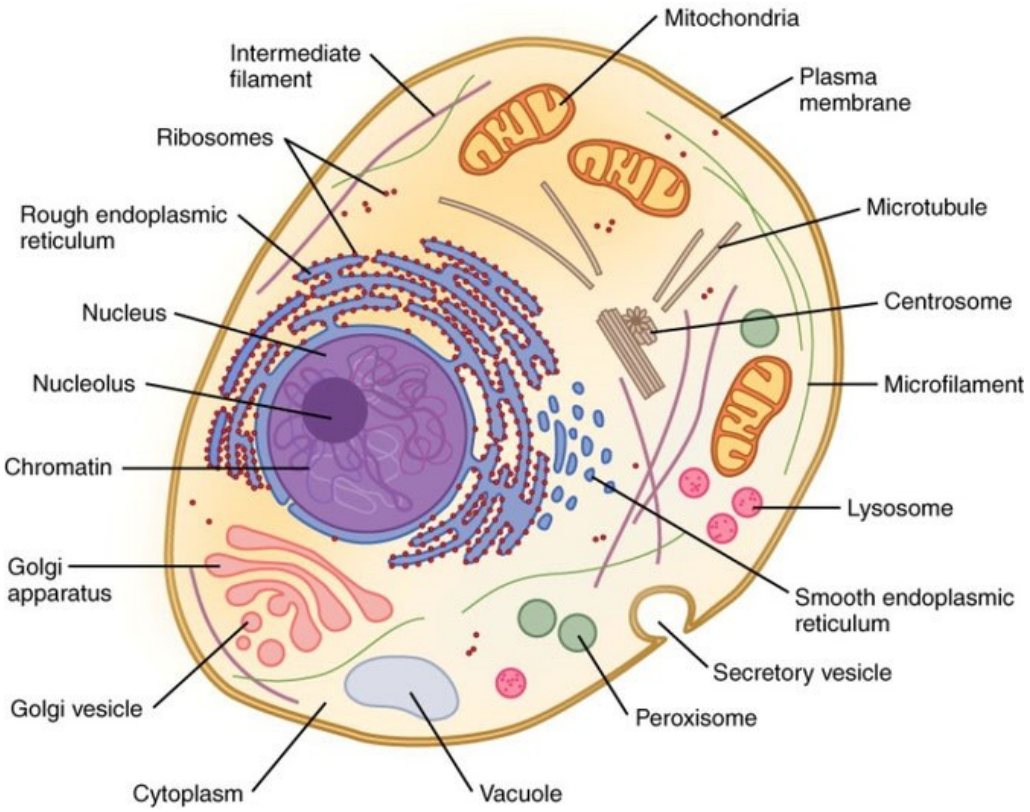
Prepared slide of Blood cells (animal cells)

Microscopic slide showing the animal epidermal cells

Labelled structures:



Detailed plant cell structure



Labelled animal cell

References and further reading:

https://www.microscopemaster.com/onion-cells-microscope.html

https://www.pathpedia.com/education/eatlas/histology/blood_cells/Images.aspx?bb60f786-ded0-4b44-82ea-598ad55108d4

https://www.microscopemaster.com/cheek-cells-microscope.html

https://micro.magnet.fsu.edu/cells/plantcell.html

https://www.withcarbon.com/2014/12/13/animal-cell-parts-and-functions/

Practical 9

Preparation of temporary whole mount (human cheek cells)

Observing human cheek cells under a light microscope is a simple way to quickly view a human cell structure. Many educational facilities use the procedure as an experiment for students to explore the principles of microscopy and the identification of cells. Observation uses a wet mount process that is straight forward to achieve by following an effective preparation method. You can replicate the observational experiment at home with any standard light microscope with magnification settings of X-40 and X-100.

Materials required:

Tooth pick, slide, cover slip, microscope, methylene blue, distilled water, paper towel.

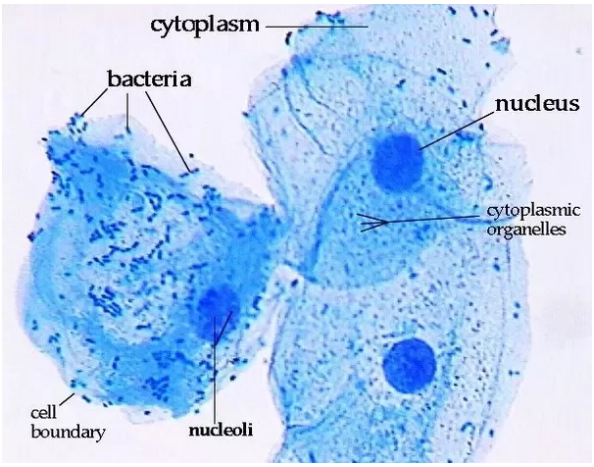
Procedure:

1. Swab the inside of your cheek with the non sharp end of a toothpick.
2. Place the toothpick at the bottom of the cheek and move the toothpick up horizontally to collect cheek cells. Be careful not to scrape the inside of the cheek too hard because the epithelial lining is delicate.
3. Place the swabbed end of the toothpick onto the middle of a microscope slide.
4. Add a single droplet of water squeezed from a plastic pipette onto the center of the slide.
5. Rotate the toothpick in the water to release the human cheek cells.
6. Add one drop of methylene blue onto the water and cell solution to stain the cheek cells for observation.
7. Position a cover slip at a 45 degree angle just inside the left edge of the solution.
8. Move your fingers down and to the right to place the cover slip over the cheek cell mixture.
9. Check for tiny air bubbles under the cover slip and lightly push the cover slip downwards to release any air bubbles you find.
10. Place the edge of a paper towel on any solution outside of the cover slip to absorb the excess moisture.
11. Mount the human cheek cell slide on the light microscope viewing platform.
12. Choose the X-40 magnification setting on the light microscope and look through the

viewing lens. Turn the focusing dial to adjust the focus until you see a clear and crisp image. Observe the human cheek cells by looking for irregularly-edged circular structures with a dark center, or nucleus. Tip: Use iodine as an alternative to methylene blue. Warning: Be careful when handling cover slips because they break easily.

Note down the results observed in practical note book.

Results:



Human cheek cell on temporary mount

References and further reading:

https://www.quora.com/When-we-see-a-cheek-cell-through-a-microscope-can-we-see-the-mitochondria

https://microcosmos.foldscope.com/?p=91783

Watch procedure at: https://www.youtube.com/watch?v=q6wJVoojWOc

Practical 10

Preparation of permanent whole mount

A permanent slide is a slide can be fixed and locked into place. While temporary slides usually have a top and a bottom with the object secured in-between, permanent slides have a top and bottom that are glued or sealed together for longer time.

• Permanent slides have the advantage of being vacuum-sealed within frame to prevent movement or leakage.

• Ideal for transport or repeated presentational viewings.

• With permanent slides we can preserve the specimen for longer than temporary slide.

Materials required:

Fresh water, Sample of algae, Formaldehyde (40%), paper, Slide, Cover slip, Oven, Microscope, Glycerine.

Procedure:

1. Wash the slide and cover slip properly so that there are no particles.
2. Put a drop of fresh water on slide.
3. Wash the algae (any specimen can be used) with PBS 10m for 2 times.
4. Place the algae on the slide.
5. Add a drop of formalin (40%).
6. Use filter paper to drain away excess liquid.
7. Put some glycerine on algae.
8. Keep the slide on oven for 2-3 minutes at 60°c.
9. Put washed cover slip on it.
10. Again keep the slide in oven for 2-3 minutes.
11. Remove the excess glycerine.
12. Seal it properly with glu or nail polish.

Results:

Identification and morphology is characterised under microscope.

References and further reading:

https://www.slideshare.net/sunirmaldas/morphology-of-micro-algae-ad-permanent-slide-preparation-of-micro-algae-and-cyanobacteria

Practical 11

Squash preparation of onion root tip for mitotic stages

The genetic information of plants, animals and other eukaryotic organisms resides in several (or many) individual DNA molecules, or chromosomes. For example, each human cell possesses 46 chromosomes, while each cell of an onion possesses 8 chromosomes. All cells must replicate their DNA when dividing. During DNA replication, the two strands of the DNA double helix separate, and for each original strand a new complementary strand is produced, yielding two identical DNA molecules. DNA replication yields an identical pair of DNA molecules (called sister chromatids) attached at a region called the centromere.

DNA replication in eukaryotes is followed by the process called mitosis which assures that each daughter cell receives one copy of each of the replicated chromosomes. During the process of mitosis, the chromosomes pass through several stages known as prophase, metaphase, anaphase and telophase. The actual division of the cytoplasm is called cytokinesis and occurs during telophase. During each of the preceding stages, particular events occur that contribute to the orderly distribution of the replicated chromosomes prior to Cytokinesis.

The stages of Mitosis

Prophase:

During prophase, the chromosomes supercoil and the fibers of the spindle apparatus begin to form between Centrosome located at the pole of the cells. The nuclear membrane also disintegrates at this time, freeing the chromosomes into the surrounding cytoplasm.

Prometaphase:

During Prometaphase, some of the fibers attach to the centromere of each pair of sister chromatids and they begin to move toward the center of the cell.

Metaphase:

At metaphase the chromosomes have come to rest along the center plane of the cell.

Anaphase:

During anaphase, the centromeres split and the sister chromatids begin to migrate toward the opposite poles of the cell.

Telophase:

During telophase, the chromosomes at either end of the cell cluster begin to cluster together,

which facilitates the formation of a new nuclear membrane. This also is when cytokinesis occurs, leading to two separate cells. One way to identify that telophase has begun is by looking for the formation of the cell plate, the new cell wall forming between the two cells. :

Why use onion roots for viewing mitosis?

• The roots are easy to grow in large numbers.

• The cells at the tip of the roots are actively dividing, and thus many cells will be in stages of mitosis.

• The tips can be prepared in a way that allows them to be flattened on microscopes slide ("squashed") so that the chromosomes of individual cells can be observed.

• The chromosomes can be stained to make them more easily observable.

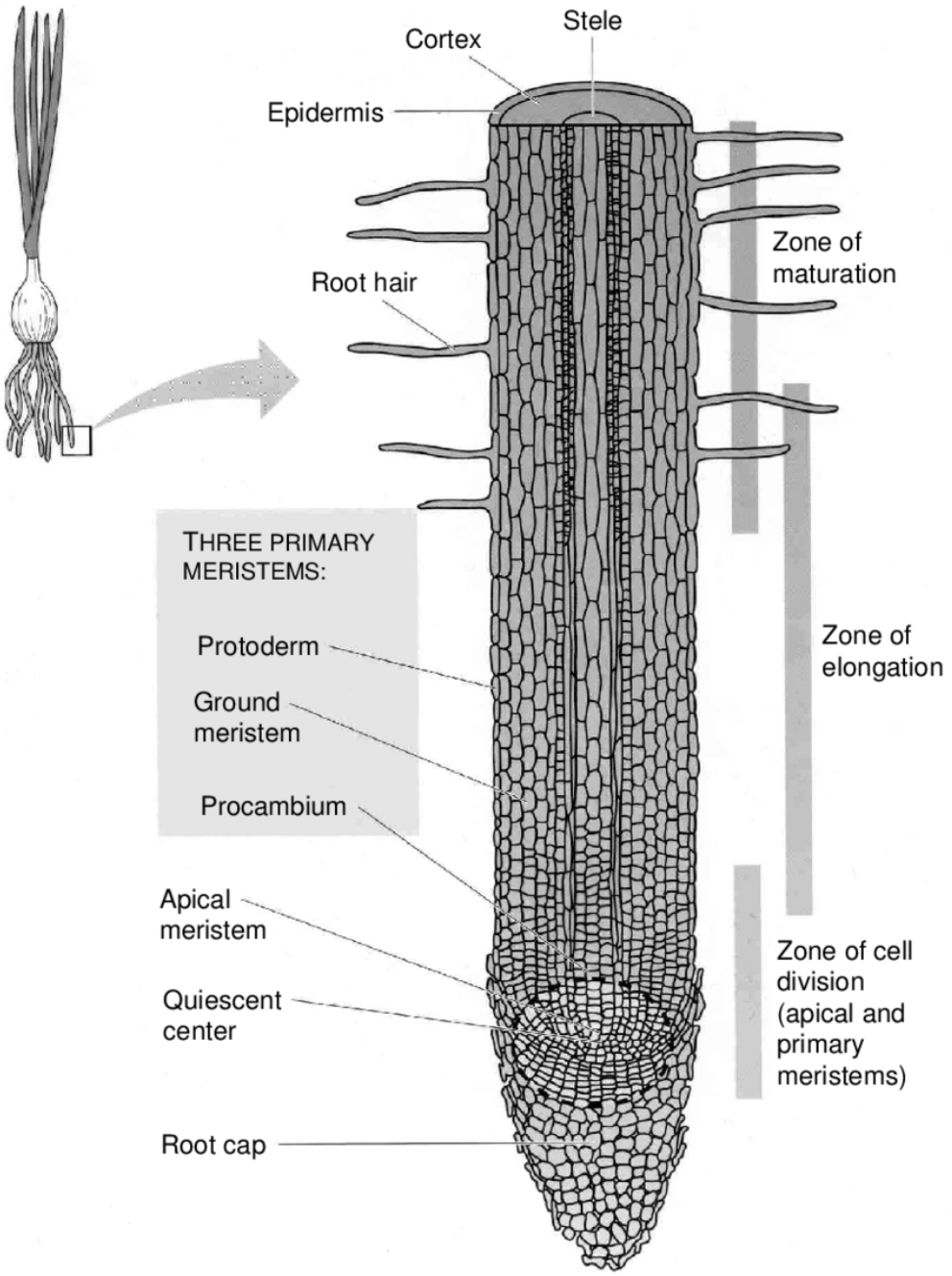
Regions of Onion Root tips

There are three cellular regions near the tip of an onion root.

 The root cap contains cells that cover and protect the underlying growth region as the root pushed through the soil.

 The region of cell division (or meristem) is where cells are actively dividing but not increasing significantly in size.

 In the region of cell elongation, cell are increasing in size, but not dividing.



Structure of onion root tip

Viewing Chromosomes

Chromosomes generally are not visible as distinct entities in non-dividing cells, since the DNA is uncoiled, but the process of mitosis is facilitated by supercoiling of the chromosomes into a highly compacted form. Supercoiled chromosomes can be visualized in cells, particularly if they are treated with a DNA-specific stain, such as the Feulgen stain.

Materials required:

Onion plant with root, feulgen stain, 1 N HCl, scissors, forceps, razor blade, pasture pipette,

1.5 ml microfuge tubes, dissection probe with wooden back, microscopic slides and cover slips, water bath, light microscope.

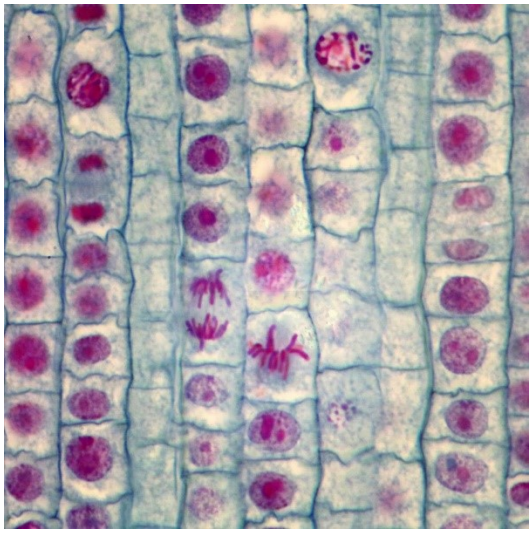
Procedure:

1. While actively growing onions are present in the lab for you to observe, you will be provided with roots that have been previously harvested and treated with a fixative to stabilize the cells. You will work in groups of two for this lab exercise. The first step will be to 'soften' the roots so that they later can be spread on a microscope slide.
2. Using scissors, cut 2 roots tips about 1 cm long, and transfer them into a plastic microtube (One of the rots will be an extra one).
3. Fill the tube about 2/3 full with 1N HCl from a dropper bottle. *** Caution: Work with the HCl carefully, it is a strong acid.
4. Place the tube in a 60OC water bath, and allow the roots to incubate for 12 minutes. 4. After the 12-minute incubation period, remove the tube from the water bath.□
5. Rinse the roots in H2O.
6. Using forceps carefully transfer the root tips to a small petri plate.
7. Using a plastic 'squeeze' pipet, carefully remove the HCl from the micro-tube and transfer it to the "discard flask".
8. Rinse the root tips 3 times with water from the dropper bottle, disposing of the rinses in the discard flask.
9. After removing the water from the third rinse, cover the root with the Feulgen stain. Caution: Although the Feulgen stain does not appear colored, it will strongly stain skin and clothing.
10. Incubate the roots in the stain for 12 minutes. During this time the very tip of the root will begin to turn red as the DNA stains the numerous small actively dividing cells at the tip.
11. Remove the stain and again rinse the roots.
12. Using a plastic 'squeeze' pipette, carefully remove the Feulgen stain and discard it in the discard flask. Again, rinse the root tips 3 times with water.
13. Transfer a root to the centre of a clean microscope slide and add a drop of water.
14. Using a razor blade cut off most of the unstained part of the root, and discards it.
15. Cover the root tip with a cover slip, and then carefully push down on the cover slide with the wooden end of a dissecting probe. Push hard, but do not twist or push the cover slide sideways. The root tip should spread out to a diameter about 0.5 – 1 cm.

16. Scan the microscope under the 10x objective. Look for the region that has large nuclei relative to the size of the cell; among these cells will be found cells displaying stages of mitosis. Examples are shown in the figure to the right. Switch to the 40X objective to make closer observations.

Results:

The prepared slide will show results as shown in figure below. The student will be made to identify different stages of mitosis.



Microscopic examination of prepared onion root tip slide

References and further readings:

https://www.researchgate.net/publication/200736502_Biological_Approaches_to_Sustainable_Soil_Systems_Books_in_Soils_Plants_and_the_Environment/figures?lo=1

https://vlab.amrita.edu/?sub=3&brch=188&sim=1102&cnt=2

https://www.youtube.com/watch?v=VJ678ceiiV0

https://www.southernbiological.com/biology/prepared-slides/botany/pms27-11c-onion-allium-cepa-root-tip-for-plant-mitosis-ls-quadruple-stain/

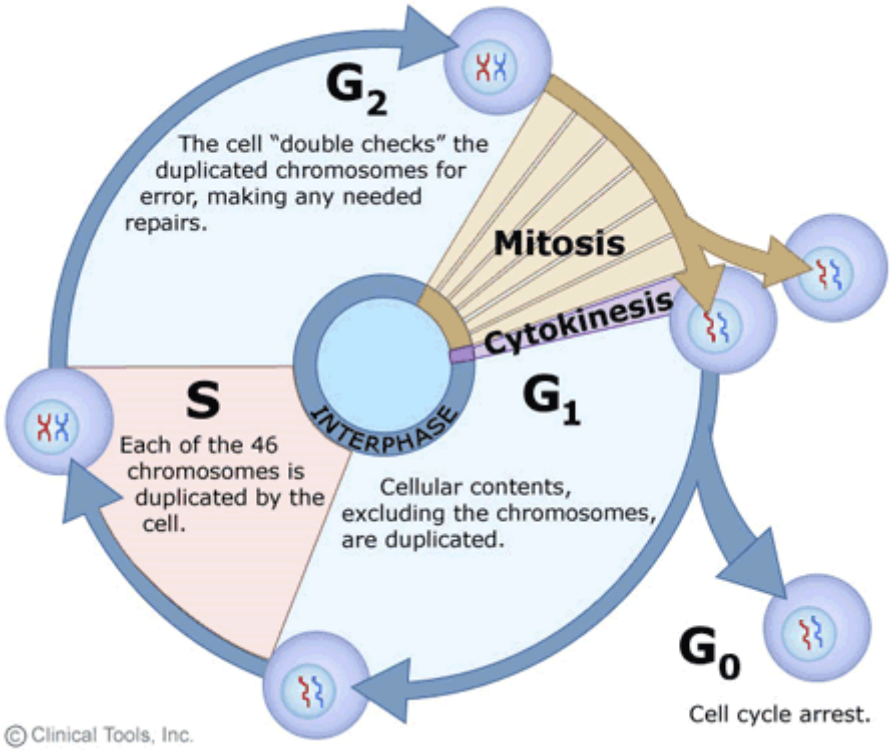
Practical 12

Study of mitotic and meiotic stages (prepared slides)

A cell cycle is a series of events that takes place in a cell as it grows and divides. A cell spends most of its time in what is called interphase, and during this time it grows, replicates its chromosomes, and prepares for cell division. The cell then leaves interphase, undergoes mitosis, and completes its division.

The cell cycle:

- **G1 phase.** Metabolic changes prepare the cell for division. At a certain point - the restriction point - the cell is committed to division and moves into the S phase.
- **S phase**. DNA synthesis replicates the genetic material. Each chromosome now consists of two sister chromatids.
- **G2 phase.** Metabolic changes assemble the cytoplasmic materials necessary for mitosis and cytokinesis.
- **M phase.** A nuclear division (mitosis) followed by a cell division (cytokinesis).



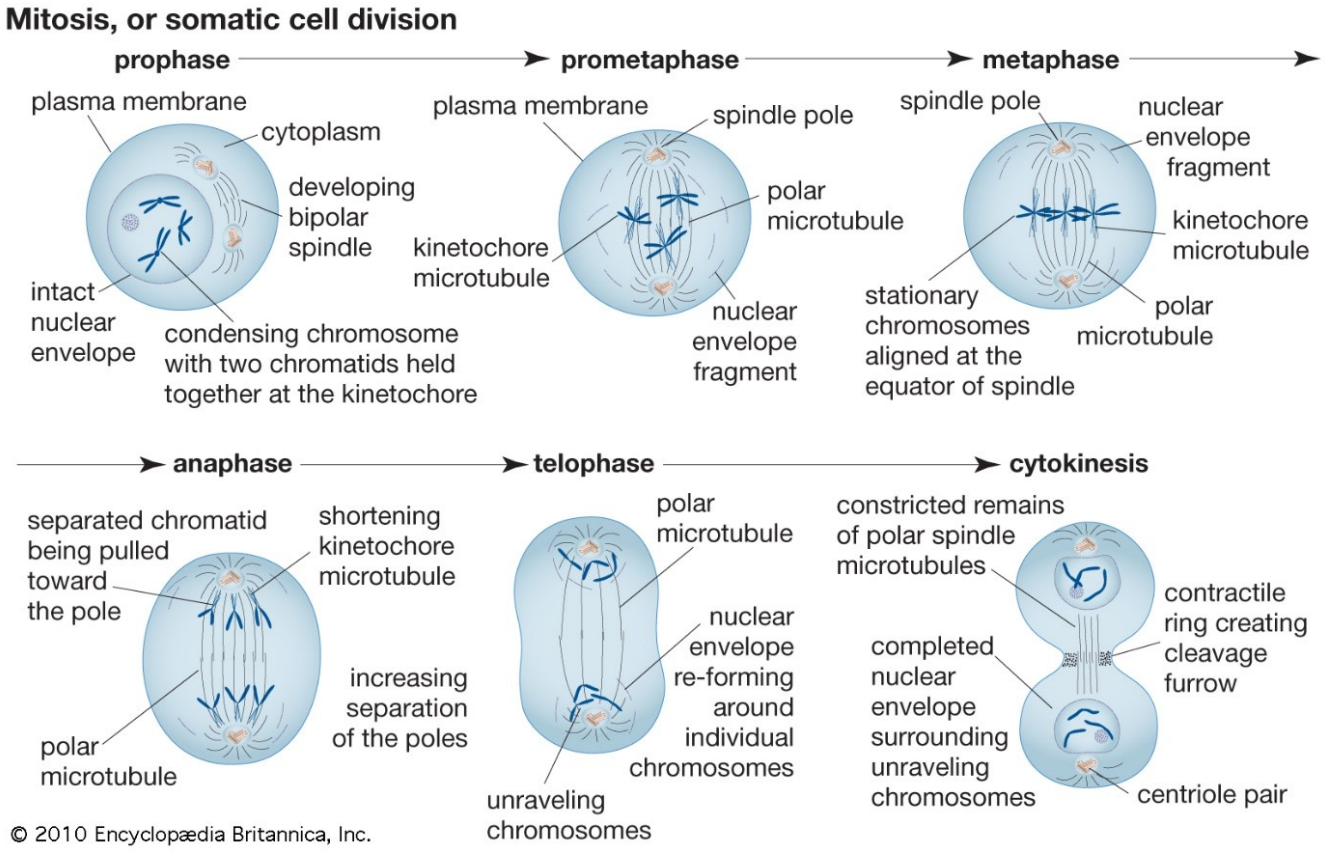
Cell cycle

What is mitosis:

Mitosis is the vegetative cell division in eukaryotes, which divides parent cell's replicated

genome between two daughter cells. The two cells are genetically identical, bearing an approximately equal number of organelles and cytoplasm. The mitotic phase is called the M phase of the cell cycle. Eukaryotes have a large number of chromosomes. These chromosomes are replicated during S phase of the interphase of the cell cycle, prior to the entering of the M phase. Replicated chromosomes contain two sister chromatids joined together at their centromeres. Two types of mitosis can be identified among organisms: open mitosis and closed mitosis. During open mitosis in animals, nuclear envelope is broken down in order to separate the chromosomes. But in fungi, chromosomes separated in the intact nucleus, which is called closed mitosis.

What are the stages of mitosis?



Stages of mitosis

What is meiosis?

Meiosis is the process by which sex cells (gametes) are made in the reproductive organs. It involves the reduction division of a diploid germline cell into four genetically distinct haploid nuclei.

The process of meiosis consists of two cellular divisions:

1. The first meiotic division separates pairs of homologous chromosomes to halve the chromosome number (diploid → haploid).
2. The second meiotic division separates sister chromatids (created by the replication of DNA during interphase).

What are the stages of meiosis?

Meiosis consists of two divisions, both of which follow the same stages as mitosis (prophase, metaphase, anaphase, telophase)

Meiosis is preceded by interphase, in which DNA is replicated to produce chromosomes consisting of two sister chromatids

A second growth phase called interkinesis may occur between meiosis I and II, however no DNA replication occurs in this stage

Meiosis I

The first meiotic division is a reduction division (diploid → haploid) in which homologous chromosomes are separated

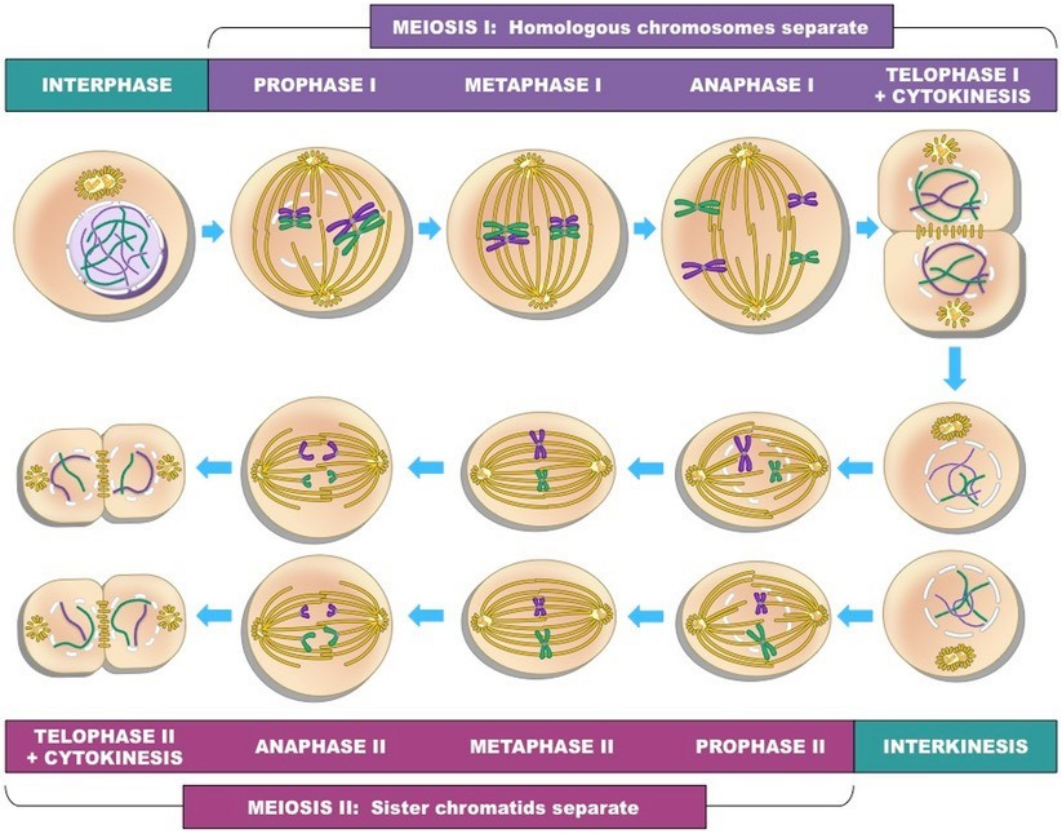
1. P-I: Chromosomes condense, nuclear membrane dissolves, homologous chromosomes form bivalents, crossing over occurs
2. M-I: Spindle fibres from opposing centrosomes connect to bivalents (at centromeres) and align them along the middle of the cell
3. A-I: Spindle fibres contract and split the bivalent, homologous chromosomes move to opposite poles of the cell
4. T-I: Chromosomes decondense, nuclear membrane may reform, cell divides (cytokinesis) to form two haploid daughter cells

Meiosis II

The second division separates sister chromatids (these chromatids may not be identical due to crossing over in prophase I)

1. P-II: Chromosomes condense, nuclear membrane dissolves, centrosomes move to opposite poles (perpendicular to before)

2. M-II: Spindle fibres from opposing centrosomes attach to chromosomes (at centromere) and align them along the cell equator
3. A-II: Spindle fibres contract and separate the sister chromatids, chromatids (now called chromosomes) move to opposite poles
4. T-II: Chromosomes decondense, nuclear membrane reforms, cells divide (cytokinesis) to form four haploid daughter cells



Stages of meiosis

Materials required:

Prepared slides, microscope.

Procedure:

Student will be made to focus the available prepared slides in microscope.

Results:

Student will made to identify the cells at different stages of mitosis and meiosis according to the descriptions given in the start of experiment.

References and further reading:

https://ib.bioninja.com.au/standard-level/topic-3-genetics/33-meiosis/stages-of-meiosis.html

file:///C:/Users/zaeem/Downloads/WhataretheStagesofMitosis_ProphaseMetaphaseAnaphaseTelophase.pdf

https://www2.le.ac.uk/projects/vgec/highereducation/topics/cellcycle-mitosis-meiosis
https://www.britannica.com/science/mitosis