AVIT
AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY

VINAYAKA MISSION'S RESEARCH FOUNDATION
(Deemed to be University under section 3 of the UGC Act 1956)

Accredited by NAAC

Approved by AICTE

DEPARTMENT OF BIOTECHNOLOGY

NAME OF THE LAB

17BTCC82- CELL BIOLOGY LAB

HOD

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DEPARTMENT O BIOTECHNOLOGY

17BTCC82-CELL BIOLOGY LAB

LIST OF EXPERIMENT

1. Introduction to principles of sterilization techniques and cell propagation.
2. Principles of Microscopy.
3. Isolation of Cell organelle – Mitochondria, Microtubules, Actin and Myosin filaments.
4. Cell Fractionation – Separation of peripheral blood mononuclear cells from blood.
5. Cell staining - Gram's staining, Leishman staining
6. Cell counting - Tryphan blue assay, Alamar blue assay.
7. Osmosis and Tonicity.
8. Staining for different stages of mitosis in *Allium cepa* (Onion).

HOD

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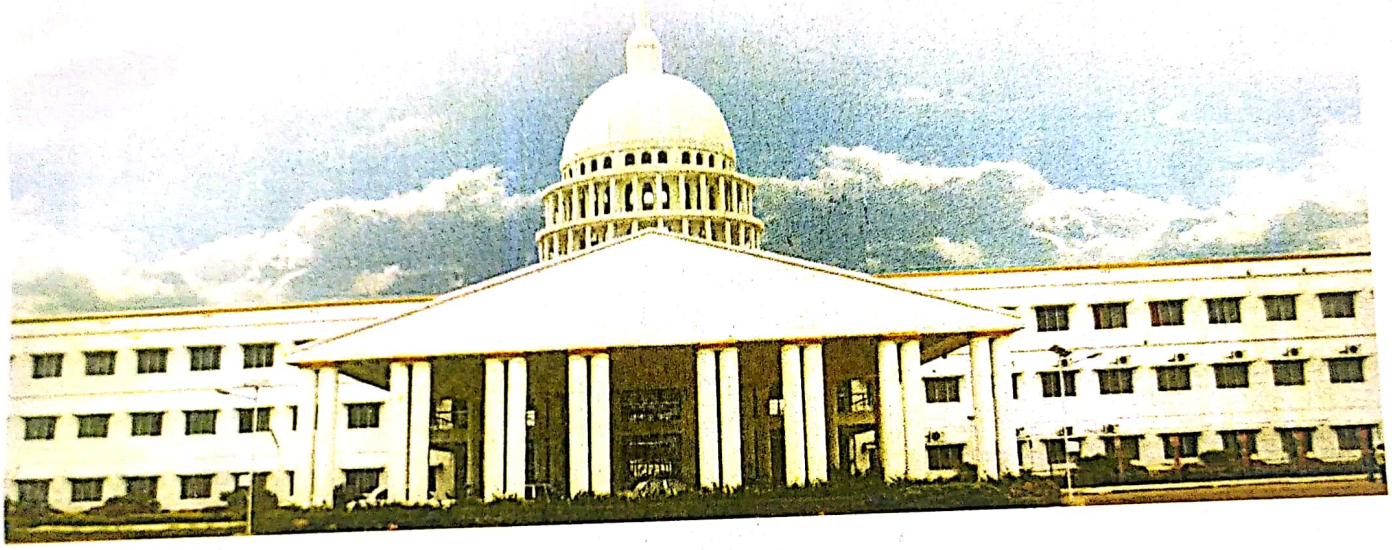

B.Tech - Biotechnology

CELL BIOLOGY LABORATORY MANUAL

17BTCC02

DEPARTMENT OF BIOTECHNOLOGY

AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY
VINAYAKA MISSION'S RESEARCH FOUNDATION
(Deemed to be University)
Paiyanoor, Chennai

TABLE OF CONTENT

LABORATORY RULES AND REGULATIONS

Upon entering the laboratory, place the bag, books and other materials in the specified locations.

. Always wear lab coat before entering the laboratory for protecting clothes and your body from burning, contamination or accidental discolouration by staining solution.

i. Familiarize yourself with the exercise to be performed.

. Always perform the exercise in sequence, and as you perform the exercise, record the data in the observation note book.

. Wash your hands with soap and water upon entering and prior to leaving the laboratory.

i. Before and after each laboratory session wipe your work bench with disinfectant like lysol or ethanol.

ii. Label all plates, tubes, cultures and solutions properly before starting the exercise.

iii. All microbial cultures should be handled as potential pathogens.

. Never pipette any broth or cultures or any chemical reagents by mouth.

. Always keep the test tubes containing cultures in an upright position in the rack. When moving around the laboratory, carry the test tubes containing the cultures in a rack.

i. If a live culture is spilled over the area, leave the area with disinfectant solution for 15minutes and then clean it.

ii. In case of accidental splash of hazardous chemical, immediately rinse the area thoroughly with water and inform the instructor.

iii. Materials such as stains, reagents, bottles, test tubes, Petri plates, pipettes etc must be returned to the original location after use.

EXPT. NO. :

DATE :

STERILIZATION TECHNIQUES

Sterilization techniques include all the means used to completely eliminate or destroy living microorganisms on any object, including tools used to test or treat patients. The objective of sterilization is to remove the contaminating or the infecting microorganisms from the materials or from areas. The methods of sterilization employed depend on the purpose for which it is carried out, the material that has to be sterilized and the nature of the microorganisms that are to be removed or destroyed.

Sterilization is defined as the process of killing of all microorganisms in a material or on the surface of an object. **Disinfection** means reducing the number of viable microorganisms present in a sample. Different disinfectants have different mechanisms of action. The various agents used in sterilization can be classified as physical or chemical agents.

SPECIFIC PHYSICAL ANTIMICROBIALS

Specific physical antimicrobial agents include Heat, Ultraviolet radiation, Ionizing radiation, strong visible light, Ultrasonic waves, Filtration and Osmotic pressure.

(i) Heat

Heat is a highly efficient means of sterilization so long as the material to be sterilized is resistant to heat. Different types of heat application include dry heat, moist heat etc. Factors that affect sterilization by heat are nature of heat, temperature and time, number of microorganism, characteristics like spore formation etc.

(a) Dry Heat

To effect sterilization, dry heat typically requires higher temperature than moist heat. It is less penetrating and requires longer exposure. Nevertheless, application of dry heat is cheap and easy. Typically one bakes materials in an oven at 171°C for at least one hour or 160°C for at least two hours or 121°C for at least 16 hours. The length of exposure necessary is inversely proportional to the

temperature (lower temperatures require longer exposures). Dry heat leads to protein denaturation and oxidative damage.

(b) Moist Heat

Moist heat is more effective than dry heat at a given temperature or length of exposure. Moist heat is also more penetrating than dry heat. The advantage of steam lies in the latent heat liberated when it condenses on a cooler surface, raising the temperature of that surface. In the case of spore, steam condenses on it, increasing its water content, resulting in hydrolysis and breakdown of the bacterial protein. However, to achieve sterilization employing moist heat require rather elaborate equipment, i.e., the autoclave. An autoclave is a high pressure device used to allow the application of moist heat above the normal-atmosphere boiling point of water. Exposure to 121°C for 15+ minutes is typically sufficient to sterilize.

ii) Ultraviolet (UV) Radiation – Non-ionizing Radiation

UV light is not terribly penetrating but is good for disinfecting surfaces and air. This is accomplished by a long-term exposure to UV light

(ii) Ionizing Radiation

Ionizing radiations includes X-rays, Gamma rays and Cosmic rays. Different types of ionizing radiation display different degrees of penetration. Mainly it causes DNA damage.

(iii) Sun Light

Sunlight has bactericidal activity due to the combined effect of ultraviolet and heat rays.

(v) Filtration

Filtration is a common means of antimicrobial treatment used when materials are heat labile such as sera and solution of sugars or antibiotics used for preparations. Filters used are candle filters, asbestos filters, sintered glass filters and membrane filters.

SPECIFIC CHEMICAL ANTIMICROBIALS

Specific chemical antimicrobial agents include Surfactants, various Organic acids and bases, Heavy metals, Halogen-containing compounds, Alcohols, Phenol and phenol derivatives, Oxidizing agents, Alkylating agents, Certain dyes and other agents.

Mechanism of action of chemical agents include :

- Protein denaturation
- Membrane disruption
- Nucleic acid damage
- Inhibition of metabolism

(i) Surfactants

Surfactants are substances that are soluble in water but are able to dissolve lipids. They increase the solubility of lipids in water solution. Surfactants additionally increase the ability of water solutions to wet, i.e., move along or penetrate lipid surfaces. Examples of surfactants are soaps and detergents. Soaps are sodium or potassium salts of fatty acids. Consequently soaps are alkaline (pH greater than 7). Soaps exert their antimicrobial effects by harming bacteria that are sensitive to high pH or by removing pathogens from surfaces by cleaning the surface. Detergents are synthetic surfactants. A detergent may be cationic (positively charged) or anionic (negatively charged). Cationic detergents are better at killing bacteria than anionic detergents

(ii) Organic Acids

Various organic acids are employed especially as inhibitors of fungi and molds in foods. For example, benzoate of soda is a sodium salt of benzoic acid, an organic acid.

(iii) Heavy Metals

Various metals and metal salts are commonly employed to prevent microbial growth or kill microbes. E.g. Silver nitrate

(iv) Halogens

Two halogens are regularly employed as antimicrobials – Iodine and Chloride. Drinking water is commonly disinfected using hypochlorite. Bromine sometimes is employed as a substitute for chlorine. Iodine is often employed as a tincture or as an iodophor. Iodine tinctures may be employed as antiseptics. Iodine is an active bactericidal with moderate action against spores.

(v) Alcohols

It is most frequently used. Alcohols work best as 70 to 99% mixtures with water. Alcohol-water mixtures are additionally more penetrating than pure alcohols. Either ethanol or isopropyl alcohol may be employed for disinfecting.

(vi) Phenol and Phenol Derivatives (Phenolics)

Phenol and their derivatives (called phenolics) are especially useful for disinfecting materials contaminated with organic materials. Lysol employs phenolics. Some phenolics are mild enough for use as antiseptics while others are too harsh or otherwise dangerous to be employed on living tissue.

(vii) Oxidizing Agents (Hydrogen Peroxide)

Hydrogen peroxide (HOOH) is a typical oxidizing agent. The oxygen released upon inactivation can help oxygenate deep wounds and thus kill strict-anaerobic contaminants.

(viii) Alkylating Agents

Formaldehyde, glutaraldehyde and ethylene oxides are alkylating agents. They add carbon-containing functional groups to biological molecules. Formaldehyde is employed to inactivate viruses and toxins to produce whole-killed vaccines and toxoid vaccines. Glutaraldehyde is capable of sterilizing equipments, though to effect sterilization. It often requires many hours of exposure. Ethylene oxide is a gaseous chemosterilizer that is especially useful due to its extraordinary penetrating power.

EXPT . NO :

DATE :

INTRODUCTION TO MICROSCOPY

Microscope is an optical instrument that uses a lens or a combination of lenses to produce magnified images of small objects, especially of objects too small to be seen by the unaided eye. The science of investigating small objects using such an instrument is called microscopy.

The microscope has been the most valuable contribution to the field of medicine. The simplest form consists of a single glass lens mounted in a metal frame – a magnifying glass. All modern microscopes are made up of more than one glass lens in combination, hence referred as a compound microscope. The first compound microscope was constructed in Holland by Hans and Zacharias Janssen over 400 years ago (1590).

The microscopes are broadly classified into two different types of microscopes – the light microscope and the electron microscope. Light microscope use a series of glass lenses to focus light in order to form an image whereas electron microscope use electromagnetic lenses to focus a beam of electrons.

Major Components of a Microscope –

Base – Metal or plastic part on which microscope rests

Arm – Somewhat C shaped pillar arising from base that support the stage and ocular components

Stage – Flat platform attached to lower portion of arm on which slide or sample is placed. The stage enable the specimen to be moved and positioned in fine and smooth increments both horizontally and vertically in the X and Y directions. It has an opening in the center that allows the passage of light from an illuminating source below to the lens system above the stage.

Condenser – Contains two sets of lens system that collects illuminator light rays and focuses as it passes upward from the source into the lens system.

Substage Condenser – Originally designed by Ernst Abbe in the late 1800s and is usually referred to as the Abbe condenser. The substage condenser diaphragm is used to control the solid angle of the light emerging from the condenser, illuminating the specimen and filling the objective.

Condenser or Iris Diaphragm Lever – Lever beneath opening in the stage consists of a shutter-like group of metal leaves which regulate the amount of light coming through the slide on the stage.

Condenser Adjustment Knob – Raises and lowers the condenser.

Turret – Revolving plate which bears the objective. It is attached at the lower end of tube and can be turned to change the objective lens.

Coarse Focus – Larger knob which moves the tube up or down rapidly to get the sample into coarse focus.

Fine Focus – Smaller knob which moves the tube through short distances slowly and is used to get the sample into sharp focus.

Transformer or Illuminator – Controls the amount of light transferred to the sample.

The Microscope Lamp – Positioned below the condenser lens in the upright microscope. The lamps can be a low-voltage tungsten filament lamp or quartz-halogen bulb.

Eyepiece (Ocular Lens) – Removable short metal tube that contains lens that fit into the top of the tube - generally 10 -15x magnification. The eyepiece relays to the eye, an image projected by the objective into the plane of the eyepiece diaphragm further magnifying it in the process. More advanced microscopes have eye piece for both eyes and are called binocular microscopes.

Objective Lens – Small metal tubes screwed into the turret which increases the magnification of the sample. (Often referred to as just objective). The light that either passes through the specimen (transmitted) or reflected back from the specimen (reflected) is focused by the objective lens into the eyepiece lens. Available in different varieties – 4x, 10x, 20x, 40x, 60x, 100x.

Mirror – Used to reflect light through a sample

Principle of Microscopy – When a ray of light passes from one medium to another, refraction occurs i.e. the ray is bent at the interface. The refractive index is a measure of how greatly a substance slows the velocity of light. The direction and magnitude of bending is determined by the refractive indexes of the two media forming the interface. When light passes from air into glass, a medium with a greater refractive index, it is slowed and bent towards the normal, a line perpendicular to the surface. As light leaves glass and returns to air, a medium with a lower refractive index, it accelerates and is bent away from the normal. Thus a prism bends light.

Lenses act like a collection of prism operating as a unit. When the light source is distant so that parallel rays of light strike the lens, a convex lens will focus these rays at a specific point, the focal point (F). The distance between the center of the lens and the focal point is called the focal length. Our eyes cannot focus on objects nearer than about 25cm or 10 inches. This limitation may be overcome by using a convex as a simple magnifier and holding it close to an object. A magnifying glass provides a clear image at much closer range and the object appears larger.

Magnification – A measure of the ability of the microscope to enlarge an image is called magnification. It is the ratio of the size of retinal image seen with optical microscope to the size of the retinal image seen with unaided eye. The total magnification is equal to the product of the magnification of the objective and the magnification of the eyepiece.

RESOLUTION OR RESOLVING POWER

It is the ability of a lens to show two closely situated points as two separate points. The practical resolving power of the microscope is limited by that of the human eye. From a distance of 25 cm, which is approximately the distance of the optical tube in a microscope, the human eye can distinguish two small objects that are 0.1mM apart. Shorter the wavelength of illuminating light, the higher is the resolving power of the microscope. Greatest resolution is obtained with light of the shortest wavelength (blue). The limit of resolution for a microscope that uses visible light is about 300nm with a dry lens (in air) and 200nm with an oil immersion lens. By using UV as light source the resolution can be improved to 100nm because of the shorter wavelength of light (200 - 300nm).

Aberration – To form a clear image, a lens must focus each ray of light from a point in a specimen into a point in the image. The failure to do so is called as an **aberration.** **Chromatic aberration** occurs because the focal length of a simple lens varies noticeably with wavelength. Blue rays are shorter in wavelength and focus closer to the lens than green or red rays. The single lens is unable to bring all colours to a common focus, resulting in a slightly different sized image for each wavelength at slightly different focal points. **Achromatism** can be achieved through the combination of two or three lenses of different optical properties cemented together to form a doublet or triplet, respectively. **Spherical aberration** occurs when light rays passing through the central and outer portion of a lens are not brought to focus at the same distance from the lens. This condition arises because light is refracted more at the edge of the lens. Spherical aberration causes fuzzy images by focusing some rays of light closer to the lens and some rays further from the lens.

Ernst Abbe derived an equation for calculating approximate resolving power. Thus the quality of lenses could be compared numerically. Abbe's equation may be stated as :

Resolving power = Wavelength of light used 1 / 2 (numerical aperture of objective) (minimum distance).

The variable other than wavelength of light that determines resolving power is called **numerical aperture** (N.A.). **Numerical aperture:** $n \sin \theta$ in the equation above is called the numerical aperture (NA) of the lens and is a function of its light-collecting ability.

Light that strikes the microorganism after passing through a condenser is cone shaped. When this cone has a narrow angle and tapers to a sharp point it does not spread out much after leaving the slide and therefore does not adequately separate images of closely packed objects. The resolution is low. If the cone of light has a very wide angle and spread out rapidly after passing through a specimen, closely packed objects appear widely separated and are resolved. The angle of the cone of light that can enter a lens depends on the refractive index of the medium in which the lens works as well as upon the objective itself. The refractive index for air is 1.00. Since Sine θ cannot be greater than 1, no lens working in air can have a numerical aperture greater than 1.00. The only practical way to raise the N.A. above 1.00 and therefore achieve higher resolution is to increase the

refractive index with immersion oil, a colourless liquid with the same refractive index as glass. If air is replaced with immersion oil, many light rays that did not enter the objective lens and slide will do so resulting in an increased numerical aperture and better resolution.

Staining – Cell staining is a technique is used to visualize cells and cell components under a microscope. By using different stains, one can preferentially stain certain cell components, such as a nucleus or a cell wall, or the entire cell. Most stains can be used on fixed or non-living cells, while only some can be used on living cells ; some stains can be used on either living or non-living cells.

Staining involves the following steps

(i) **Permeabilization** -treatment of cells generally with a mild surfactant that dissolves cell membranes in order to allow larger dye molecules to enter inside the cell.

(ii) **Fixation** – serves to "fix" or preserve cell or tissue morphology through out the preparation process. Common fixatives include formaldehyde, ethanol, methanol and or picric acid.

(iii) **Mounting** – involves attaching samples to a glass microscope slide for observation and analysis.

(iv) **Staining** – application of stain to a sample to colour cells, tissues, components, or metabolic processes.

Some of the common stains are carmine, coomassie blue, crystal violet, eosin, ethidium bromide, malachite green, methylene blue and safranin.

Different Types of Microscopy

(i) **Bright Field Microscopy** (Fig. 1) – This system contains 2 lens systems for magnifying specimens - the ocular lens in the eye piece and the objective lens located in the nose piece. The specimen is illuminated by a beam of tungsten light focused on it by a substage lens called condenser. The specimen appears dark against a bright background.

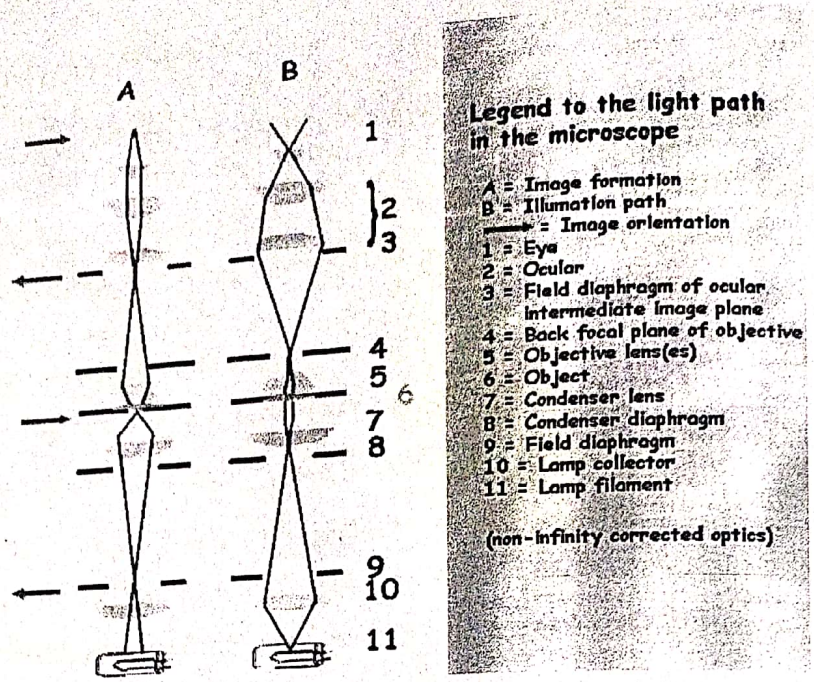

Fig. 1: Bright field microcopy

ii) **Dark Field Microscopy** (Fig. 2) – Dark field microscopy is an optical microscopy illumination technique used to enhance the contrast in unstained samplcs. Thc condcnscr is modificd by placing a dark ficld stop in the centre of the substage condenser. This blocks the direct light and does not permit it to reach the objective but allows a hollow cone of light to illuminate the specimen. Thus while no direct light enters the objective, the specimen is seen by scattering of reflected light against a dark background.

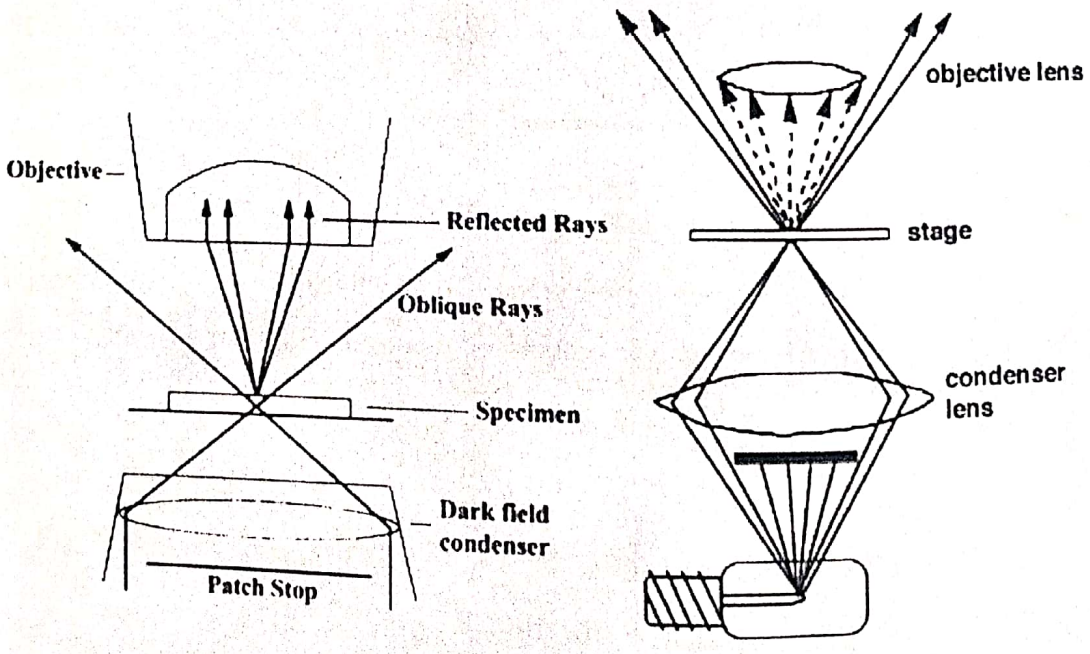

Fig 2: Dark field microscopy

Dark field microscopy is a very simple yet effective technique and well suited for uses involving live and unstained biological samples such as a smear from a tissue culture or individual water borne single-celled organisms. The main limitation of dark field microscopy is the low light levels seen in the final image. This means the sample must be very strongly illuminated and can cause damage to the sample.

(iii) **Phase-contrast Microscopy** (Fig. 3)/– Phase contrast microscopy is an optical microscopy illumination technique in which small phase shifts in the light passing through a transparent specimen are converted into amplitude or contrast changes in the image. The technique was invented by Frits Zernike in the 1930s for which he received the Nobel prize in physics in 1953. Phase contrast microscopy is achieved by mounting an annular aperature diaphragm below the substage condenser so that the specimen is illuminated with a ring of light. It avoids hitting of bright light rays on the object. A phase plate or phase ring is placed in the rear focal plane of the objective lens. It blocks the unretarded rays entering into the objective lens. So the primary image formed by the objective lens has only retarded light rays.

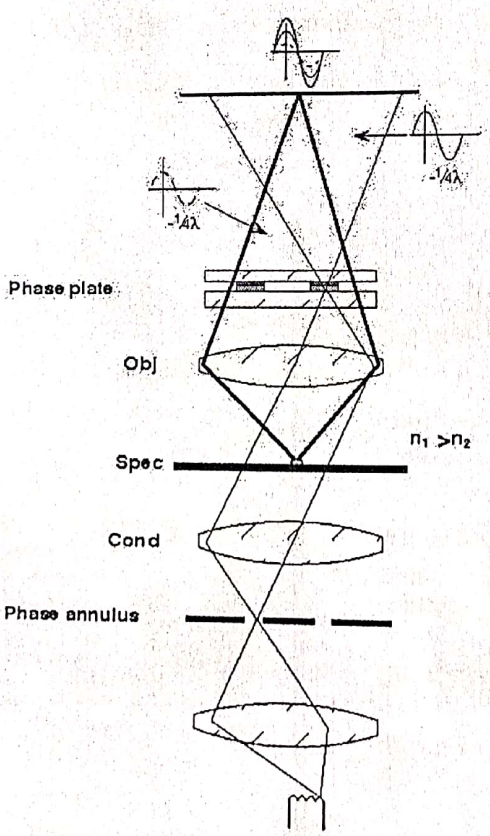

Fig 3: Phase Contrast Microscope : The Optical Path for Phase Contrast Illumination.

Since the specimen and the surrounding medium have different indices of refraction, light waves passing through the specimen and entering the objective lens are somewhat refracted and out of phase compared with those coming from the medium. These differences in refractive indexes are converted into variations in light intensity based on the interference of light wave passing through the specimen and the surrounding medium. Phase contrast microscopy is used to examine the living cells in their natural state without being killed, fixed or stained.

(iv) **Fluorescence Microscope** (Fig. 4) - It is a light microscope used to study properties of organic or inorganic substances using the phenomena of fluorescence and phosphorescence. The fluorescent dye absorbs at wavelengths between 230nm and 350nm and emits orange, yellow or greenish light. A component of interest in the specimen is specifically labelled with a fluorescent molecule called a fluorophore such as green fluorescent protein (GFP), fluorescein or DyLight 488. The specimen is illuminated with light of a specific wavelength (or wavelengths) that is absorbed by the fluorophores, causing them to emit longer wavelengths of light (of a different colour than the absorbed light). The illuminated light is separated from the much weaker emitted fluorescence through the use of an emission filter.

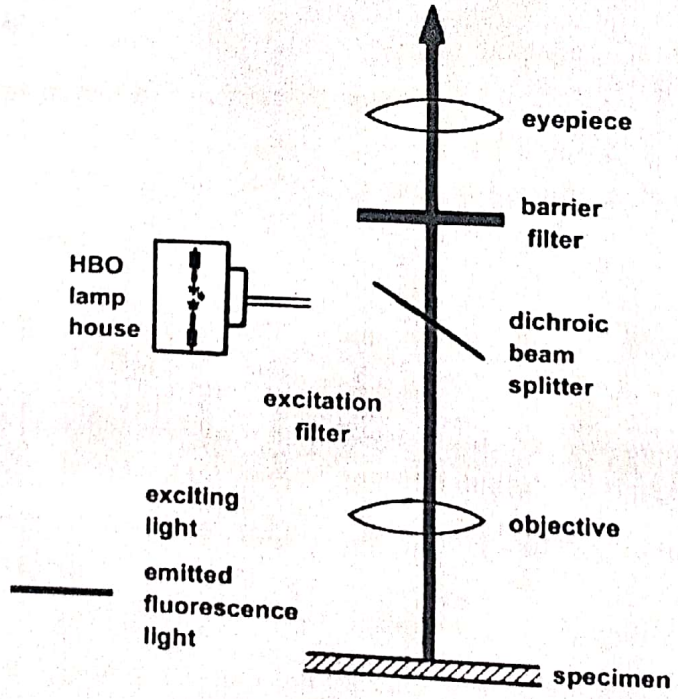

Fig. 4: Fluorescence microscopy

Typical components of a fluorescence microscope are the light source (xenon arc lamp or mercury-vapour lamp), the excitation filter, the dichroic mirror (or dichromatic beamsplitter), and the emission filter. The filters and the dichroic mirror are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen. In Epifluorescence microscopy the excitatory light is passed from above through the objective onto the specimen instead through the specimen into the objective. Since only reflected excitatory light filters through and the transmitted light is filtered out, a much higher intensity is obtained. Fluorescence microscope is primarily used for the detection of antigen-antibody reactions.

USE AND CARE OF MICROSCOPE

Perform the following functions before using a Microscope

(i) When you pick up the microscope and walk with it, grab the arm with one hand and place your other hand on the bottom of the base.

(ii) Revolve the coarse adjustment knob about a half turn to see if it works freely. Repeat the above step with the fine adjustment knob. Revolve the nosepiece around carefully to see if it works properly. Check the mechanical stage to see if it works easily. Open and close the iris diaphragm.

(iii) Clean the lens surfaces with "optically safe" lens paper. Do not use "kim- wipes", Kleenex type tissues or paper toweling.

(iv) Never turn or tilt the microscope towards or upside down position. Keep the microscope level.

(v) Do not attempt to disassemble the ocular or any other part of the microscope unless so authorized.

(vi) If you remove the ocular (eyepiece) from the instrument, be sure to cover the open end of the barrel with a dust cap or other appropriate cover (such as a piece of lens paper).

(vii) If the lamp surface is touched, clean the touched surface with alcohol. Fingerprints, if left on glass will etch the surface, reduce brightness, damage the lamp, and shorten its service life.

(viii) Keep the condenser at its highest point. The iris diaphragm should be almost wide open. Being fully opened will cause over-brightness of the field. Remember that as you close the diaphragm the numerical aperature of the diaphragm will decrease.

(ix) Check the bottle of immersion oil and observe it for clarity. This oil should always be clear (never cloudy).

(x) Avoid over-crowded conditions when using a microscope.

(xi) Keep unneeded materials off the desk.

(xii) If the microscope has a long electrical cord, keep the cord on the table. Coil the cord into a compacted oval and secure with a tie. Never allow a cord to dangle over the side of a work area. The cord could "smag" a foot, cart, or something and causes the microscope to fall over.

(xiii) Never work with or leave the microscope close to the edge of the table.

(xiv) Never carry more than one microscope at a time.

(xv) When using solvents on the lenses, use only those provided by the lab instructor. Do not allow the solvent to remain on the lens surface, but quickly remove with a lens tissue.

(xvi) Clean the slides, materials and the work area when you are done. Please, be careful with the slides and cover slips. They are made of glass and if broken, may result in cuts and you will bleed.

(xvii) **Use the following check list when you have finished with your lab assignments and are preparing to return the microscope to the cabinet.**

- Remove the slide from the stage.
- If oil has been used, wipe it off from the lens and stage with lens paper.
- Rotate the low power objective (10X) into the down position.
- Adjust the mechanical stage so that it does not project too far on either side of the platform.
- Replace the dust cover on the instrument.

ISOLATION OF MITOCHONDRIA FROM POTATO TUBERS

AIM:

To isolate mitochondria from potato tubers.

PRINCIPLE:

To study the function of plant mitochondria is to extract them from plant tissues in an uncontaminated, intact and functional form. The reductionist assumption is that the components present in such a preparation and the *in vitro* measurable functions or activities reliably reflect the *in vivo* properties of the organelle inside the plant cell. Here, we describe a method to isolate mitochondria from a relatively homogeneous plant tissue, the dormant potato tuber. The homogenization is done using a juice extractor, which is a relatively gentle homogenization procedure where the mitochondria are only exposed to strong shearing forces once. After removal of starch and large tissue pieces by filtration, differential centrifugation is used to remove residual starch as well as larger organelles. The crude mitochondria are then first purified by using a step Percoll gradient. The mitochondrial band from the step gradient is further purified by using a continuous Percoll gradient. The gradients remove contaminating amyloplasts and peroxisomes as well as ruptured mitochondria. The result is a highly purified, intact and functional mitochondrial preparation, which can be frozen and stored in liquid nitrogen in the presence of 5% (v/v) dimethylsulfoxide to preserve integrity and functionality for months.

REAGENTS:

1. Mannitol
2. 3-morpholinopropane-1-sulfonic acid (MOPS)
3. Potassium hydroxide (KOH)
4. Bovine serum albumin (BSA)
5. Ethylenediaminetetraacetic acid (EDTA)
6. Cysteine
7. Percoll

8. Sucrose
9. Dimethylsulfoxide (DMSO)
10. Liquid nitrogen
11. Extraction medium (see Reagent preparation)
12. Wash buffer (see Reagent preparation))
13. Gradient buffers (see Reagent preparation))
14. Percoll gradients (see Reagent preparation))

PROCEDURE:

a. *All steps should be performed at 0-4 °C using detergent-free glass-ware or plastic!*

b. *We will here describe the isolation of mitochondria from 1 kg of peeled tubers of the cultivar Folva. This will give a final yield of 8-12 mg mitochondrial proteins. Using other cultivars can give different yields. Total time from start of the homogenization (point 3) to freezing about 6 h.*

1. Pre-cool centrifuge rotor.

2. Add cysteine and BSA to extraction buffer, adjust to pH 7.3. Cysteine is an antioxidant and BSA binds fatty acids and phenolics, which can interfere with mitochondrial function, BSA also works as a protease substrate to help protect the mitochondrial proteins from damage.

3. Peel potatoes and homogenize the peeled potato using a juice extractor (1 kg gives approximately 500 ml juice) and let the juice run directly into about 1/2 volume extraction medium (250 ml here). Adjust to pH 7.2 using 2 M KOH immediately afterwards or, for larger preparations, after each kg homogenized.

4. Leave homogenate (total volume about 750 ml) standing for 5 min allowing starch to sediment.

5. Filter through two layers of cotton (or similar) using a funnel and transfer to centrifuge tubes.

6. Transfer ~190 ml filtrate to each of four 250 ml centrifuge tubes. Balance tubes, and centrifuge at 3,000 *x g* for 5 min in a 6 x 250 ml precooled angle rotor.

7. Pour supernatants carefully into fresh centrifuge tubes (avoid transferring the pellets), balance and centrifuge at 18,000 *x g* for 10 min.

8. Discard the supernatants gently without disturbing the pellets and resuspend each pellet in 1 ml of 1x mannitol gradient buffer using a paint brush. Total volume of resuspended pellets 8-10 ml.

9. Prepare two Percoll step gradients using plastic Pasteur pipettes. Avoid mixing the bands by gently layering first the 50%, then the 28% and finally the 20% Percoll (all in mannitol) on top of each other in 50 ml centrifuge tubes.

10. Gently layer the crude fraction (4-5 ml maximum per gradient) on top of the two Percoll step gradients

11. Balance tubes and centrifuge at 40,000 *x g* for 30 min using an 8 x 50 ml pre-cooled angle rotor .

12. Transfer the mitochondrial band from each tube using a Pasteur pipette to new 50 ml tubes, fill up to 40 ml with wash buffer and mix.

13. Balance the two tubes against each other and centrifuge at 18,000 *x g* for 10 min.

14. Carefully remove supernatant, resuspend the very loose pellets with wash buffer, fill up to 40 ml with wash buffer and mix.

15. Balance and centrifuge at 18,000 *x g* for 10 min .

16. Remove supernatant and resuspend the very loose pellets using a paint brush in 1 ml 1 x mannitol buffer and gently layer on top of two 28% Percoll sucrose gradients.

17. Balance and centrifuge at 40,000 *x g* for 30 min using the 8 x 50 ml rotor .

18. Transfer the mitochondrial bands to two fresh 50 ml centrifuge tubes, fill up to 40 ml with wash buffer and mix.

19. Centrifuge at 18,000 *x g* for 10 min.

20. Carefully remove supernatant and repeat wash (as steps 13-14) of pellets .

21. Remove supernatant and resuspend each pellet in 500 µl wash buffer. Add 5% (v/v) DMSO for freezing of the intact organelles. The final volume from each pellet is around 800 µl containing 4-6 mg (total 8-12 mg) mitochondrial protein.

22. Snap freeze and store aliquots of 100-200 µl in liquid nitrogen. In this way, the mitochondria maintain their intactness and respiratory function (see below) for months, if quickly thawed shortly before use.

PROPERTIES OF THE ISOLATED MITOCHONDRIA

1. The mitochondria isolated from potato tubers using this protocol have highly intact outer membranes as judged by >97% latency of cytochrome C oxidase and are highly purified as judged by the high specific activity of cytochrome C oxidase (3.5 µmol mg^{-1} min^{-1}). The respiration rates in state 3 (presence of ADP) using single substrates (NADH, succinate and malate) are 150-350 nmol O_2 mg^{-1} min^{-1}, the respiratory control ratio of 2.5-3.5 and the

ADP/O ratio 1.7-2.0 all depending on the substrate, the preparation and the cultivar. This is fully consistent with previous studies (Neuburger *et al.*, 1982; Rasmusson and Moller, 1990; Struglics *et al.*, 1993).

2. The mitochondria were judged to be >95% pure by western blotting (Salvato *et al.*, 2014), which is consistent with measurements of marker enzymes and marker compounds in previous studies (Neuburger *et al.*, 1982; Struglics *et al.*, 1993).

3. Using one-dimensional polyacrylamide gel electrophoresis to separate the mitochondrial proteins according to size followed by tryptic digestion, separation of the tryptic peptides by liquid chromatography and identification by mass spectrometry a total of 1,060 different proteins were identified including about 500 proteins not previously identified in plant mitochondria (Salvato *et al.*, 2014).

4. Similar methods can be used to isolate mitochondria from other tissues and species. However, the homogenization methods will probably have to be changed as does the Percoll concentration used to collect the mitochondria and purify them further (28% here). See Meyer and Millar (2008) for a method for isolating mitochondria from Arabidopsis cell cultures. Isolating mitochondria from green leaves is more difficult because of the large amount of chloroplasts and thylakoid membranes released from the tissue, and Arabidopsis leaves are particularly difficult (Keech *et al.*, 2005).

PREPARATION OF REAGENT

EXTRACTION MEDIUM [600 (300) ml, pH 7.3]

Chemical	Concentration	g/L
Mannitol	0.9 M	163.95
MOPS	30 mM	6.28
EDTA	3 mM	0.87
L-Cysteine	25 mM	3.03
BSA	0.3 % (w/v)	3.00

Dissolve mannitol, MOPS and EDTA in double-distilled water (ddH_2O)

Adjust pH to 7.2 and transfer 300 ml to new container to use as wash buffer

On day of use add cysteine and BSA to the remaining 300 ml, adjust to pH 7.3 with KOH.

WASH BUFFER (900 ml, pH 7.2)

Chemical	Concentration	g/L
Mannitol	0.3 M	54.65
MOPS	10 mM	2.07
EDTA	1 mM	0.29

Use extraction medium (no cysteine and BSA) and dilute 3-fold by adding ddH_2O
Include 0.1% BSA if the mitochondria are to be used in a non-proteomics context.

GRADIENT BUFFERS

a. **2x mannitol buffer** (150 ml, pH 7.2)

Chemical	Concentration	g/L
Mannitol	0.6 M	109.3
MOPS	20 mM	4.14
BSA	0.2 % (w/v)	2.0

Use 35 ml for each of the two step gradients and dilute the remaining 115 ml 2-fold to use for resuspending pellets.

b.2x sucrose buffer (100 ml, pH 7.2)

Chemical	Concentration	g/L
Sucrose	0.6 M	205.4
MOPS	20 mM	4.14
BSA	0.2 % (w/v)	2.0

Percoll gradients
Total volume 35 ml without samples

a. Step gradient

ercoll, % (v/v)	ddH_2O, %	2x mannitol buffer, %	Volume per gradient, ml
20	30	50	17.5
28	22	50	11.67
50	-	50	5.83

b. Continuous gradient

Percoll, % (v/v)	ddH_2O, %	2x mannitol buffer, %	Volume per gradient, ml
28	22	50	35

Very carefully add the three layers of the step gradient one by one (highest first) holding the 50 ml tube at a 45 degree angle to avoid mixing.

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SEPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM BLOOD

AIM:

To separate the peripheral blood Mononuclear cells from freshly collected blood. Immune responses can be evaluated using human peripheral blood mononuclear cells (PBMCs).

PRINCIPLE:

Density gradient centrifugation is the most common method for isolating peripheral blood mononuclear cells (PBMCs), namely lymphocytes and monocytes. For this isolation procedure, PBMCs are centrifuged in the presence of density gradient media, such as Ficoll or Ficoll-Paque medium. Each cell population exhibits a unique migration pattern through the medium (related to the density of the cell), creating distinct layers that each contains specific cell populations. The cells can then be isolated by extracting the respective layer.

MATERIALS AND REAGENTS

1. Freshly collected heparinised blood
2. Ficoll Histopaque
3. Sterile PBS or Dulbecco's modified eagle medium
4. Pencillin-streptomycin solution
5. Heparin vials
6. Dulbecco's modified eagle medium supplemented with 1% of Pencillin-streptomycin solution
7. Centrifuge machine with swing-out bucket rotor
8. Sterile 15 ml centrifuge tube.
9. Auto pipettes and 1 ml tips

PROCEDURE

1. Collect 4 ml of human venous blood sample in heparinised vials (BD biosciences) and mix well by gently inverting the tube several times.

2. Take 4 ml of Ficoll Histopaque in a 15 ml centrifuge tube.

3. Gently layer the blood on the top of Ficoll Histopaque using a 1 ml auto pipette. The layering should be done very slowly that blood and Ficoll Histopaque should stay as two different layers.

4. Centrifuge the tubes (without any delay) for 30 min at 100 *x g* in 4 °C in a swing-out bucket. Fixed angle rotors also can be used but would require more caution when separating cells in interphase.

5. Aspirate the whitish buffy coat (about 1 ml) (PBMCs) formed in the interphase between histopaque and medium.

6. The cells in interphase need to be aspirated without delay. If the tubes are kept standing for more than 10 min, PBMCs from the interphase will get disturbed and start settling down.

7. Wash (centrifuge in 100 *x g* for 10 min) twice with 10 ml of sterile PBS or sterile Dulbecco's modified eagle medium. The approximate yield of cells from 4 ml of blood varies between 10^7-10^8.

NOTE

1. Use freshly collected heparinised blood. If plasma needs to be used for any other purpose, then remove the plasma and add equal volume of sterile Dulbecco's modified eagle medium before proceeding further.

2. The ratio between Ficoll Histopaque and blood should be 1:1 for human blood. The ratio could be different for purification of PBMC of other species.

3. Ficoll Histopaque is stored at 4 °C. Before use the tube needs to be kept at room temperature for 1-2 h since PBMCs will get cold shock or sometimes aggregate if layered in pre-chilled Ficoll Histopaqueon blood sample.

EXPT. NO. :

DATE :

GRAM'S STAINING BY KIT METHOD

AIM

To identify the given bacterial cell by performing Gram's staining (Kit method).

PRINCIPLE

Gram's staining is a method of differentiating bacterial species into two large groups – Gram-positive and Gram-negative, based on the chemical and physical properties of their cell walls. The method is named after its inventor, the Danish scientist Hans Christian Gram (1853 – 1938) who developed the technique in 1884 to discriminate between *Pneumococci* and *Klebsiella pneumoniae* bacteria. By Gram's staining, the size and shape of both types of cells can be more easily observed under a microscope. At the same time they can be differentiated by the imparted colour. Examples of Gram-negative bacteria are *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae* and *Bordetella pertussis*. Examples of Gram-positive bacteria are *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Actinomyces odontolyticus* and *Clostridium tetani*.

KIT COMPONENTS REQUIRED

(i) SO12 – Gram's Crystal Violet

(ii) SO13 – Gram's Iodine

(iii) SO32 – Gram's Decolourizer

(iv) SO27 – Safarin 0.5% W/V (Gram's Counter Stain)

MATERIALS REQUIRED

(i) Microscope

(ii) Microscope slide

(iii) Cover slips

(iv) Inoculation loop

(v) Bunsen burner

(vi) Wash bottle

(vii) Sample applicator

(viii) Slide staining stand

(ix) Other standard lab ware

PROCEDURE

(i) Prepare a thin smear, air dry and fix by gentle heat.

(ii) Flood the smear with SO 12 reagent (Gram's crystal violet) for 1 minute.

(iii) Wash the smear with water and flood with SO 13 reagent (Gram's Iodine) for 1 minute.

(iv) Wash off the stain and decolourize the stain with SO 32 reagent (Gram's decolouriser) until no further violet colour comes off.

v) Wash the smear with water and counterstain with SO 27 (0.5% Safranin) for about 1 minute.

vi) Wash the excess stain, air dry the slide and observe under the microscope.

PRECAUTIONS

i) Over decolourisation should be avoided as it will result in the loss of crystal violet causing Gram positive organism to appear as gram negative.

ii) Between applications of each reagent the slides must be thoroughly washed with running tap water to remove excess reagents.

OBSERVATION

Pink coloured Bacilli and violet coloured Cocci are observed (Fig 1).

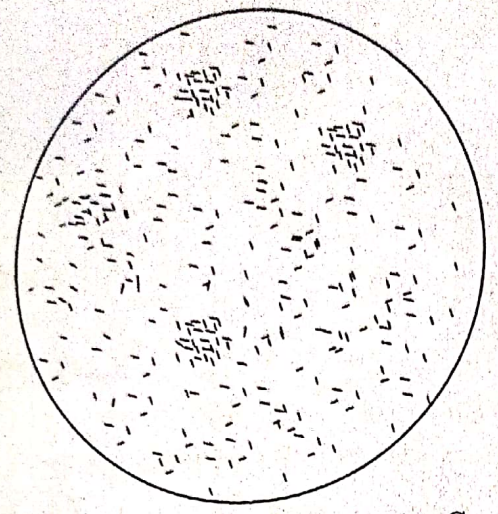

Fig 1 (a) . Gram Positive Cocci

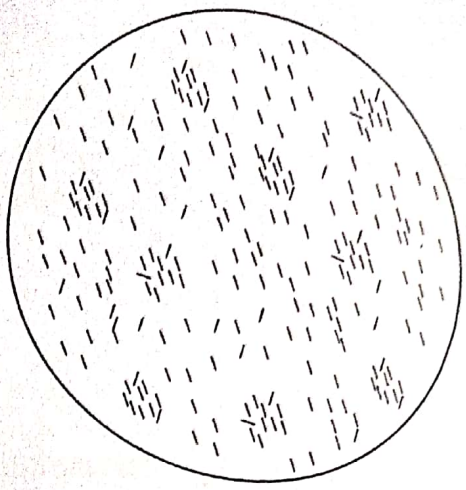

Fig 1 (b). Gram Negative Bacilli

RESULT

The pink coloured Bacilli are Gram negative and the violet coloured Cocci are Gram positive organisms.

EXPT. NO. :

DATE :

GRAM'S STAINING BY MANUAL METHOD

AIM

To identify the given bacterial cell by performing Gram's staining (Manual method).

PRINCIPLE

Gram staining is a method of differentiating bacterial species into two large groups – Gram-positive and Gram-negative, based on the chemical and physical properties of their cell walls. The method is named after its inventor, the Danish scientist Hans Christian Gram (1853 – 1938) who developed the technique in 1884 to discriminate between *Pneumococci* and *Klebsiella pneumoniae* bacteria. There are four basic steps in Gram staining, which include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram's iodine), rapid decolourisation with alcohol or acetone and counter staining with safranin or basic fuchsin.

Crystal violet (CV) dissociates in aqueous solutions into CV^+ and chloride (Cl^-) ions. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50 – 90% of cell wall). Gram-negative bacteria also have an additional outer membrane which contains lipids and is separated from the cell wall by the periplasmic space. The CV^+ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Iodine (I^- or I_3^-) interacts with CV^+ and form large complexes of crystal violet and iodine (CV – I) within the inner and outer layers of the cell.

When a decolourizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the peptidoglycan layer is left exposed. Alcohol increases the porosity of the cell wall by dissolving the lipids in the outer layers. The CV–I complexes are washed from the Gram-

negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large CV–I complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan.

The decolourization step is critical and must be timed correctly. The crystal violet stain will be removed from both Gram-positive and negative cells if the decolourizing agent is left on for too long time (a matter of seconds). After decolourization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple colour. The counterstain which is usually positively – charged safranin or basic fuchsin, is applied last to give decolourized Gram – negative bacteria a pink or red colour. Some bacteria after staining with the Gram stain, yield a *Gram-variable* pattern – a mix of pink and purple cells. The genera *Actinomyces*, *Arthobacter*, *Corynebacterium*, *Mycobacterium* and *Propionibacterium* have cell walls particularly sensitive to breakage during cell division, resulting in Gram – negative staining of these Gram-positive cells.

By Gram's staining, the size and shape of both types of cells can be more easily observed under a microscope. At the same time they can be differentiated by the imparted colour. Examples of Gram-negative bacteria are *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae* and *Bordetella pertussis*. Examples of Gram-positive bacteria are *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Actinomyces odontolyticus* and *Clostridium tetani*.

REAGENTS REQUIRED

(i) Gram's Crystal Violet Solution

2g of crystal Violet was dissolved in 120mL of Ethanol. To the above solution, 0.8 g of ammonium oxalate was added.

(ii) Gram's Iodine Solution

2g of Potassium iodide was added to 10mL of double distilled water. To the above solution, 1g of Iodine was added and made up to 300mL by double distilled water (290 mL).

(iii) Gram's Decolourizer Solution :

95% Ethanol or Acetone

(iv) Gram's Safranin Solution :

1 %g of Safranin was dissolved in 100mL of double distilled water.

(v) O/n Bacterial Culture

MATERIALS REQUIRED

(i) Microscope

(ii) Microscope slide

(iii) Cover slips

(iv) Inoculation loop

(v) Bunsen burner

(vi) Wash bottle

(vii) Pasteur pipette

(viii) Slide staining stand

(ix) Other standard lab ware

PROCEDURE

(i) Transfer a drop of the suspended culture to be examined onto a slide with the help of a sample applicator.

(ii) Spread the culture with the sample applicator to an even thin film over a circle of 1.5cm in diameter, approximately the size of a dime.

(iii) Air dry and fix the culture by passing over a gentle flame, while moving the slide in a circular fashion to avoid localized overheating.

(iv) 5 drops of crystal violet stain is poured over the fixed culture.

(v) Keep the fixed culture undisturbed for 2 minutes.

(vi) Discard the stain and the gently rinse the excess stain with a stream of water from a faucet or a plastic wash bottle.

(vii) Add about 5 drops of the Gram's iodine solution on the smear, enough to cover the fixed culture.

(viii) Keep the culture undisturbed for 30 seconds.

(ix) Discard the iodine solution and rinse the slide with running water.

(x) Add a few drops of decolourizer to the smear so that the solution trickles down the slide. Rinse the decolourizer with water after 5 seconds.

(xi) Add a few drops of safranin solution on the smear, enough to cover the fixed culture.

(xii) Leave it undisturbed for 30 seconds.

(xiii) Wash the excess safranin solution with double distilled water.

(xiv) Blot the slide with a filter paper gently to remove the excess water.

(xv) Dry the slide by air and observe under a microscope.

PRECAUTIONS

(i) Over decolourisation should be avoided as it will result in the loss of crystal violet causing gram positive organism to appear as gram negative.

(ii) Between applications of each reagent the slides must be thoroughly washed with running tap water to remove excess reagents.

OBSERVATION

Pink coloured Bacilli and violet coloured Cocci are observed (Fig 1).

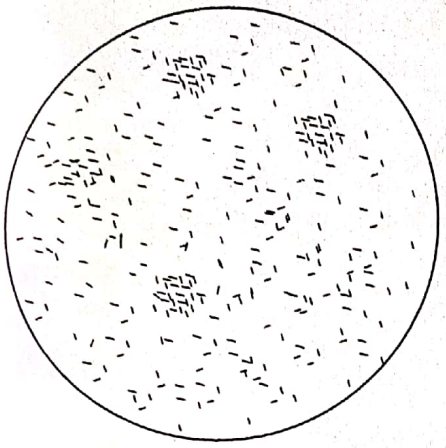

Fig 1 (a) . Gram Positive Cocci

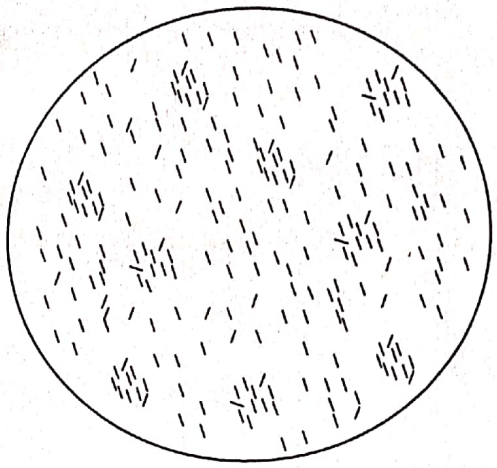

Fig 1 (b). Gram Negative Bacilli

RESULT

The Pink coloured Bacilli are Gram negative and the violet coloured Cocci are Gram positive organisms.

EXPT. NO. :

DATE :

LEISHMAN'S STAINING

AIM

To demonstrate different types of leucocytes in human blood by Leishman's staining.

PRINCIPLE

Leishman's stain is used in microscopy for staining blood smears. It is used to differentiate blood cells and to stain intracellular parasites in red blood cells and plasma, e.g. *Plasmodium falciparum* (malaria parasite). Leishman stain is named after its inventor, the Scottish pathologist William Boog Leishman. It is similar to and partially replaceable with Giemsa stain, Jenner's stain and identical to Wright's stain. Like them, it is a version of Romanowsky stain.

Romanowsky staining was a prototypical staining technique that was the forerunner of several distinct but similar methods including Giemsa, Jenner, Wright, and Leishman stains which are used to differentiate cells in pathological specimens. Romanowsky and Malakowsky independently developed a technique using a mixture of Eosin Y and oxidized Methylene blue.

The original Romanowsky stain was made by dissolving in methyl alcohol, the precipitate formed by the interaction of watery solutions of eosin and zinc free methylene blue. The stain imparts a reddish purple colour to the chromatin of malaria and other parasites.

REAGENTS REQUIRED

(i) Blood sample

(ii) Leishman's stain

(iii) Double distilled water

MATERIALS REQUIRED

(i) Microscope

(ii) Microscope slides

(iii) Lancet

(iv) Wash bottle

(v) Other standard lab ware

PROCEDURE

(i) Place a sterile 1by 3 – in. glass microscope slide on a horizontal surface.

(ii) Add a drop (30 to 40 µL) of blood onto one end of the slide about 0.5 inches. from the end.

(iii) Place a second glass slide 45 degree to the first slide and make a blood smear from one end to the other end.

(iv) Allow the film to air dry for at least 2 – 3 minutes.

(v) Pour the undiluted stain on the film and allow it to act for 1 minute.

(vi) Add double the volume of double distilled water with the help of a pipette to the slide and mix.

(vii) Leave the slide undisturbed for 12 minutes.

(viii) Wash off the stain by gently flooded in double distilled water until the film appeared bright pink in colour (usually in 30 seconds).

(ix) Remove the excess water with a blotting paper.

(x) Air dry the slide and observe under a microscope

OBSERVATION

Microscopically the RBC's appear as pinkish grey, platelets appear as deep pink and WBC's show blue nuclei and lighter cytoplasm. The nuclei of neutrophils appear as

dark purple multi-lobed nucleus with pale pink cytoplasm, bilobed blue nuclei for eosinophils, trilobed purple to dark blue nucleus for basophils and a dark purple to deep bluish purple nuclei for the lymphocytes (Fig. 1).

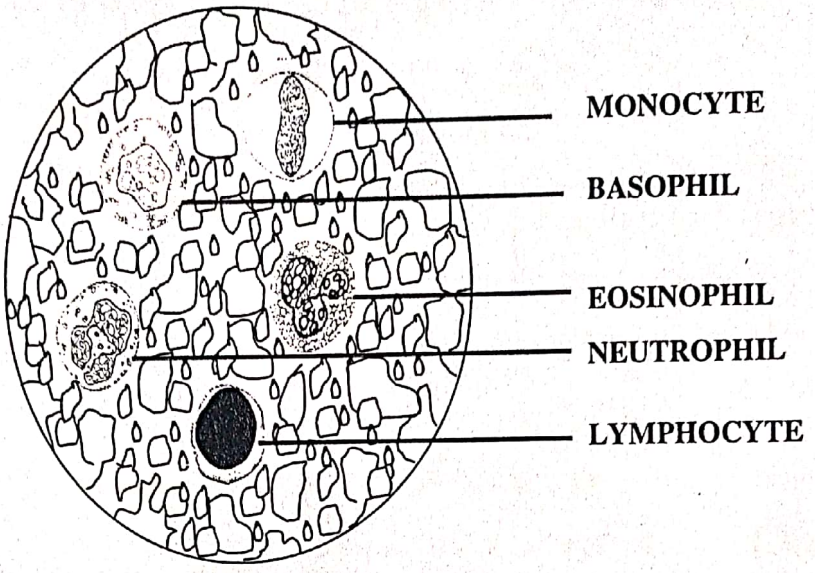

Fig 1. Geimsa stained WBCs

RESULT

In the slide Neutrophils, Eosinophils, Lymphocytes and Monocytes are identified.

EXPT. NO. :

DATE :

TRYPAN BLUE ASSAY

AIM

To count the number of viable cells in the given RBC population by Trypan blue assay.

PRINCIPLE

Viability assays measure the percentage of a cell suspension that is viable. The measurement of cell viability and growth is a valuable tool in a wide range of research areas. This is generally accomplished by a dye exclusion stain, where cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the colouring agent. A dye uptake stain can be used to measure viability as well. In this case the dye is normally taken up by viable cells but not by the non-viable cells.

The dye used for exclusion stain is usually trypan blue but erythrosin and naphthalene black have also been used. Diacetyl fluorescein is an example of a dye used for dye uptake assays. The cells are very selectable when it comes to allowing or baring compounds to pass through the cell membrane. In viable cells, trypan blue stain is not absorbed. However, in nonviable cells the damaged membrane allows trypan blue to become absorbed. Hence, dead cells are shown as a distinctive blue colour under a microscope.

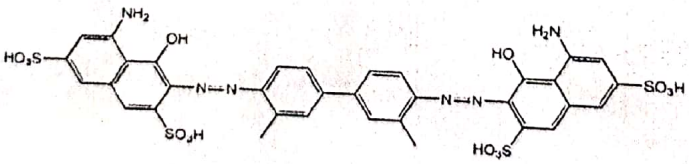

Fig 1. Chemical Structure of Trypan Blue

Trypan blue is derived from Toludine. Trypan blue is so-called because it can kill trypanosomes, the parasites that cause sleeping sickness. The dye is also known as diamine blue or Niagara blue. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with cells unless the cell

membrane is damaged. The blue stain is easily visible and the cells can be counted using a light microscope.

REAGENTS REQUIRED

(i) Blood sample

(ii) RBC diluting fluid

(iii) 0.4% Trypan blue solution

MATERIALS REQUIRED

(i) Microscope

(ii) Haemocytometer

(iii) Test tubes

(iv) Microscope slides

(v) Slide staining stand

(vi) Cover slips

(vii) Other standard lab ware

PROCEDURE

(i) Dilute 0.5mL of the given RBC sample with RBC diluting fluid to a concentration of 2×10^5 to 4×10^5 cells / mL.

(ii) Aseptically transfer the diluted suspension to a fresh tube.

(iii) Add 0.5mL of trypan blue solution (0.1% w/v) to the diluted blood sample and mix well.

(iv) Allow the cells to remain in the dye solution for not less than 3 minutes and no longer than 10 minutes.

(v) Apply a drop of the above sample to the haemocytometer or a slide.

(vi) Count the total number of cells, keeping a separate count of blue cells.

(vii) Determine the frequency of the blue cells (that is cells that are not excluded the dye).

(viii) The viability of the given cell population was determined by the following calculation.

CALCULATION

Total number of cells counted = X

Number of cells failed to exclude and are blue = Y

Number of cells that excluded the dye and are white in colour = Z

$$\text{Percentage Viability} = \frac{Z}{X} \times 100$$

OBSERVATION

Blue coloured non-viable cells and white coloured viable cells are observed (Fig.1).

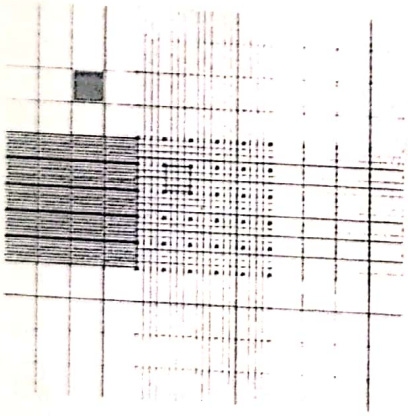

Fig 2. Haemocytometer grid

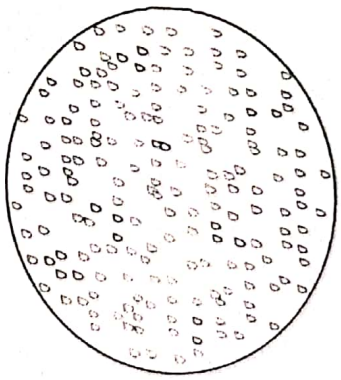

Fig 3. Blue coloured non viable and white coloured viable cells

RESULT

The percentage viability of the given cell population is ----------%

CELL VIABILITY ASSAY – BY ALAMARBLUE

AIM:

To find viable cell using Alamar Blue

PRINCIPLE:

The Alamar Blue, Cell Viability Assay Reagent is used to quantify cellular metabolic activity and in turn determine the concentration of viable cells in a given sample. AlamarBlue Cell Viability Reagent quantitatively measures the proliferation of mammalian cell lines, bacteria and fungi. The dye incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and change colour in response to the chemical reduction due to cell growth. Since, the Alamar Blue is very stable and nontoxic to the cells, continuous monitoring of cultures over time is possible.

The alamarBlue dye in its oxidized form is blue in colour and non-fluorescent. In Alamar Blue Reagent assay, the growing cells cause a chemical reduction of the alamarBlue dye from non-fluorescent blue to fluorescent red. The continued growth of viable cells maintain a reducing environment (fluorescent, red) and inhibition of growth maintains an oxidized environment (non-fluorescent, blue), which can be detected using a fluorescence or absorbance detector.

REAGENTS:

Cat. #	Description	Size
786-921	AlamarBlue Cell Viability Reagent	10ml
786-922	AlamarBlue Cell Viability Reagent	25ml
786-923	AlamarBlue Cell Viability Reagent	50ml

- Fluorescence plate reader with excitation 530-570nm and emission 580-620nm
- Microtiter plate reader for reading absorbance at 570nm and 600nm
- Multi-channel Pipettor
- 96 well tissue culture plates, compatible with fluorometer
- Culture plate shaker

ASSAY CONTROL

The following assay controls are recommended for each assay plate set up:

Negative Control	Untreated Control	Positive Control (100% Reduced)
No Cell Control may be set up without the cells. This will serve as the Negative Control to determine background fluorescence that might be present.	Untreated Control may be set up with Untreated cells. Same solvent used to deliver the test compound may be added to the control wells.	Positive Control with 100% Reduced alamar. Blue is necessary to assay viability using fluorescence values. To prepare 100% reduced form of alamarBlue Reagent, autoclave a sample containing cell culture media and alamarBlue for 15 minutes

PROCEDURE:

1. When cells are in log phase of growth, harvest them and determine the cell count. The suggested optimal cell count is 1 x 104 cells/ml (cell density), which may vary between the cell lines.

2. To appropriate wells of a 96 well plate, add the test compound and vehicle controls so that the final volume is 100µl in each well. Culture cells at 37°C in a cell culture incubator for desired test compound exposure period.

3. Remove the assay plates from 37°C incubator and mix by gently shaking it.

4. Aseptically add 10µl of alamarblue Reagent in an amount equal to 10% of the volume in the well. In the Positive Control well, add 10µl of ultrapure sterile water.

5. Incubate cultures with alamarblue Reagent for the predetermined time in a cell culture incubator at 37°C (the optimum incubation time may vary between the cell types).

6. After the incubation time ends, remove the plate and measure the fluorescence with Excitation wavelength at 530-560nm and Emission wavelength at 590nm.

7. Alternatively, absorbance at 570nm and 600nm wavelengths can be measured.

CALCULATION OF RESULTS

1. To calculate the % Reduction of Alamar Blue Reagent with fluorescence based readings, subtract the average fluorescence reading (RFU) of the Untreated (UT) Control from fluorescence values (RFU) of experimental wells as below:

$$\% \text{ Reduction of alamarBlue} = \frac{\text{(Experimental RFU value- Untreated Control RFU value)}}{\text{100\% Reduced (+) control RFU – Untreated control RFU value}} \times 100$$

2. To calculate % Difference between Treated (T) & Untreated (UT) Control cells based on fluorescence (RFU values), divide Experimental RFU value with Untreated Cell Control RFU value as below:

$$\% \text{ Difference between T \& UT} = \frac{\text{Experimental RFU value with test compound}}{\text{Untreated Control RFU value}} \times 100$$

3. To calculate the % Reduction of alamarBlue Reagent with absorbance based readings, follow the equation below and use Molar Extinction Coefficient from the table.

$$\% \text{ Reduction of alamarBlue} = \frac{(O2 \times A1) - (O1 \times A2) \times 100}{(R1 \times N2) - (R2 \times N1)}$$

Where:
O1 = Molar Extinction Coefficient of OXIDIZED alamarBlue at 570nm
O2 = Molar Extinction Coefficient of OXIDIZED alamarBlue at 600nm
R1 = Molar Extinction Coefficient of REDUCED alamarBlue at 570nm
R2 = Molar Extinction Coefficient of REDUCED alamarBlue at 600nm
A1 = Absorbance value of test wells at 570nm
A2 = Absorbance value of test wells at 600nm
N1 = Absorbance value of Negative Control well at 570nm
N2 = Absorbance value of Negative Control well at 600nm

Molar Extinction Coefficient of alamarBlue at different wavelengths:

Wavelength	Reduced (R)	Oxidized (O)
540nm	104395	47619
570nm	155677	80586
600nm	14652	117216
630nm	5494	34798

To calculate % Difference between Treated (T) & Untreated (UT) Control cells based on absorbance readings, follow the equation below and use Molar Extinction Coefficient from the table on previous page (6).

$$\% \text{ Difference between T \& UT} = \frac{(O2 \times A1) - (O1 \times A2) \times 100}{(O2 \times P1) - (O1 \times P2)}$$

Where:
O1 = Molar Extinction Coefficient of OXIDIZED alamarBlue at 570nm
O2 = Molar Extinction Coefficient of OXIDIZED alamarBlue at 600nm
A1 = Absorbance value of test wells at 570nm
A2 = Absorbance value of test wells at 600nm
P1 = Absorbance value of Untreated Control well at 570nm
(Cells + alamarBlue Reagent and NO Test Agent)
P2 = Absorbance value of Untreated Control well at 600nm
(Cells + alamarBlue Reagent and NO Test Agent)

EXPT. NO. :

DATE :

OSMOSIS USING POTATO

AIM

To demonstrate osmosis in living plant membrane using potato.

PRINCIPLE

Osmosis is the net diffusion of water molecules from a dilute solution or pure water itself to a more concentrated solution across a semipermeable membrane that permits the diffusion of water but not of a solute. Thus if an aqueous solution of sucrose is separated from water by a semipermeable membrane, water flows into the sucrose solution across the membrane. Osmosis requires (a) semipermeability of the membrane separating the two solutions, so that the membrane is permeable to water but not to the solute and (b) a difference in concentration of the solute on the two sides of the membrane.

Water continues to flow into the more concentrated solution across the membrane until the hydrostatic pressure rises so high on the concentrated side of the membrane as to cause a transmembrane diffusion of water in the opposite direction at the same rate as the osmotic inflow. This excess of hydrostatic pressure, which exactly balances the osmotic influx of water from pure water to the concentrated solution is called the osmotic pressure of that solution. Osmotic pressure may also be defined as that pressure that has to be exerted on the concentrated solution, separated from pure water by a semipermeable membrane in order to counteract and stop the osmotic inflow into that solution. Osmotic pressure is a colligative property of a solution and not dependent on their nature or shape of the solutes. According to vant Hoff's, the osmotic pressure of a solution is directly proportional to the molar concentration (c) of the solute as long as the temperature is maintained constant. The more concentrated a solution is, the higher is its osmotic pressure.

REAGENTS REQUIRED

(i) 1% Sucrose solution

(ii) Coloured water

MATERIALS REQUIRED

(i) Potato

(ii) Beaker

(iii) Other standard lab ware

PROCEDURE

(i) Take a large size potato tuber.

(ii) Remove its skin and cut the base to make it flat.

(iii) Make a hollow cavity and fill with concentrated sugar solution.

(iv) Place the tuber in a beaker filled with coloured water.

OBSERVATION

After few hours the level of the solution within the potato cavity rises and turns pink colour (Fig. 1).

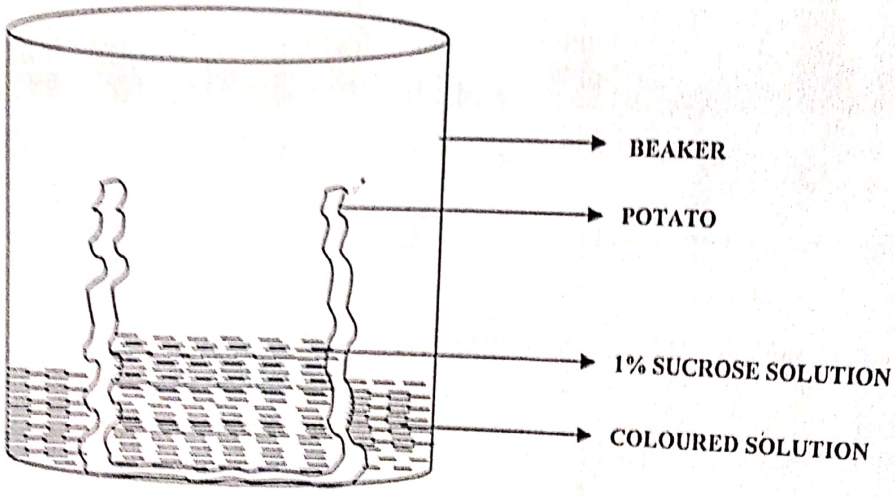

Fig 1 : Demonstration of Osmotic inflow

RESULT

The level of solution within potato cavity is raised. The raise in the level of the solution inside the potato cavity is due to the net movement of water through the living cell membrane of potato tubers.

EXPT. NO. :

DATE :

MITOSIS

AIM

To demonstrate the different stages of mitosis in *Allium cepa* (Onion)

PRINCIPLE

During normal cell activity the chromosomes are unwound and too thin to be seen. Chromosomes of most plant and animal cells are visible with light microscopy only in the most condensed state i.e. during the various phases of cell division (mitosis or meiosis). Mitosis is the process by which a cell duplicates the chromosomes in its cell nucleus of eukaryotes, in order to generate two identical daughter nuclei. It is generally followed immediately by cytokinesis, which divides the nuclei, cytoplasm, organelles and cell membrane into two daughter cells containing roughly equal shares of these cellular components. The process of mitosis is complex and highly regulated. The sequence of events is divided into stages, corresponding to the completion of one set of activities and the start of the next. These stages are prophase, metaphase, anaphase and telophase.

In plants, the root tips are meristematic regions; i.e. root tips continue to grow as they search for water and nutrients. The meristematic region is located just behind the root cap, a protective covering on the root. This can be seen as the bright white tip in the preserved onion root tips. These regions of growth are good for studying the cell cycle because at any given time, cells that are undergoing mitosis can be found.

Onions are excellent material for studying mitosis, because lot of roots can be generated in a few days simply by placing an onion in a dish of water and it is fairly easy to see mitosis in onion root tips. The root tips are fixed prior to staining. The fixation brings about the death of the cells in such a way that the structure of the living cells is preserved in the same form as it was before death therefore the cells do not undergo any alterations. The fixative used is an acetic acid: ethanol solution. Acetic acid is a powerful precipitant of nuclear protein and it prevents cell shrinkage. Ethanol acts as a dehydrant (removes water form tissues) which hardens the tissue. 1 N HCl breaks down the calcium

pectate and magnesium pectate (which cement or attach the cells to each other) in the root tip tissues to pectic acid which cannot cement cells together. As a result after gently tapping, the cells separate out and form a mono layer in which cells are easily observable. The stain carmine is a pigment of a bright red colour. It is a basic dye extracted from the *Coccus* cacti and is taken up well by nucleic acids. Ferric chloride added to the stain acts as a mordant.

REAGENTS REQUIRED

(i) Freshly grown onion root tip

(ii) 1N Hydrochloric acid

(iii) Carnoy's fixative solution (Absolute alcohol : Glacial acetic acid – 3 : 1)

(iv) Acetocarmine stain (45 mL of glacial acetic acid and 55 mL of distilled water was taken into a 1 liter flask. Heat to boiling and add 0.5 grams of carmine, shake well, cool and filter).

(v) Ferric chloride

(vi) Double distilled water

MATERIALS REQUIRED

(i) Petri plates

(ii) Plastic pipettes

(iii) Razor blade

(iv) Microscope

(v) Microscope slides

(vi) Coverslips

(vii) Forceps

(viii) Other standard lab ware

PROCEDURE

(i) Using forceps take an onion root and remove the root tip.

(ii) Place the root tip in the 1N HCl kept in the Petri plate for 3 minutes (agitate the plate during fixation).

(iii) Transfer the hydrolyzed root tip to a Petri plate containing a small amount of Carnoy's fixative.

(iv) Fix the root tip for 3 minutes (agitate the plate during fixation).

(v) Place the fixed root tip in a Petri plate containing double distilled water (washing) for 30 seconds.

(vi) Transfer the root tip on a sterile glass slide.

(vii) Add a few drops of acetocarmine dye to the root tip and allow to stand for 5 minutes.

(viii) Place a cover slip over the stained tissue.

(ix) Remove the stain with a blotting paper.

(x) Press the cover slip firmly onto the slide to spread the cells in a very thin layer.

(xi) Observe the slide under a microscope

OBSERVATION

The various stages of mitosis are observed. In the interphase, chromosomes (DNA) exist as chromatin. During prophase, the chromatin is condensed. In metaphase, the duplicated chromosomes each consisting of two sister chromatids are lying midway between the two poles. In the early and late anaphase – the sister chromatids are seen separating and moving towards the poles. In the telophase, the chromosomes are located at opposite poles (Fig 1).

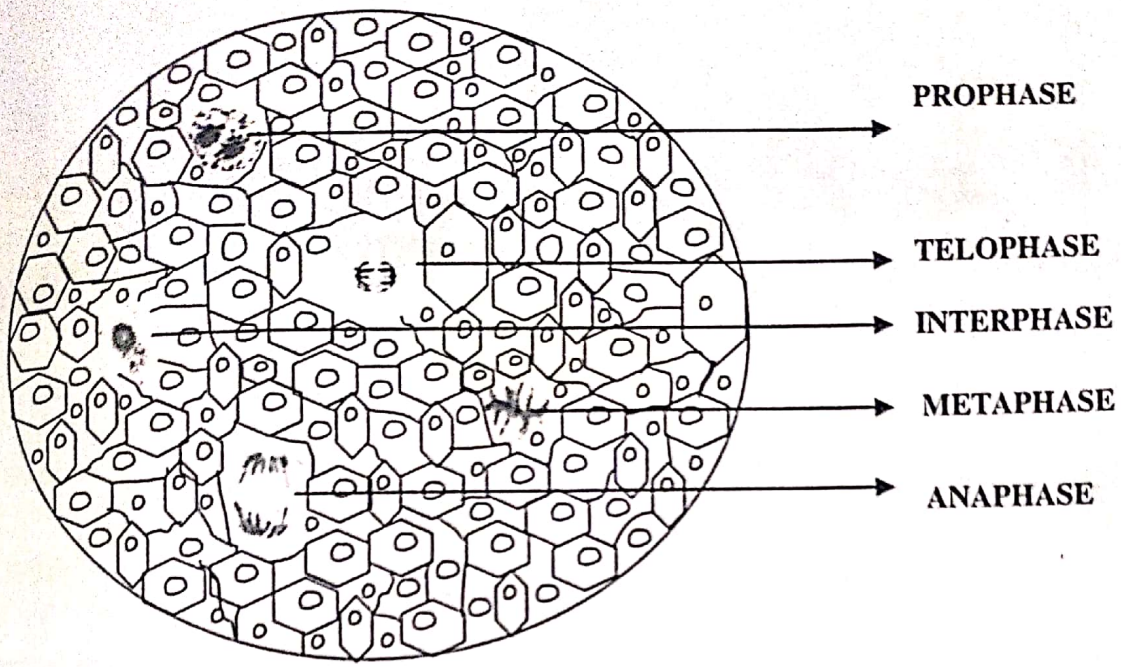

Fig 1. Mitosis – Different steps

RESULT

The interphase, prophase, metaphase, anaphase and telophase stages are identified.

EXPT. NO. :

DATE :

GIEMSA STAINING

AIM

To differentiate the different types of WBC's using Giemsa Staining

PRINCIPLE

Giemsa stain named after Gustav Giemsa (1867 – 1948), an early malariologist is used for the histopathological diagnosis of malaria and other parasites. It is a member of the "Romanowski" group of stains and consists of a mixture of a stock glycerol methanol solution of eosinates of Azure B and methylene blue with some excess of the basic dyes. The stain is usually prepared from commercially available Giemsa powder.

Giemsa stain is a differential stain. It is used to differentiate nuclear and / or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites. Erythrocytes stain pink, platelets show a light pale pink, lymphocyte cytoplasm stains sky blue, monocyte cytoplasm stains pale blue, and leukocyte nuclear chromatin stains magenta. It is also used to study the adherence of pathogenic bacteria to human cells. It differentially stains human and bacterial cells with purple and pink respectively. Hence used for histopathological diagnosis of malaria, some other spirochete and protozoan blood parasites.

It is also specific for the phosphate groups of DNA and attaches itself to regions of DNA where there are high amounts of adenine – thymine bonding. Hence used in Giemsa banding commonly called G-banding to stain chromosomes and often used to create a karyotype. It can identify chromosomal aberrations such as translocations and interchanges.

REAGENTS REQUIRED

(i) Blood sample

(ii) Giemsa stain – 1: 20 dilution (2 mL of Cuimsa + 40 mL of buffered water)

(iii) Fixative – (Ethanol : Acetic acid in 3:1 ratio)

(iv) Double distilled water

MATERIALS REQUIRED

(i) Microscope

(ii) Microscope slides

(iii) Cover slips

(iv) Staining trough

(v) Lancet

(vi) Other standard lab ware

PROCEDURE

(i) Place a sterile 1by 3 – in. glass microscope slide on a horizontal surface.

(ii) Add a drop (30 to 40 µL) of blood onto one end of the slide about 0.5 inches. from the end.

(iii) Place a second glass slide 45 degree to the first slide and make a blood smear from one end to the other end.

(iv) Allow the film to air dry for at least 30 minutes to 1 hour.

(v) Fix the air-dried film in the fixative for 2 – 3 minutes.

(vi) After fixing, air dry the slide for 2 – 3 minutes.

(vii) Stain the air dried film with diluted Giemsa stain (1 : 20, v/v) for 30 min.

(viii) Briefly wash the slide by dipping the slide in and out of a Coplin jar of buffered water (one or two dips) for one or two seconds. Note : Excessive washing will decolourize the film.

(ix) Air dry the slide in a vertical position.

(x) Observe the slide under a microscope

OBSERVATION

Microscopically the RBC's appear pinkish grey, platelets appear deep pink and WBC's show blue nuclei and lighter cytoplasm. The nuclei of neutrophils appear as dark purple multi-lobed nucleus with pale pink cytoplasm, bilobed blue nuclei for eosinophils, trilobed purple to dark blue nucleus for basophils and a dark purple to deep bluish purple nuclei for the lymphocytes (Fig 1).

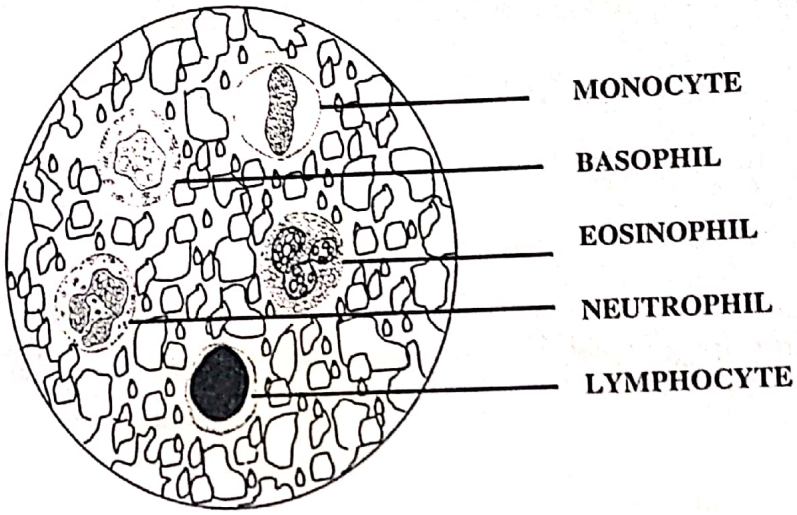

Fig 1. Geimsa stained WBCs

RESULT

In the slide, Neutrophils, Eosinophils, Lymphocytes and Monocytes are identified.

EXPT. NO. :

DATE :

THIN LAYER CHROMATOGRAPHY

AIM

To separate the lipids of the given egg yolk by thin layer chromatography

PRINCIPLE

Thin layer chromatography (TLC) is a chromatographic technique used to separate chemical compounds. The process is similar to paper chromatography with the advantage of faster runs, better separations and the choice between different stationary phases. Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase.

TLC involves a stationary phase consisting of a thin layer of adsorbent material, usually silica gel, aluminium oxide, or ceilulose immobilised onto a flat inert carrier sheet. A liquid phase consisting of the solution to be separated is then dissolved in an appropriate solvent and is drawn up the plate via capillary action. The given sample mixture is separated based on the polarity of the components of the compound.

Silica is the most widely used adsorbent in TLC. Silica is present as SiO_2. TLC plates are made by mixing the adsorbent with a small amount of inert binder like calcium sulphate (gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic, and the resultant plate is dried and activated by heating in an oven for 30 min at 110 °C. The thickness of the adsorbent layer is typically around 0.1–0.25 mm for analytical purposes and around 1–2 mm for preparative TLC.

A small spot of solution containing the lipid sample is applied to a plate, about one centimeter from the base. The plate is then dipped into a suitable solvent such as ethanol or methanol - water mixture and placed in a sealed container. The solvent moves up the plate by the capillary action. The sample mixture is dissolved and is carried up the plate by the solvent. Different lipids are absorbed onto the activated silica gel with varying degrees

of strength. Those components which are not adsorbed or adsorbed less strength tend to move faster along with the moblie phase (solvent system) where as those which are held more firmly by the adsorbent (stationary phase) travel slowly.

The distance the solute (analyte) moves in relation to the distance the solvent moves serves as a convenient means for identifying the solute and is called as Rf value for the compound under the specified conditions of the experiment.

$$Rf = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by the solvent from origin}}$$

REAGENTS REQUIRED

(i) 1 % NaCl

(ii) Chloroform : Ethanol (2 : 1)

(iii) Developing Mixture – Chloroform : Ethanol : Water (65 : 25 : 4)

(iv) Iodine granules

MATERIALS REQUIRED

(i) Egg yolk

(ii) Activated TLC plates

(iii) Beakers

(iv) Eppendorf tubes

(v) Pipettes

(vi) Microtips

(vii) Vortexer

(viii) Centrifuge

(ix) Other standard lab ware

PROCEDURE

(i) Add 0.6mL of 1% NaCl to 0.2mL of egg yolk taken in an eppendorf tube.

(ii) Vortex briefly the above mixture and spin at 10000 rpm for 5 minutes.

(iii) Transfer 0.2mL of clear supernatant to another eppendorf tube.

(iv) To the above supernatant, add 0.2mL of 1% NaCl and 0.2mL of ethanol – chloroform mixture and mix well.

(v) Spin the above sample at 10000 rpm for 5 minutes.

(vi) Discard the upper phase without disturbing the lower phase.

(vii) Using a pipette tip, Spot 10 – 20µL of lower lipid phase (yellow coloured) at a distance of 2cm starting from the left bottom edge of the activated TLC plate.

(viii) Place the plate in the beaker containing the developing mixture.

(ix) Develop the plates till the solvent front moves up to 1 cm below the top edge of the plates.

(x) Take out the developed plates and air dry.

(xi) Transfer the dried plate to another beaker containing iodine granules and keep till the yellow coloured spot is developed.

(xii) Measure the distance travelled by the individual component.

(xiii) Calculate the corresponding Rf values.

(xiv) Compare the obtained Rf values with the standards.

OBSERVATION

On exposure to iodine vapours, yellow coloured spots are observed. The Rf values are measured from the developed chromatogram (Plate 1).

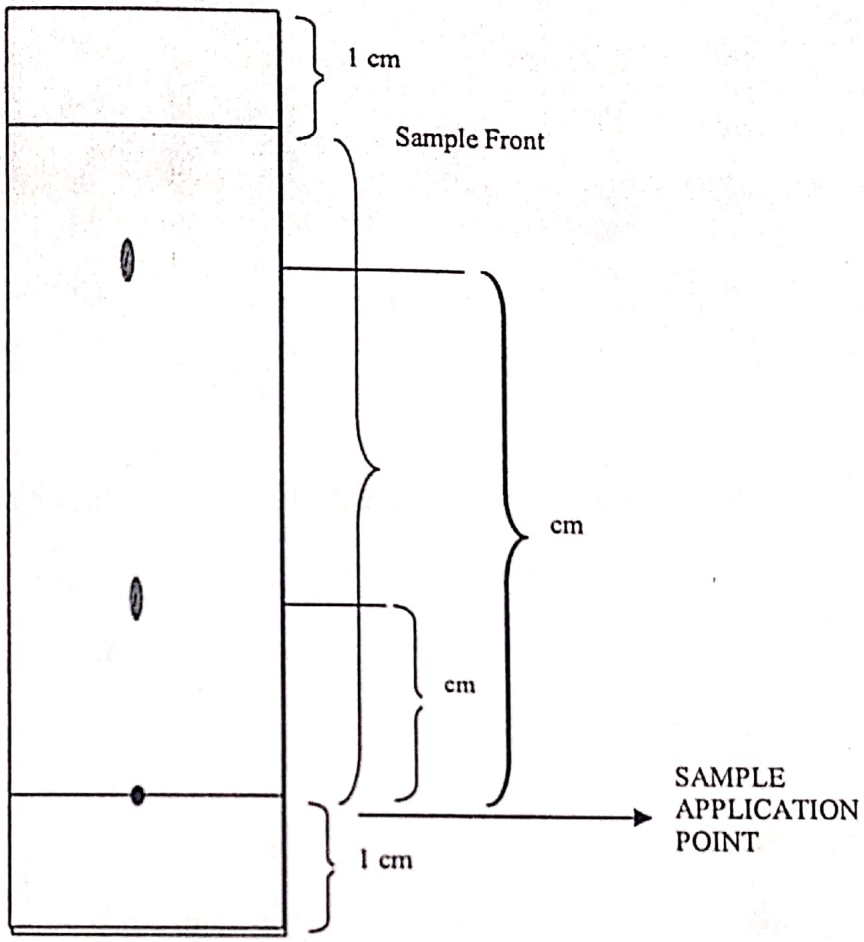

Plate 1 : Developed Chromatogram showing two peaks

INFERENCE

The Rf value should not be more than 1. The Rf values of various lipid components are:

Phosphatidyl Ethanolamine – 0.9

Phosphatidyl Choline – 0.5

Lysophosphatidyl Choline – 0.1

Sometimes the lysophosphatidyl choline not be detected but seems to be appear as a slight movement upward of the original spot. Two components which are not sufficiently well separated from phosphatidyl choline are phosphatidyl serine and phosphatidyl inositol. This gives the phosphatidyl choline a streaky spot.

CALCULATION

Distance moved by the analyte =

Distance moved by the Solvent =

$$\text{Retarding factor (Rf)} = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by the solvent from origin}}$$

RESULT

The compound 'a' is identified as Lysophopshatidyl Choline – Rf =

The compound 'b' is identified as Phosphatidyl Choline – Rf =

The compound 'c' is identified as Phosphatidyl Ethanolamine – Rf =