May 2026 Volume 49, Issue 2 ISSN 2349-8307

INDIAN SOCIETY OF CELL BIOLOGY

Cell Biology Laboratory Manual

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PREFACE

Cell biology forms the foundation of all biological sciences, offering insights into the structure, organization, and function of the basic unit of life—the cell. Understanding cellular mechanisms is essential for exploring physiological processes, developmental biology, genetics, immunology, and molecular medicine. Through cell biology, students learn how complex life arises from simple cellular interactions and how alterations in cellular mechanisms lead to disease and dysfunction. For students of life sciences at both undergraduate and postgraduate levels, hands-on experimentation is vital to link theory with biological reality.

This manual presents a comprehensive series of experiments designed to illustrate fundamental and advanced concepts in cell biology. The initial protocols—such as microscopy, cell measurement, and basic staining techniques—train students to visualize cells and subcellular components. Exercises on bacterial staining and human epithelial cell observation reinforce the diversity of cell types and structures. Studies on mitosis and meiosis deepen understanding of the cell cycle and chromosomal behaviour, while colchicine treatment and chromosomal banding techniques introduce cytogenetic principles.

Advanced experiments such as cell culture, cell viability assays, apoptosis detection, and immunostaining expose learners to modern tools in cellular physiology and pathology. Techniques like in situ hybridization, immunofluorescence, and fluorescence-activated cell sorting bridge molecular biology with cellular visualization, revealing gene expression patterns and cell population dynamics. Biochemical approaches including SDS-PAGE, Western blotting, and co-immunoprecipitation highlight the importance of proteins in cellular signalling and structure.

The Inclusion of model systems such as *Drosophila*, *Dictyostelium*, *Hydra*, *Caenorhabditis elegans* and Zebrafish demonstrates how diverse organisms aid in deciphering conserved cellular processes like development, regeneration, and morphogenesis. Experiments involving CRISPR/Cas9 genome editing and assays for mitochondrial activity, phagocytosis, and cytotoxicity integrate modern molecular and cellular tools used in contemporary research.

Collectively, these protocols not only reinforce conceptual understanding but also cultivate technical skills necessary for independent investigation in biological sciences. The exercises are designed to encourage observation, critical thinking, and experimental precision—qualities essential for every budding biologist. This compilation thus serves as both a teaching resource and a guide for exploring the fascinating world within cells.

We are grateful to the all the authors who are the researchers or faculty members in various institutions and actually using these methodologies in their laboratory. We are certain that help and guidance will be provided by the authors if any student or scientist find difficulty in doing any of the experiments at their place. We pay sincere gratitude to the reviewers, Professor Subhash C Lakhotia (BHU, Varanasi), Professor Shweta Saran (JNU, New Delhi) and Professor Sathees C Raghavan (IISc, Bengaluru), who helped in making these chapters more comprehensive and user-friendly by their constructive and critical inputs. We sincerely thank one of the young researchers, Mr Saurabh Chand Sagar (BHU, Varanasi) for his kind help in organizing figures in this book. Also sincere gratitude to Professor S Ganesh and his team members for final formatting, adding the attractive cover page and publishing this book.

We will be happy to receive critical feed-back from the users of this book and from the Cell Biology community about any error or omission in this Protocol Book, to improve this compilation dynamically.

May, 2026

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Chapter 1: PEEPING INTO A STUDENTS' MICROSCOPE

Recommended level: UG

INTRODUCTION

Students' microscope is the basic compound microscope to observe **Magnified Image** of a tissue, cell or subcellular component (Spencer, 1982). It contains a horse shoe shaped base on which the components of optics are fitted. To start with (Fig.1) it has a Concave mirror at the base, which reflects the scattered day light

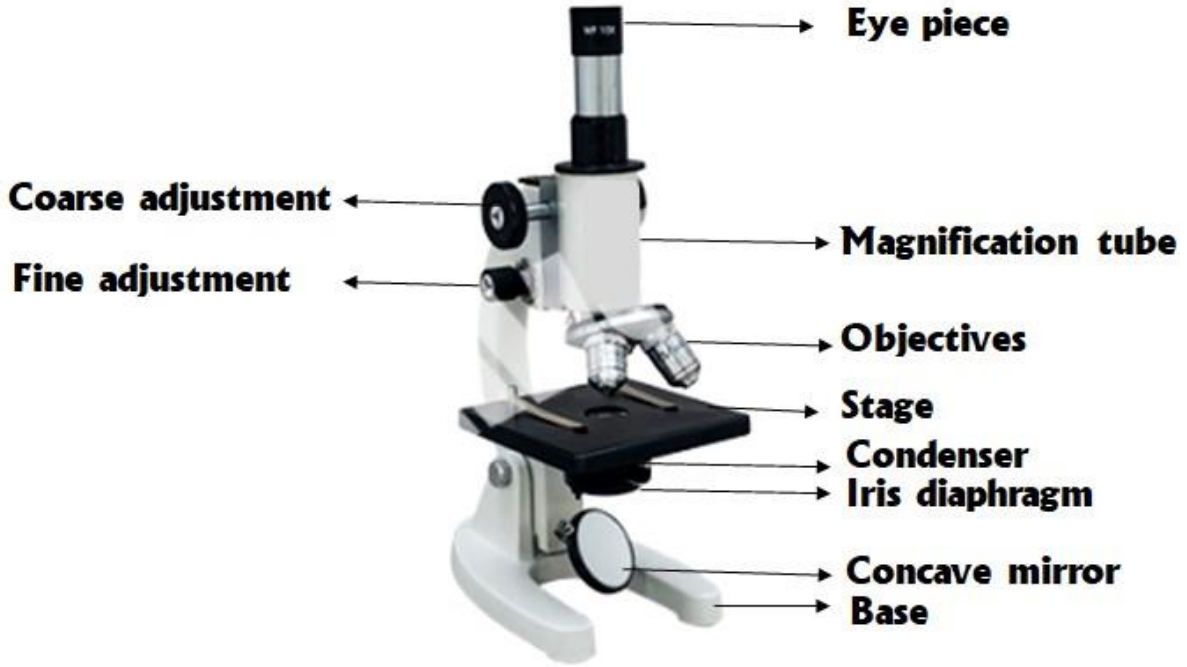

Figure 1. A students' microscope

or electric light of the room towards the iris diaphragm in the form of a parallel beam of light rays. As the name Iris diaphragm, it can be closed or opened to allow a narrow or wide beam of light (the required intensity) to pass, like the diaphragm functions in our eyes. The light is then received by a Condenser, which converges the parallel rays on to the object present on a glass slide on the stage. So now the object is illuminated and light from the object is passed to the Objective lens, a convex lens, of low or high magnification. If the object is between F and 2F of the objective lens (adjusted with the help of the Focusing knobs [coarse and fine adjustment]), then a real, inverted image of the object is formed within the Magnification tube. The Eye piece, a convex lens, is fixed at a distance on the other side of the magnification tube, such that the first image falls within the F and O of the eye piece. As a result, a virtual, erect and magnified image is formed (Spencer, 1982), which is seen through our eyes. As the first image is inverted, finally we observe the magnified inverted image of the object (see Fig. 2).

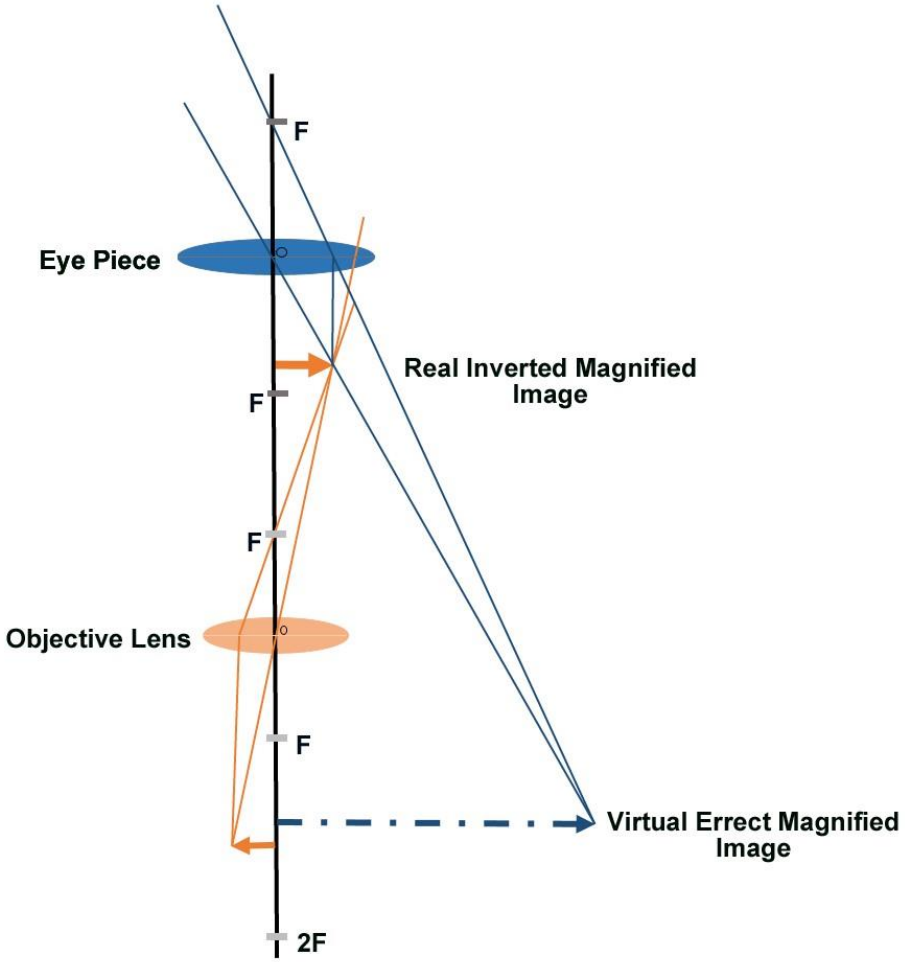

Figure 2. Image formation in a students' microscope

Besides magnification, the most important component of a microscope is, its power of **Resolution** (Sarikaya, 1992), to be able to distinctly visualize (resolve) the two closely placed points. The minimum distance (D) between the two distinguishable objects, is almost fixed for a given objective lens and it depends on the wavelength of the incident light (λ) being used, the refractive index (N) of the medium between the object and the objective lens where image formation is initiated and the half angle (α) of the cone of light that is formed between the object and the objective lens (also called as angular aperture, Fig. 3).

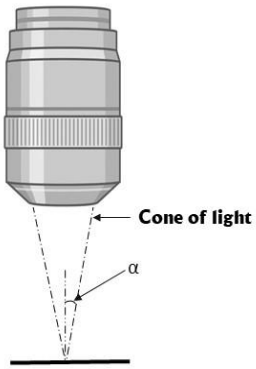

Figure 3. Cone of light that is formed between object and objective lens showing the half angle α

Their relations are as given below:

$$D \propto \frac{\lambda}{N \sin \alpha} \quad \text{or} \quad D = \frac{0.61\,\lambda}{N \sin \alpha}$$

(0.61 is a constant)

If one uses shortest wave length of light in visible range (450 nm), refractive index of air (1) where image formation is initiated and best possible angular aperture of a lens Sin 70^{o} then

$$D = \frac{0.61 \times 450 \text{ nm}}{1 \times 0.94} = 292 \text{ nm or } 0.3 \text{ µm}$$

This means the given lens with these properties and conditions can distinctly visualize the two points or two organelles in a cell if they are 0.3 µm apart. By using an oil immersion lens (refractive index 1.5) the D can be further brought down to 0.2 µm.

OBJECTIVE

To observe English letter 'L' under the low and high power of a Student microscope.

REQUIREMENTS

A glass slide on which English letter `L' is written, a Student microscope.

PROCEDURE

1. Prior to using the microscope, make sure that the low-power objective lens is facing the condenser and there is sufficient gap between the stage and the low power lens to place a slide.
2. Turn the concave mirror in such a way that sufficient light is being reflected on to the iris diaphragm. Do not allow direct Sun rays to get reflected by the mirror. This may badly affect the eyes.
3. Place the slide with the object on the stage. Observing through the eye piece, adjust the iris diaphragm such that sufficient but soothing light is passing through the slide and the objective lens to the eye piece.
4. Adjust the height of the condenser such that it is close to the slide but not touching it and the light is condensed on to the object. If the iris diaphragm is almost closed and observed through the eye piece, a small circular beam of light is seen. Now open the iris diaphragm so much that the entire field is uniformly illuminated. That ensures proper adjustment of light and right position of the condenser.
5. While observing through the eye piece bring the object (English letter L) to the center and sharply focus using coarse adjustment. The microscope is ready for observation in low power.
6. If high-power observation is desired, turn the high-power lens without changing the focus. Now focus the object sharply using fine adjustment. If needed, move the slide a bit to bring the object in the middle. Very little focusing or bringing the object to the center will be required. The microscope is ready for high power observation.
7. Once high-power observation is over, turn the low-power lens towards the object. The slide can now be removed from stage.

OBSERVATION

In the slide the object, English letter `L' was in straight readable direction. The low-power image had vertical and lateral inversion. The High power image was further magnified image of the same.

QUESTIONS FOR FURTHER STUDIES

1. Are Phase contrast microscope and Fluorescence microscope work in same principle?
2. Can you calculate the power of resolution of the high power lens of the microscope that you used?
3. How a stereobinocular microscope gives a 3-dimentional image?

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Chapter 2: MEASUREMENT OF THE SIZE OF CELLS UNDER STUDENTS' MICROSCOPE

Recommended Level: UG, PG

INTRODUCTION

The diameter of a cell or length/diameter of subcellular components can be easily measured using an ocular micrometer which has graduation in arbitrary units. This arbitrary graduation of the ocular micrometer is calibrated using a stage micrometer by superimposing the two scales.

OBJECTIVE

To measure the diameter of a cell

MATERIALS REQUIRED

Light microscope, ocular and stage micrometer, slide having cell preparations whose size is to be estimated.

PROCEDURE

1. The ocular micrometer is placed on the circular shelf inside the eyepiece in such a way that the graduations sketched on the ocular, is visible when an observation is made using the microscope (Fig. 1).
2. Place the stage micrometer on the stage of a microscope and focus the graduations using low power objectives. The graduations on stage micrometer are spaced 0.01mm (10 µm) apart.
3. Superimpose the two scales and record the number of ocular divisions coinciding exactly with the number of divisions of the stage micrometer. The calibration factor or the least count of ocular micrometer is calculated as follows:

   If 13 ocular divisions coincide with 2 divisions (2X10 µm=20 µm) of stage micrometer
   Then 1 ocular division = 20 µm

   $$\frac{20}{13 \text{ divisions}} = 1.54 \text{ µm}$$

4. Now remove the stage micrometer from the stage and place the slide having cell preparation under low power magnification. Position the cell being observed in such a way that the ocular micrometer is able to measure the diameter of a cell or the length/diameter of a cell component in arbitrary units. All the steps are shown in Fig. 1. Calculate the size as shown below:

   If the diameter of a cell is occupying 5 divisions of ocular, the diameter of the cell will be: 5 divisions X 1.54 µm = 7.7 µm
5. Similarly for high power objective, the ocular micrometer calibration has to be done again following the same procedure and then cell diameter is can be measured focusing the cell in high magnification.

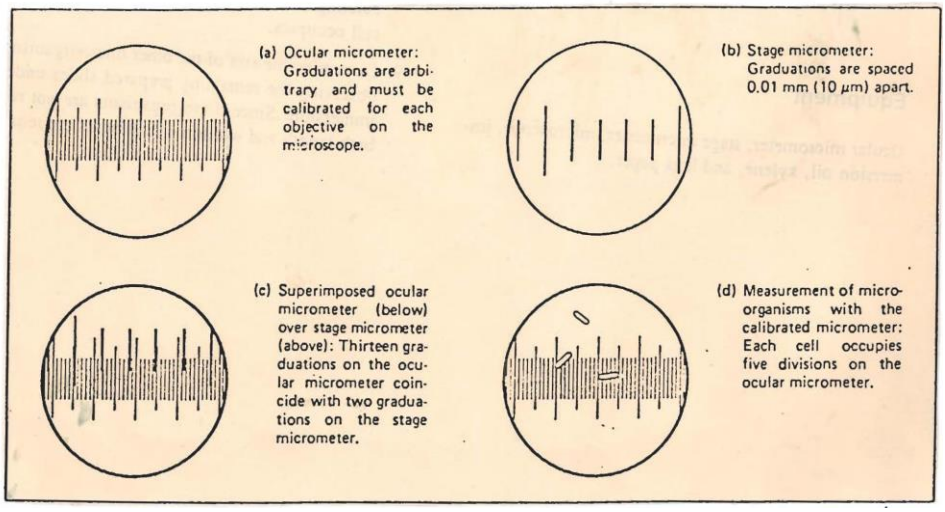

Figure 1. Steps in measuring the size of a cell

QUESTIONS FOR FURTHER STUDIES

1.What is the diameter of your RBC and different WBCs?

2. What is the average diameter of your cheek epithelial cells?

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Chapter 3: GRAM STAINING FOR BACTERIAL CELLS

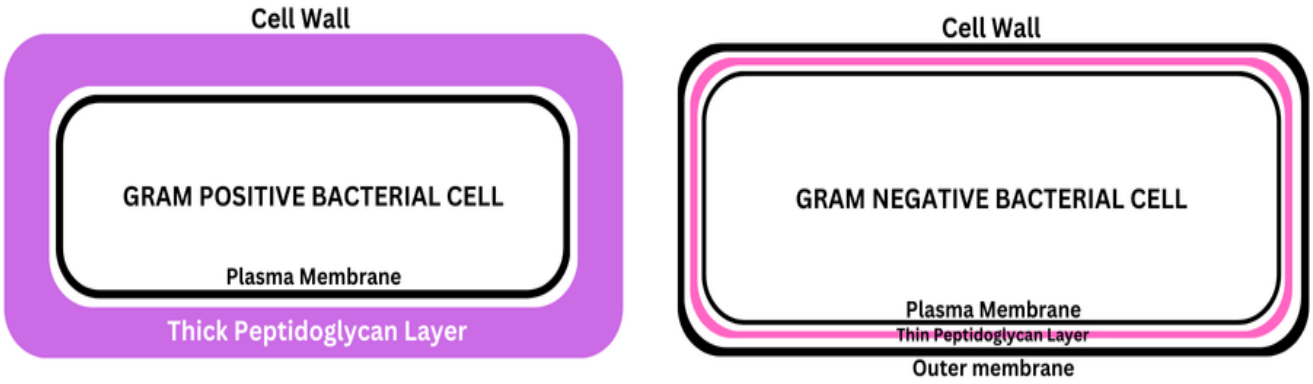
Recommended Level: UG, PG

INTRODUCTION

The Gram stain, introduced by Hans Christian Gram in 1884, is the most commonly used differential stain in bacteriology. This technique classifies bacteria into two main groups—**Gram-positive** and **Gram-negative**—based on their cell wall composition (Fig. 1). The staining results reveal differences in the cell wall structure: Gram-positive bacteria have thick peptidoglycan layers in their cell walls (90%), composed of carbohydrate and protein subunits, low lipid content and they also contain teichoic acids. In contrast, Gram-negative bacteria have a thin peptidoglycan layer (10%), high lipid content, and lack teichoic acids in their cell walls, but they possess an outer membrane similar to the phospholipid bilayer of the plasma membrane.

Principle: The Gram staining technique uses four stains/ reagents: Crystal violet, Gram's iodine, Ethanol, and Safranin, to differentiate between Gram-positive and Gram-negative bacteria based on their cell wall structure.

- First, crystal violet, **the primary stain**, is added which penetrates the peptidoglycan of both types of bacterial cells, giving them a purple colour. In aqueous solutions, crystal violet dissociates into CV+ and Cl- ions, which pass through the cell wall and membrane of both Gram-positive and Gram-negative bacteria. The CV+ ions interact with negatively charged components within the cells, staining the cells purple.
- Then, Gram's iodine (I- or I3-), **the mordant,** is added which interacts with CV+ to form a large complex with crystal violet in the peptidoglycan wall, making the primary stain more difficult to remove, in a step referred to as "Fixing" the dye.
- After iodine treatment, the cells are treated with ethanol or ethanol and acetone solution, **the decolourizer,** which interacts with the lipids of both gram-positive and gram-negative bacteria.
- Alcohol dissolves the lipid present in the outer membrane (lipopolysaccharide layer) of Gram-negative cells, exposing the peptidoglycan layer. Gram-negative cells have thin peptidoglycan layers, only one to three layers deep, with a slightly different structure compared to Gram-positive cells, which do not offer much resistance. When treated with ethanol, the cell walls of Gram-negative bacteria become leaky, and it leaches the large CV-I complexes out of the cells.
- Gram-positive cell wall contains a thick layer of peptidoglycan with numerous teichoic acid cross-linking which resists the decolorization. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. The highly cross-linked and multi-layered nature of the peptidoglycan along with the dehydration from the ethanol treatment traps the large CV-I complexes within the cell and the cell does not get decolorized.
- This step, referred to as "Decolorization," causes the crystal violet-iodine complex to be retained in Gram-positive cells, which hold onto the purple crystal violet colour, while being washed out from Gram-negative cells, losing the purple colour and be transparent.
- Finally, safranin, **the counterstain,** is added, staining the now colorless Gram-negative cells pink, while the Gram-positive cells remain purple. This results in Gram-positive bacteria appearing purple and Gram-negative bacteria appearing pink under a microscope, allowing for easy observation and differentiation.



(A) (B)

Figure 1. Images showing bacteria stained using the Gram stain.
(A) Gram-positive bacteria appear purple, have a thick peptidoglycan layer outside the plasma membrane. (B) Gram-negative bacteria appear pink, have a thin peptidoglycan layer outside the plasma membrane, with an additional outer membrane. Lipopolysaccharides (LPS) are embedded in the outer membrane of Gram-negative bacteria which are not depicted in the diagram.

OBJECTIVE

To make a preparation of bacterial cells (*Lactobacillus*) on slide and to identify if the cells are Gram positive or Gram negative.

REQUIREMENTS

Freshly prepared bacterial culture (e.g., a sample of curd)
Gram staining reagents

a. Crystal Violet
b. Gram's iodine solution
c. 95% ethanol
d. Safranin

Wash bottle with distilled waters
Droppers, glass slides, needle or thin glass rod, blotting paper, Bunsen burner/ spirit lamp, microscope

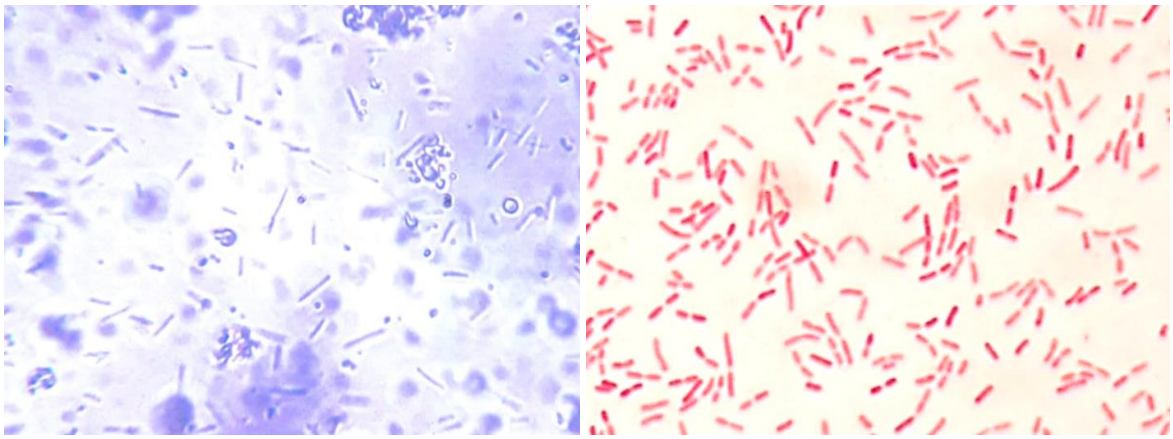
PROCEDURE

1. A strip or small drop of curd is taken on a clean dried slide by using a needle or thin glass rod. A thin film was made by spreading the sample strip of curd over the slide by using the smooth edge of another glass slide.
2. The smear is air dried and heat fixed by passing the glass slide swiftly over the blue flame of the burner 2-3 times (heat fixing kills the bacterial cells in the smear, firmly attaches the smear to the slide, and allows the sample to more easily take up stains).
3. The smear is then stained with crystal violet for 40-60 sec.
4. The slide with the stained smear on it is rapidly rinsed with distilled water for a few sec.
5. Smear is then covered with Gram's iodine solution (which acts as a mordant) for 30 sec.

6. Ethanol is added drop by drop to wash iodine until no more colour flows from the smear. The gram-positive bacteria are not affected while all gram-negative bacteria are completely decolorized.
7. The slide is rinsed with distilled water and drained (this step is not mandatory because after washing with ethanol, if the slide becomes dry, one can directly go to step 8, if not, step 7 should be followed before counterstaining).
8. The smear is counterstained with Safranin for 30 sec.
9. The slide is washed with distilled water and blot-dried with absorbent paper.
10. The stained slide is left to air dry and observed under a microscope.

OBSERVATION

A large number of purple-coloured rod-shaped structures were observed. These were the cells of *Lactobacillus* which belong to Gram positive bacterial group (Fig. 2A). On the other hand pink coloured Gram negative *E. coli* bacterial cells are seen as rod shaped structures (Fig. 2B).



A **B**

Figure 2: Microscopic images showing bacterial cells after gram staining. (A) Gram-positive bacterial cell *(Lactobacillus)*; (B) Gram-negative bacterial cell *(E. coli)*

Significance of the study:

- **Screening for Harmful Bacteria**: Gram staining is essential for the initial screening of bacterial samples to determine if any harmful bacteria are present. This is particularly important in clinical settings where identifying pathogenic bacteria before initiating any medical process is crucial.
- Gram staining provides a quick and reliable method to classify bacteria into two major groups based on their cell wall composition. This classification aids in the preliminary diagnosis of bacterial infections and guides further microbiological testing and treatment decisions.
- Gram staining technique is an invaluable tool in developing a foundational understanding of prokaryotic cells. By observing the differences in staining patterns, students can grasp key concepts related to bacterial cell wall structures, functions, and the biochemical basis for Gram staining.
- **Antibiotic Selection**: The Gram reaction informs the choice of antibiotics, as Gram-positive and Gram-negative bacteria have different susceptibilities to various antibiotics. This is crucial for effective patient management and combating antibiotic resistance.

- **Study of Microbial Communities**: Gram staining helps in the study of microbial communities and their interactions within different environments, including soil, water, and the human body.

General DON'Ts –

- DO NOT overheat the smear during heat fixing, as it can distort bacterial shapes and affect staining.
- DO NOT over-decolorize with alcohol or acetone, as this can remove the crystal violet stain from Gram-positive bacteria, making them appear Gram-negative.
- DO NOT under-decolorize, as this can leave the crystal violet stain in Gram-negative bacteria, making them appear Gram-positive.
- DO NOT apply stains unevenly or excessively, as it can cause improper staining and make interpretation difficult.

QUESTIONS FOR FURTHER STUDIES

1. How would you distinguish between Gram-positive and Gram-negative bacteria under the microscope? What color and shapes would you expect to see for each type?
2. Why is it important to heat-fix the bacterial smear before staining?
3. How does the structure of the peptidoglycan layer influence the retention of crystal violet in Gram-positive bacteria?
4. How does the decolorization step differentiate between Gram-positive and Gram-negative bacteria?
5. What are the roles of each of the reagents used in Gram staining (crystal violet, iodine, alcohol/acetone, and safranin)?
6. If you observed a sample that contained both purple and pink cells, what conclusions could you draw about the bacterial population in that sample?

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Chapter 4: OBSERVATION OF HUMAN CHEEK EPITHELIAL CELLS

Recommended level: UG

INTRODUCTION

Loose cells from our body can be obtained either from shedding epithelial cells or from blood. In order to obtain a few cells without any pain from our body, cheek epithelium is the tissue of choice. The outer epithelial layer of dying and shedding cells can be easily obtained by gentle scraping with the help of a tooth pick or a brush. These cells can be easily layered on the slide and stained to view the general organization of an animal cell.

Methylene blue is a cationic basic dye used as a vital stain, enters in cells or tissues without killing (fixing). It binds strongly to negatively charged groups (stains acidic components) of the cell, like nucleus and thus serves as a good stain to quickly observe the cell shape and measuring the size of a cell or nucleus (Barbosa and Peters, 1971).

OBJECTIVE

To make a temporary stained preparation of cheek epithelial cells and to observe them under low and high power of a Student microscope

MATERIALS REQUIRED

Ethanol-soaked tooth pick, slide, methylene blue stain (2% in normal saline)

PROCEDURE

1. Dry the ethanol-soaked tooth pick in air and scrape gently the inner side of cheek. A large number of cells will come on the tooth pick.
2. Gently rub the tooth pick on slide in one direction to make a spread of cells. Dry the cells on slide so that the cells will not get washed away while staining.
3. Put a few drops of methylene blue stain and leave for 5 min. Methylene blue is a vital stain and thus even a diluted solution of stain can be easily picked up by the living cells. In this case we are not staining the living cells.
4. After 5 min of staining, rinse cells once with distilled water in such a way that complete stain is not gone and a diluted stain remains. Mount the cells in a drop of distilled water with a cover glass and observe under the bight field students' microscope.

OBSERVATION

A thin, irregular, flat sheet like squamous epithelial cells with small nucleus in each can be observed (Fig. 1). The live deeper layer of cheek cells has slightly larger nuclei.

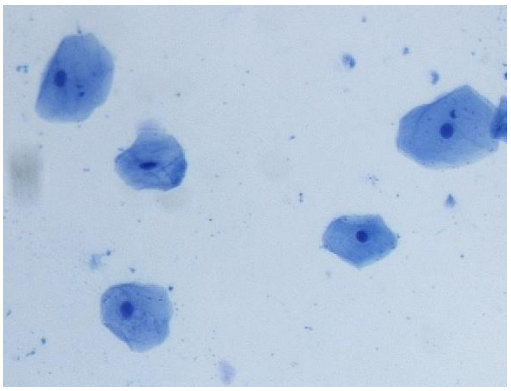

Figure 1: Methylene blue stained human cheek epithelial cells

QUESTIONS FOR FURTHER STUDIES

1. What kind of shape these cells have?
2. Did you get any idea about the thickness of the cells and how are they organized with respect to each other when you are observing several cells in a group?

REFERENCE

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Chapter 5: STAINING OF MITOCHONDRIA IN HUMAN CHEEK EPITHELIAL CELLS

Recommended level: UG, PG

INTRODUCTION

Mitochondria are considered as power houses of a cell as it produces ATP by a process called oxidative phosphorylarion. Each cell contains large number mitochondria and they can be observed under a light microscope if stained with Janus green. This stain is bluish green in colour when oxidized and colourless when reduced. When a dilute solution of the stain is applied to stain the cells, it enters in the cytoplasm as well as in mitochondria. Since mitochondrial inner membrane contains cytochrome oxidase enzyme, which can keep the stain in oxidized state, the mitochondria appear stained while in rest of the cytoplasm the stain gets reduced and thus appears colourless (Cooperstein and Lazorow, 1953; Lazorow and Cooperstein, 1953).

OBJECTIVES

To stain and visualize mitochondria in cheek epithelial cells

MATERIALS REQUIRED

Ethanol-soaked tooth pick, slide, cover glass, 0.01% Janus green B stain in normal saline (dissolve, leave for 48 h and then filter)

PROCEDURE

1. Dry the ethanol-soaked tooth pick in air and scrape gently the inner side of cheek. A large number of cells will come on the tooth pick.
2. Gently rub the tooth pick on slide in one direction to make a spread of cells. Dry the cells on slide so that the cells will not get washed away while staining.
3. Put a few drops of Janus green stain and leave for 5-10 min for staining.
4. After 5 min of staining, rinse cells once with distilled water in such a way that complete stain is not gone and a diluted stain remains. Mount the cells in a drop of distilled water with a cover glass and observe under the bright field students' microscope. The cells can alternatively be mounted in the stain itself. A few air bubbles remaining inside the cover glass give a background stain that makes the viewing easy. The slide can be observed under the high magnification of a student microscope.

OBSERVATION

Each cell is seen to contain a large number of tiny round or elongated bacteria like bodies in the cytoplasm mainly around the nucleus. Generally, they are not strongly stained thus appear like pimples on a face. Mitochondrion can be easily distinguished from a bacterium as bacterial cells become more prominently stained and appear sharper than mitochondria. Also, as bacteria are on the surface of cells, they will be focused at a slightly different level than mitochondria and can be distinguished.

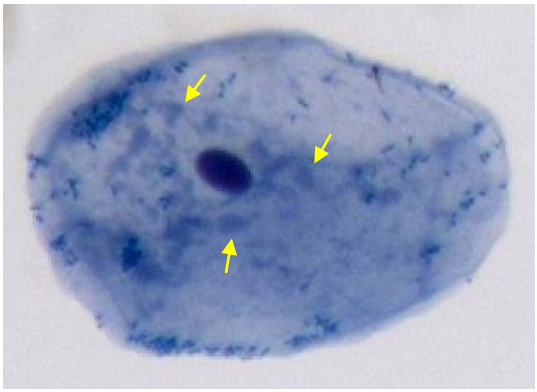

Figure 1. Janus Green stained human cheek epithelial cell showing mitochondria (a few of them shown by arrow). The fine small needle like or dotted structures on the cell, especially in peripheral region, are bacteria.

QUESTIONS FOR FURTHER STUDIES

1. Could you distinguish between mitochondria and bacteria present on the same cell?
2. Did you get any idea about where the mitochondria are located in a cell?
3. Whether all cells have a fixed number of mitochondria?

REFERENCES

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Lazorow A, Cooperstein S J (1953). Studies on the enzymatic basis for Janus Green B staining reaction. Journal of Histochemistry and Cytochemistry 1: 234

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Chapter 6: IDENTIFICATION OF DIFFERENT CELL TYPES IN MAMMALAIAN BLOOD SMEAR

Recommended level: UG, PG

INTRODUCTION

Blood is a heterogeneous mixture of different cell type bathing in the serum. The different cells types are - Red Blood Cells (RBCs), White Blood Cells (WBCs) and the Platelets.

There are 4,000,000-6,000,000 RBCs per µl of blood. Since a mammalian RBC does not have nucleus, it appears like a biconcave disc of approximately 7 µm in diameter.

WBCs are usually larger in size compared to RBCs, 12 to 15 µm in diameter and have a nucleus. Their number ranges between 5000 to 10,000 per µl of blood. They are classified into Granulocytes (cells having granular cytoplasm) and Agranulocytes (cells having non granular cytoplasm). Granulocytes are further classified into - Neutrophils (40-70% of total WBCs), Eosinophils (1-4%) and Basophils (0-1%). Agranulocytes are of two types - Monocytes (the largest WBCs, 4-8% in number) and Lymphocytes (20-45%).

Platelets are small disc shaped 1.5 to 3 µm in diameter. There are 150,000 to 400,000 platelets per µl of blood.

By using Leishman stain, which is a mixture of two stains, methylene blue and eosine, the different WBCs can be differentially identified based on the nuclear shape and the types of granules present in the cytoplasm (Mathur et al, 2013).

OBJECTIVES

To prepare a slide of stained a blood smear and to identify all the cell types of human blood.

MATERIALS REQUIRED

Slides, disposable needles, Leishman stain (0.2% in methanol or ethanol, leave for 48 h and then filter), water pH 7 (pH of water is adjusted with the help of a few drops of 1N NaOH)

PROCEDURE

1. Clean the tip of a finger with spirit-soaked cotton and allow the tip to dry.
2. Prick the finger-tip with a sterile disposable needle and press the finger for a blood drop to come out.
3. Take a small drop of blood on one side of a clean slide and with the help of the edge of another slide held at 45^{o}, make a thin smear. Allow the smear to dry for 5 to 10 min.
4. Put a few drops of Leishman stain on the blood smear and stain for 1 min. The stain should not dry.
5. Immediately after 1 min dilute the stain with equal volume of pH 7 water and allow the differential staining to occur for 10 min.
6. Rinse the slide in water (pH 7) and air dry. Observe the cells under a microscope.

OBSERVATION

The different cell types are identified under a compound microscope as described below and shown in Fig. 1:

RBCs – These cells can be easily identified as they are plenty in number, usually unstained or slightly redish stained as seen in the figure. These cells contain the respiratory pigment, hemoglobin, which receives oxygen from the lungs and carries in the dissolved form to all parts of the body. They bring back the carbon di-oxide from all body parts to the lung.

Neutrophils – They are major type of WBCs (40-70%), identified by the presence of polymorphic nuclei (violet stained nucleus can be seen in Fig A, D). They contain fine neutral granules and thus cytoplasm remains unstained. They respond first following microorganism infection, reach to the site, engulf the infecting agent and also kill them by releasing enzymes. They help the other immune cells in boosting immunity.

Eosinophils - Identified by presence of bilobed nucleus and coarse basic granules in cytoplasm that gets red stained with eosin (Fig B, E). They are very few in number (1-4%) but very prominently detected by red cytoplasmic granular staining. They function as effector cells in immune system and defends hosts against parasitic infestation. They get also involved in allergic reactions.

Basophils – They have U shaped or lobed nucleus and coarse granular acidic cytoplasm that gets bluish stained by basic stain, methylene blue (Fig C). Often not seen in blood smears due to very low in number (0-1%). They defend body from allergens, pathogens and parasites.

Monocytes - The largest WBCs, 4-8% in number, having a characteristic kidney or horse shoe shaped nucleus with no cytoplasmic granules (Fig J, K). These are major antigen presenting cells as macrophages and dendritic cells.

Lymphocytes - 20-45% in number, nucleus covering almost the entire area of the cells in small lymphocytes (Fig G, H), while a clear rim of cytoplasm (no granules) is seen in large lymphocytes (Fig I). The T-lymphocytes have roles in direct cell mediated killing of microbes and also involved in helping the B-lymphocyte response while, B-lymphocytes produce antibodies (immunoglobulins).

Platelets – Small cells usually seen in aggregates in blood film and their main function is to make a clump to form a clot that stops bleeding once a vessel is damaged.

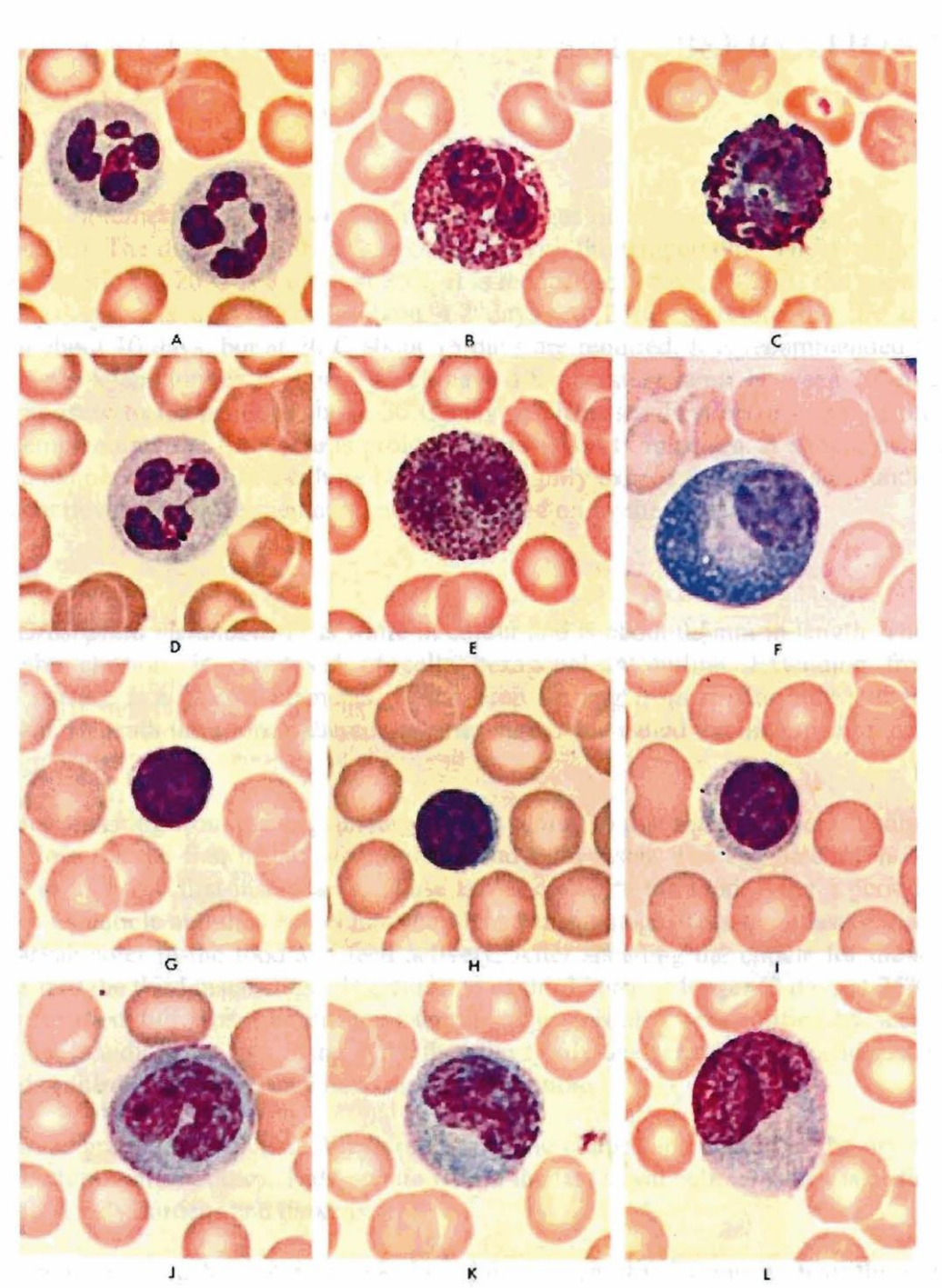

Figure 1. Human blood cells from a smear after Leishman staining. A and D, Neutrophils; B and E, Eosinophils; C, Basophil, F, Plasma cell (this is not a normal constituent of the peripheral blood but is included here for comparison with the non-granular leukocytes; G and H, Small lymphocytes; I, Medium lymphocytes; J, K, L, Monocytes (characteristic nucleus seen in J, K)

QUESTIONS FOR FURTHER STUDIES

1. One of the cell types is not seen in figure. Which cell type?
2. Can you do differential leucocytes count (DLC) using this technique? You may give a try.

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Chapter 7: CELL VIABILITY ASSAY BY TRYPAN BLUE EXCLUSION

Recommended level: UG, PG

INTRODUCTION

The dye, Trypan blue is not permeable through intact cell membrane and thus do not stain the live cells, however it stains the dead cells as the membrane of the dead cell is not intact. This is also called as dye exclusion, because live cells exclude the dye and remain unstained.

Determining the approximate number of viable cells by dye exclusion involves mixing an aliquot of cells with a volume of buffer or balanced saline containing a water-soluble (membrane lipid-insoluble) dye (trypan blue) that is visible when it leaks into cells that have damaged plasma membranes.

OBJECTIVE

To estimate live cell population in a cell suspension.

MATERIALS REQUIRED

Any type of single cell suspension in PBS, 0.4% trypan blue in PBS.

PROCEDURE

1. Make single cell suspension of any cell type in PBS in a tube.
2. Add equal volume of 0.4% trypan blue prepared in PBS to the cell suspension.
3. Immediately take a drop of cell suspension on slide, cover with a cover glass and observe under a microscope. Mixing of trypan blue to the cell suspension should be done just before making the observation otherwise after a while (2-3 min) even the live cells start dying and showing blue colour.
4. If cell counting is to be done, place the cover glass on the hemocytometer first, transfer a small amount of trypan blue with immediately mixed cell suspension cell suspension to both the chambers of the hemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action. Do not overfill or under fill the chambers.
5. Starting with chamber 1 of the hemocytometer, count all the cells in 1 mm central square and four 1 mm corner squares. Keep a separate count of viable and non-viable cells. Similarly count stained and not stained cells in chamber 2.

   Each large square of the hemocytometer, with cover glass in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1ml, the total number of cells per ml will be determined using the following calculations:

   Cells/ml = average cell count per square x dilution factor x 10^4;

   Total cells = cells/ml x the original volume of fluid from which the cell sample was removed;

   % Cell viability = Total viable cells (unstained)/total number of cells X 100.

OBSERVATION

Live cells will not be stained while dead cells will be brightly stained and they can be counted using a haemocytometer (as shown in Fig. 1) within 2-3 min. In hemocytometer each large square, with cover

glass in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1ml, the total number of cells per ml will be determined using the following calculations:

Cells/ml = average cell count per square x dilution factor x 10^4

% Cell viability = Total viable cells (unstained)/total number of cells X 100

Total cells in the sample = cells/ml x the original volume of fluid from which the cell sample was removed

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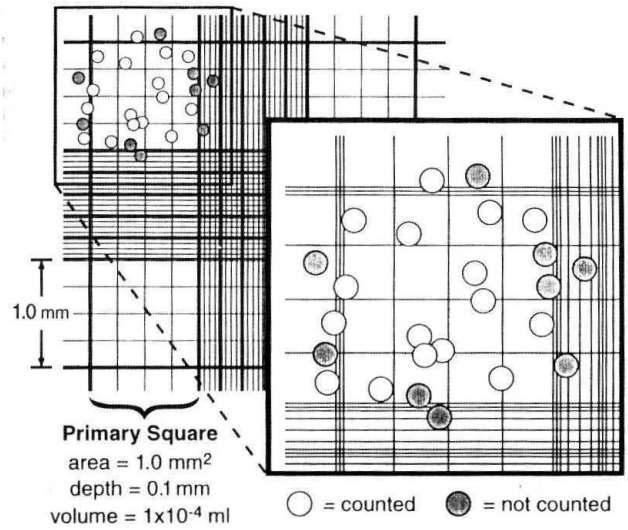

Figure 1. Counting of viable cells using a hemocytometer

QUESTIONS FOR FURTHER STUDIES

1. What is the advantage of using this method over other available methods?
2. Can Trypan blue be considered as a vital dye?

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Chapter 8: STUDY OF MITOSIS IN ONION ROOT TIP CELLS

Recommended level: UG, PG

INTRODUCTION

Onion root tip has meristematic tissue just behind the root cap, hence this serves as a good material for studying various stages of mitosis (Vanderlyn, 1948; González-Fernández et al, 1968). The roots can be easily grown if an onion is placed (after shaving its disc) on a water filled conical flask in such a way that the onion disk touches water, or else making the onion to sit on wet sand in such a way that the bulb is buried partially in sand. In two days nearly 1 cm long roots develop, which can then be cut, fixed and stored. Longer grown roots are not good as it will have more of non-dividing tissue.

OBJECTIVES

To make a temporary squash preparation of onion root tip cells and to study the different stages of mitosis.

MATERIALS REQUIRED

Onion root tips, 1N HCl, 1:3 aceto-methanol fixative, 70% and 90% ethanol, 2% acetocarmine stain (2 g of carmine mixed with 100 ml of 45% acetic acid and boiled using a reflux condenser for 1 h to dissolve carmine), 45% acetic acid, slide, cover glass, sealing wax or nail polish.

PROCEDURE

Fixation of root tips:

1. Fix the freshly cut ~1 cm long root tips in aceto-methanol fixative for overnight in a specimen tube.
2. Remove fixative and add 90% ethanol, leave for 2 h.
3. Decant 90% ethanol and add 70% ethanol. The root tips can be stored in 70% ethanol for a long period of time if the tube is tightly closed. Storing at 4^{o}C is even better.

Staining and making squash preparation:

1. Treat the root tips with 1N HCl for 1 min. This will soften the cell wall.
2. Rinse the tips once in water, transfer to acetocarmine stain and stain for 30 min.
3. Take a drop of 45% acetic acid on slide, place a root tip on the drop, leave for 1-2 min. If acetic acid drop becomes coloured, it can be decanted and a fresh 45% acetic acid drop can be added.
4. Place a cover glass on the root tip and squash it using a rubber-end pencil under the folds of a blotting paper.
5. Seal the edges of the cover glass with molten wax or with nail polish immediately to prevent drying of acetic acid film and entry of air bubbles.
6. The slide is ready for observation under a microscope.

OBSERVATIONS

Different phases of mitosis are as follows (also can be observed in the Fig. 1 below):

Prophase – The first phase of mitotic division where chromatin condensation begins. The already divided centrosomes start moving apart due to polymerization of tubulin dimers elongating the microtubules. The nucleolus starts disappearing as the rRNA synthesis ceases. The nuclear membrane also starts fragmenting into small vesicles due to phosphorylation of nuclear lamins, these fragments (vesicles) remain associated with the chromosomes,

Metaphase – Chromosomes reach to the maximum level of condensation, the two chromatids of each chromosome appear clearly and as the nuclear membrane disappeared, the centromeric region (kinetochore) having high affinity for microtubules, get associated with the + end of the microtubule. The kinetochores of each chromosome attached to microtubules from both the poles get oriented such that that the kinetochore of one chromatid faces one pole while that of another chromatid faces the opposite pole. All chromosomes associated with microtubules (spindle fibers) appear to be present in a circular plane called as equatorial plane.

Anaphase – The depolymerization of microtubules begins mainly at + end. The shortening of microtubules exerts a pulling force that allows the two chromatids of a chromosome to start their movement towards opposite poles.

Telophase – The two chromatids of each chromosome reached the opposite pole. The fragmented nuclear membrane in the form of vesicles that remained associated with the chromatids, start getting reassembled due to dephosphorylation of nuclear lamins, thus nuclear membrane reappears. The chromatids start decondensing and cytokinesis occurs, thus two daughter cells get separated.

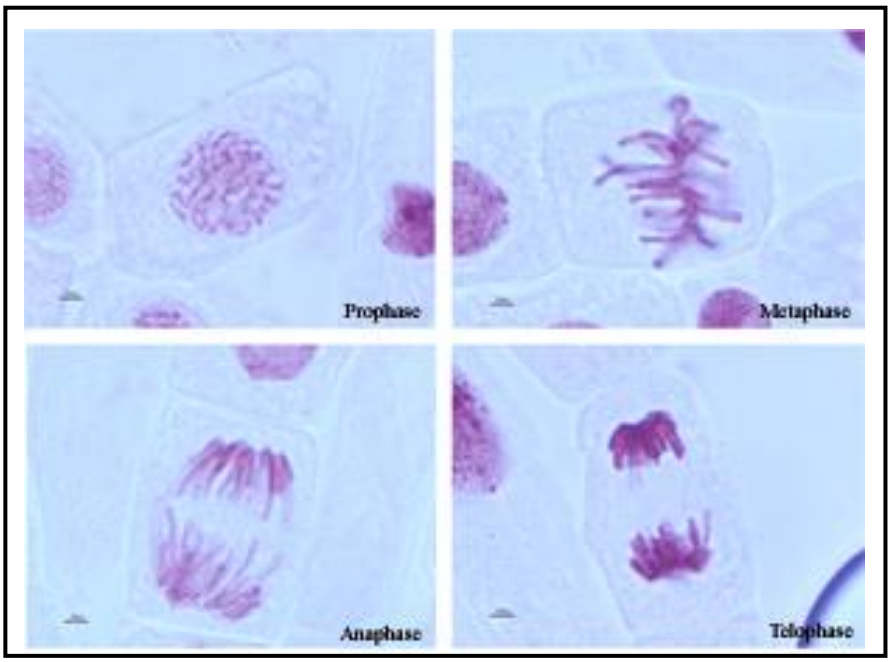

Figure 1. The different phases of mitosis seen in cells of onion root tip

QUESTIONS FOR FURTHER STUDIES

1. Did you observe any cell which is in interphase? If yes, what features did you observe there?
2. What will happen if a mitotic poison, colchicine is given to a batch of these cells before fixation and to another batch after fixation?

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Chapter 9: STUDY OF THE EFFECT OF COLCHICINE ON MITOSIS IN ONION ROOT TIP CELLS

Recommended level: UG, PG

INTRODUCTION

Colchicine is very well known as mitotic poison which inhibits tubulin polymerization causing a block on spindle fiber assembly and thus cell division remains blocked at metaphase stage. The chromosomes are most condensed at this phase and thus chromosome morphology can be best studied at this stage.

OBJECTIVE

To study the effect of colchicine on mitosis in onion root tip cells.

MATERIALS REQUIRED

Onion root tips, 0.05% colchicine in water, 1N HCl, 1:3 aceto-methanol fixative, 70% and 90% ethanol, 2% acetocarmine stain, 45% acetic acid, slide, cover glass, sealing wax or nail polish.

PROCEDURE

1. The roots can be easily grown if an onion disc is a bit shaved and is placed on a water filled conical flask is such a way that the onion disk touches water or else making the onion to sit on wet sand in such a way that the bulb is buried partially in sand. In a day time nearly 0.5 cm long roots develop.
2. Take one onion out from sand, wash the roots and place the onion on a small container containing 0.05% colchicine in such that the roots are now exposed to colchicine. Let it be exposed to colchicine for 4 h to overnight.

Staining and making squash preparation:

1. Cut the root tips and treat them with 1N HCl for 1 min. The onion which is not exposed to colchicine (control), also taken out and the roots are cut and treated with 1N HCl for 1 min.
2. Rinse the tips once in water, transfer to acetocarmine stain and stain for 30 min.
3. Take a drop of 45% acetic acid on slide, place a root tip on the drop, leave for 1-2 min. If acetic acid drop becomes coloured, it can be decanted and a fresh 45% acetic acid drop can be added.
4. Place a cover glass on the root tip and squash it using a rubber-end pencil under the folds of a blotting paper. Seal the edges of the cover glass with molten wax or with nail polish immediately to prevent drying of acetic acid film and entry of air bubbles.
5. The slide is ready for observation under a microscope.

OBSERVATIONS

Different phases of mitosis can be observed in control samples while only metaphases or randomly dispersed chromosomes in cells but no anaphases will be observed in the colchicine treated samples.

QUESTIONS FOR SELF STUDY

1. What do you understand by control sample? Why is this essential?

2. What will happen if you forget to add colchicine in your experimental sample?
3. Name some other drugs that has similar function. How do they function?

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Chapter 10: STUDY OF DIFFERENT STAGES OF MEIOSIS IN GRASSHOPPER TESTIS CELLS

Recommended level: UG, PG

INTRODUCTION

Grasshopper testis is an ideal material for studying various stages of meiosis (Perry and Jones, 1974). Grasshopper is of good choice because it is easily available in lawns and fields, males can be easily distinguished from female and testis is easy to dissect. In addition, it has fewer number of chromosomes (locally available species contain 17 or 19 or 21 chromosomes in males; odd number of chromosomes due to XX/XO sex chromosome system) and all chromosomes are of one type, i.e., acrocentric, facilitating unambiguous identification of division stages (Taylor, 1965).

OBJECTIVE

To make stained temporary squash preparation of grasshopper testes and to observe the different phases of meiosis.

MATERIALS REQUIRED

Male grasshopper, insect saline (0.67% NaCl), 1:3 aceto-methanol fixative, 70% and 90% ethanol, 2% acetocarmine stain (2 g of carmine mixed with 100 ml of 45% acetic acid and boiled using a reflux condenser for 1 h to dissolve carmine), 45% acetic acid, slide, cover glass, sealing wax or nail polish.

PROCEDURE

Fixation of grasshopper testes:

1. Hold a male grasshopper in hand, give a small incision with scissors at the junction of thorax and abdomen and press the abdomen gently. The testes covered in yellow fat bodies will pop out. Dissect them out and put in insect saline. Remove yellow fat with the help of forceps as much as possible. A pair of testes (each having a bunch of white tubules) will be seen.
2. Transfer the tubules in a tube and fix in aceto-methanol fixative, close the tube and leave for overnight.
3. Remove fixative and add 90% ethanol, leave for 2 h.
4. Decant 90% ethanol and add 70% ethanol. The testes can be stored in 70% ethanol for a long period of time if the tube is tightly closed. Storing at 4^{o}C is even better.

Staining and making squash preparation:

1. Stain the fixed testis in acetocarmine for 30 min.
2. Take a drop of 45% acetic acid on slide, place a few (2 to 3) tubules of testis in the drop, leave for 1-2 min. If acetic acid drop becomes coloured, it can be decanted and a fresh 45% acetic acid drop can be added.
3. Place a cover glass on the tubules and squash using a rubber-end pencil under the folds of a blotting paper.
4. Seal the edges of the cover glass with molten wax or with nail polish immediately to prevent drying of acetic acid film and entry of air bubbles.

5. The slide is ready for observation under a microscope.

OBSERVATIONS

Different phases of meiosis can be observed in the Fig. 1. Also, a spermatogonial (mitotic) metaphase can be seen in Fig. 2.

Division I (reductional division)

Division I have been divided into four phases:

Prophase I (takes a longer period and has been divided into five different sub-phases for convenience in understanding the processes occurring – Leptotene, Zygotene, Pachytene, Diplotene and Diakinesis)

Metaphase I

Anaphase I

Telophase I

Division II (equational division)

Division II has been divided into four phases:

Prophase II

Metaphase II

Anaphase II

Telophase II

Leptotene (thin thread) – The first phase of meiosis when chromosomes started condensing and becomes visible as thin thread.

Zygotene (paired thread) – Homologous chromosomes pair from one end to the other, point by point helped by synaptonemal complex. Chromosome condensation also continues. Thus, at this stage the chromosome threads are slightly thicker and their number got reduced to half as they are paired.

Pachytene (thick thread) – Chromosome condensation continues, so the paired threads appear more thicker. Crossing over, i.e., cutting and joining of the two chromosomes occur at recombination nodules (appear as beaded structure on synaptonemal complex) in this stage. Continuous condensation drives separation of the paired chromosomes at both the ends towards the end of this sub-phase.

Diplotene (double thread) – Chromosome condensation continues and the separation of paired chromosome continues, remaining together at crossing over points or chiasmata. The two chromatids of each chromosome also become visible, more so at chiasmata points.

Diakinesis (going apart) – Chromosome condensation still continues and that gives a force for chiasmata to move to the terminal end (terminalization of chiasmata). As the two chiasmata touch each other, they get resolve into one, and thus chiasmata number reduces. Paired chromosomes maximally separated, only remaining associated at one or two or with a few chiasmata gives various shapes like a plus or a circle or alpha. Spindle fibers are formed by the end of this sub-phase and nuclear membrane becomes fragmented and remain in the form of vesicles associated with chromosomes. This ends the prophase I.

Metaphase I – Paired chromosomes with terminalized chiasmata gets associated with the spindle fibers (microtubules) in such a way that kinetochores of one chromosome (with two chromatids) finds the microtubules of one pole while, its homologue gets associated with the microtubules of the opposite pole. Chromosome condensation is maximum at this stage.

Anaphase I – The paired chromosomes are separated due to pulling force generated by depolymerization (shortening) of microtubules and the homologues move to opposite poles, thus each pole receiving half the number of chromosomes. As all the chromosomes of grasshopper are acrocentric, the chromosomes moving to opposite poles appear as V shaped.

Telophase I – Half the number of chromosomes reached at each pole, starts decondensing a bit and nuclear membrane appears due to fusion of the nuclear membrane fragments (vesicles) with the dephosphorylation of nuclear lamins.

Prophase II – A very short prophase to organize the spindle fibers, to condense the chromosomes and to allow disappearance of nuclear membrane. No DNA replication prior to this phase occurs but only duplication of centrosomes occurs in a very short interphase or at the beginning of this prophase.

Metaphase II - The half number of chromosomes get associated with spindle fibers in such a way that the kinetochores of the two chromatids face opposite pole.

Anaphase II – The chromatids move to opposite poles due to shortening of the microtubules. As the chromosomes are acrocentric in grasshopper, each chromatid appears like I or rod shaped.

Telophase II – The chromatids reached the opposite poles, nuclear membrane reappears, cytokinesis occurs. This way four haploid daughter cells formed at the end of Division II from one diploid cell that had entered in Division I.

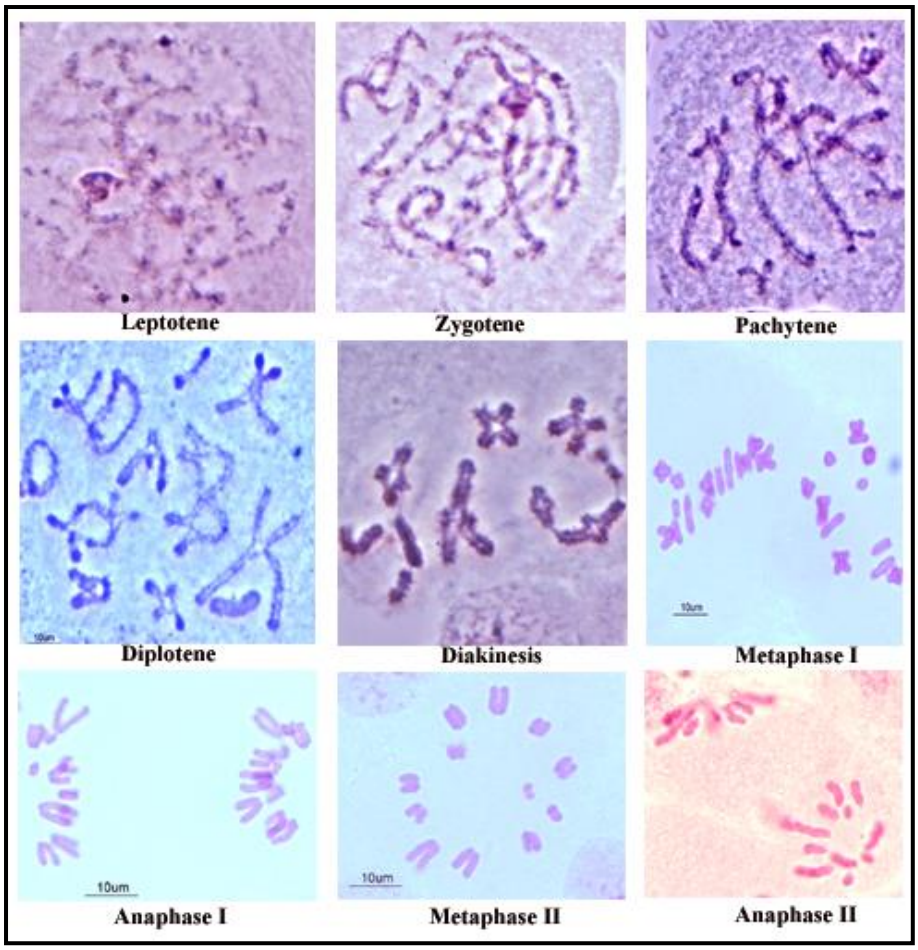

Figure 1. Different stages of meiosis seen in grasshopper testis

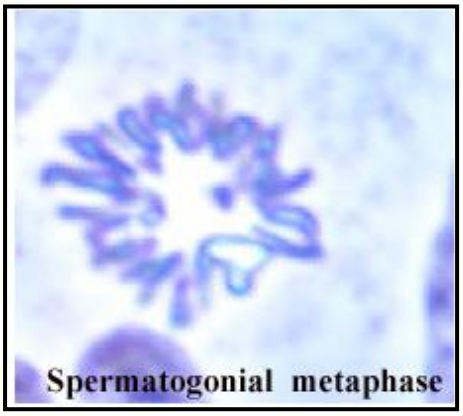

Figure 2. A mitotic spermatogonial metaphase seen in grasshopper testis

QUESTIONS FOR FURTHER STUDIES

1. How did you see a mitotic metaphase in testis?
2. Can you line draw the chromosomes and explain all the shapes that you observed in different phases in figure or on your slide?
3. When exactly the chromosome number became half?
4. Can you do an exercise to represent chromosome number (N) and DNA content (C) at each phase of meiosis?
5. Can you make a correlation of the results obtained in a monohybrid cross with the meiosis that you studied?

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Taylor J H (1965). Distribution of tritium labelled DNA among chromosomes during meiosis: I. Spermatogenesis in the grasshopper. Journal of Cell Biology 25, 57-68

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Chapter 11: HAEMATOXYLIN SQUASH TECHNIQUE FOR PLANT AND ANIMAL CHROMOSOMAL PREPARATIONS

Recommended level: UG, PG

INTRODUCTION

Haematoxylin is a nuclear stain, widely used to stain nuclei in tissue sections. This stain has also been used successfully in chromosomal squash preparations. These chromosomal preparations are superior to those obtained employing other procedures, namely those stained with aceto-carmine or aceto-orcein. Chromosomes appear blue with a clear background, as the cytoplasmic basophilia is removed through hydrolysis. The preparations do not fade even after two years of storage, and the chromosomes retain the blue colour.

OBJECTIVE

To study mitotic and meiotic cell divisions in plant and animal tissue using Haematoxylin stain.

PROCEDURE

Studying mitosis in onion root tip cells

1. Root meristematic tissues are fixed in a mixture of methanol and glacial acetic acid (3:1) for overnight.
2. Fixed roots are brought in 90% ethanol for 1-2 h and then transferred to 70% ethanol.
3. Rinsed in water and then hydrolyzed in 1N HCl at 60° C for 10 - 12 min.
4. Washed in distilled water and then treated with 4% solution of iron alum (ferric ammonium sulphate) for 15 min, after which they are repeatedly washed in distilled water to remove the traces of the mordant.
5. Stained with 0.5% solution of haematoxylin for 20 - 25 min.
6. The stained roots are washed thoroughly in water.
7. Each root tip is softened with 45% acetic acid and teased into fragments in a drop or two of 45% acetic acid on a slide and squashed under a coverslip. The coverslip is then sealed with paraffin wax to obtain a temporary preparation.

Note: Roots may be treated with colchicine, if a large number of metaphases are to be examined from the meristematic region.

Studying meiosis in grasshopper testis cells

1. Testes from grasshopper is dissected in Ringer's solution and is fixed in mixture of methyl alcohol and glacial acetic acid (3:1) for 24 h
2. They are brought in 90% ethanol, kept for 1 – 2 h and then transferred and stored in 70% ethanol.
3. The tissue is hydrolysed in 1N HCl at 60ºC for 6 min.
4. After thorough washing in distilled water, the tissue is treated with 4% iron alum (Ferric Ammonium sulphate) for about 25 min.

5. It is washed repeatedly in distilled water and stained with 0.5 % solution of haematoxylin for 30 min.
6. The tissue is teased into small fragments in a drop or two of 45% acetic acid on a slide and squashed under a coverslip.
7. The preparation is sealed with paraffin wax or nail polish to have a temporary preparation.

OBSERVATION

All the different stages of mitosis and meiosis are seen under the microscope.

QUESTIONS FOR FURTHER STUDIES

1. If you know the chromosome number of a given organism, by observing an anaphase plate can you identify whether the plate is of a mitotic anaphase or anaphase I or anaphase II?
2. Your aim is to study all the phases of meiosis of a given organism. If you have treated the cells with colchicine for 1 h prior to fixation and staining, how successful your studies will be?

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Chapter 12: PREPARATION OF METAPHASE CHROMOSOMES FROM BRAIN GANGLIA OF *DROSOPHILA* LARVAE

Recommended level: UG, PG

INTRODUCTION

Dipteran insects contain two types of cells at larval stages of development, one those get histolysed at the end of larval period, and the other that do not get histolysed. The tissue those get histolysed, stop their division during third instar larval period. Brain is the tissue of the second type that does not get histolysed, keep dividing during larval stages and thus the tissue of choice for mitotic chromosome preparations (Kaufman, 1934).

MATERIALS REQUIRED

Healthy late 3rd instar larvae, droppers, dissecting needles and fine forceps, cavity block/Maximov slide, clean glass slides, cover glasses, stereo-binocular microscope, cloth for cleaning slides, diamond marker, marker-pen, slide box, microscope for examination of preparations.

Solutions

Poels' Salt Solution (pH 6.8)

NaCl	86 mg
KCl	3l3 mg
$CaCl_2.2H_2O$	ll6 mg
$NaH_2PO_4.2H_2O$	88 mg
$KHCO_3$	l8 mg
$MgSO_4.7H_2O$	5l3 mg
Distilled H_2O to make	l00 ml

Adjust pH to 7.0 with 1 M NaOH and filter.

Hypotonic solution: 0.67% tri-sodium citrate

Fixative: Aceto-Methanol 1:3 - freshly prepared

60% acetic acid

Giemsa stain

Stock solution -	Giemsa Powder	380 mg
	Methanol	25 ml
	Glycerol	25 ml

Leave overnight at 37°C. Filter the stain and store.

Working solution -	Stock solution	2.5 ml
	Methanol	1.5 ml
	Giemsa water	50.0 ml

Giemsa water -	0.2 M Na_2HPO_4	80 ml
	Distilled water	800 ml

Adjust pH to 6.8 with 0.1 M Citric acid (roughly 24 ml) and then make up the volume to l000 ml.

DPX mountant

PROCEDURE

1. Take late 3rd instar larvae (about 5 days old if grown at 24ºC) from a healthy culture (late 3rd instar larvae crawl out of the food medium and move actively on food-free surface), wash them free of adhering food particles with water and transfer to a slide/cavity slide containing a small amount of Poels' salt solution (simple insect saline/Ringer's solution can also be used).

2. Using fine forceps and/or dissecting needles, pull forward the mouth parts of a larva to rupture its skin. This forces out internal organs. Insect brain contains a pair of dorsal lobes and a ventral lobe and this is found near the place where the two salivary glands (prominent structures seen as a pair of whitish translucent elongated structures) are connected at their anterior ends with a common salivary duct. Brain lobes are associated with a large number of disc shaped structures, called imaginal discs from which most of the adult epithelial structures, like eye, antenna, proboscis, legs, wings, halteres, etc., are formed. The associated imaginal discs can be easily separated out (if needed), with the help of a pair of needles.

3. Using tips of the dissecting needles, transfer the cleaned brain to Poels' salt solution on a clean cavity block or Maximov slide. Add colchicine 1 µg/ml to brain ganglia in the Poels' solution and leave for 1 h for the dividing cells to get blocked at metaphase stage. After 1 h replace Poels' salt solution with hypotonic solution and incubate at room temperature (24ºC) for 30 min. Hypotonic treatment helps in cells to swell and makes the chromosomes to move apart from each other Hypotonic treatment time can be standardized according to lab temperature and other conditions.

4. Drain out hypotonic solution and add drops of freshly prepared fixative. Fix the tissue for 15 min at room temperature. Change the fixative once and leave for 5 min. In first fixation step still some water, i.e., drops of hypotonic, remain, hence a second time fixation is required to completely remove water and to preserve best cell/chromosome morphology.

5. Take a drop of 60% acetic acid on a clean slide and transfer a brain complex in that drop. After 1 min the tissue will become transparent and the cells will get gradually dissociated. The tissue can be smashed gently with a needle or forceps to make single cell suspension (pre heating of 60% acetic acid to 50ºC and heating the slide under an ordinary 40W bulb light or on a 50ºC hot plate while smashing also helps in getting better suspension). Now pour the fixative slowly on slide from all the sides of the cell suspension in acetic acid drop. The cells will get adhered to the slide in the form of a ring where fixative touched the acetic acid drop. Acetic acid drop can be drained and the slide can be air dried.

6. Stain the slide in Giemsa stain for 10 min. Rinse the slide in tap water twice and air dry. The stained slides after complete drying can be mounted with a cover glass on a drop of DPX and can be observed under a Student compound microscope.

OBSERVATIONS

Drosophila melanogaster contains 4 pairs of chromosomes (a pair of first chromosomes or sex chromosomes [acrocentric XX in females and XY in males, Y-chromosome is submetacentric], a pair of metacentric chromosome 2, a pair of metacentric chromosome 3 and a pair of small dot like chromosome 4). All of them can be clearly visualized in a metaphase plate (Henderson, 2004) as seen in the Fig. 1.

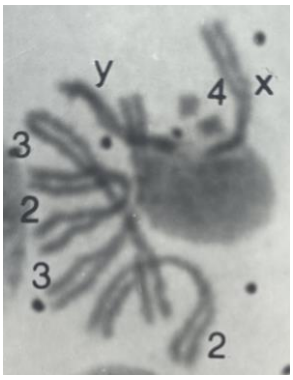

Figure 1. A metaphase plate from brain ganglion cell of *Drosophila melanogaster*

QUESTIONS FOR FURTHER STUDIES

1. Which chromosome pair is designated as Chromosome 1?
2. How do you classify chromosomes as metacentric, submetacentric and acrocentric?

REFERENCES AND FURTHER READING

Henderson D S (2004). The chromosomes of *Drosophila melanogaster*. In: *Drosophila* Cytogenetic protocols, Ed. D S Henderson, Humana Press, Totowa

Kaufmann B P (1934). Somatic mitosis of *Drosophila melanogaster*. Journal of Morphology 56, 125-155

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Chapter 13: PREPARATION OF METAPHASE CHROMOSOMES FROM RAT BONE MARROW CELLS

Recommended level: UG, PG

INTRODUCTION

The marrow from long bones, like the limb bones, provides the most-handy material for analysis of karyotype of small animals (Dowd et, 1968). In animals like snakes, which do not have limbs, ribs can provide the marrow. Bone marrow also provides useful material in diagnosis of leukemia. The present technique of air-dry chromosome preparation can be used for other soft tissues, viz., spleen. liver, kidney and gills. This is useful in cases where marrow may be difficult to obtain, e.g., fish, new born mammals, etc. Most importantly, bone marrow cells can be incubated in vitro for at least one cell cycle without any exogenous mitogenic stimulation. Thus, these cells may be used for certain in vitro experiments (mutagenesis, cell metabolism, cell cycle) as very accurate representative of in vivo conditions. Such preparations can be made even in field, where power supply is not available. A hand centrifuge and a burner can substitute for incubator and power-driven centrifuge.

In bone marrow cells or even in other tissues all the cells are not actively dividing under normal conditions. The frequency of dividing cells in a cell population (number of dividing cells per 100 cells) is represented as mitotic index. In certain disease conditions, like cancers, many more cells enter in the division cycle and thus a large number of dividing cells are seen under the microscope or in other words the mitotic index becomes high. Thus, this index serves as one of the important prognostic markers in diseases with high or low cell proliferation and also helps in assessing the efficacy of drug treatments.

The protocol used in preparing mitotic chromosomes often lose one or more chromosomes. Sometimes some of the chromosomes fall some distance apart from the whole bunch of chromosomes. Therefore, counting chromosome number of one plate may not give the actual representation of the chromosome number of the given species, if it is not already known. Hence chromosome number from a large number of plates are counted to establish the chromosome number. The details are given in the observation section.

OBJECTIVE

To make permanent slide of metaphase chromosomes from bone marrow cells of rat/mouse, to calculate mitotic index and to establish the chromosome number of the given species.

MATERIALS REQUIRED

Rat or mouse, dissection set, centrifuge tubes (15 ml graduated), Pasteur pipettes, agitator, syringe (5 ml) and needle (#22, #18), incubator (37-38^{o}C), clinical centrifuge
Colchicine (0.1 mg/ml in normal saline),
Hypotonic Solution: 0.56% KCl (should be kept pre-warmed to 37^{o}C before use)
Fixative: Glacial acetic acid and methanol 1:3
Giemsa stain

Stock solution -	Giemsa Powder	380 mg
	Methanol	25 ml
	Glycerol	25 ml

Leave overnight at 37^{o}C. Filter the stain and store.

Working solution -	Stock solution	2.5 ml
	Methanol	1.5 ml
	Giemsa water	50.0 ml
Giemsa water -	0.2 M Na_2HPO_4	80 ml
	Distilled water	800 ml

Adjust pH to 6.8 with 0.1 M Citric acid (roughly 24 ml) and then make up the volume to l000 ml

PROCEDURE

1. Inject colchicine (0.2 mg/kg body weight) to the animal 2-3 h prior to sacrifice. Colchicine inhibits microtubule assembly and thus those cells which reach at metaphase cannot proceed to anaphase
2. Sacrifice by cervical dislocation and dissect out femur bone (any long bone will do)
3. Cut both ends of the bone. Take about 0.5 ml of prewarmed hypotonic solution in a 5 ml syringe (with #18 needle for rat and #22 for mouse) and by inserting through one of the cut ends of the bone flush the marrow into a centrifuge tube
4. Agitate the marrow with a rubber agitator so that it breaks into a uniform cell suspension. Make up the volume with the pre-warmed hypotonic solution to 10 ml and keep in incubator (37°C) for 25-30 min. This treatment makes the cells to swell and makes the chromosomes to move apart from each other
5. Centrifuge the cell suspension (1,000 rpm) for 5 min to obtain a cell pellet at the bottom of the tube. Discard the supernatant
6. Fix the cells by adding the fixative drop-by-drop (upto about 0.5 ml) and agitating the tube to make a suspension. Make up the volume to 10 ml with the fixative. Keep for 15 min. In this fixation step still some water, i.e., drops of hypotonic, remain, hence a second time fixation is required to completely remove water and to preserve best cell/chromosome morphology.
8. Centrifuge the cell suspension (1,000 rpm) for 5 min to obtain a cell pellet again at the bottom of the tube. Discard the supernatant and resuspend cells in a small volume of fixative (0.3 - 0.5 ml).
9. Using a Pasteur pipette place 2-3 drops of fixed cell suspension on a clean slide and gently flame it. Fleming helps in charring the cytoplasm, which subsequently gets removed during staining and water rinsing and thus show a clean chromosome preparation.
10. After drying, stain the slide with Giemsa for 3-4 min and following 2 rinses in distilled water (pH 6.8-7.2) or clean tap water, air dry the slide. When completely dried, mount with DPX mountant, using a large cover glass.
11. To calculate mitotic index, observing the slide in low power count the number of interphase nuclei and number of metaphases observed in one area of the slide in one focus. Now change the area (random area selection such that the same nuclei are not counted again) and again take similar observation until a total of approximately 1000 nuclei are counted. Tabulate the observation and calculate mitotic index as follows:

$$\frac{\text{Number of metaphases observed}}{\text{Total number of nuclei observed including the metaphases}} \times 100$$

12. To establish chromosome number in the given species, count the number of chromosomes from at least 20 well spread plates in 40X objective and plot a histogram taking different chromosome

numbers observed on X-axis and the frequency of each number observed on Y-axis. The chromosome number with highest frequency is taken as chromosome number of that species.

Note: To begin with, prepare only one slide and observe under the microscope to judge the density of cell suspension (an unstained slide can be examined for this purpose). If there are too many cells on the slide, dilute the suspension by adding more fixative. If the cells are too few, re-spin the tube and suspend the cells in a smaller volume of fixative.

OBSERVATION

A well spread metaphase plate with countable chromosomes is shown in Fig. 1 below:

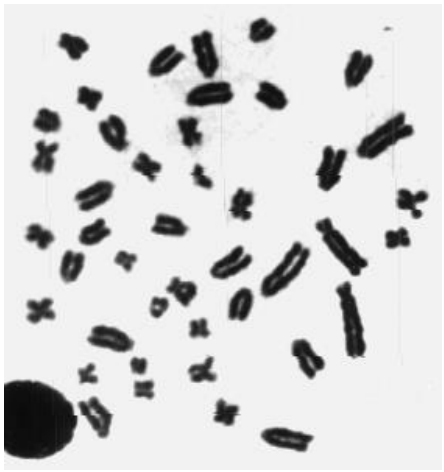


Figure 1. A metaphase spread from bone marrow of Rat

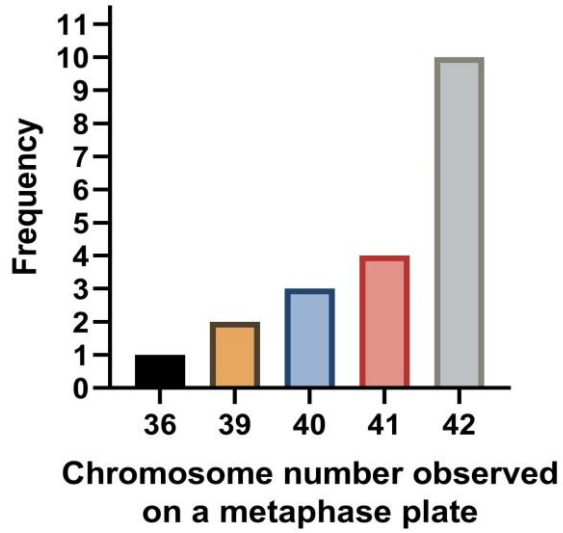
The observations on mitotic index (Table 1) and chromosome count (Table 2) hypothetical number given here) are presented in the Table below:

Table 1. Mitotic index

Area of focus in slide	Number of metaphases	Number of nuclei including the metaphases	Mitotic index
1	2	200	
2	3	300	
3	4	250	
4	1	250	
Total	10	1000	10/1000 X 100 = 1%

Table 2. Chromosome number count based on 20 observed plates

Number of chromosomes counted on metaphase plate	Frequency
36	1
39	2
40	3
41	4
42	10



Bar diagram drawn based on the observation to establish the chromosome number of the given species

Chromosome number of the given species, *Rattus norvegicus*, established as 42 as this number was observed in highest frequency.

QUESTIONS FOR FURTHER STUDIES

1. Why do we observe varying chromosome number in different metaphase plates?
2. Can we not make chromosome preparation from rat blood, so that we would have saved the rat?
3. Why do we use centrifugation to collect cells after every step of treatment?

REFERENCE

Dowd G, Dunn K, Moloney W C (1968). Chromosomal studies in normal and leukemic rats. Blood 23, 564-571

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Chapter 14: G and C- BANDING OF METAPHASE CHROMOSOMES

Recommended level: PG, Research

INTRODUCTION

Chromosomes as seen at metaphase stage appear uniformly stained all through their length. However, they are made up of different structural compartments. These compartments or domains manifest various structural and functional attributes of chromatin. The most obvious distinct domains are EUCHROMATIN and HETEROCHROMATIN. At interphase, euchromatic regions of chromatin decondense while heterochromatic regions remain condensed. In genetic terms, while euchromatin comprises potentially active parts of genome, heterochromatic regions are generally transcriptionally inert. In structural terms, heterochromatin is generally enriched in highly repeated base sequences. Euchromatin, in contrast, harbors unique sequences of DNA. The euchromatin, however, is structurally not uniform. Through its length, different structurally as well as functionally distinct domains are encountered; these domains are constant for any given species. These domains, though invisible in routinely stained metaphase chromosomes, can be resolved if the chromosomes are treated with certain agents like trypsin. Trypsin treated chromosome preparations elicit transverse bands on chromosomes following staining with Giemsa. They are called "G-bands". Heterochromatin region, on the other hand, can be distinctly visualized by treating chromosome preparations first with a denaturing agent (e.g., an alkali) and then with saline-sodium citrate solution (SSC) followed by Giemsa staining. The darkly stained heterochromatin regions in such preparations are called "C-bands".

Besides unraveling certain aspects of chromosome structure and function, these techniques have been particularly useful in clinical cytogenetics and evolutionary studies.

A. G-BANDING OF METAPHASE CHROMOSOMES

MATERIALS REQUIRED

Good chromosome preparations, 1 ml Trypsin (30 mg/ml in 0.9% NaCl, stored at –20°C), 0.9% NaCl (stored at 4°C), phosphate buffer (stored at 4°C), Giemsa stain, distilled water

Phosphate buffer

Equal parts of solution 1 and solution 2

Solution 1 : 9.073 g KH_2PO_4 in 1000 ml distilled water

Solution 2 : 11.87 g $Na_2HPO_4.2H_2O$ in 1000 ml distilled water

Giemsa stain

Stock solution -	Giemsa Powder	380 mg
	Methanol	25 ml
	Glycerol	25 ml

Leave overnight at 37°C. Filter the stain and store.

Working solution -	Stock solution	2.5 ml
	Methanol	1.5 ml
	Giemsa water	50.0 ml
Giemsa water -	0.2 M Na_2HPO_4	80 ml
	Distilled water	800 ml

Adjust pH to 6.8 with 0.1 M Citric acid (roughly 24 ml) and then make up the volume to l000 ml.

PROCEDURE

1. Thaw 1 ml of trypsin stock solution and make up to 50 ml with 0.9% NaCl in a Couplin jar. Adjust pH to 7.5-7.8 using pH paper (usually 1-2 drops of 1 M NaOH required).
2. Add 50 ml phosphate buffer to a second Couplin jar.
3. Prepare another Couplin jar containing 7-10% Giemsa stain in phosphate buffer.
4. Dip the slide in trypsin for 5 sec (take 3-4 slides, but process only one slide at a time and go to the next only when results of the first one is clear)
5. Rinse the slide in phosphate buffer immediately after trypsin exposure.
6. Stain the slide for 3-5 min in Giemsa
7. Rinse in water, and monitor under the microscope. If stain is less, put again in Giemsa; if treatment is under, put again in trypsin for a few seconds and repeat these steps until satisfactory bands emerge. If the slide gets overtreated (i.e., chromosome will look hollow and chewed up), take a new slide and repeat the exercise giving shorter trypsin treatment
8. Dry the slide, mount in DPX and observe.

OBSERVATION

A G-banded metaphase plate is seen (see Fig. 1).

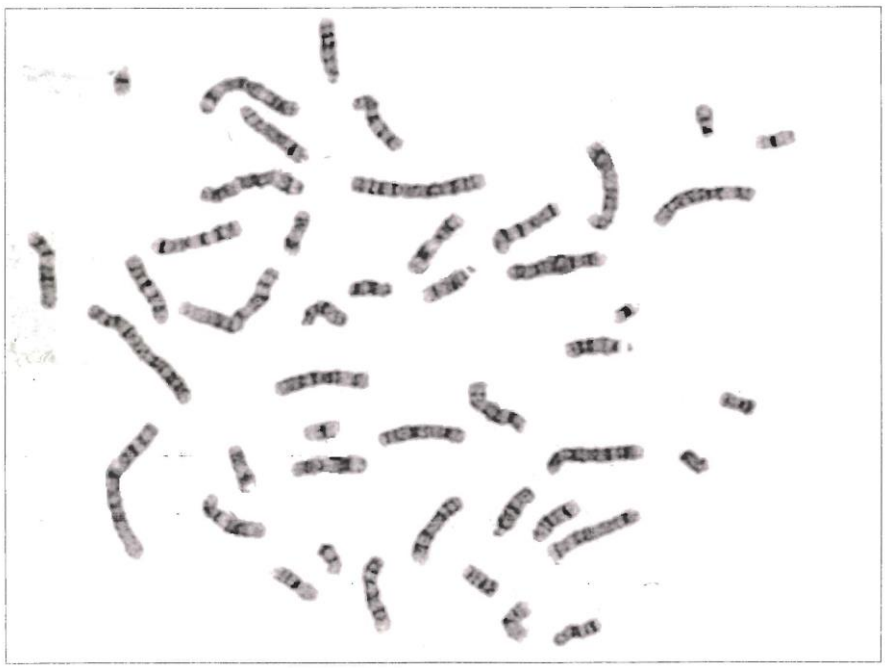

Figure 1. A G-banded metaphase plate of human

B. C-BANDING OF METAPHASE CHROMOSOMES

MATERIALS REQUIRED

Good chromosome preparations, Water baths set at 50°C (for $Ba(OH)_2$ solution) and at 60°C (for 2xSSC); DPX mountant; 500 ml conical flask; Couplin jars

Solutions

5% Barium Hydroxide

Barium Hydroxide	5 g
Dist. water	100 ml

Boil 100 ml of dist. water in a conical flask and add 5 g $Ba(OH)_2$ while the water is steaming. Stir vigorously to get as much $Ba(OH)_2$ in solution as possible. Filter in a Couplin jar and maintain the solution at 50°C in a wate rbath

0.2 N HCl

Conc. HCl	1 ml
Dist. water	54 ml

2xSSC (pH 7.2)

Giemsa stain and Giemsa water

PROCEDURE

1. Make chromosome preparations by the standard air-drying technique and keep the slides for nearly a week before use
2. Dip the slides in 0.2N HCl in a Couplin jar for 30min followed by two rinses in dist. water
3. Air dry and put the slides in $Ba(OH)_2$ at 50°C; treat different slides for varying time intervals ranging from 1min to 5min (remove the precipitate on the solution, before placing slides in the $Ba(OH)_2$ solution,). Rinse in 2 changes of dist. water and air dry
4. Keep the air-dried slides in 2xSSC in a water bath maintained at 60°C for 1-2 h
5. Rinse in dist. water, air dry and stain with Giemsa for 15-20 min (monitor the staining after different time intervals). After appropriate staining, air dry and mount with DPX.

OBSERVATION

A C-banded metaphase plate is seen in Fig. 2.

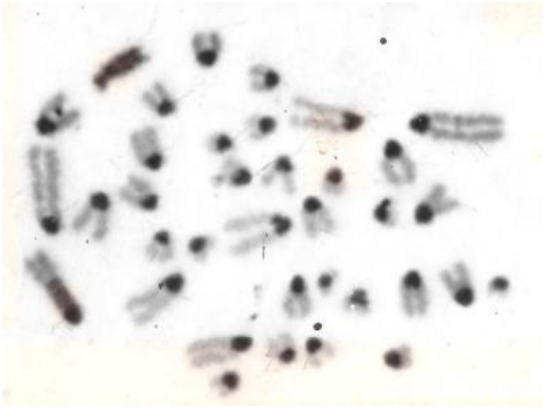

Figure 2. C-banded metaphase plate of mouse

QUESTIONS FOR FURTHER STUDIES

1. How many different types of chromosome banding are known? Understand the mechanism that makes a chromosome appear banded.
2. How G-bands are given numbers (nomenclature of G-bands)?
3. How chromosome bandings help in studies/diagnosis?

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Chapter 15: LOCALIZATION OF NUCLEOLAR ORGANIZER REGION (NOR) ON CHROMOSOMES

Recommended level: UG, PG

INTRODUCTION

Nucleolar Organizer Region (NOR) is a chromosomal region containing rRNA gene repeats. As the cell enters in G1 phase after cell division, the transcription of rRNA genes start and the newly formed rRNAs get associated with proteins forming RNPs which gets accumulated near the site of the transcription forming the nucleolus. NOR can be visualized on chromosome spreads by silver nitrate staining. Silver nitrate gets associated with NOR associated proteins and gets reduced to metallic silver (appearing black) after formalin fixation.

OBJECTIVE

To localize NOR containing satellite chromosomes in the given organism.

MATERIALS REQUIRED

Chromosome spreads, 5% silver nitrate, 3% formalin, ammonical silver nitrate (dissolve 4 gm $AgNO_3$ in 5 ml of concentrated ammonium hydroxide, add slowly 7.5 ml distilled water. No precipitate should appear. Store at 4^{o}C in dark bottle).

PROCEDURE

1. Place 3 drops of 5% $AgNO_3$ on the chromosome spread, gently place a cover glass on the drop and incubate the slide in an incubator at 60^{o}C until $AgNO_3$ is crystalline.
2. Rinse slide in distilled water to remove cover glass. Place 3 drops of 3% formalin solution and 3 drops of ammonical silver nitrate on slide and place a cover glass. Monitor staining reaction under low magnification of a microscope. When nuclei develop a golden brown colour (30-60 sec), rinse the slide in distilled water. The cover glass will fall off.
3. Air dry the slide and observe NOR on chromosomes under high magnification of microscope.

OBSERVATION

The NOR appears as two dots on two chromatids of metaphase chromosomes (Fig. 1). On polytene chromosomes NOR appears as a black line or band.

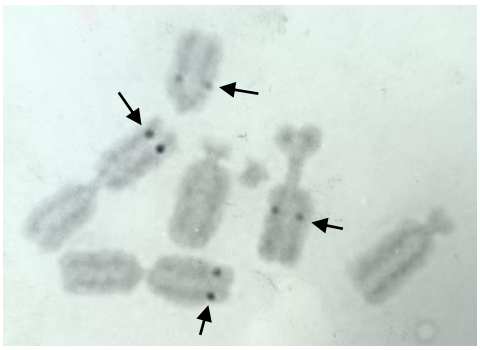

Figure 1. NOR staining as black dots (arrows) on metaphase chromosomes of barking deer, *Muntiacus muntjak*

QUESTIONS FOR FURTHER STUDIES

1. In the interphase nuclei do you expect to get the silver staining?
2. Why do you call NOR containing chromosomes as satellite chromosomes?
3. Is satellite DNA located on satellite chromosomes only?

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Chapter 16: STAINING OF SEX CHROMATIN

Recommended level: UG, PG

INTRODUCTION

Sex chromatin or Barr body is the inactivated X chromosome in a mammalian female somatic cell by the process called lyonization. All the X chromosomes except one are inactivated randomly during mammalian early embryogenesis. In men and women with more than one X chromosome, the number of Barr bodies visible at interphase is always one less than the total number of X chromosomes. For example, female with 46,XX chromosomes and men with 47,XXY chromosomes have a single Barr body, whereas a female with 47,XXX chromosomes have two sex chromatins.

OBJECTIVE

To observe sex chromatin in cheek epithelial cells of human.

MATERIALS REQUIRED

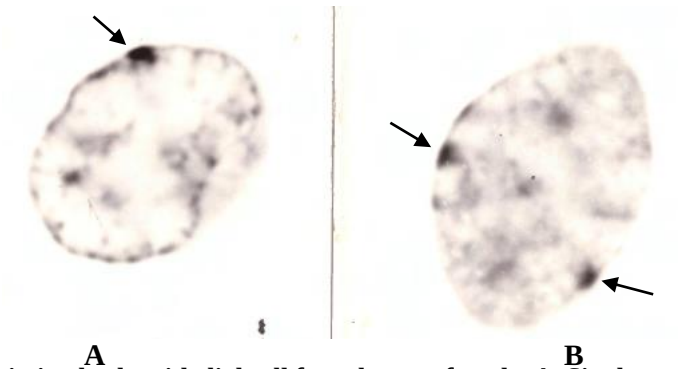
Ether-Ethanol (1:1) fixative, 2% acetocarmine stain (2 g of carmine mixed with 100 ml of 45% acetic acid and boiled using a reflux condenser for 1 h to dissolve carmine).

PROCEDURE

1. Dry the ethanol-soaked tooth pick in air and scrape gently the inner side of cheek. Discard the first scrape and take the second scrape to get rid of many bacteria coming along with the epithelial cells. A large number of cells will come on the tooth pick.
2. Gently rub the tooth pick on slide in one direction to make a spread of cells. Dry the cells on slide so that the cells will not get washed away while staining.
3. Put a few drops of acetocarmine stain and leave for 2 min. Cover with a cover glass, soak extra stain with a blotting paper and seal the edges of cover glass with nail polish or DPX.
4. Observe under high magnification of microscope.

OBSERVATIONS

All nuclei will be red stained. If a light stained nucleus is carefully observed, a slightly darker red stained body can be seen at the periphery on many nuclei. The dark stained body is sex chromatin. The dirt particles and presence of bacteria also sometime mimic sex chromatin. But the peripheral localization and not very sharply stained body is the identification of sex chromatin (see Fig. 1).



A **B**

Figure 1. Sex chromatin in cheek epithelial cell from human female. A. Single sex chromatin (arrow) is seen in cells from normal female (XX) individual. B. An individual with XXX showing two sex chromatin.

QUESTIONS FOR FURTHER STUDIES

1. Why sex chromatin is also called as Barr body?
2. Why a female does not get converted to a male when its one X-chromosome is inactivated?
3. Can you think of some modern molecular method for visualizing sex chromatin?

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Chapter 17: ASEPTIC TECHNIQUE AND GOOD CELL CULTURE PRACTICES

Recommended level: UG, PG, Research

INTRODUCTION

Cell or tissue culture is a process to grow cells in a medium outside the living body. The medium used for this purpose is rich in nutrients to sustain growth of any cell types, prokaryotes or eukaryotes. There are plenty of microbes in air, water or on surfaces of any container or platform used. In order to make sure that only the desired cell types grow and contaminants do not find a chance to enter and contaminate the culture, a very strict culture room rules and good cell culture practices are to be followed. This chapter gives a glimpse of rules and practices to be followed for cell culture.

OBJECTIVE

To ensure all cell biology procedures are performed to a standard that will prevent contamination from bacteria, fungi and mycoplasma and cross contamination with other chemicals.

MATERIALS REQUIRED

Chloros / Presept solution (2.5 g/l), 1% formaldehyde based disinfectant, e.g., Virkon, Tegador, 70% ethanol in water, Personal protective equipment (sterile gloves, laboratory coat, safety visor), Laminar Flow Hood/ Microbiological safety cabinet at appropriate containment level (Usually Level II Safety Cabinet)

PROCEDURE

1. Sanitize the cabinet using 70% ethanol before commencing work.
2. Sanitize gloves by washing them in 70% ethanol and allowing to air dry for 30 seconds before commencing work.
3. Put all materials and equipment into the cabinet prior to starting work after sanitizing the exterior surfaces with 70% ethanol.
4. Whilst working do not contaminate gloves by touching anything outside the cabinet (especially face and hair). If gloves become contaminated re-sanitize with 70% ethanol as above before proceeding.
5. Discard gloves after handling contaminated cultures and at the end of all cell biology procedures.
6. Equipment in the cabinet or that which will be taken into the cabinet during various procedures (media bottles, pipette tip boxes, pipette aids) should be wiped with tissue soaked 70% ethanol prior to use.
7. Movement within and immediately outside the cabinet must not be rapid. Slow movement will allow the air within the cabinet to circulate properly.
8. Speech, sneezing and coughing must be directed away from the cabinet so as not to disrupt the airflow.

9. After completing work disinfect all equipment and material before removing from the cabinet. Spray the work surfaces inside the cabinet with 70% ethanol and wipe dry with tissue. Dispose of tissue by autoclaving.
10. Discard cultures in bleach (10,000 ppm), they must be kept in the cabinet for a minimum of two hours (preferably overnight) in a bucket prior to discarding down the sink with copious amounts of water.

Sterilization

Autoclaving is a technique to kill any contaminant that might contaminate a cell culture. This process is commonly called as sterilization. The word `auto' in Greek means `self' and `clavis' in Latin means `key', i.e., a self-key or self-locking. The equipment that is used to for this purpose is an autoclave, a machine that uses heat to build steam under pressure to kill any pro or eukaryotic cells, viruses and spores.

Autoclaving is needed for solutions, media and equipment required for growing microorganisms, culturing cells and tissues *in vitro.* Solutions which are not heat sensitive are only autoclaved. Used cultures and waste materials prior to disposal are also autoclaved.

During autoclaving the contents (liquid or solid) become exposed to saturated steam at the required temperature for appropriate length of time.

For efficient sterilization exclusion of air is necessary. If air is present, steam may become superheated with a relative humidity less than 100% and sterilization efficiency is decreased.

Air removal is achieved by:

1. Downward displacement of steam, or
2. Evacuation by pump prior to sterilization cycle

Downward Displacement Sterilizer

As steam enters the chamber, it fills the upper areas as it is less dense than air. This compresses the air at the bottom, forcing it out through the strainer and drain pipe past the temperature sensing device to waste. Only when air evacuation is complete the discharge should stop. This can be done manually or automatically.

High Vacuum Autoclaves

High Vacuum Autoclaves are not suitable for the sterilization of liquids and are primarily used for non-aqueous materials or porous loads where air is likely to be trapped in cavities or gaps. The vacuum line in pre-vacuum sterilizers should be fitted with appropriate air filters to prevent the release of infectious aerosols into surrounding areas.

Sterilization Times

The sterilization time must be the sum of the steam penetration time (time required for the entire load to reach the set temperature) and the holding time (minimum time required for complete sterilization at the set temperature). The time-temperature profile of infectious waste being autoclaved varies with the load and type of container used in the process.
The temperature attained by the contents and the holding time are critical parameters in sterilization.

In order to eliminate bacterial and fungal contaminants, media must be submitted to heat and high pressure. Fungal spores may survive if only heat is used. Therefore, media is sterilized by heating to 121°C at 105 kPa (15 psi [pounds per square inch]) for ~20 min. The time required for sterilization varies depending on the volume of medium being sterilized. See Table 1. for the autoclaving times according to volume. To autoclave, one can also use a pressure cooker, which is convenient and economic autoclaving equipment for small laboratories. It attains 15 psi pressure when first whistle comes in a pressure cooker. 20 min autoclaving after first whistle ensures proper autoclaving.

Table 1. Minimum autoclaving time for tissue culture medium

Volume of medium per vessel (ml)	Minimum Autoclaving (min)*		Volume of medium per vessel (ml)	Minimum Autoclaving (min)*
25	20		500	35
50	25		1000	40
100	28		2000	48
250	31		4000	63

***Minimum autoclaving times include the time required for the medium to reach 121°C. Nevertheless, autoclaving times may vary due to autoclave differences and may require your validation.*

Container Selection

Polypropylene bags: Commonly called biohazard or autoclave bags, these bags are tear resistant, but can be punctured or burst in the autoclave. Therefore, **place bags in a rigid container during autoclaving**. Bags are available in a variety of sizes, and some are printed with an indicator that changes color when processed.

Polypropylene bags are impermeable to steam, and for this reason should not be twisted and taped shut, but gathered loosely at the top and secured with a large rubber band or autoclave tape. This will create an opening through which steam can penetrate.

Polypropylene containers and pans: Polypropylene is plastic capable of withstanding autoclaving, but resistant to heat transfer. Therefore, materials contained in a polypropylene pan will take longer to autoclave than the same materials in a stainless-steel pan. To decrease the time required to sterilize material in these containers,

- remove the lid (if applicable)
- turn the container on its side when possible
- select the container with the lowest sides and widest diameter possible for the autoclave.

Stainless steel containers and pans: Stainless steel is a good conductor of heat and is less likely to increase sterilizing time, though is more expensive than polypropylene.

Preparation and Loading of Materials

1. Fill liquid containers only half full.
2. Loosen caps or use vented closures.
3. Always put bags of biological waste into pans to catch spills.

4. Position biohazard bags on their sides, with the bag neck taped loosely.
5. Leave space between items to allow steam circulation.
6. Household dishpans melt in the autoclave. Use autoclavable polypropylene or stainless-steel pans.

Handling Autoclave

Cycle Selection

- Use liquid cycle (slow exhaust) when autoclaving liquids, to prevent contents from boiling over.
- Select fast exhaust cycle for glassware.
- Use fast exhaust and dry cycle for wrapped items.

Time Selection

- Take into account the size of the articles to be autoclaved. A 2-liter flask containing 1 liter of liquid takes longer to sterilize than four 500 ml flasks each containing 250 ml of liquid.
- Material with a high insulating capacity (animal bedding, high sided polypropylene containers) increases the time needed for the load to reach sterilizing temperatures.
- Autoclave bags containing biological waste should be autoclaved for 50 min to assure decontamination.

Removing the Load

- Check that the chamber pressure is zero.
- Wear lab coat, eye protection, heat insulating gloves, and closed-toe shoes.
- Stand behind door when opening it.
- Slowly open door only to a small extent for the steam to go out first. Beware of rush of steam.
- After the slow exhaust cycle, open autoclave door and allow liquids to cool for 20 min before removing.

Personal Safety Precautions

- When unloading an autoclave, wear heat resistant gloves, eye protection, and lab coat.
- To prevent steam burns, make sure that the autoclave pressure is near zero before opening the door.
- Allow steam to escape gradually by slowly cracking open the autoclave door. Allow load to cool for 10 min before removing.
- Do not autoclave sealed containers or full bottles with narrow necks as they may explode.
- Do not autoclave materials containing solvents, volatile or corrosive chemicals (such as phenol, chloroform, bleach, etc.), or radioactive materials.

Alternate methods of sterilization and heat labile compounds

Dry Sterilization

Glassware can be sterilized in an oven by placing them at 200°C for 1-4 h. Be sure to cover glassware with aluminum foil to maintain aseptic conditions after removing the glassware from the oven. Avoid the use of any plastic caps, paper (labeling tape), or other flammable materials as they are fire hazards.

Microwave Sterilization

Rapid sterilization of media can be achieved by using microwave ovens. Most plant tissue culture media can be sterilized using a microwave, although it may not be suitable with some media types (medium containing complex additives like oatmeal)

Filter Sterilization

Certain media components are susceptible to heat denaturation and therefore must be added to the media after autoclaving. To do so, you must filter the components using a 0.22 µm pore size filter. Filters are available from Whatman, Fisher Scientific, Titan, and VWR Scientific. Be sure to consult with the sales person regarding the solvent and application you are intending to use for the filters.

Coconut water is sometimes filter sterilized instead of autoclaved. To filter sterilize coconut water, filter the water using a 0.45 µm pore size filter, before using a 0.22 µm pore size filter for the final sterilization. If you are unsure of what components of the medium are heat labile, consult the table of thermolabile components.

Sterile filtration with syringe-tip filter

Sterile:

- Plastic or glass syringe (10-50 ml capacity)
- Syringe-tip filter (Disposable; e.g., Millipore Millex, Acrodisk, etc., of 0.22 µm pore size or a reusable filter)
- Receiver vessel (e.g., milk dilution bottles or universal containers)

Nonsterile:

- Solution for sterilization

1. Swab down hood and assemble materials
2. Uncap receiver vessel and the filter-assembly
3. Place the bottom nozzle of the filter in the receiver vessel without touching the nozzle with the fingers
4. Fill the syringe with the solution to be filtered and attach the tip of the syringe to the top nozzle of the filter assembly
5. Expel solution through filter into the receiver vessel by applying moderate positive pressure by pressing the plunger with the palm
6. The syringe may be refilled several times by carefully detaching it from the filter assembly.
7. After filtering, cap the receiver vessel and seal it with parafilm.
8. Discard the used filter and syringe.

Key points

1. If the pressure increases, take new filter and do not apply excessive pressure because it may lead to damaging of membrane and also spillage.
2. Although disposable filters are expensive than reusable, they are less time consuming and give fewer failures.
3. Similar to bottle-top filter are filter flasks. Medium added to upper chamber and collected in the lower. Lower chamber can be used for storage also.

Monitoring Sterilization

In most states autoclaves are classified as boilers or pressure vessels and require registration and inspection under state law. An accredited inspector must inspect large autoclaves every 3 years.

Temperature controllers, recording charts and timers must be calibrated every six months (e.g., use NATA calibrated thermometers to calibrate thermocouples). Pressure gauges need not be calibrated but must read true with respect to the required pressure at the nominated calibrated temperature.

Autoclave Tape

Autoclave tape only shows that the tape has been exposed to heat (80ºC). No information on time, steam penetration or temperature/pressure can be inferred.

Biological Indicators

Biological indicators are recommended as an adjunct to the daily monitoring of autoclave cycle parameters.

Biological indicators can be used to confirm thermocouple data when checking heating profiles and validating autoclave operational parameters. They should not be used in isolation as a measure of sterilization efficacy.

Manufacturers of biological indicators using *Bacillus stearothermophilus* claim that spores will survive 5 min at 121ºC but not 15 min at 121ºC.

Check each lot by confirming survival for 5 min at 121ºC.

Biological indicators have several limitations**:**

- Results are retrospective (several days). Autoclaved materials may be required earlier.
- They cannot be used for checking the center of large liquid loads.
- They are less accurate in determining holding times than temperature measurement.
- They cannot be used for sterilization cycles below 121ºC.

Chemical Indicators

There are many types of chemical indicators in use and one must check the performance of a particular type and use it in conjunction with temperature and time measurements only.

QUESTIONS FOR FURTHER STUDIES

1. From where microbes come and enter in the cultured cells?
2. How does flaming help in preventing infections?
3. What is an autoclave and how does it function?

REFERENCE

Biological Safety Principles and Practices. Ed. Diane Fleming & Debra Hunt, ASM Press, Washington D.C., pp 393-395 (2000).

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Chapter 18: SHORT TERM CULTURE OF WHOLE BLOOD AND PREPARATION OF METAPHASE CHROMOSOMES

Recommended level: PG, Research

INTRODUCTION

One of the most vital limitation in understanding many physiological, genetic and other functions in the body system is the difficulty in devising suitable experiments directly on the organism. The worst sufferer of this limitation has been the human system. Tissue culture techniques, long as well as short term, have therefore been developed to simulate *in vitro* the *in vivo* conditions so that various molecular, cellular and organic functions could be better understood. Short-term lymphocyte culture is the simplest form of tissue culture in which genetically inert lymphocytes are stimulated to proliferate by using a lectin as a mitogen, viz., phytohaemagglutinin, concanavalin A, pokeweed mitogen, etc. Its most obvious application is diagnostic with regard to chromosomal defects in man and variety of other systems. Usage of various banding techniques has made it possible to identify small fragments of chromosomes which allows detection of even minor chromosomal rearrangements. It is also possible to address several cell biological and immunological questions through lymphocyte cultures. These cells can also be used in somatic cell fusion and hybridization for gene mapping and studies on differentiation.

MATERIALS REQUIRED:

Sterilized glass ware: Pipettes (10 ml, 5 ml, 1 ml), culture vials (universal containers), conical flask 100ml, Syringes (5 ml, 1 ml), needles (#22, #26), Millipore filter assembly

Chemicals: Tissue culture media (RPMI 1640 + L-glutamine to be added at the time of setting up the culture), fetal calf serum (FCS) (alternatively, heat inactivated human AB serum), phytohaemagglutinin-m (PHA), heparin, 0.2 μg/ml colcemid, 1N HCl, 90% alcohol

> **It is ideal to check sterility of different solutions at least 24 h prior to setting up a culture by keeping each one of them at 37^{o}C for one night and examining if any of them becomes turbid the next day. Sterile solutions remain clear.**

Centrifuge tubes, Pasteur pipettes, 0.56% KCl (Hypotonic), Acetic acid methanol (1:3-Fixative),

Acid-cleaned slides (maintained in 70% alcohol), Giemsa stain

Incubator, centrifuge, clean-sterile work bench,

Giemsa stain (see page 29)

PROCEDURE

Setting the culture

1. About 5 ml of blood is collected in a sterile, heparinised syringe in suitably clean environment. Blood is kept in refrigerator until used
2. Arrange TC medium, FCS, PHA, L-glutamine, pipettes, beakers and flasks etc. on the alcohol-swabbed work bench before setting up the culture
3. Prepare the TC media by adding antibiotics and L-glutamine and 10% fetal bovine serum (10 ml serum to 100 ml medium - serum can be added separately to the culture). Since commercial media have phenol red as indicator, colour of the medium indicates its pH which must be 7.2

(light pink to orangish). If the pH is alkaline (indigo), use CO_2 or a few drops of 1N HCl to bring it to the proper range

4. Prepare a working solution of PHA (5 ml in sterile distilled water). This can be stored under sterile conditions for 1-2 months at 4ºC

5. Before using pipettes, container, culture vial, etc., flame them gently but use only after cooling

6. For each culture, add the following in the order given:

TC medium	5 ml
Fetal bovine serum	1 ml (if not already added)
Blood	0.3 ml
PHA	0.1 ml

7. In order to buffer the pH, blow CO_2 from a CO_2 cylinder or bubble exhaled air orally through a cotton-plugged-pipette

8. Culture is kept in an incubator at 37°C for 48 to 72 h

9. Culture must be inspected every morning and evening for change in pH and infection and shaken to break the clumps of RBCs

10. About 2-3 h prior to harvesting the culture for chromosome preparation, colcemid (working conc. 0.02 μg/ml) is added.

Chromosome Preparation

1. Transfer the culture to a centrifuge tube and centrifuge at 1000 to 1200 rpm for 5 min

2. Decant the supernatant and make a fresh suspension of cells in prewarmed 0.56% KCl (hypotonic). Initially add a small volume and agitate the sediment. Once the cells come in suspension, make up the volume to 8-10 ml. Keep in incubator (37ºC) for 18-20 min

3. Immediately before centrifugation, add 3-4 drops of fixative to the tube and mix. Spin (1000 - 1200 rpm) the tubes and decant the hypotonic completely. Add the fixative drop-by-drop to fix the cells and to keep them in suspension. Make up the volume to ~8-10 ml. Keep for 15 min

4. Re-centrifuge (1000-1200 rpm) for 5 min, decant the supernatant and resuspend the pellet in fresh fixative (~ 8-10 ml). Keep for 10 min.

5. Re-centrifuge (as in #4) and discard the supernatant. Add only about 0.2-0.4 ml of fixative. Resuspend the cells well by gentle agitation.

6. Take out a slide from 70% alcohol and wipe it with a clean piece of cloth. Add 2-3 drops of the cell suspension on the slide and either blow it dry (air-drying) or expose to a flame for instant drying (flame drying)

7. Stain the slide with Giemsa stain for 3-4min and rinse in 2 changes of distilled water (pH 6.8-7.2) or clean tap water. Dry it fully and mount with DPX mountant using a 24x60 mm cover glass

PRECAUTIONS

Success of tissue culture work depends largely on the personal habits of the user. Tissue culture medium is rich not only for the cells that have been provided but also to any other kind of cells, like bacteria and fungi, that can find access to it. Therefore, standard of hygiene and sterilization has to be extremely high.

Hygiene - The place where blood culture is to be done, and its near-abouts, must be thoroughly swabbed every day with a disinfectant like alcohol or dettol (a laminar flow is an ideal work station but for short-term cultures like that of blood, any suitably secluded, clean bench should do). A germicidal lamp must be installed over the working bench and switched on ~30min prior to starting the culture. The lamp must be switched off while working. No part of the body should be exposed to the UV, since exposure to UV is harmful.

The user must wash himself/herself thoroughly, swab hands with alcohol and depending upon the conditions may or may not have to take off shoes and wear aprons etc. Such habits as of scratching skin or hair, growing long (often unkept) nails, touching all kinds of things are sure prescriptions to attracting infections. These habits must be banished.

Sterilization - In our lab, most of the glass-wares are kept in concentrated nitric acid over-night and then washed in running tap water for 3 h. They are rinsed first in steamed distilled water and then in two changes of double distilled water. They are dried in incubator at ~60^{o}C.

Dried glass-wares are sterilized either dry or wet. For the former they are kept in an oven at 160^{o}C to 200^{o}C for 2 h. Wet sterilization is done in an autoclave in which the sterilization is done by the pressure created by steam. The contents are autoclaved for 20-30 min at 15-20 lbs/inch^{2} pressure. If an autoclave is not available, a domestic pressure cooker will do. Components which cannot stand high temperatures, like solutions, plastic ware, etc., should not be autoclaved or kept in oven. Solutions (medium, PHA, etc.) should be membrane filtered using Millipore or locally available assemblies. The filter should have a pore size of 0.22 μm which prevents most of the microbes from being filtered. Plastic ware, if used, should generally be disposable. During and after the work the glass-wares must be discarded in a detergent solution. After the work is over, they must be gently cleaned with the detergent and then deposited in HNO_3 for subsequent washing.

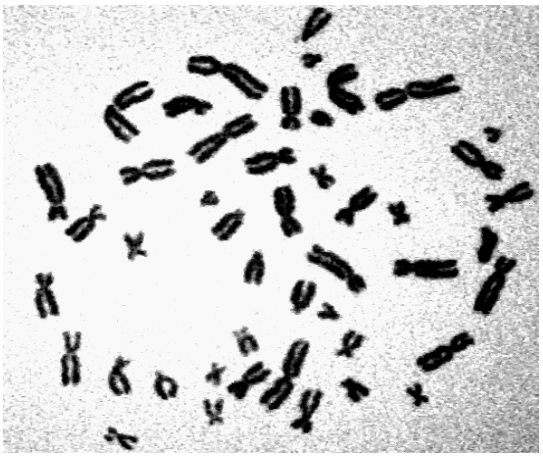

Fig. A metaphase spread from human lymphocyte culture

QUESTIONS FOR FURTHER STUDIES

1. Why do we need to culture blood cells for 2 to 3 days before making chromosome preparation?
2. How colchicine or colcemid function on dividing cells?
3. What will happen if hypotonic solution is not completely drained and fixative is added?

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Chapter 19: CHROMOSOME PREPARATION FROM CELL LINE

Recommended level: PG, Research

INTRODUCTION

Cell culture is the process of growing cells outside of their original environment in a media containing essential nutrients. Cells taken from the body and grown initially in a medium is called as primary culture. Primary culture may grow for a limited number of cell cycles but if some of the cells from that culture acquire growth potential and can grow for an extended or indefinite period, then those cell populations are maintained as cell line. As these cells are repeatedly dividing, chromosomes from them can be prepared and studied.

MATERIALS REQUIRED

Centrifuge tubes (15 ml graduated), Pasteur pipettes, agitator, colchicine, incubator (37-38ºC), clinical centrifuge

Solutions

Hypotonic Solution: 0.56% KCl (should be kept pre-warmed to 37ºC before use)

Fixative:

Glacial acetic acid	25 ml
Ethanol or Methanol	75 ml

Giemsa stain

Stock solution -	Giemsa Powder	380 mg
	Methanol	25 ml
	Glycerol	25 ml

Leave overnight at 37ºC. Filter the stain and store.

Working solution -	Stock solution	2.5 ml
	Methanol	1.5 ml
	Giemsa water	50.0 ml
Giemsa water -	0.2 M Na_2HPO_4	80 ml
	Distilled water	800 ml

Adjust pH to 6.8 with 0.1 M Citric acid (roughly 24 ml) and then make up the volume to l liter.

PROCEDURE

1. Treat 48-72 h old culture with colchicine (0.5 µg/ml) for 2-3 h at 37ºC
2. Shake the culture bottle to dislodge the colchicine arrested metaphase cells and collect the growth medium containing the metaphase cells in a centrifuge tube and centrifuge at 1000 rpm for 5 min.
3. Discard supernatant, add 1ml of hypotonic solution, agitate with an agitator to resuspend the pellet and make up volume to 10 ml with hypotonic solution. Incubate for 30 min at 37ºC.
4. Re-centrifuge (1000-1200 rpm) for 5 min, decant the supernatant and resuspend the pellet in fresh fixative (~ 8-10ml). Keep for 20 min.
5. Re-centrifuge (as in #4) and discard the supernatant. Add only about 0.2-0.4 ml of fixative. Agitate to resuspend the cells.

6. Take out a slide from 70% ethanol and wipe it with a clean piece of cloth. Add 2-3 drops of the cell suspension on the slide and either blow it dry (air-drying) or expose to a flame for instant drying (flame drying)
7. Stain the slide with Giemsa stain for 3-4 min and rinse in 2 changes of distilled water (pH 6.8-7.2) or clean tap water. Dry it fully and mount with DPX mountant using a 24x60mm cover-glass

OBSERVATION

A small darkly stained dot like body in nucleus, generally on one side touching the edge of the nucleus, is seen in several cells.

QUESTIONS FOR FURTHER STUDIES

1. Why sex chromatin is not visible in all the cells with clarity?
2. Do cells from male possess sex chromatin in some conditions?
3. Do cells from female posses zero, one or two sex-chromatin under some conditions?

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Chapter 20: COLONY FORMATION ASSAY TO EVALUATE CELLS' DIVISION POTENTIAL

Recommended Level: PG/Research

INTRODUCTION

The clonogenic assay, or colony formation assay, is an *in vitro* cell survival assay that measures a single cell's ability to grow into a colony of at least 50 cells. This assay evaluates each cell's capacity for "unlimited" division and is primarily used to assess a cell's inability to divide after ionizing radiation treatment or to evaluate the effectiveness of a cytotoxic agent, overexpression, or loss of function of a gene. Typically, only a fraction of seeded cells can form colonies. Cells are seeded in appropriate dilutions before or after treatment to form colonies over 1-3 weeks. The colonies are then fixed with paraformaldehyde or glutaraldehyde, stained with crystal violet, and counted using a stereomicroscope. The assay also includes a method for analysing radiation dose-survival curves. In this assay, phenotype due to the overexpression of the gene from stably transfected cells has been evaluated.

OBJECTIVE

To evaluate the division potential of cultured cells

MATERIALS REQUIRED

Cell culture medium serum, Phosphate-buffered saline (PBS) pH 7.2, Trypsin–EDTA (0.5% trypsin with EDTA). The trypsin solution is freshly prepared before the experiment from a stock solution containing 0.5 g/l trypsin, 0.2 g/l EDTA, and 0.85 g/l NaCl, Isotonic buffer for cell counting when using a Coulter counter, Colony fixation-staining solution (paraformaldehyde 4.0% vol/vol, crystal violet 0.5% weight/vol in H_2O), Pipettes, Culture dishes or six-well plates, Tubes for dilution, Automatic countess cell counter or hemocytometer, Stereomicroscope, Colony counting pen, Statistical analysis software (SPSS) or Fiji.

PROCEDURE

Initial Handling of Cells

1. Gather all necessary sterile materials: pipettes, test tubes, culture dishes, and six-well plates. Warm the medium, PBS, and trypsin to 37°C. Plan cell dilutions and label dishes/plates. Work efficiently to minimize time and avoid pH and temperature changes.
2. Harvest cells using trypsinization:
   Remove the medium from the cells.

   Wash the cells with PBS.

   Trypsinize cells to form a single-cell suspension. Inspect under a microscope until cells round up.

   When cells round up, indicating detachment, resuspend them in a medium with serum to neutralize trypsin. Pipette up and down to detach cells.
3. Count the cells. Accurate cell numbers are crucial for correct plating efficiency (PE) and survival calculations.
4. Dilute cell suspension to the desired concentration and seed into flasks or plates. Accurate dilutions are essential for correct cell seeding.

Clonogenic Assay Setup

There are two methods:

Plating before treatment (A)

1. Harvest exponentially growing cells, re-plate appropriate numbers, and allow time for attachment (a few hours at 37°C). Check attachment with a microscope.
2. Treat the cells, then incubate dishes with necessary conditions (e.g., excess CO_2, humidity) until control cells form large clones (more than 50 cells).

Plating after treatment (B)

1. Harvest cells post-treatment, count, and dilute in sterile tubes. Pipette 100 to 10^4 cells into test wells. For potentially lethal damage repair, re-plate cells immediately (IP) or after a delay (DP) of 6-24 h. Keep cells on ice if not processed immediately
2. Incubate dishes until control cells form large clones

Plating after the transfection (C):

1. Harvest the cell that is stably transfected with the help of antibiotic selection, count, dilute it in sterile tubes. Pipette 100 to 10^4 cells into test wells. Keep cells on ice if not processed immediately
2. Incubate dishes until control cells form large clones

Fixation and Staining of Colonies

1. Remove the medium above the cells
2. Rinse carefully with PBS
3. Add 2-3 ml of 4.0% paraformaldehyde and 0.5% crystal violet mixture
4. Leave for at least 30 min
5. Carefully remove the mixture, rinse with tap water (do not use running tap) and immerse dishes in water
6. Let dishes dry in normal air at room temperature (20°C). Colonies can be counted up to 50 weeks after staining

Counting the Colonies

Count colonies using a stereomicroscope and an automatic colony counter pen. Determine the PE of control cells (cells not exposed to treatment/un-transfected). Calculate the surviving fraction of cells after treatment based on the PE of control cells.

OBSERVATION

Colony Counting and Plating Efficiency:

1. The number of colonies formed from the initial seeding of cells is counted using a stereomicroscope and an automatic counting "colony counter pen." Or Fiji imaging app.
2. Plating Efficiency (PE) is calculated as the ratio of the number of colonies to the number of cells seeded, expressed as a percentage.
3. Quantification is done by ImageJ software using analyze particles tool (Fig. 4).

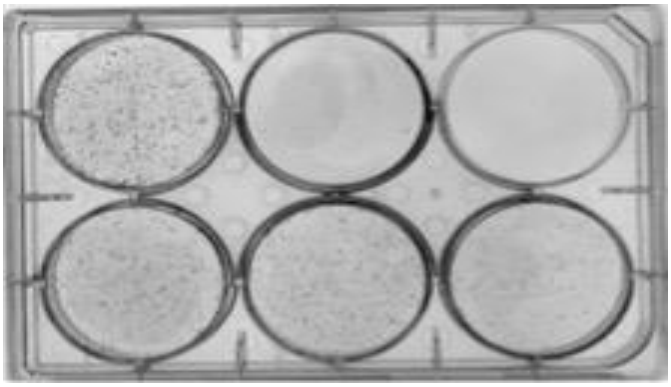

Figure 1. Representative image showing the chemidoc image of colonies.

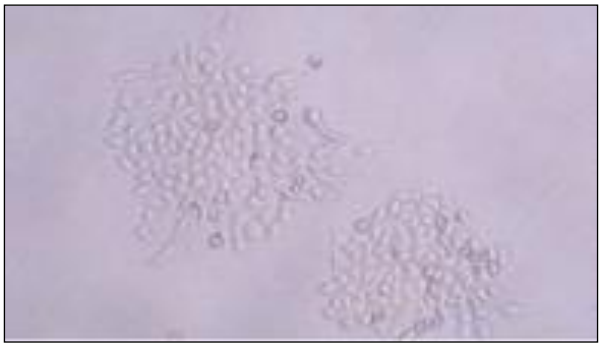

Figure 2. Representative photograph showing microscopic image of colonies

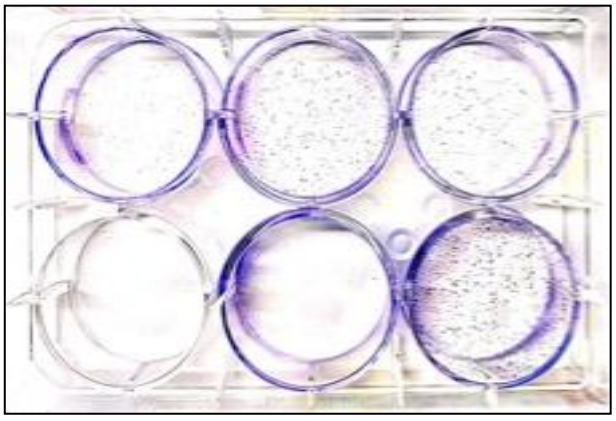

Figure 3. Representative image showing normal images of colonies (In each well there is stable transfection of different Gene of interest. 1- Empty vector, 2- ORF1p, 3- EN, 4- RT, 5- Un-Transfected.

Using ImageJ software quantification of the colonies formed can be shown as histograms (Fig. 4).

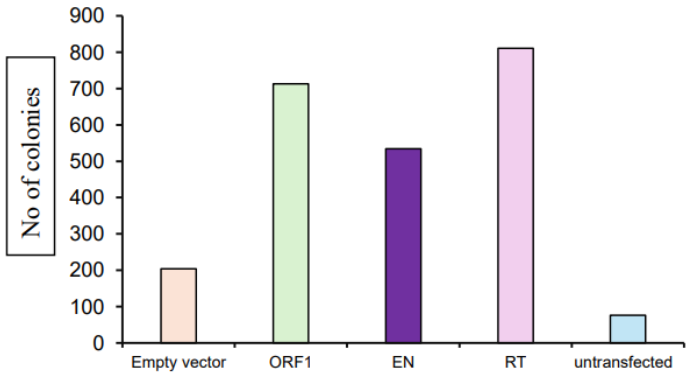

Figure 4. Representative plot showing the number of colonies per sample

QUESTIONS FOR FURTHER READING

1. How does seeding density optimization ensure accurate quantification of clonogenic potential in the colony formation assay?
2. Why is methanol based fixation followed by crystal violet staining critical for reliable visualization of colonies?
3. How do incubation duration and nutrient availability affect colony size and reproducibility in the assay?

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Chapter 21: MTT ASSAY FOR CELL VIABILITY

Recommended level: PG, Research

INTRODUCTION

The Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to cell death, apoptosis or necrosis, the reduction in cell viability.

OBJECTIVE

To carry out a quantitative estimation of cell viability in the given cell suspension samples following a treatment given to the cells.

MATERIALS REQUIRED

Any type of single cell suspension in PBS, MTT (5 mg/ml in PBS), DMSO, colorimeter, and any agent which can cause cell death for example Dexamethasone in case of lymphocytes.

PROCEDURE

1. Make single cell suspension of any cell type in PBS in a tube.
2. Subdivide the suspension in 5 microfuge tubes. Add dexamethasone (10 µM or any other agent which causes cell death) to the four tubes and incubate them for 0, 1, 2 and 3 h, respectively, in water bath at 37ºC to induce apoptosis. The untreated tube will have live cells.
3. Take 200 µl of suspension from each tube in fresh microfuge tubes. Add 20 µl of MTT (5 mg/ml in PBS) in each and incubate at 37ºC for 2 h.
4. Centrifuge the incubation mixtures at 5000 rpm for 5 min in a microfuge, remove the supernatant and resuspend the pellets in 100 µl of DMSO. The cells will get lysed and the mixture will appear bluish due to the formation of formazan crystals in live cells. In the mixture that had dead cells, no formazan crystals will form and thus the mixture will remain colourless.
5. Take OD at 540 nm to measure the intensity of colour developed.

OBSERVATION

Higher OD will be observed for the samples having live cells.

QUESTIONS FOR FURTHER STUDIES

1. Can you write down the chemical reactions that produced blue colour?
2. How colour intensity is measured by a colorimeter?

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Chapter 22: DETECTION OF SENESCENCE

Recommended Level: PG/Research

OBJECTIVE

To detect senescence by senescence associated β-galactosidase assay

INTRODUCTION

The senescence-associated β-galactosidase (SA β-Gal) assay is a standard method to quantify or visualize senescent cells. In this assay, cells are incubated in a pH 6.0 buffer containing X-Gal. Eukaryotic cells express β-galactosidase, a lysosomal enzyme that is active at a pH of approximately 4.5 but not at a neutral pH. SA β-Gal activity, responsible for cleaving X-Gal and staining cells blue at pH 6.0, is frequently found in cultured cells undergoing replicative and induced senescence (Dimri et al., 1995).

Most normal human cells have a limited replicative capacity and eventually undergo cellular senescence, where it stops to proliferate/ divide further. The SA-βgal biomarker identifies individual senescent cells *in vitro* and *in vivo*. SA-βgal activity reflects increased lysosomal mass and the expression of the GLB1 gene, encoding a lysosomal enzyme. Elevated lysosomal enzyme levels and activity are hallmarks of cellular senescence.

MATERIALS REQUIRED

Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum
2. 100× penicillin–streptomycin
3. 35-mm plates or 6-well plates
4. Mammalian cells

Solutions to prepare

- Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO4; adjust to pH 7.4 with HCl if necessary).
- Fixing solution: 4% formaldehyde
- Staining solution: 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside (X-gal), 1X citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$.
- Mounting medium.
- 22 × 22 mm cover glasses

SA-β −gal staining solution

Component	Stock Solution	Amount for 10 ml	Final Concentration
Citric acid/sodium phosphate buffer (pH 6.0)	5x	2 ml	1x
Potassium Ferricyanide	50 mM	1 ml	5 mM
Potassium Ferrocyanide	50 mM	1 ml	5 mM
NaCl	5 mM	0.33 ml	150 mM
$MgCl_2$	1 M	20 µl	2 mM
X-gal	20 mg/ml	0.5 ml	1 mg/ml
H_2O	-	5.2 ml	-

PROCEDURE

1. Seed 2–5×10^4 cells in either a 35-mm plate or 6-well plate, and culture for 2–3 days or more
2. Wash cells twice with PBS
3. Fix cells with neutral buffered 4% formaldehyde for 3 min at room temperature
4. Wash cells twice with PBS
5. Add SA-βgal staining solution (2 ml per 35-mm plate).
6. Incubate cells with staining solution at 37°C (NOT in a CO_2 incubator).
7. Blue colour is detectable in some cells within 2 h, but staining is generally maximal in 12–16 h.
8. After blue colour is fully developed wash cells twice with PBS. Add one drop of mounting medium, and place cover glasses either on a 35-mm plate or 6-well plate.
9. Count the blue SA-βgal-positive cells under a microscope. In general, human normal fibroblast cultures are senescent if >80% of cells are SA-βgal positive.

OBSERVATION

The image below displays the result of a senescence associated β-galactosidase (SA-β-gal) used to detect senescent cells. Cells that have entered senescence stain blue due to β-galactosidase activity at pH 6. In this example cells were treated with doxorubicin (which induces senescence post drug treatment) show strong blue staining compared to control cells.

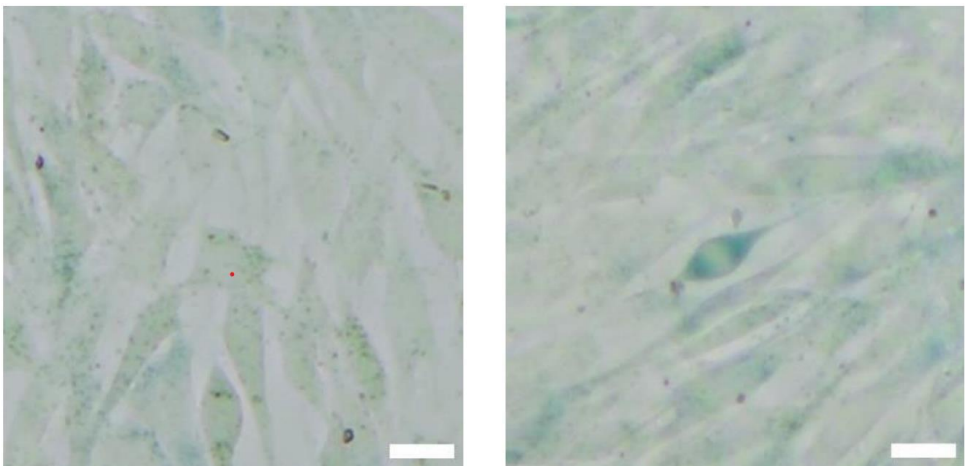

Figure 1. Representative image showing β-galactosidase assay. Left panel is control in which cells do not show characteristic blue-coloured cells, on the other hand right panel shows cells with characteristic blue coloration depicting senescence. The cells were treated with doxorubicin drug (10 µM) for a brief period, washed and left for 10 days to induce senescence and then harvested.

QUESTIONS FOR FURTHER READING

1. How is the working pH adjusted and maintained during the staining step to ensure specificity for senescence-associated β-galactosidase?.
2. What factors determine optimal fixation time to preserve cell morphology without inactivating β-galactosidase enzyme activity?
3. How do incubation duration and X-gal substrate concentration affect the intensity and reproducibility of senescence staining?

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Chapter 23: ASSAYING APOPTOSIS IN MOUSE THYMUS CELLS BY ACRIDINE ORANGE AND PROPIDIUM IODIDE STAINING

Recommended level: PG, Research

INTRODUCTION

Cell death occurs mainly in two ways: Apoptosis and Necrosis. In apoptosis as the cell has made commitment to die, a large number of enzymes become active digesting the cellular components slowly leaving almost nothing after the death. During the process cell membrane gets blebbed and apoptotic bodies are formed. The nuclear DNA also gets fragmented, condensed and are packaged.

There are two kinds of stain, one inclusion stain which can enter the live cell whereas; exclusion stains are unable to permeate through intact membrane. Acridine orange is an inclusion stain and can stain the living cells (green), whereas, propidium iodide can enter the cell only if the membrane permeability is disturbed and the DNA in the nucleus or apoptotic bodies gets stained orange. If a cell population is stained with mixture of these two stains (without fixation) then we observe counter staining for live (green) and dead cells (orange; Necrotic and apoptotic). With careful examination one can clearly distinguish between apoptosis and necrosis. In apoptosis the cells would appear comparatively smaller and condensed fragmented DNA would be very clearly seen. On the other hand, in necrosis the size of the stained dead cell (orange) will be larger and the nucleus would be homogeneously stained.

OBJECTIVE

To induce apoptosis in mouse thymus cell suspension and to identify the apoptotic cells.

MATERIALS REQUIRED

Mouse (less than 2 months of age), Phosphate buffered saline (PBS), Dexamethasone (10 µM final concentration), Acridine orange and Propidium iodide mixture (10 µg/ml each, in water or PBS).

PROCEDURE

1. Dissect out thymus from mouse and put in a watch glass having PBS. Make single cell suspension by agitation in a tube and acclimatize the cells at 37^{o}C for 10 min.
2. Treat the cells with dexamethasone (10 µM) for 0, 1, 2 and 3 h to induce apoptosis.
3. Wash the cells in PBS and resuspend in PBS and stain them with 1:1 mixture of acridine orange and propidium iodide (10 µg/ml each, in PBS) for 5 to 10 min without any fixation (fixation disrupts membrane and even the live cells take up Propidium iodide and stain orange).
4. Observe cells under a fluorescence microscope.

OBSERVATION

Live cells would stain green while the apoptotic cells would show orange fluorescence.

If DNA is isolated from these cells and run on 1.5% agarose gel, a ladder of bands will be seen instead of a single band at high molecular weight range. The appearance of a ladder is indicative of fragmentation of DNA in the nucleus and this is one of the main features of apoptosis.

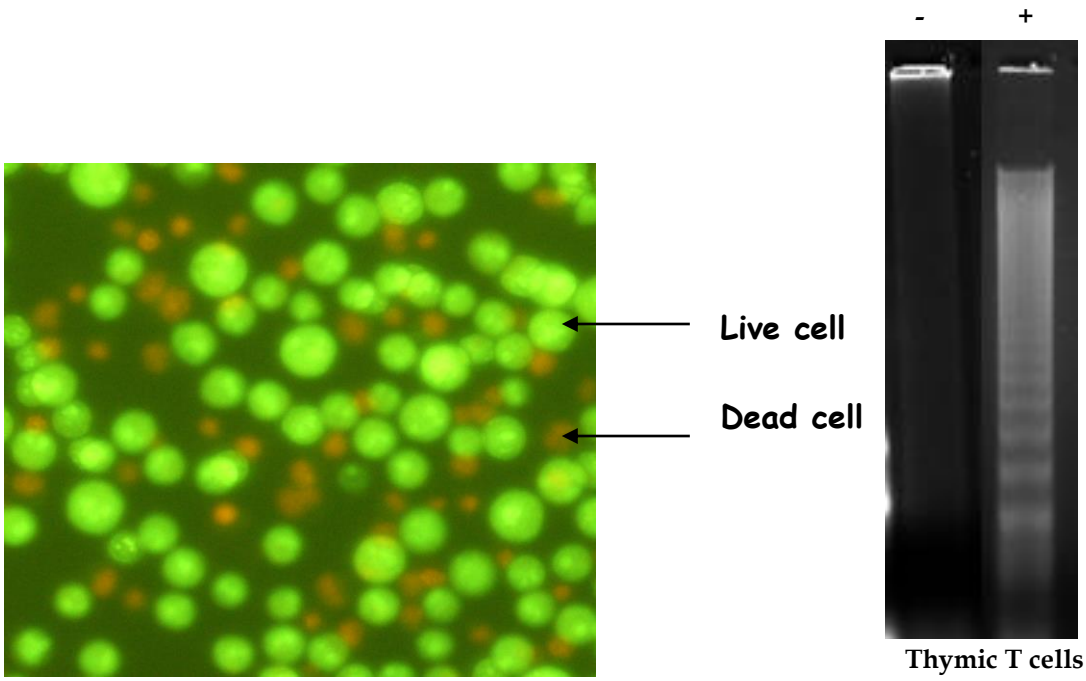

Fig. a. Photomicrograph showing counter staining of live (green) and apoptotic cells (red) with acridine orange/propidium iodide staining of T-cells exposed to Dexamethasone; **b. Agarose gel electrophoresis showing apoptosis in T cells exposed to Dexamethasone (+) for 2 h (thymic T cells)**

QUESTIONS FOR FURTHER STUDIES

1. If cell death has occurred by necrosis in the same tissue, which colour fluorescence it will show?
2. What is the mechanism of action of dexamethasone on thymocytes?
3. If you stain cells with propidium iodide alone, can you identify dead cells?

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Chapter 24: ANNEXIN V-FITC / PROPIDIUM IODIDE STAINING ASSAY FOR THE ANALYSIS OF APOPTOSIS AND NECROSIS

Recommended Level: PG, Research

INTRODUCTION

Apoptosis is a critical biological process of programmed cell death that plays a vital role in maintaining homeostasis and regulating development in multicellular organisms (Green, 2005, 2022).

Discerning and quantifying apoptotic cells with precision is paramount for understanding various physiological and pathological processes. Among the methodologies developed for this purpose, the Annexin V/Propidium Iodide (PI) assay has emerged as a paragon of sensitivity in discriminating between viable, apoptotic, and necrotic cells [Crowley et al, 2016].

The Annexin V/Propidium Iodide assay leverages two complementary components with distinct functionalities. Annexin V, a 35-36 kD calcium-dependent phospholipid-binding protein, exhibits a remarkable affinity for phosphatidylserine (PS) in the presence of calcium ions. During the early stages of apoptosis, PS undergoes translocation from the inner to the outer leaflet of the plasma membrane, while the integrity of the cellular membrane is still maintained. This externalization of PS provides the fundamental basis for employing fluorochrome-conjugated Annexin V to detect and quantify apoptotic cells [Green 2022].

Propidium Iodide (PI) is a red-fluorescent dye that binds to DNA and RNA. It is cell-impermeable, meaning it can only enter cells that have lost membrane integrity, as seen in late-stage apoptosis and necrosis. In aqueous solution, the dye has *excitation/emission maxima of 493/636 nm. However*, upon binding to nucleic acids, PI exhibits a substantial 20- to 30-fold fluorescence enhancement with excitation maxima of ~535 nm and an emission maximum of ~615 nm. The wide range of excitation and emission wavelengths allows PI to be used with a diverse array of fluorescent probes, facilitating multicolour analyses [Green 2005, 2022; Kari 2022].

The Annexin V-PI assay typically categorizes cells into four populations:

1. Viable cells: Annexin V-negative and PI-negative
2. Early apoptotic cells: Annexin V-positive and PI-negative
3. Late apoptotic cells: Annexin V-positive and PI-positive
4. Necrotic cells: Annexin V-negative and PI-positive

The Annexin V-PI assay has found myriad applications in the realms of biological and medical research, spanning fields such as cancer biology, immunology, and drug discovery.

OBJECTIVES

(i) To quantitate the apoptosis
(ii) To discriminate apoptotic and necrotic cell death

MATERIALS REQUIRED

Buffers and Reagents

Phosphate Buffered Saline (PBS), pH 7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4

Annexin V Binding Buffer/HEPES binding buffer: 10 mM HEPES (pH 7.4), 150 mM NaCl, 2.5 mM $CaCl_2$. Available as 5X or 10X concentrate in commercial apoptosis detection kits. Dilute to 1X working solution with distilled water and filter-sterilize before use. Prepare fresh 1X solution for each experiment to ensure optimal results

Annexin V-FITC Conjugate: Available as a ready-to-use solution or can be purchased from several manufacturers along with propidium iodide. This protocol utilizes the Annexin V-FITC kit from Thermo-Fisher Scientific's Dead Cell Apoptosis Kit (V13242), though comparable reagents from other sources are suitable. Gently homogenize the solution before each use. Store at 4°C, maintain on ice during experiments, and protect from direct light exposure.

PI: It can be purchased in powder or solution form. It can also be obtained along with annexin V as components of apoptosis detection kits. Make a stock solution or working solution using Milli-Q water and store it at 4°C in the dark.

Cell Culture Reagents and Equipment:

Humified 37°C incubator with 5% CO_2.

Cell line: Select the appropriate cell line for experimental objectives.

Culture medium: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum and 1X antibiotic-antimycotic solution.

Trypsin-EDTA: 0.25% (w/v) Trypsin and 0.53 mM EDTA. Commercially available, ready-to-use solutions also can be used. Ensure the solution is at room temperature before application.

Culture vessels: Utilize appropriate cell culture flasks and plates.

Test compound: Prepare stock solution of a drug or compound of interest according to experimental design.

Additional Equipment and Materials:

FACS tubes: 5 ml FACS tubes for sample preparation and analysis under flow cytometer.

Flow cytometer: An instrument capable of 488 nm laser excitation and fluorescence detection at 530 nm (for FITC) and 617 nm (for PI)

Centrifuge with a rotor for 15 ml tubes.

Haemocytometer for cell counting while seeding the cells (not essential)

PROCEDURE

Day 1: Cell Seeding

Harvest cells from a quasi-confluent culture flask and inoculate the required number in a 12 well culture plates (~ 40-50% confluency). Allow the cells to grow and proliferate till 48 h in a 5% CO_2 incubator at 37°C.

Day 2: Cell Treatment

1. Prepare a concentration series of test compounds in the fresh cell culture media.

2. Remove old media and add fresh drug-containing media to the cells. For compounds needing a vehicle, include vehicle-only controls.
3. Visualize the cells under a phase-contrast microscope to observe cell death and accordingly, period of drug treatment shall be decided.

Day 3: Cell Staining

*Prior to staining, examine cells under a bright-field microscope. This critical step assesses morphology and identifies issues that could affect cytometric results.

1. Carefully collect media from each cell sample in the pre-labelled FACS tubes to ensure the retention of any detached cells.
2. To harvest the adherent cells, perform a gentle rinse with PBS to remove all traces of culture media as it will interfere with trypsin activity.
3. Immediately, add trypsin solution just to cover the cell surface and incubate at 37°C typically 1-3 min. Monitor the cells under the microscope if cells are rounded. If so, gently tap the plate to detach the cells from the surface.
4. Immediately, neutralize trypsin by adding serum-containing media (approximately 5 times the trypsin volume). Mix the cell suspension by gentle pipetting and transfer to labelled FACS tubes as per the samples (step 1).
5. Centrifuge the sample tubes at 2000 rpm for 3 min to pellet down the cells and carefully discard the media.
6. For washing, gently resuspend the pellet in cold 1X PBS and centrifuge at 3000 rpm for 3 minutes followed by discard of the supernatant carefully.
7. Prepare an adequate volume of 1X HEPES binding buffer from the stock solution according to number of sample tubes. Each sample tubes will require approximately 200 µl of buffer. Dilute the 5X or 10X stock solution precisely with Milli-Q water to achieve 1x binding buffer.
8. Prepare staining solution**:** 10 µl Annexin V-FITC and 2 µg/ml PI per 200 µl 1X HEPES buffer for each sample to be stained.
   (Annexin V and PI concentrations may vary as per the context of specific assays).
   Note: Staining solution should be ready while performing step 6. Staining solution should be added immediately in cell samples once the step 6 is over.
9. Add 200 µl staining solution in sample tubes and gently mix the pellet by pipetting. Following array of controls and testing samples can be included for experiment validation:

Sample	Group	Stain
1	Media only	No Stain
2	Untreated cells	No Stain
3	Untreated cells	Annexin V, Propidium iodide
4	Treated cells	No Stain
5	Treated cells	Annexin V
6	Treated cells	Propidium iodide
7	Treated cells	Annexin V, Propidium iodide

*Few compounds, e.g., doxorubicin or epirubicin, may exhibit inherent fluorescence. Include an unstained treated sample to differentiate between drug-induced signals and specific staining.

10. Incubate samples for 30-45 min at room temperature in complete darkness for optimal staining.
11. Post-incubation, add 300 µl 1X HEPES buffer to each stained sample. Mix gently to ensure homogeneity. Maintain on ice and protect from light to preserve staining integrity. Analyze these samples by employing flow cytometry.

Flow Cytometer System Preparation and Analysis

Instrument Initialization:

Power on and thoroughly clean the flow cytometer.

Configure excitation (488 nm) and emission settings: Annexin V-FITC: Ex 488 nm / Em 520 nm Propidium Iodide: Ex 535 nm / Em 617 nm

Note: Both fluorophores are efficiently excited by a 488 nm laser for simultaneous detection. Basically, FL1 and FL2 channels are used for FITC and PI respectively.

PROCEDURE

1. Create a new experiment file.
2. Establish primary dot plot: Forward scatter (FSC) vs. Side scatter (SSC) on linear scale.
3. Create an FSC-Height vs. FSC-Area plot to exclude doublets and isolate singlets.
4. Create a secondary dot plot: Propidium iodide vs. Annexin V-FITC on log scale for apoptosis analysis.

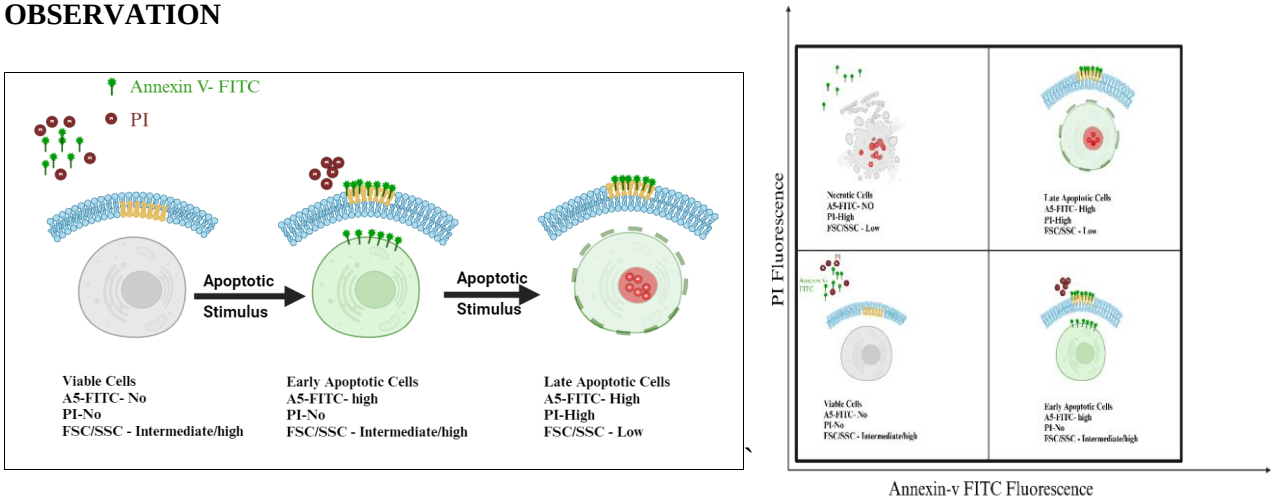
Sample Calibration:

1. Analyze an untreated sample, adjusting FSC and SSC voltage and gain to detect live cells, set broad gates to include both live and dead cell populations, unlike typical flow cytometry protocols that focus solely on live cells.
2. Set broad gates encompassing both live and dead populations.
3. Establish FSC and SSC exclusion gates to eliminate cellular debris. While small particles in the FSC-SSC plot's lower left quadrant are typically non-cellular and should be excluded, monitor this region for apoptotic bodies. These cellular fragments, though not counted as intact cells, offer valuable insights into the culture's overall cell death levels.
4. Analyze PI-only cells: Calibrate voltage to position dead cells at quadrant apex.
5. Examine Annexin V-only cells: Adjust voltage to concentrate apoptotic cells in the lower quadrant region.
6. Evaluate dual-stained sample: Verify proper distribution of live, apoptotic, and necrotic cells.

Data Acquisition:

Collect a minimum of 10,000 events per sample for optimal peak distribution and statistical robustness.

OBSERVATION



a **b**

Fig.1: Diagrammatic illustration of Annexin V-mediated apoptosis detection

(a) In homeostatic cells, phosphatidylserine (PS) is asymmetrically distributed, residing exclusively on the cytoplasmic face of the plasma membrane, maintained by Mg^{2+} and ATP-dependent amino phospholipid translocase. During apoptosis, PS translocate to the outer leaflet of the plasma membrane, serving as a signal for phagocytic recognition. FITC-conjugated Annexin V binds specifically to this externalized PS in a Ca^{2+} dependent manner.

(b) The dual-staining approach, analysed by flow cytometry, allows precise discrimination between cellular states: (i) viable cells (Annexin V-/PI-) exclude both markers (ii) early apoptotic cells (Annexin V+/PI-) bind Annexin V but maintain membrane integrity, excluded PI (iii) late-phase apoptotic cells (Annexin V+/PI+) show both the markers (iv) necrotic cells reflect only PI positivity.

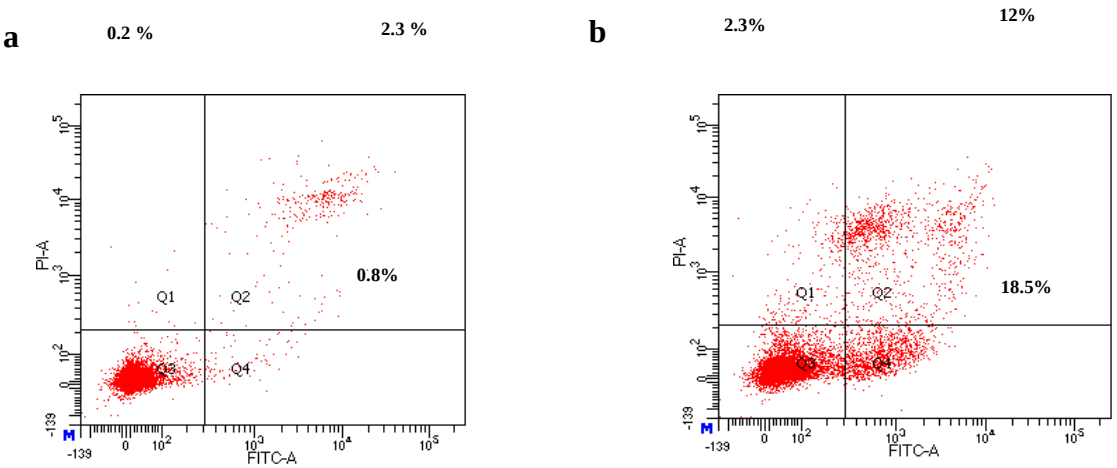


Figure 2. Typical flow cytometry-based quadrant analysis of annexin V-FITC/PI staining for the detection of apoptosis and necrosis- A549 cells treated with either (a) vehicle control (DMSO) or (b) 50 µM Etoposide for 24 h, 10,000 cells were analysed per sample. The proportion (%) of cell number is shown in each quadrant. The proportion of viable cells is shown in Q3 quadrant (FITC -/ PI -) (lower left), early apoptotic cells shown in Q4 quadrant (FITC +/ PI -) (lower right), late-stage apoptotic cells shown in quadrant Q2 (FITC +/PI +) and lastly, the upper left Q1 quadrant (FITC-/PI+) reflects necrotic cells.

QUESTIONS FOR FURTHER STUDIES

1. What causes the translocation of phosphatidylserine during apoptosis?
2. Why is propidium iodide unable to enter early apoptotic cells?
3. How do macrophages recognize the "eat-me" signal on apoptotic cells?
4. What factors can lead to false positive results in Annexin V-PI staining?
5. How does the annexin V/ PI assay distinguish between apoptosis and necrosis?

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Chapter 25: DETECTION OF APOPTOSIS IN *DROSOPHILA* LARVAL EYE DISC BY TUNEL STAINING

Recommended Level: PG/Research

INTRODUCTION

In multicellular organisms, the number of cells is strictly controlled by regulating the balance between the rate of cell division and cell death (Argyle et al., 2007). Cells initiate an intracellular program to induce cell death when they are no longer required or when they are stressed. This programmed cell death, initiated by a series of intracellular signals inside the cell, is called apoptosis (Wyllie, 1997). DNA fragmentation is one of the important events that can be detected in a cell undergoing apoptosis (Bortner et al., 1995). The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay uses the enzyme terminal deoxynucleotidyl transferase (TdT), which catalyzes the addition of deoxyribonucleotides to the 3'-hydroxyl (3'-OH) termini of the fragmented DNA strand (Heatwole, 1999). The deoxyribonucleotides are directly tagged with a fluorescent dye like fluorescein (FITC), TMR-Red, or can be tagged with biotin that is then bound by streptavidin-HRP and detected using a chromogenic HRP substrate, such as 3, 3′-Diaminobenzidine (DAB) (Gao et al., 2017). The fluorescent signal or the color changes can be detected and quantified for cell death. In this protocol, we use the *In Situ* Cell Death TMR-Red TUNEL System to detect the apoptotic cells in the *Drosophila* eye disc by catalytically incorporating TMR-Red-dUTP at 3´-OH of the fragmented DNA ends by TdT. The TMR-Red-dUTP labeled eye disc is visualized under a Confocal microscope for detection of cell death.

OBJECTIVES

To dissect the *Drosophila* third instar eye disc and detect programmed cell death using the TUNEL assay kit.

MATERIALS REQUIRED

In Situ Cell Death Detection Kit, TMR-Red (12156792910, Roche, Switzerland)
Proteinase K (P4850, Sigma, USA)
Third instar *Drosophila* larvae
Brush (Round 4), Cavity Block, Dissecting needles, Micropipettes, Petri dish (960010, Tarsons), Aluminium foil, Glass slides, Transparent nail polish, Coverslip (18x18 mm)
Prolong Gold Antifade containing DAPI (4',6-diamidino-2-phenylindole) (P36931, Thermo Fisher Scientific, USA)
Fluorescence Microscope

Reagents

1XPBS (Phosphate Buffer Saline [130 mM NaCl, 9 mM Na_2HPO_4, 2 mM $NaH_2PO_4{\cdot}2H_2O$ in distilled water. pH 7.4 adjusted using HCl or NaOH and then final volume is made.

4% PFA (Paraformaldehyde, 28906, Sigma): 16% paraformaldehyde is diluted with dH_2O (Warning: Paraformaldehyde is toxic and flammable).

0.1% PBT (phosphate-buffered saline with Triton-X): 1x PBS with 0.1% Triton-X 100.

PROCEDURE

1. **Obtaining Third Instar *Drosophila* Larvae**: Wandering third instar *Drosophila* larvae are collected using a round brush and transferred to a glass slide with a drop of PBS (Fig. 1A).
2. **Dissection of Eye Discs**: Third-instar larval eye discs are dissected in PBS using dissecting needles. The larva is secured by one dissecting needle by positioning it over the abdominal region while the other needle is carefully positioned on the mouth hook. A gentle tug of the mouth hook facilitates the isolation of the eye disc along with the larval brain from the remainder of the larval body. Employing the needles, precise incisions are made, leading to the separation of the eye discs from the larval brain. The eye disc is transferred to a cavity block filled with PBS (Fig. 1B).
3. **Fixation**: The eye disc samples are fixed in 4% PFA for 20 min at room temperature (Fig. 1C).
2. **Washing:** The fixed tissues are then subjected to three washes at 10 min intervals with PBS to remove excess fixative.
3. **Permeabilization**: The dissected eye discs are permeabilized by treatment with 20 µg/ml Proteinase K for 2 min on ice. Then they are washed three times in PBT for 5 min each (Fig. 1D).
4. **Secondary fixation:** This is carried out by incubating the tissues in 4% PFA for 15 min, followed by another three washes in PBT at 10 min intervals.
5. **TUNEL Labelling**: The permeabilized and fixed eye discs are then incubated overnight with a TUNEL reaction containing TdT and TMR-Red-dUTP mixture at 4°C. Following incubation, the tissues are washed five times in PBT for 15 min each to remove unbound TUNEL reagents (Fig. 1E).
6. **Mounting for Imaging**: Finally, the prepared tissues are put in a drop of mounting media and placed on a glass slide. The DAPI in mounting media stains the nuclei of the cell. The samples are covered with coverslips, and the edges are sealed with transparent nail polish. Finally, the slides are imaged under confocal microscope (Fig. 1F).

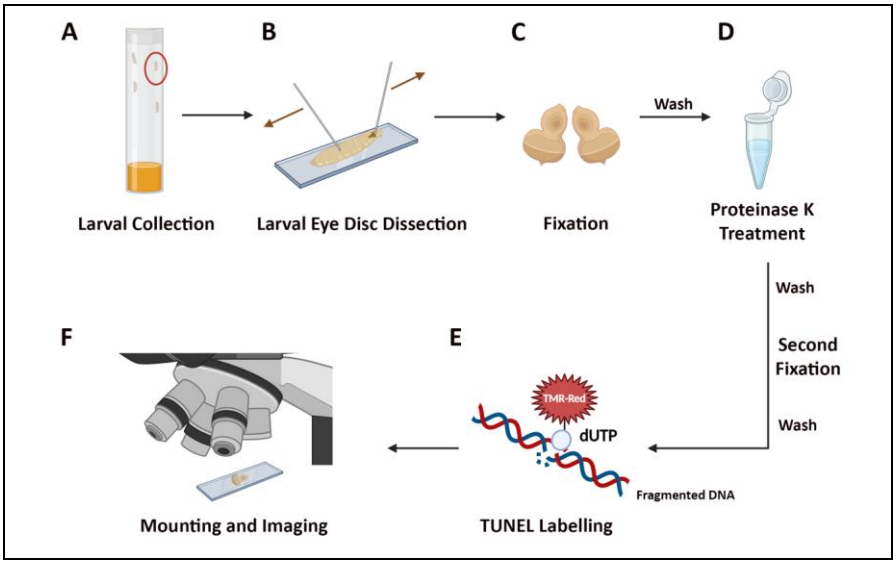

Figure 1. Schematic representation of the TUNEL assay for *Drosophila* Larval eye disc. (A) Collection of 3rd instar wandering larvae using a brush. (B) Larval eye disc dissection using two dissecting needles positioned over the abdominal region of larvae and on the mouth hook. (C) Fixation of dissected eye discs in 4% PFA. (D) Permeabilization using Proteinase-K. (E) TUNEL labelling of eye disc overnight at 4^0C. (F) Mounting and imaging of the eye discs.

OBSERVATIONS

In this experiment, larval eye discs are subjected to TUNEL staining, as described above. Following staining, the eye discs are carefully mounted using antifade DAPI media, which preserves fluorescence signal, stains the nuclei in the sample and marks the boundary of the imaginal disc. The morphogenetic furrow, eye disc region, and antennal region are seen in DAPI staining (Fig. 2A). The TUNEL positive puncta are observed in the third instar eye disc (Fig. 2B, see arrow). The apoptosis in the eye disc is seen below the morphogenetic furrow in the merged image (Fig. 2C). This basal level of programmed cell death in the developing eye disc is required for proper tissue patterning (Rusconi et al., 2000). Lastly, the protocol will be useful for biologists who are interested in detecting apoptosis in different model organisms and different disease models.

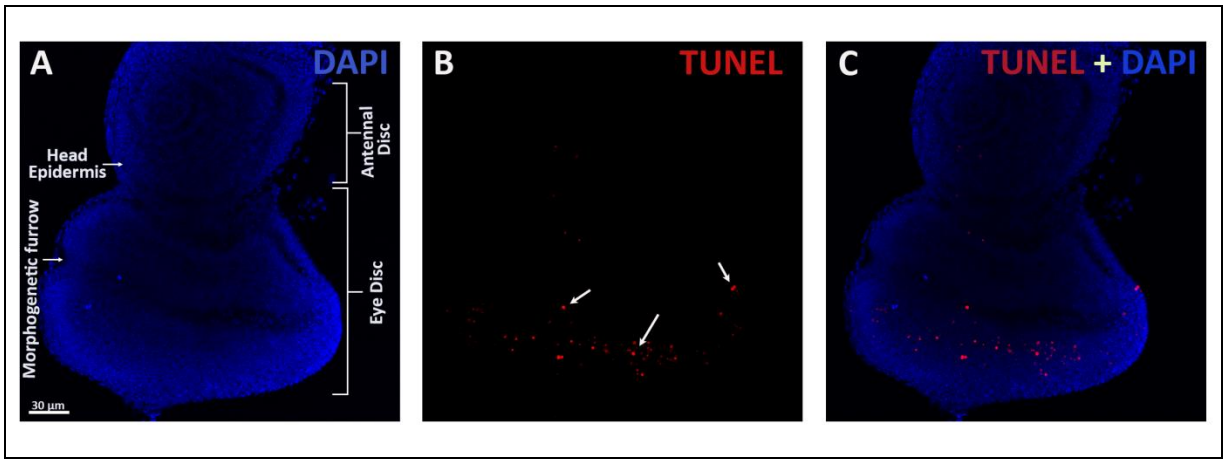

Figure 2. Programmed cell death in third instar *Drosophila* Eye disc. (A) The eye disc is stained with DAPI (Blue) to define the overall nuclei of the eye disc. (B) TUNEL positive cells (Red puncta) indicated by arrows. The red puncta mark the cells undergoing apoptosis as the TUNEL stain detects only the fragmented DNA. (C) Merged Image showing the co-immunostaining of TUNEL and DAPI. Scale Bar 30 µm.

QUESTIONS FOR FURTHER STUDIES

1. What is apoptosis, and why is it important in multicellular organisms?
2. What is the principle of TUNEL assay?
3. What is the importance of Proteinase K treatment in TUNEL assay?

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Chapter 26: COMET ASSAY TO ASSESS DNA DAMAGE

Recommended Level: PG/Research

INTRODUCTION

The comet assay, also known as single-cell gel electrophoresis, is a sensitive technique for measuring DNA damage in individual cells. This assay relies on the ability of denatured, cleaved DNA fragments or damaged DNA to migrate out of the cell under electrophoresis, forming a "comet tail," while undamaged DNA remains within the cell membrane, creating the "comet head." The comet assay is most performed under alkaline conditions to detect both single- and double-stranded DNA breaks but can also be conducted under neutral conditions to detect only double-stranded DNA breaks.

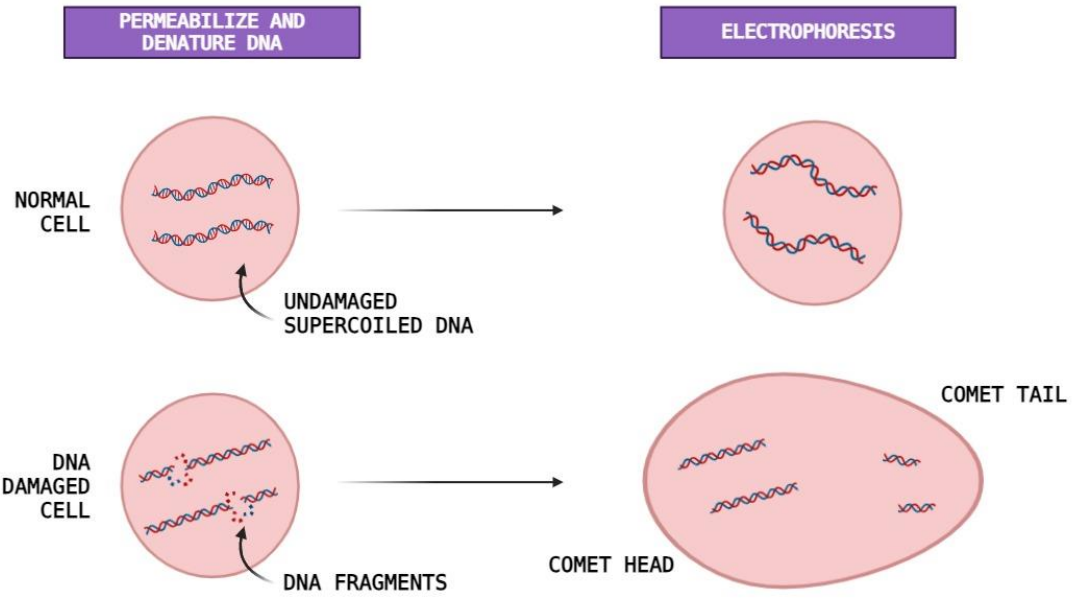

Figure: Representative image showing permeable & denature DNA before electrophoresis and after electrophoresis

OBJECTIVE

To assess DNA damage in cells by using Comet assay technique

MATERIALS REQUIRED

Slide preparation

- Add 1 g of agarose in 100 ml dd H_2O, microwave to dissolve agarose to make 1% agarose gel, cool down to around 60°C before use.
- Dip the slides in 1% NMPA and wipe one side of slide, keep in oven (65°C) less than 1 h
- (10X PBS, Ca++ and Mg++ free* (Prepare 1X PBS in autoclave water and filter)
- 2.1% or 0.8% 0r 0.6% Low melting Agarose (LMA)

- Prepare low melting agarose and keep in a 37°C water bath
- 0.6% LMA: We prepared for our samples, LMA in 1X PBS* (filtered)
- For 6-8 samples do not prepare more than 10 ml. Heat 5 min at 100°C using a water bath/ microwave oven to dissolve agarose (in glass bottle), aliquot the agarose into 15 ml tubes (~3 ml/ tube) before gelation and store the tubes under 4°C.
- Incubate the tubes with aliquoted agarose at 37°C for at least 2 h prior to mixing with cells.

Alkaline lysis solution (1 Liter)

Take 900 ml dH_2O (autoclaved) and add following reagents

Working Conc	**Stock**
NaCl- 2.5 M	3 M
Disodium EDTA- 100 mM	0.5 M (pH-8.0)
Tris-base- 10 mM	1 M (pH-8.0)
NaOH- 200 mM	5 M

Adjust the pH-10, adjust the final volume to 1000 ml, cool it to 4°C.
Keep the solution in 4°C till further use.
Add 1% Triton-X-100 before lysis
10% DMSO (For our cells of concern we used 1% DMSO in Lysis solution).
Filter the solution in aliquots and add just before lysis, 1% Triton-X-100 and 1% DMSO.

Alkaline Electrophoresis Solutions (pH>13)

Take 1800 ml dH_2O (autoclaved) and add the following reagents
NaOH- 200 mM
Disodium EDTA- 1 mM
Adjust the final volume to 2000 ml with autoclaved water.

Note: The same solution is used as alkaline unwinding solution. Wear gloves when preparing and handling the Alkaline Unwinding Solution as the solution becomes hot during its preparation. Allow to cool to room temperature before use.

Staining solution

Propidium Iodide STOCK 1 mg/ml (aliquot small volume and store it in 4°C dark). Prepare a working solution of 5-10 μg/ml before use.

PROCEDURE

Control and Standardization for Comet Assay

Control Sample Preparation:

Include untreated cells as a negative control, for assay variability, endogenous damage levels, and additional damage during sample preparation. Handle control and treated cells identically. For UV damage studies, keep cells in low yellow light during processing.

Positive Control Generation

To generate samples positive for comet tails, treat cells with 100 μM hydrogen peroxide or 25 μM $KMNO_4$ for 20 min at 4°C.

Standardization Steps:

1. Lysis Solution: Cool the lysis solution and melt the low melting Agarose while preparing cell and tissue samples.

2. Large Sample Batches: For convenience, perform cell lysis overnight (Alkaline step 5) when dealing with many samples.
3. Cryopreservation: Use cryopreservation to process experimental samples concurrently.
4. Avoid natural light and direct artificial light during the process to prevent DNA damage and bias in the comet assay.
5. Remove the media from cells after treatment. Wash once with 1X PBS and EDTA.
6. To harvest cells, Trypsinize it (trypsin 0.125%, for MCF7 and UC3 cells). Make single suspension of cells in ice cold 1X PBS (200-500 µl). Rigorous pipetting should be avoided.
7. Scrape (only if high level of damage is seen in healthy population, reduce cell exposure to Trypsin) the cells (for adherent cells) by scraper and collect in a microfuge tube.
8. Keep the tube in ice bucket and take out for slide preparation
9. Combine cells at 1 x 10^{5}/ml with molten LM Agarose (at 37°C) at a ratio of 1:10 (v/v) and mix gently
10. Add 80 µl of the solution prepared in step 6 on to the slides and keep cover slide on it.
11. Place slides flat at 4°C in the dark (in refrigerator) for at least 10-15 min. Increasing gelling time to 30-60 min improves adherence of samples in high humidity environments.
12. After 1 h dip the slide in alkaline solution at 4°C in a coupling jar and leave for 1 h.
13. For added sensitivity or convenience incubate overnight at 4°C.
14. After 1 h drain excess buffer and keep in alkaline solution (pH>13) for 30 -60 min
15. Then start electrophoresis 21Volt, 0.3A, for 20-30 min in Alkaline Unwinding Solution at 4°C, in the dark.

Since the Alkaline Electrophoresis Solution is a non-buffered system, temperature control is highly recommended. To improve temperature control, the use of a large electrophoresis apparatus (20-30 cm between electrodes) is recommended. Performing the electrophoresis at cooler temperatures (e.g. 4°C) will diminish background damage, increase sample adherence at high pHs and significantly improves reproducibility. After electrophoresis the slides were removed and neutralized 1-3X times with 0.4 M Tris at pH 7.5.

Staining

1. Add propidium iodide (5-10 µg/ml) 25 µl into each gel.
2. Carefully put the cover slide onto it.
3. Wait for 5 to 7 min and observe under a microscope.

OBSERVATION

The image below shows the result of a comet assay, which is used to detect DNA damage in individual cells. Cell with damaged DNA form a tail when subjected to electrophoresis resembling a comet. The longer and brighter the tail, the greater the DNA damage (Fig. 1).

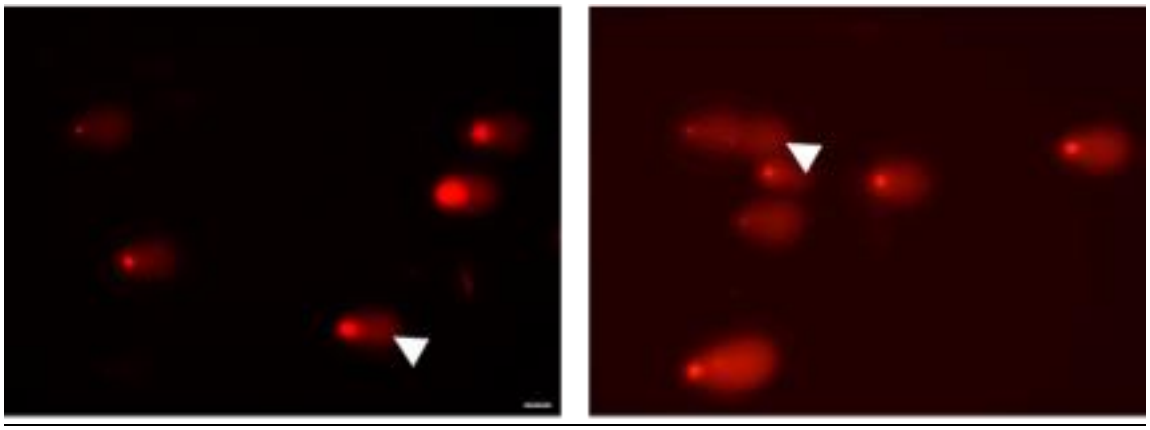

Figure: Representative image shows a comet like picture indicated by white arrow. The length the tail denotes the degree of cell damage caused. In the above result, we observed that the tail is longer in the cells with greater DNA damage by overexpressing specific gene (right panel) than in the control cells (left panel).

QUESTIONS FOR FURTHER READING

1. How does the comet assay detect and quantify DNA damage at single cell level?
2. Why is alkaline comet assay preferred over the neutral version for assessing single strand breaks?
3. What are the key parameters influencing comet tail length?

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Chapter 27: STUDYING STRUCTURAL CHROMOSOMAL ABERRATIONS IN MAMMALIAN CELLS

Recommended Level: UG, PG, Research

INTRODUCTION

The chromosome aberration test is responsible for the determination of chromosomal disabilities that may further cause genotoxicity. It is usually observed because of exposure to certain chemical compounds causing chromosomal deformation and thus proved to be genotoxic. It is a sensitive test for DNA damage (Greenwood et al., 2004). Double-stranded DNA damage is also one of the factors responsible for chromosome aberration. It can be direct damage due to exposure to a genotoxic agent. In contrast, it can be indirect damage too, which can result from an error in the replication of DNA or an error in repair, finally leading to a double-strand break (Obe et al., 2002).

Chromosomal aberrations can be produced experimentally by exposing active cells to mutagens, such as ionizing radiation or chemical mutagens. Structural chromosomal aberrations occur when part of a chromosome is missing (deletion), turned upside down (inversion), duplicated, or attached to another chromosome (translocation) and produce microscopically visible fragments or structural rearrangements). The aberrations are best observed at the metaphase stage of cell division when chromosomes are condensed.

The in vitro chromosome aberration test uses cell lines based on growth ability, karyotype stability, chromosome number, diversity, or human peripheral blood lymphocytes. The in vivo chromosome aberration studies can be performed in mouse or rat bone marrow cells in the laboratory.

OBJECTIVES

To study structural chromosome aberrations in human peripheral blood lymphocytes.

MATERIALS REQUIRED

All materials required for short term cell culture and chromosome preparation

PROCEDURE

Setting the culture and chromosome preparation

4. After the collection of human blood, distribute 1ml blood in a culture vial as untreated and another 1 ml put in a vial as a treated (treated with either radiation or chemicals). If treated with chemicals, then treat the sample for 2 h or more as per requirement. After chemical treatment, 8 ml culture medium is added to both the culture vials, centrifuge at 1400 rpm and then discard the supernatant. Proceed for culture setting for 54 to 72 h (as per requirement). If radiation is used for the treatment, then proceed for culture setting 1 h after radiation. All the samples are kept at 37^{o}C till the culture setting process starts.

5. For each culture, add the following in the order given:

TC medium	5 ml
Fetal bovine serum	1 ml (if not already added)
Blood	0.3 ml
PHA	0.1 ml

4. In order to buffer the pH, blow CO_2 from a CO_2 cylinder or bubble exhaled air orally through a cotton-plugged-pipette.

4. Culture is kept in an incubator at 37^oC for 54 to 72 h and inspected every morning and evening for change in pH and infection and shaken to break the clumps of RBCs.

5. About 2-3 h prior to harvesting the culture for chromosome preparation, colcemid (working conc. 0.02 μg/ml) is added.

Chromosome Preparation

1. Transfer the culture to a centrifuge tube and centrifuge at 1000 to 1200 rpm for 5 min

2. Decant the supernatant and make a fresh suspension of cells in prewarmed 0.56% KCl (hypotonic). Initially add a small volume and agitate the sediment. Once the cells come in suspension, make up the volume to 8-10 ml. Keep in incubator (37^oC) for 18-20 min

3. Immediately before centrifugation, add 3-4 drops of fixative to the tube and mix. Spin (1000 - 1200 rpm) the tubes and decant the hypotonic completely. Add the fixative drop-by-drop to fix the cells and to keep them in suspension. Make up the volume to ~8-10 ml. Keep for 15 min

4. Re-centrifuge (1000-1200 rpm) for 5 min, decant the supernatant and resuspend the pellet in fresh fixative (~ 8-10 ml). Keep for 10 min.

5. Re-centrifuge (as in #4) and discard the supernatant. Add only about 0.2-0.4 ml of fixative. Resuspend the cells well by gentle agitation.

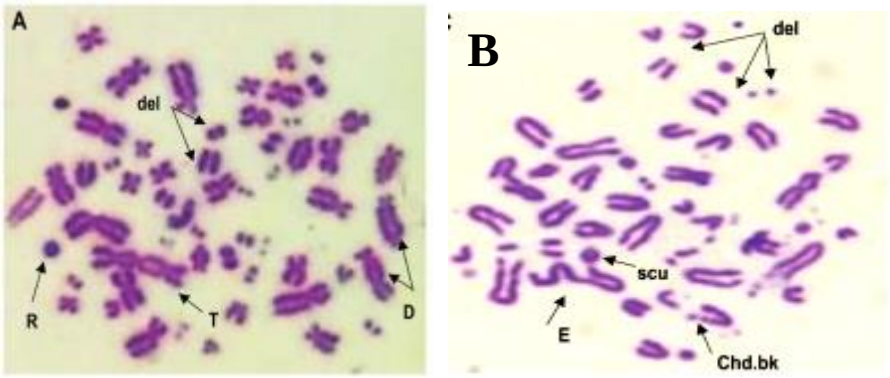
6. Take out a slide from 70% alcohol and wipe it with a clean piece of cloth. Add 2-3 drops of the cell suspension on the slide and either blow it dry (air-drying) or expose to a flame for instant drying (flame drying).

7. Stain the slide with Giemsa stain for 3-4 min and rinse in 2 changes of distilled water (pH 6.8-7.2) or clean tap water. Dry it fully and mount with DPX mountant using a 24x60 mm cover glass.

Note: The same experiment can be done in vivo using rat or mouse also.

OBSERVATIONS

Score chromosomal aberrations from 100 metaphases spread each from untreated and treated slides. The two photographs (Fig. 1A, B) show different types of aberrations induced by radiation.



A. Microphotograph shows radiation-induced chromosome aberrations in human blood lymphocytes. D, dicentric; del, deletion; R, ring; T, tricentric (3 centromeres).

B. Radiation-induced chromosomal aberrations in mouse bone marrow cells. Mouse has 40 acrocentric chromosomes. chd.bk, chromatid break; del., deletion; E, exchanges (note: in mouse cells exchanges are mostly chromatid exchange since cells are dividing during exposure; therefore, clear dicentric and ring of chromosome type exchanges are difficult to obtain).

The aberration scoring data can be tabulated in the following manner:

Experimental condition	Total metaphases scored	Aberrant metaphase (%)	Aberration %		
			Exchanges	Chromatid breaks	Deletions
Untreated	94	2	0	2	0
Treated	104	45**	28[aa]	38[aa]	22[aa]

Exchanges include dicentric, ring and chromatid break
**** p < 0.001 2 × 2 contingency χ2-test compared with respective control.**
aa p < 0.001 χ2-test at d.f. = 2 compared with respective control.

From the table, the data indicate a higher frequency of aberrant metaphases and different types of aberrations in the treated samples compared to the untreated control. Therefore, the treatment with the chemical/radiation induces a significant chromosomal damages in the cell.

Dicentric chromosome: An abnormal chromosome with two centromeres (by fusion of two chromosome broken ends), produced by chromosomal aberrations and are used for radiation damage assessment.

Ring : A ring chromosome is a circular structure formed when a chromosome breaks in two places and its broken ends fuse together.

Chromatid break: is a discontinuity of a single chromatid.

Chromosome break or deletion: is a discontinuity of both the chomatids of a chromosome.

QUESTIONS FOR FURTHER STUDIES

1. How one will be sure that the chromosome damages seen in the study are not due to technical issues like harsh procedure used in the study?
2. How do dicentric and ring chromosomes appear?
3. By seeing chromosome or chromatid breaks can one imagine in which phase of cell cycle the break actually occurred?

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Chapter 28: ASSAYING PHAGOCYTOSIS IN MOUSE MACROPHAGES

Recommended level: PG, Research

INTRODUCTION

The Phagocytes are large white cells that can engulf and digest foreign invaders. They include monocytes, which circulate in the blood and macrophages, which are found in tissues throughout the body, as well as neutrophils, cells that circulate in the blood but move into tissues where they are needed. Monocytes are the precursors of macrophages. They are larger blood cells, which after attaining maturity in the bone marrow, enter the blood circulation where they stay for 24-36 h. Then they migrate into the tissue, where they become macrophages and move within the tissues. In the presence of an inflammation, monocytes quickly migrate from the blood vessel and start an intense phagocytic activity.

Macrophages are ideal to show phagocytosis. Another important point is that these cells can be easily separated from other cells because of their adhering property. Macrophages adhere to surface (glass slide, culture plates) when incubated at optimal temperature and can be detached from the surface by lowering the temperature. This property is utilized for obtaining the macrophage from the peritoneal cavity of the mice by injecting chilled PBS and then making them adhere again on glass slide for studying phagocytosis.

OBJECTIVE

To observe the phagocytic activity of the macrophages.

MATERIALS REQUIRED

Mouse, dissecting tray & tools, Petri-dish, slides, coverslips, BOD incubator at 37^{o}C, Water bath at 37^{o}C, PBS (Phosphate buffered saline, pH 7.4), Crystal violet stain (0.2%; 0.2 g of crystal violet mixed with 2 ml of ethanol to dissolve and finally make the volume to 100 ml with distilled water), Compound microscope at least with 40X magnification, mounting medium DPX.

PROCEDURE

1. Sacrifice a mouse by cervical dislocation. Inject 5 ml chilled PBS and 1 ml air in the peritoneal cavity and agitate the mice for 5 min.
2. Aspirate back the PBS injected from the peritoneal cavity. This is called peritoneal lavage.
3. Place a clean slide on a Petri-dish and pour 200-500 µl of the collected lavage on the slide and incubate the slide at 37^{o}C in moist chamber (water bath or BOD) for 1.5 to 2 h.
4. In the mean while prepare yeast cell suspension. Heat kill the yeast cells by boiling Baker's yeast (3 mg/ml) in PBS in a test tube in a boiling bath for 15 min, wash 2-3 times with PBS. Keep the heat killed yeast cells for further use.
5. After incubation wash the slide gently with PBS to remove the non-adherent cells. Majority of adherent cells will be macrophages and it will remain adhered on the slide.
6. Flood the macrophages on slide with yeast cell suspension prepared.

7. Incubate the macrophages (on slide) with heat killed yeast cells for 1 to 1.5 h again in moist chamber as above.
8. After incubation wash the cells vigorously with PBS three times to remove all the yeast cells that have not been phagocytosed by the macrophages.
9. Fix the cells with methanol for 2- 5 min and then air dry.
10. Stain the cells with crystal violet for 5 min or with Giemsa for 20 min and observe under the microscope.

OBSERVATION

Search for flattened cell showing several processes radiating out. Those are macrophages. Several of them are seen at different stage of engulfing dead yeast cells (Fig. 1). Score the macrophages showing phagocytosis and calculate the % phagocytosis and phagocytic index:

$$\% \text{ Phagocytosis} = \frac{\text{Number of macrophage showing phagocytosis}}{\text{Total number of macrophages}} \times 100$$

$$\text{Phagocytosis index (PI)} = \frac{\text{Total number of yeast phagocytosed}}{\text{Total number of macrophage showing phagocytosis}} \times 100$$

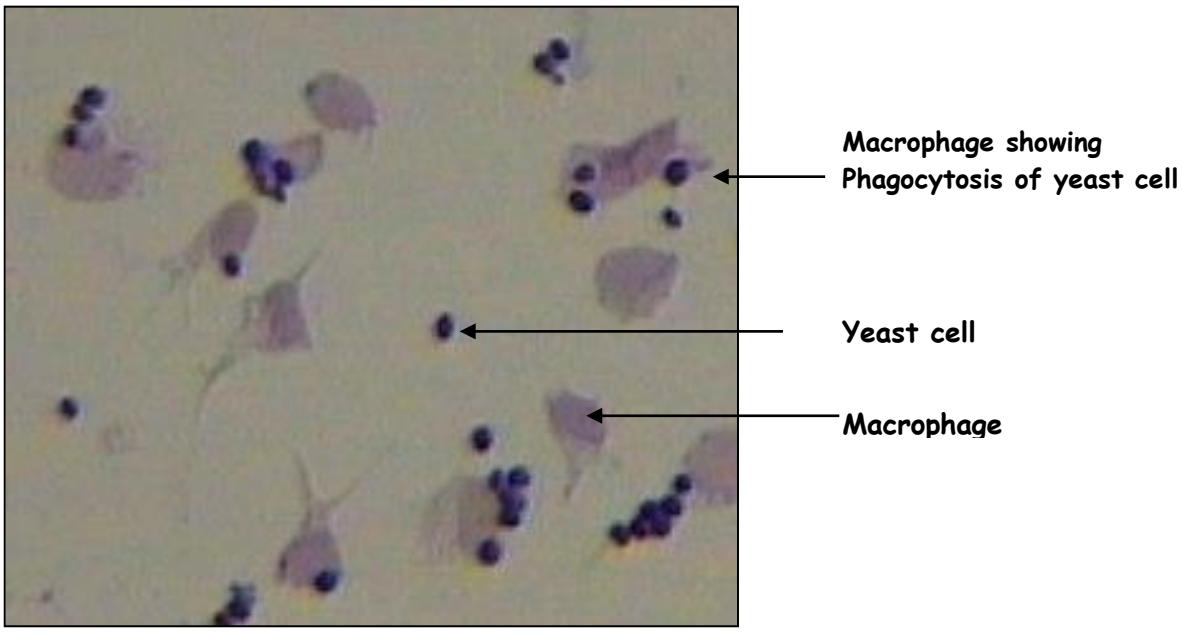

Figure 1. Phagocytosis as seen on the slide

QUESTIONS FOR SELF STUDY

1. Why heat killed yeasts and not live yeast cells were provided?
2. Why other cells along with macrophages are not seen? Is this because of macrophages adhesion property?

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Chapter 29: PREPARATION OF POLYTENE CHROMOSOMES FROM *DROSOPHILA* LARVAE

Recommended level: UG, PG, Research

INTRODUCTION

Polytene chromosomes are well known for their use in a variety of genetic, cytogenetic (Bridges 1935) and molecular studies. These chromosomes remain in permanent interphase but due to repeated cycles of endoreduplication and tight lateral association of all the chromatids, each chromosome becomes thicker and distinctly visible as a cable-like structure with alternating dark and light regions, the bands and interbands, respectively. Polytene chromosomes are most commonly found in dipteran insects. In addition, they are also seen in certain other insects, macronucleus of some ciliates and in certain plant tissues.

Drosophila has been very widely used for studies on polytene chromosomes. Salivary glands of late third instar larvae provide cytologically excellent polytene chromosome preparations due to a high level of polyteny achieved by cells in this tissue. Polytenization in *Drosophila* begins at early stage of development in specific tissues with homologous chromatin pairing (somatic pairing), fusion of centromeric heterochromatin and then endoreplicaion cycles. With increasing larval age, the level of polyteny of chromosomes in cells of salivary glands increases. Each salivary gland has about 120 cells. Of these, the more posterior cells endoreplicate more often than the anterior ones so that posterior cells provide better chromosome preparations. By late 3rd instar stage, many of these cells have completed 9 extra rounds of replication (1 normal round + 9 extra round = 10 round of replication beginning from 2 DNA double helix in a homologous chromatin pair). All the resulting 2^{10} DNA double helix (i.e., 2^{10} chromatins) maintain their lateral association in such a way that their differentially coiled regions remain in tight register, this results in the characteristic banding pattern consisting of more dense band regions alternating with light stained interbands. The chromatin is more densely packed in bands while it is less coiled in interband regions. Transcriptionally active regions can be easily identified under light microscope as "puffs". The chromatin fibrils are more loosely arranged in a puff and the newly synthesized RNA also accumulates here. These events result in the enlarged diameter and lighter staining of puff regions compared to the transcriptionally inactive regions (bands). The fused pericentromeric heterochromatic regions of different chromosomes form the chromocenter. The euchromatic arms of different chromosomes appear to radiate from this common chromocenter (in some dipterans, like *Chironomus*, chromocenter formation does not occur in polytene cells). A major part of the DNA in chromocenter region does not participate in endoreplication, i.e., it remains under-replicated.

Drosophila melanogaster has an acrocentric X-chromosome, two pairs of metacentric chromosomes (chromosomes 2 and 3) and a pair of very small dot-like 4th chromosomes. Females have two X-chromosomes while males have one X and a large sub-metacentric Y-chromosome. Y-chromosome, like the other heterochromatic regions, remains buried within the chromocentre mass. As mentioned above, the homologous chromosomes in polytene cells remain tightly synapsed, a polytene nucleus of *D melanogaster* shows a common chromocentre (formed by centromeric and pericentromeric heterochromatic regions of all chromosomes) from which 5 long and a very short euchromatic banded chromosome arms radiate out. The 5 long arms represent the X-chromosome, left and right arms of chromosomes 2 and 3, respectively, while the very short arm is formed by the 4th chromosome. Each chromosome arm has a characteristic banding pattern due to which each region of every chromosome

can be very easily distinguished and identified. Every band is given a specific number identity: for *D melanogaster*, the polytene chromosome maps prepared by C B Bridges and P N Bridges in 1930s and 1940s are followed to identify each of the approximate 5000 bands seen in a salivary gland polytene nucleus. As the salivary glands are histolysed after pupation, polytene chromosome preparations can be prepared from salivary glands of larvae only. Salivary glands of adult flies do not contain polytene cells.

For cytological studies, polytene chromosome preparations are made by the classical squashing technique following a brief fixation and staining. These preparations could be temporary or permanent, depending upon how they are made.

OBJECTIVE

To make a temporary squash preparation of polytene chromosomes from salivary glands of late third instar larva of *Drosophila melanogaster*.

MATERIALS REQUIRED

Healthy late 3rd instar larvae, blotting paper, droppers, dissecting needles, fine forceps, cavity slides, clean glass slides, cover glasses, stereo-binocular microscope, dipping jars, microscope for examination of preparations.

Solutions

Poels' Salt Solution (pH 6.8) – see page

Fixative - Aceto-Methanol 1:3 (freshly prepared)

2% Aceto-Orcein (2 g Orcein dissolved in 45% acetic acid by boiling for 30 min under a reflux condenser; filter when cool. It is strongly desirable to filter the stain every time before use)

2% Aceto-Carmine (preparation same as acetocarmine)

45% acetic acid

Cleaning of slides and cover-glasses

To obtain good squash preparations, it is absolutely essential that the slides and cover-glasses are totally free of any dust-particles, fibers and greasy material. A simple way to achieve this is to store the fresh (or soap-cleaned, if desired) slides and cover-glasses in 90% ethanol in suitable containers and wipe them dry, immediately before use, with a clean soft silken cloth.

PROCEDURE

1. Take late third instar larvae (about 5 days old if grown at 24^{o}C) from a healthy culture (this stage larvae crawl out of the food medium and move actively on food-free surface), wash them free of adhering food particles with water and transfer to a cavity slide containing a small amount of Poels' salt solution (insect saline (0.67% NaCl)/Ringer's solution can also be used).

2. Using fine forceps and/or dissecting needles, pull forward the mouth parts of larvae to rupture larval skin. This forces out internal organs. Salivary glands are seen as a pair of whitish translucent elongated structures connected at their anterior ends with a common salivary duct. Remove fat bodies adhering to glands.

3. Using tips of the dissecting needles, transfer the cleaned salivary glands to a drop of Poels' salt solution on a clean slide. Drain out the salt solution (do not let the glands dry). Keeping the slide in a slanting position, add drop-by-drop freshly prepared fixative. Wipe out excess fixative with a piece of filter paper (the total duration of fixation should not exceed 1 min since longer fixation

makes chromosomes brittle and difficult to spread). Add a few drops of aceto-orcein or aceto-carmine or a mixture of both stain and leave the slide covered with a watch glass for 10 min.

4. Drain out the stain and add a few drops of 45% acetic acid to remove excess stain. Finally place a drop of 50% acetic acid, cover with clean cover-glass.

5. For squashing, put the slide with its cover-glass between folds of a clean filter paper and lightly tap the cover-glass either with the rubber-end of a pencil or with the blunt end of needle-holder or even with the needle (tapping breaks the cell and nuclear membranes and releases chromosomes free in cytoplasm; a very slight movement of cover-glass on the slide may be desirable but too strong a tapping would break chromosomes in pieces). Hold the cover-glass in position with fingers of one hand placed over the filter paper such that they press on two diagonal corners of cover-glass. Apply firm pressure of thumb of the other hand on the cover-glass. This act of squashing spreads the polytene chromosome arms of a nucleus and makes them flat in one plane. Any lateral movement of cover-glass relative to the slide at this stage is likely to cause "rolling" of chromosomes making them totally unsuitable for study. Too strong a thumb pressure may cause the chromosomes unduly stretched ("optimum" thumb-pressure is learnt with experience only).

6. After squashing, seal the edges of cover-glass with nail-polish or DPX (to prevent evaporation of acetic acid and drying of the slide) and observe under microscope (these preparations will stay for a few days only.

OBSERVATIONS

A good squash preparation reveals many polytene nuclei with well spread polytene chromosome arms connected to a common chromocenter. The chromocenter is an irregular mass of densely stained chromatin giving a granular appearance. This granular and irregularly arranged chromatin is termed the β-heterochromatin. A small very densely stained compact region, the α-heterochromatin can often be seen within this mass. Five long (corresponding to the X, left and right arms of chromosomes 2 and 3 (2L, 2R, 3L and 3R), respectively) and one short (chromosome 4) chromosome arms radiate from the chromocenter. Each chromosome arm displays a typical pattern of dark stained bands and light interbands: this banding pattern allows identification of not only each chromosome arm but also specific chromosome region since each band has been assigned a specific number (see Fig below). Certain specific regions, the puffs, appear swollen (greater diameter) and light stained. Specific regions that are puffed and the size of each puff (the puffing pattern) are characteristic of the developmental stage of the larva.

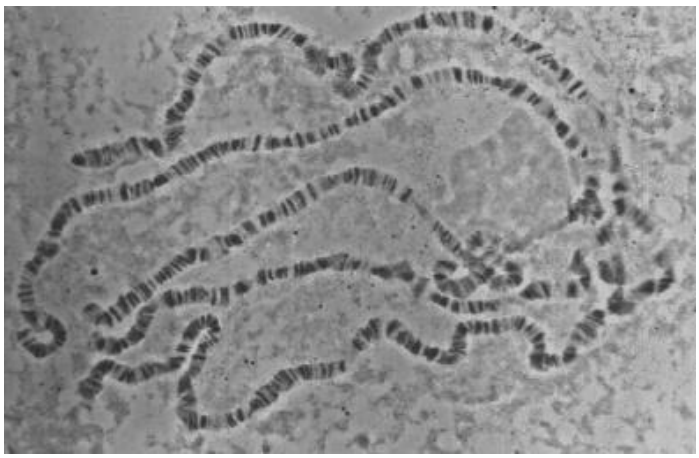

Figure. A polytene nucleus from orcein stained squash preparation of salivary glands of late third instar larva of *Drosophila melanogaster* - note the five long chromosome arms (X, 2L, 2R, 3L, 3R) and the short chromosome 4 connected to a common chromocenter (phase-contrast optics)

QUESTIONS FOR FURTHER STUDIES

1. If a *Drosophila* species has 4 pairs of metacentric chromosomes, how many arms their polytene chromosome will have?
2. How division occurs in those cells which have polytene chromosomes?
3. What is the significance of polyteny?

REFERENCE

Bridges C B (1935). Salivary gland chromosome maps. Journal of heredity 26, 60-64

Adapted from Roy and Lakhotia (2024) Study of polytene chromosomes of Drosophila melanogaster. In: Experiments with Drosophila for biology courses, Ed. Lakhotia and Ranganath, Publ. Indian Academy of Sciences, Bangalore, pp 229-238

Chapter 30: *IN SITU* HYBRIDIZATION ON CHROMOSOMAL DNA

Recommended level: PG, Research

INTRODUCTION

Nucleic acid hybridization provides a means of evaluating homology between single stranded DNA and/or RNA molecules. When one of the hybridization partners remains *in situ* in a cytological preparation, using a given labeled polynucleotide (DNA or RNA) probe, location of the homologous sequences in cells can be determined. *In situ* hybridization studies on chromosomes provide an approach to genetic mapping of the sequence of interest. The pattern of functional organization or its expression can also be studied conveniently by this technique at cellular or at organ level.

OBJECTIVE

To localize a given sequence on polytene chromosome of *Drosophila melanogaster*.

MATERIALS REQUIRED

1. Incubators set at 37^{o}C and 42^{o}C, cleaned glass slides and cover-glasses, slide racks, slide tray, Couplin jars, magnetic stirrer, micropipettes, pipette tips, plastic box, forceps

Solutions required
70%, 90% and absolute ethanol
3 M sodium acetate (pH 5.2 with the help of glacial acetic acid)
1 M Tris pH 7.5, 1 M Tris pH 8.0, 1 M Tris pH 9.5
5 M sodium chloride
0.5 M EDTA
TE (10 mM Tris, pH 8.0; 1 mM EDTA
20x SSC (3 M sodium chloride, 0.3 M sodium citrate)
Digoxigenin-dUTP labeled DNA probe
RNase 10 mg/ml (Stock solution)
20x SSC (3 M sodium chloride, 0.3 M sodium citrate)
0.1% gelatin solution (freshly prepared)
Hybridization mix
Salmon sperm DNA 10 mg/ml
Colour detection buffers

- Buffer I (100 mM Tris, pH 7.5; 150 mM NaCl)
- Buffer II (0.5% w/v Blocking reagent in Buffer I)
- Buffer III (100 mM Tris, pH 9.5; 100 mM NaCl, 50 mM $MgCl_2$)
- Buffer IV (TE)
- Colour Developing Solution (to be prepared fresh just before use)
- Nitroblue Tetrazolium (NBT, 75 mg/ml in dimethyl formamide) 4.5 µl
- 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP, 50 mg/ml in dimethyl formamide) 3.5 µl
- Buffer III to make 1 ml

Safranin stain

Safranin	100 mg
Dist. Water	100 ml

(Dissolve safranin powder in water at room temperature: the prepared stain is stable for long time and can be used repeatedly)

Entellan mountant (Merck), Immersion oil

PROCEDURE

Labeling of probe DNA with digoxigenin dUTP by random priming method

1-3 μg of DNA can be labeled per standard reaction

1. Take the required amount of DNA (linear) in a microfuge tube and denature by heating in boiling water for 10 min. Quickly chill the tube on ice.

2. Add the following in sequence to the same microfuge tube

a. Hexanucleotide mix	2 μl
b. dNTP labeled mix	2 μl
c. Distilled water to make	15 μl
d. Klenow enzyme (3-5 units)	1 μl
Final volume	20 μl

3. Incubate at 37^{o}C for 1 h

4. Stop reaction by adding 0.8 μl of 0.5 M EDTA (Final concentration 20 mM)

5. Precipitate labeled DNA by adding the following:
   2.0 μl of 10 mg/ml salmon sperm DNA (excess DNA helps in coprecipitating the small quantity of labelled DNA)
   2.5 μl of 4 M Lithium Chloride (forms lithium salt of DNA, that precipitates)
   75 μl of pre chilled (-20^{o}C) ethanol (takes away water from DNA solution)
   Leave at -70^{o}C for 2 h (chilling decreases the solubility of DNA salt, thus easily precipitated)

6. Centrifuge at 12,000 rpm for 30 min at 4^{o}C

7. Decant supernatant and wash the pellet with 70% ethanol

8. Dry at room temperature or lyophilize

9. Dissolve in required amount of TE. The labeled probe can be stored at -20^{o}C for at least 2 years.

Checking the efficiency of DIG-labeling

1. Take small piece of nylon membrane, wet it with 2XSSC and dot blot (under vacuum) 10 pg, 1 pg and 0.1 pg of the prepared probe. Let it dry at room temperature (~30 min) following which cross-link the probe DNA with the membrane either by 3-4 min exposure to UV on a transilluminator or by heating the filter at 70^{o}C for 2 h.

2. Wash the filter briefly in Buffer I.

3. Incubate in Buffer II for 30 min at room temp (to block the membrane surface for non-specific binding of the antibody used in next step).

4. Briefly rinse in Buffer I (this washing is optional)

5. Incubate in Anti-DIG Antibody-Enzyme conjugate (1 µl in 4 ml of Buffer I) for 30 min at room temp. This solution can be reused.

6. Wash twice at 15 min interval in Buffer I.

7. Briefly rinse in Buffer III (to increase the pH for bringing the normal functional environment of alkaline phosphatase).

8. Put the blot in a small polythene bag and working in dim light add colour developing solution (4.5 µl of NBT and 3.5 µl of BCIP in 1 ml of Buffer III) and seal the bag (BCIP is hydrolyzed by alkaline phosphatase to form a blue coloured intermediate. The intermediate is then oxidized by NBT to produce an intense insoluble purple dye)

9. Incubate the blot within the sealed bag in dark (by wrapping with aluminium foil) till desired level of colored signal is visible. When adequate signal is obtained, remove the blot from the bag and put in Buffer IV to stop reaction (under optimal conditions of probe labeling, 0.1 pg of probe gives a detectable signal within 30 min). The blot can be stored in Buffer IV or in dry condition.

Processing of prepared slides prior to hybridization

1. Dip the prepared slides (with the desired cytological preparation) for about 5 sec in a freshly prepared 0.1% solution of Gelatin (100 mg Gelatin dissolved in 100 ml distilled water at 70ºC for 1 h). Let the slides air dry (the gelatin coating prevents background binding of the probe and also helps keep chromosomes/cells better preserved on the slide (Lakhotia et al, 1993).

2. Arrange the slides in a moist chamber containing filter papers soaked in 2x SSC. Place 100 µl of RNase (100 µg/ml in 2X SSC) over the preparation on each slide and cover with 22 mm^2 cover-glass (no air bubbles should be trapped) to remove RNA from preparations. Incubate the slides at room temperature for 2 h.

3. Remove the cover-glass gently by dipping slides into a beaker containing 2x SSC. Coverglasses will fall in solution.

4. Wash slides in 2X SSC (3 times 5 min each), in 70% ethanol (2 times 10 min each) and in 95% ethanol for 5 min. Air dry. Slides can be stored at this stage, if required
(in certain cases when not much RNA is expected to be available for hybridization in the preparation, the RNase and subsequent washing (steps 2-4), may be omitted).

5. Place slides in 0.07 N NaOH for exactly 3 min to denature chromosomal DNA

6. Wash slides in 3 changes of 70% ethanol (10 min each) and 2 changes of 95% ethanol (5 min each). Air dry. Slides are now ready for hybridization.

Hybridization mixture

Formamide	500 µl
20X SSC	250 µl
DIG labeled probe (10-20 ng/slide)	as required
H_2O to make total volume to	1000 µl

The total volume of hybridization mix that is prepared depends upon the number of slides being processed (15-20 µl is enough for a slide when using 22 mm^2 cover-glass).

Hybridization

1. Denature labeled probe DNA by placing the tube in boiling water bath for 10 min. Add the desired amount of denatured probe to the hybridization mix.
2. Add 20 µl of hybridization mixture containing 10-20 ng of labeled probe. Place a cover-glass over the hybridization mixture and seal the edges with DPX. No air bubbles should be trapped.
3. Incubate slides at 42°C in a closed moist chamber. Allow hybridization to proceed for 12-14 h (overnight).

Note: Southern modified C-banding technique for nucleotide hybridizations. In hybridization mix formamide is used to lower the melting and annealing temperatures of hybridizing nucleotide strands. Salt (SSC) is supporter of hybridization, shields the hydrogen bond forming capability of water. 5 or 6X SSC is optimal for hybridization as 6X SSC gives nearly 1 M salt concentration (Tm = logM+0.41 X %GC + 81.5°C; M = molar salt concentration; 1 M salt is taken to make log1 = 0) so that it does not interfere in hybridization process.

Washing

1. Peel off DPX sealing with the help of forceps. Remove cover-glasses by dipping slides in 2X SSC
2. Wash slides in 1X SSC (3 times 15 min each) at 42°C (salt is the supporter to keep the hybrids; if washed in water without salt, then the probe falls off from the places of hybridization).

Colour detection

1. Rinse slides for 1 min in buffer I
2. Place them in buffer II (0.5% W/V Blocking reagent in Buffer I) and leave for 30 min
3. Wash again in buffer I for 1 min
4. Incubate in anti-Digoxigenin antibody alkaline phosphatase conjugate (diluted 1: 5000 in buffer I) for 30 min.
5. Wash in buffer I (2 times 20 min each).
6. Rinse in buffer III.
7. Prepare fresh colour reaction reagent. Put 20-30 µl of colour reaction reagent on the slide, cover with a cover-glass, seal with DPX and leave the slide in a dark chamber at room temperature for 1-2 h, depending upon the time required for optimal signal development.
8. Stop reaction in Buffer IV after observing slides under microscope.
9. Air dry and counter-stain with Safranin by dipping the slides in the staining solution for 5 to 10 sec followed by 2-3 washes in clean distilled water. Air dry.

   (Alternatively, stain the slides with 2% aceto-orcein for 5 min (filter the aceto-orcein stain immediately before use to avoid ugly stain marks on the preparation) by applying 2-3 drops of the filtered stain on the chromosome areas and covering with a cover-glass for 5 min. Following the staining, quickly rinse slides in two changes of 70% ethanol and air dry).

   Safranin staining gives better chromosome morphology and contrast so that the hybridization signal is seen more distinctly.
10. Mount dried slides with Entellan (Merck).

Alternatively, mount the slides temporarily using the stop buffer or the immersion oil; after examination the cover-glasses are removed and slides cleaned by rinsing in distilled water if mounted with the stop buffer or with xylene if immersion oil was used. Mounting with alcohol-based mountants (DPX) causes fading of colour.

OBSERVATIONS

Hybridization of the probe results in appearance of purplish-blue colour deposit at the site of hybridization (Fig. 1). The specific chromosome region that shows the hybridization signal can be identified by referring to standard polytene chromosome maps.

PRECAUTIONS

Well spread and flattened preparations with good chromosome morphology are essential for a strong hybridization signal. Denaturation of chromosomes must be precisely controlled since too long treatments would destroy chromosome morphology while too short a treatment would not permit hybridization. The probe must also be denatured just before application.

Washing after the hybridization and after antibody binding must be adequate so that all the excess probe and the antibody are removed. Incomplete washing at any step would generate undesirable background.

Staining of chromosomes must be controlled so that the hybridization signal is not masked.

Care must be taken to avoid trapping of air bubbles while mounting cover-glasses at the various steps since any trapped bubble would not permit the reaction in the local region and thereby prevent the hybridization signal.

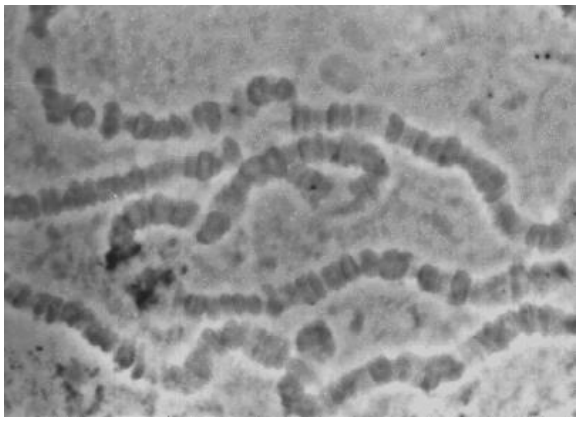

Figure 1. Hybridization *in situ* of a digoxigenin-labeled probe for *hsp70* genes to polytene chromosomes of *Drosophila melanogaster*. The *hsp70* gene probe hybridizes to 87A and 87C bands (which are puffed due to heat shock in this case)

QUESTIONS FOR FURTHER STUDIES

1. What will happen if you forget to denature the probe prior to its addition in hybridization mix?
2. What will happen if chromosomal DNA is not denatured?
3. What will happen if the temperature is lowered and salt concentration is kept high while washing?

REFERENCE

Lakhotia SC, Sharma A, Mutsuddi M and Tapadia MG (1993) Gelatin as a blocking agent in southern blot and chromosomal *in situ* hybridizations. Trends in Genetics 9: 261

Adapted from Roy and Lakhotia (2024) Localization of DNA sequence on polytene chromosomes of Drosophila melanogaster by in situ hybridization. In: Experiments with Drosophila for biology courses, Ed. Lakhotia and Ranganath, Publ. Indian Academy of Sciences, Bangalore, pp 383-392

Chapter 31: *IN SITU* HYBRIDIZATION TO STUDY PATTERN OF EXPRESSION OF A GENE

Recommended level: PG, Research

INTRODUCTION

There are several thousand genes present in all the cells of a given organism. All of them do not express at all the times, but their expressions are tissue and developmental stage or functional condition specific. Expression of a gene in a given tissue in a given time can be assessed either by observing the presence of RNA or by presence of proteins that are formed from these RNAs. In this chapter presence of RNA will be assessed on the tissue itself in their original location (*in situ*) by hybridizing with the labelled complementary nucleotide (DNA or RNA) probe. This way, *in situ* hybridization to cellular RNA provides information on temporal and spatial patterns of gene expression at single cell level in a variety of tissue preparations.

MATERIALS REQUIRED

1. Incubators set at 42^{o}C, cleaned glass slides and cover-glasses, micropipettes, pipette tips, moist chambers, forceps

Solutions required

1. 20X SSC (3 M sodium chloride, 0.3 M sodium citrate) in DEPC treated water
2. 10X PBS (175 mM NaCl, 84.1 mM Na_2HPO_4, 18.6 mM NaH_2PO_4, pH 7.4). Prepare 1X PBS by diluting 1:10 with DEPC treated water
3. 4% paraformaldehyde in PBS
4. PBT (PBS + 0.1% Tween20)
5. Proteinase K (10 μg/ml) in PBT
6. Glycine (2 mg/ml) in PBT
7. Hybridization buffer A: 50% deionized formamide + 5X SSC (pH- 5.0)
8. Hybridization buffer B:
   50% formamide
   5X SSC (pH- 5.0, adjust pH with 0.5 M citric acid, filter and autoclave)
   10 μg/ml yeast tRNA
   100 μg/ml sheared salmon sperm DNA (boiled for 10 min, chilled and added)
   50 μg/ml Heparin
   0.1% Tween20 dissolved in DEPC treated water.
9. TBS:

Tris HCl (pH 7.5)	100 mM
NaCl	150 mM
Tween20	0.1%

10. Staining Buffer:
    Buffer I (100 mM Tris, pH 7.5; 150 mM NaCl)
    Buffer II (0.5% w/v Blocking reagent in Buffer I)
    Buffer III (100 mM Tris, pH 9.5; 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween20)
    Buffer IV (TE)

11. Coloring Solution:
    NBT – 4.5 µl (7 5mg/ml in 70% DMFO),
    BCIP – 3.5 µl (50 mg/ml in water if it is a sodium salt)

PROCEDURE

1. Labelling of probe remains the same as in previous experiment.
2. Dissect the tissues in Poels' salt solution or PBS (pH- 7.4).
3. Fix the tissues in 4% paraformaldehyde in PBS for 15-20 min on ice (it is better to make fresh paraformaldehyde every time).
4. Fix again in 4% paraformaldehyde + 0.6% Triton X100 in PBS at room temperature (RT) for 20 min (in tissues like salivary glands, one can go for 0.5% NP40 for 20 min at RT in step 2 for better permeability. In this case monitor the tissues under microscope as the tissues become very much transparent).
5. Wash twice in PBT for 5 min each.
6. Wash in PBT + 0.1% active DEPC for 3 min (Carboxymethylation will help increase the signal intensity, also prevent residual RNase).
7. Wash again in PBT for 5 min.
8. Digest with proteinase K (10 µg/ml) in PBT for 2-3 min at RT (time will vary from batch to batch, one has to standardize. Do not freeze and thaw the stock many times).
9. Wash two times in chilled glycine (2 mg/ml) in PBT for 5 min each.
10. Wash three times in PBT for 5 min each.
11. Fix in 4% paraformaldehyde in PBS for 15 min at RT (use glutaraldehyde only when you go for colorimetric assay, not for fluorescence. For that only paraformaldehyde fixing will suffice).
12. Wash 5 times in PBT for 5 min each.
13. Wash in 1:1 PBT: Hybridization Buffer A for 10 min at RT.
13. Wash in Hybridization buffer B for 10 min at RT.

<u>Prehybridization and Hybridization</u>

14. Prehybridize in Hybridization buffer B for 1-2 h at 42^{o}C.
15. Hybridize with DNA probe or riboprobe in Hybridization buffer B for 24 h at 42^{o}C.

<u>Washing</u>

16. Wash in Hybridization buffer A at least for 1 h (15 min each 4 times) at the same hybridization temperature (increase in washing time will decrease the background).
17. Wash in Hybridization buffer A + PBT
    4 : 1 for 10 min at RT
    1 : 1 for 10 min at RT
    1 : 4 for 10 min at RT
18. Wash in PBT for 5 times, 5 min each.
    (The tissues can be stored in PBT at 4^{o}C overnight)

Colour detection

19. Incubate the tissues in Anti DIG antibody (1:2000 in PBT) for 2-3 h at room temperature in a shaker. The antibody should be preabsorbed in lesser dilution (1:200) on fixed tissues (steps 2-6 without active DEPC treatment) overnight at 4^{o}C.
20. Wash in PBT 5 times for 5 min each (tissues can be stored at 4^{o}C)
21. Incubate the tissues in TBS for 15-20 min, as the phosphate molecules in the PBS can decrease the phosphatase activity so as to result in weak signal, in higher pH (9-9.5) PBS tends to precipitate also to give crystal like sediments which one can see the moment you add staining buffer.
22. Incubate for 1 min in buffer I
23. Place the tissue in buffer II (0.5% W/V Blocking reagent in Buffer I) and leave for 30 min
24. Wash again in buffer I for 1 min
25. Incubate in anti-Digoxigenin antibody alkaline phosphatase conjugate (diluted 1: 5000 in buffer I) for 30 min.
26. Wash in buffer I (2 times 20 min each).
27. Rinse in buffer III.
23. Add freshly made coloring solution in darkness.
24. Observe intermittently under stereo-binoculars (avoid light) for monitoring the colour development. Once the colour develops, stop the reaction by washing in Buffer IV.
25. Incubate the tissues in 50%-80% glycerol in PBS for 2-4 h before mounting in the same solution.

OBSERVATIONS

Hybridization of the probe results in appearance of purplish-blue colour deposit at the site of hybridization (as seen in the Fig. 1).

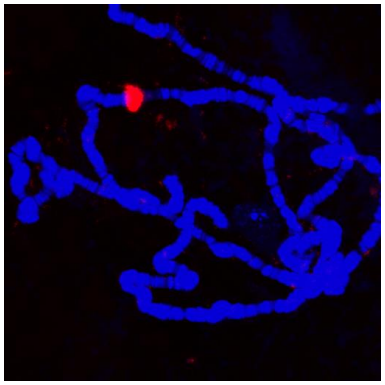

Figure 1. RNA in situ hybridization using hsr-omega repeat specific probe on 30 min heat shocked polytene chromosomes from late third instar larval salivary gland (seen as red large dot on 93D locus). Chromosome is stained with DAPI (blue).

PRECAUTIONS

If too much background appears, reduce the probe concentration, increase the Hybridization temperature, increase the proteinase concentration or treatment time.

It is always advisable to do DEPC treatment to all the solutions except the ones which has got amino group in it.

QUESTIONS FOR FURTHER STUDIES

1. Can you use labelled denatured double stranded DNA as probe in this study?
2. All nuclei have the same gene, do all nuclei show hybridization signal?
3. Why DEPC treated water was being used in all the solutions that were used up to hybridization step?

***Adapted from Singh and Lakhotia** (2024) study of polytene chromosomes of Drosophila melanogaster. In: Experiments with Drosophila for biology courses, Ed. Lakhotia and Ranganath, Publ. Indian Academy of Sciences, Bangalore, pp 393-400*

Chapter 32: IMMUNOSTAINING

Recommended level: PG, Research

INTRODUCTION

Immunostaining is based on the detection of antigens by antibodies. Antigens are in general large cellular molecules, such as proteins, polysacchrides and nucleic acids. In immunostaining the antibody that interacts with the tissue antigen is known as primary antibody. The first antigen-antibody complex can be detected by suitable markers.

Since the antigen-primary antibody complexes are small, their detection becomes often difficult. Therefore, secondary labeled antibodies specific to the primary antibodies are used to detect the primary complex. If the primary antibody is an IgG made in rabbit, then the secondary antibody used will be an anti-rabbit IgG made in goat or sheep.

The molecules used to label or tag the secondary antibody are either an enzyme or a fluorescence molecule. A chemical is said to be fluorescent if it absorbs light at one wave length (the excitation wave length) and emits light at a specific and longer wave length within the visible spectrum. Three very useful fluorescent dyes are rhodamine, Cy3, which emit red light, and fluorescein, which emits green light. These dyes have a low nonspecific affinity for biological molecules and they can be chemically coupled to purified antibodies specific to almost any desired macromolecules: a fluorescent dye-antibody complex, when added to a permeablized cell or tissue, will bind to the chosen antigens, which then light up when illuminated by the exciting wave length (Foerster, 2007).

MATERIALS REQUIRED

Poels' salt solution- [86 mg NaCl, 313 mg KCl, 116 mg $CaCl_2.2H_2O$, 88 mg NaH_2PO_4, 18 mg $KHCO_3$, 513 mg $MgSO_4.7H_2O$, 100 ml distilled water, pH6.8]

10X PBS - [175.0 mM NaCl, 84.1 mM Na_2HPO_4, 18.6 mM NaH_2PO_4, pH 7.4]
Prepare 1X PBS by diluting 1:10 with dH_2O.

PBST - [1X PBS, 0.1% Triton X-100, 0.1% BSA (Merck, Catalog No. 112018)]

PBS-PFA - [1X PBS, 4% paraformaldehyde]
Dissolve at 60^0C and cool down to room temperature.

Blocking solution - 1X PBS, 0.1% Triton X-100, 0.1% BSA, 10.0% fetal calf serum (Biological Industries, Catalog No. 04-001-1B), 0.1% Deoxycholate and 0.02% thiomersal (as anti-fungal agent).

PROCEDURE

1. Dissect tissues from crawling third instar larvae in Poels' salt solution (pH-7.0) or 1X PBS (pH-7.4).
2. Transfer tissues to cavity slide in IX PBS.
3. Fix in freshly prepared PBS-PFA for 20 mins at room temperature.
4. Wash the tissues in PBST for 10 min (x3)
5. Incubate the tissues in blocking solution at room temperature for 2 h
6. Add primary antibody at a dilution of 1:10 in blocking solution and incubate at 4^0C over-night.

7. Take off the supernatant. This can be saved for second use.
8. Rinse once with PBST and then wash 2 X 10 min each in PBST.
9. Add AlexaFluor-488 conjugated secondary antibody (or a desired secondary antibody) diluted in blocking solution and incubate for 2 h at room temperature with gentle shaking.
10. Take off the supernatant. Wash as in step 8 and counter stain in DAPI for 10 min.
11. Rinse with PBS and mount in mounting medium seal and store at –20C till further observation.
12. Observe under a fluorescence microscope at the desired excitation wave length of light.

OBSERVATIONS

Green fluorescence will be observed at those specific sites where the antigen-primary antibody complex is present when viewed at 494 nm wave length of light if AlexaFluor-488 conjugated antibody is used. Blue (DAPI) stained nuclei can be observed if excitation wave length 359 nm is used.

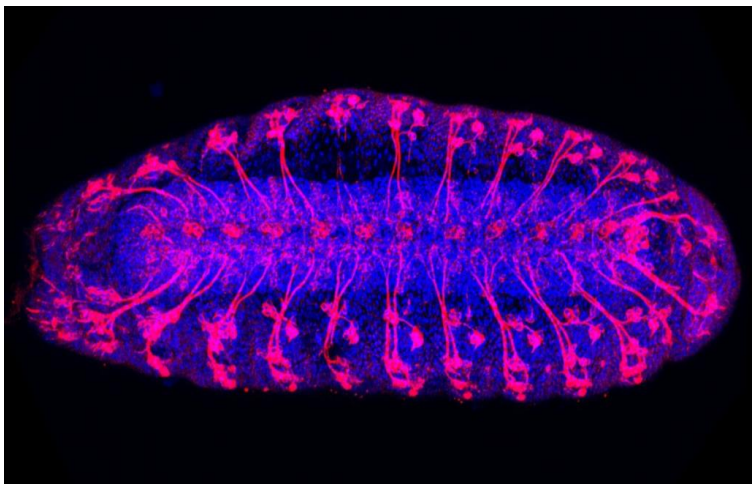

Figure 1: Confocal image showing the distribution of Futsch (red) in a late 16 stage *Drosophila* embryo. Futsch, a microtubule-associated protein, is prominently localized in the developing peripheral nervous system, highlighting neuronal cell bodies and axonal projections. Nuclei are counterstained with DAPI (blue). The image illustrates the organized pattern of Futsch-positive neurons extending along the embryonic body axis, marking the developing neural architecture.

QUESTIONS FOR FURTHER STUDIES

1. Why do we generally use a primary antibody and a labelled secondary antibody for immunostaining? Why not a labelled primary antibody?
2. Can immunostaining be replaced with in situ hybridization for studying expression of a gene?
3. If you wish to detect the localization of two different proteins, how can you achieve that?

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Chapter 33: IMMUNOFLUORESCENCE FOR ADHERENT CELLS

Recommended Level: PG/Research

OBJECTIVE

To perform immunofluorescence staining for adherent cells cultured on coverslip

INTRODUCTION

Immunofluorescence staining is an immunostaining technique used in light microscopy to detect and localize proteins within cells or tissues at a quantitative level. This method relies on the specificity of antibodies binding to antigens. To visualize target biomolecules, a pair of primary and secondary antibodies are used where secondary antibodies are conjugated to fluorophores. When the fluorophore is excited, it emits light at a specific wavelength, which is detected using a fluorescence microscope. This technique is used extensively in both scientific research and clinical diagnostics, including tissue sections, cultured cell lines, and individual cells. It helps analyze the distribution of proteins, glycans, small molecules, and visualize cellular structures such as intermediate filaments and functions.

MATERIALS REQUIRED

Cells, 10% lysine coated coverslips, 4% paraformaldehyde, Tween 20, Triton X-100, Chilled PBS, primary antibody, secondary antibody, DAPI, rocker-shaker, cell culture plates.

PROCEDURE

Cell seeding

Adherent cell lines are cultured on cover slips in a 6 or 12 well tissue culture plates aseptically. Using tweezers coverslips are washed with water followed by ethanol in the culture hood for sterilization and dried, for proper adherence, coating matrix usually poly-L-lysine is used for most of the cell lines, however, specific coating material can be used for some cell lines such as embryonic stem cells, neuronal cell lines.

Coverslip preparation

1. Coat coverslips with polyethyleneimine or poly-L-lysine for 1 h at room temperature inside a cell culture biosafety cabinet.
2. Rinse coverslips well with sterile H_2O (three times 1 h each).
3. Allow coverslips to dry completely and sterilize them under UV light for at least 4 h.
4. Seed cells on coated glass coverslips and allow it to grow until required.
5. Rinse briefly in phosphate-buffered saline (PBS).
6. For wash buffer we recommend 1x PBS with 0.1% Tween 20.

Fixation

The cells may be fixed using one of two methods:

1. Incubating the cells in 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature.

2. Or, 100% methanol (chilled at -20°C, recommended for phosphorylated and nuclear antigens) at room temperature for 5 min.
3. The cells should be washed three times with ice-cold 1x PBS.

Permeabilization

If the target protein is intracellular, it is very important to permeabilize the cells. Methanol fixed samples do not require permeabilization.

1. Incubate the cells for 10 min with PBS containing 0.1% Triton X-100. Triton X-100 is the most popular detergent for improving the penetration of the antibody. However, it is not appropriate for membrane-associated antigens since it destroys membranes.
2. Wash cells in PBS three times for 5 min in a shaking condition.

Blocking and immunostaining

Incubate cells with 1% FBS in PBST (PBS+ 0.1% Tween 20) for 30 min to block non-specific binding of the antibodies. Incubate cells in the diluted antibody in 1% FBS in PBST in a humidified chamber for 1 h at room temperature or overnight at 4°C.

1. Decant the solution and wash the cells three times in PBST, 5 min each wash.
2. Incubate cells with the secondary antibody in 1% FBS for 1 h at room temperature in the dark.
3. Decant the secondary antibody solution and wash three times with PBS for 5 min each in the dark.

Immunofluorescence Multiplexing: Multicolor Staining (Optional Step):

To examine the co-distribution of two or more different antigens in the same sample, multiplexing can be performed either simultaneously (in a mixture) or sequentially (one antigen after another).

Antibody Selection

Ensure you have primary antibodies from different species, such as a rabbit antibody against antigen A and a mouse antibody against antigen B, or vice versa. Select secondary antibodies that are raised in different hosts to avoid cross-reactivity.

Fluorophore Conjugation

Use secondary antibodies conjugated to different fluorophores for distinct and non-overlapping fluorescence signals.

Alternatively, directly conjugated primary antibodies, each labeled with a different fluorophore can also be used to simplify the staining process. Counter staining

1. Incubate cells on 0.1-1 μg/ml Hoechst 33258 or DAPI (DNA stain) for 1 min.
2. Rinse thrice with PBS, 5 mins each.

Mounting

1. Mount coverslip with a drop of mounting medium.
2. Seal coverslip with nail polish to prevent drying and movement under microscope.
3. Store in dark at 4°C.

OBSERVATION

Spatial localization of a given protein is observed in cells as shown in Fig. 1. The images show how different proteins can be found in specific parts of the cell. A marker for cell proliferation, Ki67, is seen inside the nucleus, while LINE-1 ORF1p, a protein linked to transposable elements, is mainly found in the cytoplasm. These patterns help us understand the role and location of proteins within the cell.

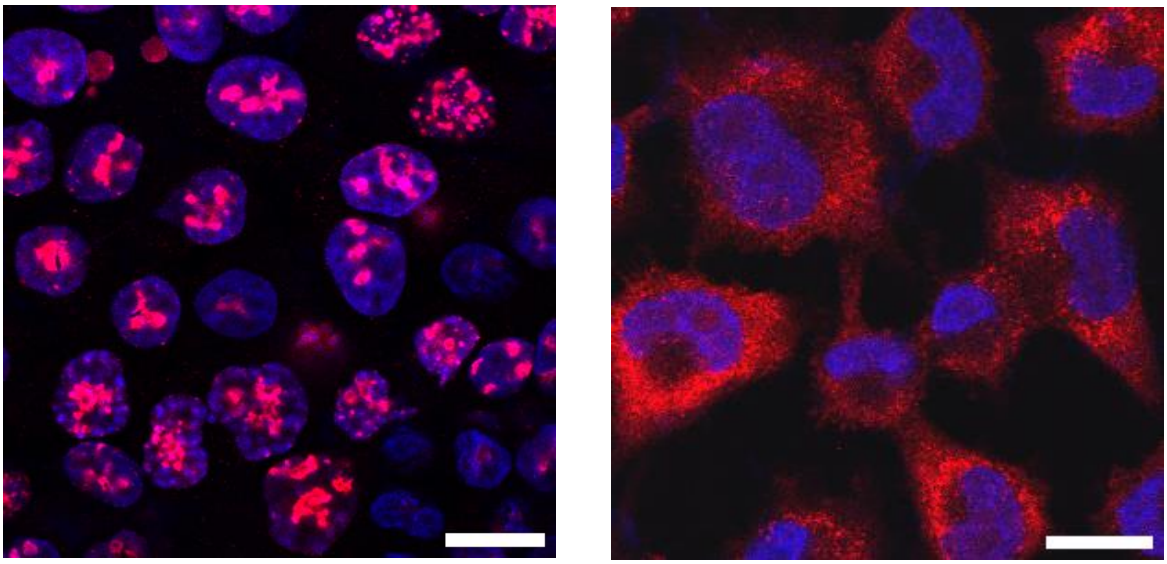

Figure 1. Representative image showing the nuclear ki67 foci (red, Alexa Fluor 568) embedded in nucleus stained with DAPI (blue) in left panel and Right panel shows the LINE-1 ORF1p (red, Alexa Fluor 568) localized at the cytoplasm surrounding nucleus DAPI (blue).

QUESTIONS FOR FURTHER STUDIES

1. How can antibody concentration and incubations be optimized to improve signal to noise ratio in immunofluorescence?
2. What are the critical controls required to confirm specificity and eliminate background staining in immunofluorescence assays?
3. How does fixation and permiabilization choice affect epitope accessibility and fluorescence intensity in immunofluorescence experiments?

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Chapter 34: DIPLOID AND ANEUPLOID CELL ANALYSIS BY FISH USING PROBES SPECIFIC FOR THE CENTROMERES OF HUMAN CHROMOSOMES

Recommended Level: PG/Research

INTRODUCTION

Diploid organisms have 2 sets of chromosomes (2n), haploid organisms have 1 set (n), and aneuploidy means abnormal number of chromosomes. If an organism has a 1 less chromosome (2n-1), known as monosomic and if having 1 more chromosome (2n+1), known as trisomic. An extra or missing chromosome is a common cause of some genetic disorders. Chromosome number abnormalities usually occur due to an error in cell division when the chromosomes do not separate properly between the two cells (nondisjunction). Changes in chromosome number can result in spontaneous abortions and, when present in newborns, to quite severe birth defects. It is also very common in cancer cells.

Fluorescent in situ hybridization (FISH) is a powerful molecular cytogenetics technique for detecting chromosome abnormalities in metaphase spreads and interphase nuclei, enabling genomic screening of tissues not accessible through routine investigation (Poddighe *et al.*, 1992; Schrock et al 1996). The FISH mapping technique utilizes two primary principles: the target and the probe. The target can be a whole chromosome or a specific chromosome region. Probes are either directly labeled with fluorochromes (fluorescein isothiocyanate-FITC, Texas Red isothiocyanate-TRITC, rhodamine) or indirectly with haptens (biotin, digoxigenin) that bind to the probe via fluorochrome-conjugated antibodies. The probe is tailored to the target through complementary DNA base pairing, enabling fluorescence-labeled probes to hybridize and produce specific fluorescent signals on specific chromosome regions. The most widely used DNA probes involve a variety of repetitive satellite DNA sequences that identify specific chromosome structures like centromeres and heterochromatic or telomeric regions. Based on the variation of repetitive sequence composition, chromosome-specific probes have been developed for almost all human chromosomes.

A fluorescence microscope is used to identify probes directly labeled with target sequences in chromosomal DNA, with the hybridization signals representing these complementary sequences. The fluorescence signal for probes that are indirectly labeled is detected through immuno-chemical reactions. To enable visualization for chromosomes and nuclei, slides are counter-stained with DNA-binding fluorochromes like propidium iodide (PI) or 4,6-diamidino-2-phenylindole DAPI.

OBJECTIVES

To assess the pattern of DNA aneuploidy by interphase FISH analysis.

MATERIALS REQUIRED

In this study, it is preferred to use isolated lymphocytes from the blood instead of whole blood. This makes the cell suspension cleaner. The rest procedure for setting the culture is the same.

Histopaque – 1077 (Sigma, USA); RPMI 1640 (Gibco, USA); 15 ml conical centrifuge tubes (Tarsons)**;** Phosphate buffer saline **(**PBS) 1X pH7.4; Clinical Centrifuge

PROCEDURE

Lymphocyte isolation

1. Take 5 ml of Histopaque–1077 in a 15 ml centrifuge tube.
2. Mix 5 ml of blood mixed with RPMI 1640 in equal proportions (2.5 ml each) and layer on to the Histopaque–1077.
3. Centrifuge at 1200 rpm for 30 min at room temperature. A mononuclear opaque ring will be formed at the Plasma - Histopaque interface that be carefully removed by aspiration.
4. Transfer the aspirate to a new centrifuge tube and resuspended in Phosphate Buffered Saline (PBS).
5. Centrifuge at room temperature at 1500 rpm for 10 min, decant the supernatant.
6. The cell pellet gently be resuspended in PBS. The step be repeated for two more times to remove any traces of the Histopaque. After the last wash the cell pellet is used for short-term culture.

Setting the culture

1. For each culture, add the following in the order given:

TC medium	5 ml
Fetal bovine serum	1 ml (if not already added)
Resuspended cell pellet	0.3 ml

2. In order to buffer the pH, blow CO_2 from a CO_2 cylinder or bubble exhaled air orally through a cotton-plugged-pipette.

   No colcemid or colchicine is needed for the observation of interphase nuclei.

4. A suspension of single cells can be obtained from the lymphocyte culture (after 52 to 56 h) by aspirating with a 21-gauge needle. Soak the cells in 0.05 M KCl solution for 2 min to disrupt the cell membranes and expose the naked nuclei, and then fix by the addition of an equal volume of methanol/acetic acid (3:1) solution (Carnoy).
5. Centrifuge at 3000 rpm for 10 min, discard the supernatant and add fresh fixative solution. Centrifugation and fixative change be repeated twice and the final resulting upper layer (0.8 ml) be transferred dropwise to glass slides and air-dry and store at -20°C until use.
6. To detect changes in the copy number of chromosomes in the cells, use two types of single-colour BAC clone probes specific for the centromeric DNA of any two chromosomes. A single-colour probe specific for chromosome 7 centromeric DNA labeled with Spectrum Green (D7Z1 for CEP7, Vysis Inc., Downers Grove; IL), and one for chromosome 8 centromeric DNA labeled with Spectrum red (CEP9, Vysis Inc.) can be used. Single-colour FISH can be carried out as follows:

   The materials on the slides are treated with 2X saline-sodium citrate (SSC) /0.1% (v/v) NP-40 at 37°C for 30 min and then dehydrated through an ethanol series (70%, 90%, and 100% for 10 min each). The slides will be denatured in 70% (v/v) formamide / 2 × SSC at 75°C for 5 min and dehydrated through an ethanol series.

Probe preparation

For FISH, labeled probe DNA is mixed with Salmon sperm DNA and human Cot1 DNA as blocking DNA. Salmon sperm DNA is added to block nonspecific binding of the probe, and human Cot1 DNA is added to block repetitive DNA sequences in the probes from binding to sites spread throughout

several chromosomes/loci. The desired combination of labeled probe is mixed using 3 µl of each probe (depending on intensity of signal) with 1 µl of human Cot-1 DNA (1 mg/ml, Invitrogen), 1 µl of salmon sperm DNA (10 mg/ml; Invitrogen), and 7 µl of the hybridization master mix (4XSSC, 0.5 mM EDTA, 10% dextran sulfate, 25% deionized-formamide in DEPC-H_2O) and thoroughly mixed and denatured at 76ºC for 10 min. The hybridization mixture is then pre-annealed by incubating at 37ºC for 30 min (allowing the Cot-1 DNA to anneal to non-chromosome-specific DNA repeats on the probes).

Hybridization

The hybridization mixture is carefully applied to the slides and then cover with a 22×22 mm^2 coverslip, and seal with rubber cement or transparent nail polish. Slides are incubated overnight in a moist chamber at 37ºC.

Washing and detection of label with antibodies

1. After removing rubber cement or nail polish and the coverslips, the slides are washed in 0.1× SSC at 43ºC for 2 min, then, when biotin or digoxigenin labels are used, incubate in PNM blocking reagent (5% nonfat dry milk powder, 1% Nonidet-P40 [Sigma], 1% sodium azide [Sigma], 0.1 M sodium phosphate buffer, pH 8.0) for 10 min at room temperature.
2. Bound probes are detected with fluorescein-conjugated avidin (avidin DCS; Vector Laboratories Inc.; Burlingame, CA) and rhodamine-labeled antidigoxigenin antibodies (Roche Diagnostics; Indianapolis, IN). In the case of direct-labeled probes, no immuno-detection step is necessary. Finally, After being washed at 45°C three times in freshly prepared 50% (v/v) formamide-2×SSC for 10 min, SSC for 10 min, and 2×SSC-0.1% (v/v) NP-40 for 5 min, the slides are counterstained with DAPI (1 µg/ml, Calbiochem; La Jolla, CA) in antifade solution (p-Phenyl-enediamine and glycerol in a PBS solution to prevent the permanent loss of fluorescence or fluorescent signal oxidation due to photobleaching).

OBSERVATION

All normal diploid cells will have each chromosome in two copies, hence the fluorescence is observed as 2 dots (on two chromosomes). Presence of 1 dot indicates monosomy while more than 2 dots are indicative of aneuploidy (3 dots for trisomy) (Fig. 1).

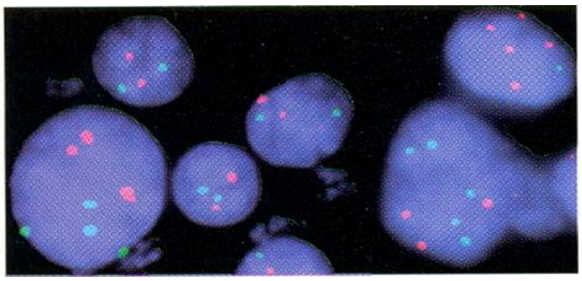

Figure 1. Fluorescence microscopy image of interphase FISH on Hct-116 cells using probes for chromosomes 7 (green) and 8 (red) showing three normal and three aneuploid cells.

QUESTIONS FOR FUTHER STUDIES

1. Can this technique be used for diagnostic purposes? If yes, then with what kind of chromosomal anomalies this FISH technique can detect
2. What do you understand by the probe and what should be the size of it?
3. How many probes can be used at a time in FISH?

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Chapter 35: VISUALIZATION OF MYOFILAMENTS IN *DROSOPHILA* ADULT MUSCLE TISSUE

Recommended Level: PG, Research

INTRODUCTION

The muscular system in the animal kingdom plays a vital role in facilitating essential activities such as locomotion, skilled movements, and flight. Across different species, muscle tissues exhibit remarkable diversity yet share fundamental structural characteristics. Among these, the *Drosophila melanogaster* muscle stands out due to its cross-striated pattern and multinucleated cells, traits that closely mirror the organization found in vertebrate muscles (Jawkar & Nongthomba, 2020; Oriel & Lasko, 2018). Leveraging its genetic proximity to humans, *Drosophila* is an invaluable model organism for investigating muscle biology (Oriel & Lasko, 2018).

The *Drosophila* muscle, like the vertebrate muscle, consists of muscle fiber which forms both direct and indirect flight muscles (IFMs), with the latter consisting of Dorsal Longitudinal Muscles (DLM) and Dorsal Ventral Muscles (DVM) (Hedenström, 2014; Fernandes & Vijay Raghavan, 1993) (Fig. 1A-B). In this protocol, we specifically dissected the DLMs to explore their structural components. Muscle fibers are made up of sarcomeres that contain myofilaments (Celestino-Montes et al., 2021) (Fig. 1C). Each sarcomere consists of an I-band (Isotropic band, thin filaments of actin) and an A-band (Anisotropic, thick filaments of myosin), bordered by Z-discs. The A-band's H-zone comprises only thick filaments, delimited by the M-line at its center (Fig. 1D). Myosin proteins act as motor proteins, interacting with actin filaments powered by ATP to generate the force required for muscle contraction (Smith, 2018).

To visualize the structural components of these muscle fibers, we employed a staining protocol using Phalloidin, which selectively binds to F-actin (Celestino-Montes et al., 2021) and Myosin Heavy Chain (MHC) antibody, which marks the M-line (Dahl- Halvarsson et al., 2020). This dual-staining approach is valuable for students to observe the intricate organization of muscle tissue (Chaturvedi et al., 2017). Furthermore, it enables researchers to analyze muscle morphology and characterize molecular mechanisms in developmental and disease models such as myotonic dystrophy and other muscular disorders (Demontis et al., 2013; Kreipke et al., 2017).

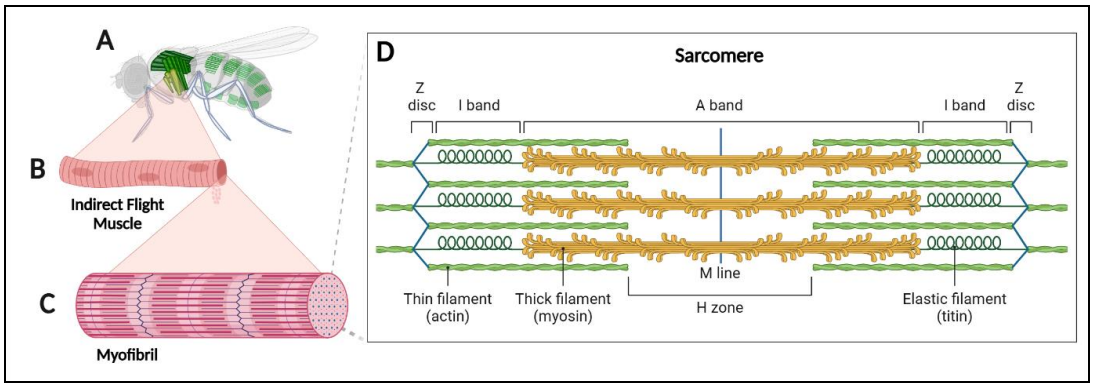

Figure 1: Schematic diagram of the *Drosophila* striated muscle with its components. (A) Adult *Drosophila*. (B) IFM from the thoracic region. (C) Muscle fiber. (D) Schematic of muscle sarcomere illustrating A-band, I-band, H-Zone, M-line, and Z-disc. (Made using BioRender).

OBJECTIVE

To dissect and stain *Drosophila* adult thoracic muscle with phalloidin and MHC antibody for visualization of the muscle structure.

MATERIALS REQUIRED

Wild-type fly stock (Canton-S/Oregon-R)
Diethyl ether, Etherizer, Brush (Round 4), 70% Ethanol, Stereo-binocular microscope, Glass Slides, Sylgard 184 (761036, Sigma-Aldrich), Vannas Spring Scissors-3 mm (15000-00, Fine Science Tools, USA), Dumont #5 Fine forceps (11254-20, Fine Science Tools, USA), Razor blade (Gillette Wilkinson Sword, Boston, USA), Liquid Nitrogen, Slide holder, Petri Dish (35 mm), Aluminium foil, Cavity Block, Micropipettes, Vectashield Plus Antifade Mounting Media (H-1900, Vector Laboratories), Transparent Clear Nail Polish, Coverslip (18x18 mm), Confocal Microscope

Reagents

1x PBS (Phosphate Buffered Saline): 130 mM NaCl, 9 mM Na_2HPO_4, 2 mM $NaH_2PO_4{\cdot}2H_2O$, distilled water pH of 7.4.
4% PFA (Paraformaldehyde, 28906, Sigma): 250 µl of commercially available 16% paraformaldehyde is added to 750 µl PBS and mixed well.
1x PBST (phosphate-buffered saline with Triton X-100): 1x PBS with 0.1% Triton X-100.
Sylgard plate: Pouring ten parts of Sylgard Base with one part of Sylgard Curing Agent into a petri dish.
Blocking Buffer: 1x PBST with 0.1% BSA (Bovine Serum Albumin)
Alexa Fluor 488 Phalloidin (A-12379, ThermoFisher Scientific, USA): Prepare 200 µl Phalloidin solution of 1:200 dilution in 1x Blocking buffer.
Primary Antibody Staining Solution: Prepare 200 µl MHC antibody (3E83D3, Developmental Studies Hybridoma Bank, Iowa) of 1:200 dilution in Blocking buffer.
Secondary Antibody Staining Solution: Prepare 200 µl Alexa 546 anti-mouse (A-11030, ThermoFisher Scientific, USA) of 1:200 dilution in 1x Blocking buffer.

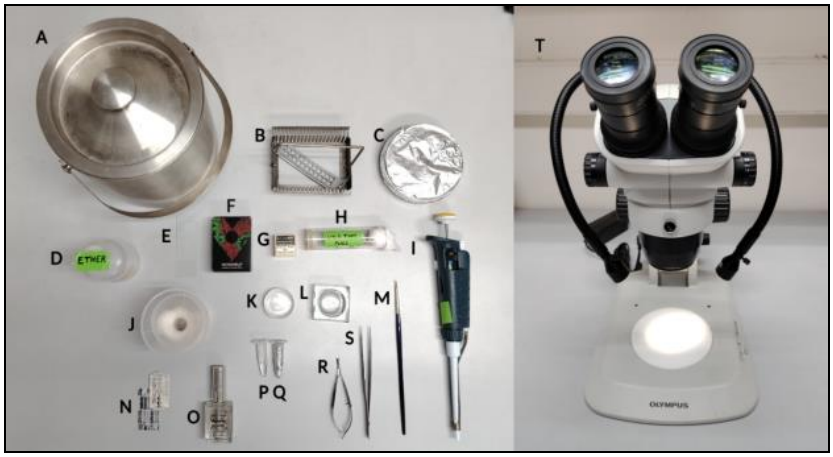

Figure 2. Materials Required for Adult *Drosophila* Muscle Dissection. (A) Liquid Nitrogen Container. (B) Slide Holder. (C) Petri Dish covered with Aluminium foil. (D) Ether in the dispensing bottle. (E) Glass Slide (F) Antifade Mounting Media. (G) Coverslip. (H) A vial containing wild-type flies. (I) Micropipette. (J) Etherizer. (K) Sylgard Plate. (L) Cavity Block. (M) Round Brush. (N) Razor Blade. (O) Transparent nail polish. (P, Q) Microfuge tubes for primary and secondary staining solutions. (R) Spring Scissors. (S) Forceps. (T) Zoom Stereo-Binocular Microscope. (Note: All required materials, including the reagents and fluorescence microscope, aren't shown).

PROCEDURE

Dissection

1. Obtain the desired *Drosophila melanogaster* flies (Fig. 3A).
2. Prepare an etherizer by placing a small funnel over a wide-mouth semi-transparent plastic bottle and wrapping a thin strip of cotton around the funnel tube with an elastic band.
3. To etherize the flies, wet the cotton strip of the etherizer with a few drops of ether, place the funnel over the mouth of the bottle, and transfer the flies to the plastic bottle through its funnel for 1- 2 min.
4. Pour 1 ml of 70% ethanol into a petri dish and transfer the etherized files into it for 30 sec.
5. Prepare a Sylgard dissection plate with a thin layer of chilled 1x PBS on top. Using a brush, transfer the above-mentioned flies to this Sylgard plate.
6. To obtain the thorax region of the fly, cut off the fly's head, abdomen, and legs using Dumont #5 forceps and spring scissors (Fig. 3B-D).
7. Collect about 10-15 thoraces in a cavity block and add 200 µl 4% PFA for fixation at room temperature (24 –25°C). Let it fix for 30 min (Critical Step: Steer clear of over-fixing, as this can impede the identification of antigens and lead to low immunostaining levels. Warning: Paraformaldehyde is toxic and flammable).
8. Wash the thoraces thrice with 1x PBST for 10 min each (Warning: Triton X-100 is toxic).
9. Using forceps, gently lift a thorax from the cavity block and place it on a glass slide with one droplet of 1x PBS (the thorax is positioned with the ventral side exposed).
10. Place this slide in a slide holder and then wholly submerge the slide holder in the container filled with Liquid Nitrogen for 30 sec (Warning: Use liquid nitrogen protective gloves, goggles, and clothing to prevent frostbite and burns).
11. Glass slides with the samples are retrieved after 30 sec once the liquid nitrogen bubbling becomes minimal.
12. Transfer these slides to the stage of a stereo-zoom binocular microscope and let it thaw for 5 sec. By looking through the microscope, the thorax is dissected using the razor blade in half, longitudinally forming two hemithorax (Fig. 3E-F) (Critical Warning: If samples are not allowed to thaw for a few sec before dissection, the muscle tissue may break).
13. Using forceps, pick up the hemithorax samples by holding their wings and transferring them to a cavity block filled with 1x PBS.
14. To mount the samples properly, cut the wings of the samples in the cavity block using spring scissors.

Immunostaining

1. Add Blocking Buffer to the samples and incubate at room temperature for 2 h.
2. After blocking, add the prepared Primary Antibody Staining Solution of MHC antibody (1:200 dilution) and incubate overnight at 4^{o}C.
3. After overnight incubation, remove and store the primary antibody staining solution.
4. Wash the thoraces thrice in 1x PBST for 10 min each.
5. Add the Alexa 546 anti-mouse Secondary Antibody Staining Solution (1:200) and incubate for 2 h on a gentle shaker. You must keep the samples in the dark until final imaging. For the

subsequent part of the protocol, you can use a petri dish covered in aluminium foil to create a dark chamber.

6. Wash the thoraces thrice in 1x PBST for 10 min each.
7. Add the prepared Alexa-488 Phalloidin solution (1:200 dilution) to the same samples and incubate overnight at 4^{o}C.
8. Repeat the wash of thoraces thrice with 1x PBST for 10 min each.
9. Store the samples in 1x PBS at 4^{o}C until mounting.

(Warning: Ensure that all samples of hemithoraces are completely submerged in the staining solutions to allow for proper uptake of the stains.)

Mounting

1. Take a glass slide and stick two coverslips parallelly, leaving a 0.5 mm gap between them, using transparent nail polish (Fig. 3G).
2. Put two drops of mounting media in the gap between the coverslips and spread it evenly.
3. Position the hemithorax samples in this gap, with the dorso-longitudinal muscles exposed with the help of forceps (Fig. 3H).
4. Take another coverslip and place it above the sample. Seal the edges of the coverslip with transparent nail polish.
5. Proceed for imaging using any appropriate fluorescence microscope.

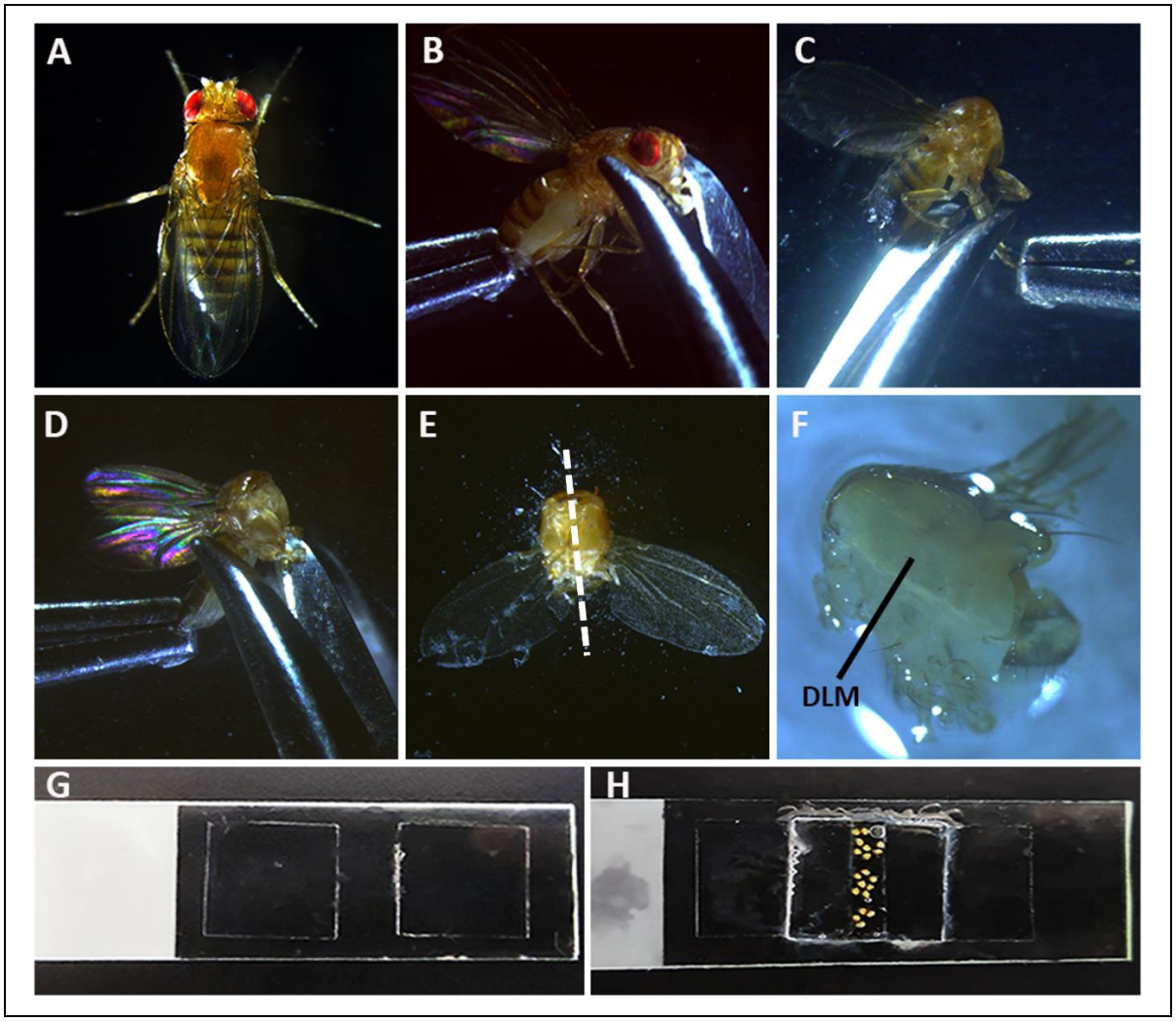

Figure 3: Procedure for the Adult Fly Muscle Dissection. (A) An etherized Adult *Drosophila melanogaster*. (B) Decapitation of the fly head with spring scissors while holding the abdomen with forceps. (C) Removal of the legs. (D) Cutting off the abdomen to isolate the thorax with wings. (E) Thorax is placed on a glass slide in PBS with its ventral side exposed. After dipping it in liquid nitrogen, a blade is used to slice it along the dotted line, as shown in (F). A hemithorax section was obtained. This dissected sample consists of Dorsal Longitudinal Muscles (DLM). (G) Prepared mounting slide with two coverslips stuck parallel to each other using transparent nail polish. (H) The final prepared slide with samples placed in the gap between the two coverslips. A third coverslip was placed on top and sealed with transparent nail polish.

OBSERVATIONS

Fluorescence imaging of *Drosophila* adult muscle tissue revealed detailed insights into the organization and localization of microfilaments essential for muscle function. The staining technique employed anti-MHC, which allowed for the visualization of the M-line (Fig. 4A). The Phalloidin labels the F-actin, which is composed of linear polymers of G-actin proteins and sarcomere Z-lines, allowing for distinct differentiation (Fig. 4B). The merged image of the MHC and Phalloidin staining illustrates the localization of the actin and myosin and shows regular arrangement of sarcomere in the muscle fiber (Fig. 4C). This protocol shows how to properly dissect the *Drosophila* adult thoracic muscle to study the arrangement of actin and myosin microfilaments within the muscle fiber.

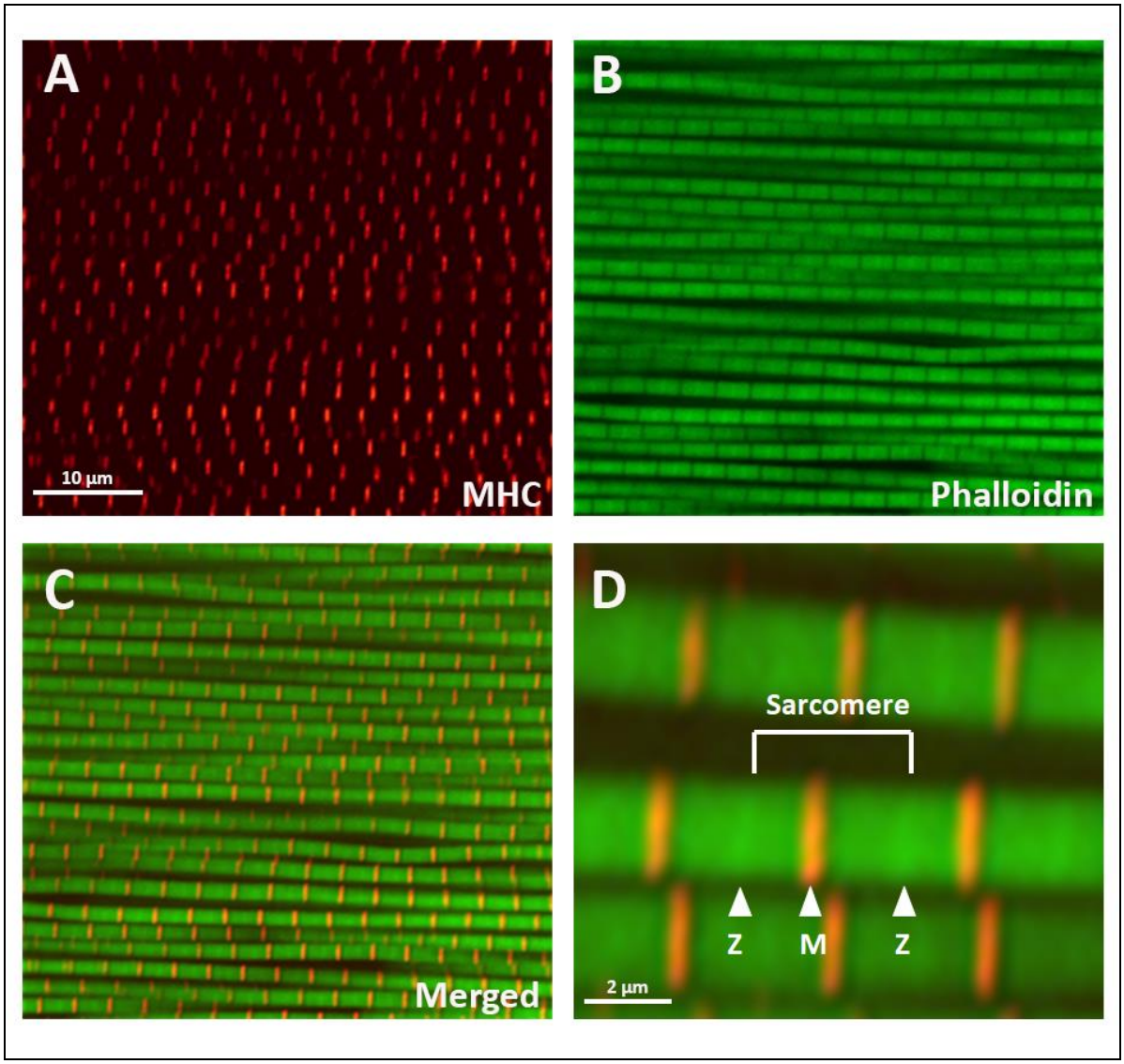

Figure 4: Confocal images of the dissected thorax of the adult *Drosophila* fly. (A) Adult DLMs stained with MHC (red) marking the M-line. (B) Adult DLMs stained with Phalloidin (green) marking the actin filaments in the hemithorax of the adult *Drosophila* fly with Sarcomere bordered by bright Z-disc. (C) Merged image of MHC and phalloidin staining. (D) Magnified image of Sarcomere's structure showing the bright green Z disc and M-line in red. Scale Bars: (A-C) 10 µm, (D) 2 µm.

QUESTIONS FOR FURTHER STUDIES

1. Was viewing under a confocal microscope essential, or viewing under any fluorescence microscope would have been fine?
2. If you had used labelled primary antibody and not secondary antibody, what results you would have obtained?

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Chapter 36: PREPARATIONS OF PARAFFIN SECTIONS TO VISUALIZE THE ARCHITECTURE OF *DROSOPHILA* BRAIN AND RETINAL PROJECTIONS

Recommended Level: UG, PG, Research

INTRODUCTION

Drosophila melanogaster is one of the key model organisms in genetic and biomedical research due to its simple genome, short life cycle, and advanced genetic tools. The *Drosophila* brain consists of around one hundred thousand neurons which constitute different domains (Zheng et al., 2018). The central body is in the central part of the brain and contains multiple neuropil processes. It involves in spatial orientation and navigation, locomotor activity, and behavioral rhythms. The optic lobes, attached on either side of the central body, are involved in visual processing. The optic lobe consists of four different neuropils: the lamina, medulla, lobula, and lobula plate. These are connected to the compound eye (Nériec and Desplan, 2016). The *Drosophila* eye is a complex and highly structured organ that originates from imaginal discs in the larvae stage. (Roignant and Treisman, 2009). The adult eye comprises a hexagonal array of a few cell types organized in a repeating pattern. It consists of approximately 800 units called ommatidia, each containing eight core photoreceptor neuronal cells (R1-R8). These are crowned by two primary pigment cells and four non-neuronal cone cells, forming an iris. This organized structure captures light and transmits visual information to the optic lobe (Cagan, 2009).

Drosophila head sectioning allows us to visualize the intricate anatomy and spatial organization of various cell types within the brain and eye, identifying cellular abnormalities and structural changes (Sunderhaus and Kretzschmar, 2016). By preserving and presenting a better view of the brain and eye's internal architecture, head sectioning is crucial for studying genetic and environmental factors that affect tissue morphology and integrity for modelling human diseases (Bilen and Bonini, 2005; Behnke et al., 2021; Wittmann et al., 2001). Thus, the importance of *Drosophila* head sectioning extends beyond basic research, offering critical contributions to developing therapeutic strategies and advancing genetic studies (Gonzalez, 2013).

OBJECTIVES

To visualize the anatomy of the adult *Drosophila* brain and retinal projections by histological staining of paraffin sections.

MATERIALS REQUIRED

Diethyl ether
Absolute ethanol (gradients 50%, 70%, 95% with 1XPBS)
Xylene
Triton X-100
Disodium hydrogen phosphate anhydrous (Na_2HPO_4)
Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$)
Sodium chloride
Bovine serum albumin
Gelatin (Sigma-Aldrich)

Paraplast
0.1% Toluidine blue in distilled water
16% paraformaldehyde
Chromium potassium sulfate dodecahydrate [$KCr(SO_4)_2 \cdot 12(H_2O)$]
DPX mountant
Microslides, cover-glasses
Hypodermic syringe 1ml
Tissue embedding ring (25x25x12 mm)
Tissue embedding mold (16x16x12 mm)
Culture vials
Couplin jars
Microtome

Solutions

1x PBS [NaCl (130 mM), Na_2HPO_4 (9 mM), $NaH_2PO_4 \cdot 2H_2O$ (2 mM)] pH 7.4

1x PBST [1x PBS (4990 µl), Triton X-100 (5 µl), 1% BSA (5 µl)]

4% PFA [16% PFA (250 µl), 1XPBS (750 µl)]

For gelatin-coated slides, immerse fresh slides in a solution containing 5 g/l gelatin and 0.05 g/l $KCr(SO_4)_2 \cdot 12(H_2O)$ for up to 6 min to achieve optimal subbing, followed by overnight drying.

PROCEDURE

Fly collection and aging

Collect about twenty wild type flies and age them for about 2 days.

Anesthetization and Dissection

1. Anesthetize the flies using diethyl Ether by placing them in a closed chamber until immobilized.
2. Decapitate the flies under a stereo-binocular microscope between the head and thorax with a syringe needle (Fig. 1A).
3. Remove the proboscis by placing an excision just above the protruding part to allow the fixative agent to penetrate the head cavity (Fig. 1B).

Fixation

1. Transfer the decapitated heads to a cavity block containing 4% PFA.
2. Incubate the heads in PFA for 90 min.
3. Decant the PFA and wash the tissue with PBST 3 times at 10 min intervals.

Dehydration

1. The tissues were dehydrated in a gradient series of alcohol dilutions starting with 50% dilution for 3 times washes in 20 min intervals.
2. Subsequently tissues were washed in 70%, 95%, and absolute alcohol three times at 20 min intervals for each of the dilutions.
3. Decant the 100% ethanol from the cavity block and wash the samples three times with xylene, each wash lasting 5 min. (**Caution**: Xylene fumes are hazardous and should not be inhaled. Experiments may be performed in a laminar flow hood.)

4. Transfer the heads to a culture vial using a wide-mouth dropper, then fill the bottle with 1 ml of xylene and 1 ml of molten wax. Incubate the bottle at room temperature overnight (Fig. 1C).

Paraffin Embedding

1. Incubate the heads in molten paraffin wax for 90 min at 60°C.
2. Carefully decant the preceding wax, ensuring the heads remain in the container, and refill the container with fresh 100% paraffin wax.
3. Repeat the washing process 4 times with 90 min incubation periods each.
4. Pour the molten wax into molds and embed the processed heads.
5. Using a dissecting needle, carefully orient the heads to face the base of the mold (Fig. 1D).
6. Store the paraffin molds in a freezer at 4°C.

Sectioning

1. Trim the paraffin mold from the side to reduce the surface area.
2. Place the molds in the microtome mold holder.
3. Cut the sections at 10 µm thickness and immediately place them onto gelatin-coated slides (Fig. 1E).
4. Using a brush, gently orient the sections on the slide.

Slide Preparation

1. Transfer slides to a 44°C incubator and remove them after 12 h.
2. Store them overnight at 4°C before subsequent staining.

Staining Protocol for *Drosophila* Heads

Rehydration of the samples

1. Wash the slides in xylene for 1 min, followed by an additional wash in fresh xylene for another 1 min.
2. Wash the slides twice in 100% ethanol for 5 min each.
3. Then, wash the slides sequentially in 95% ethanol, 70% ethanol, and 50% ethanol for 5 min each.
4. Finally, wash the slides in PBS for 5 min (Fig. 1F).

Staining and mounting

7. Dip the slides in toluidine blue stain for approximately 2 minutes (Fig. 1F).
8. Wash the slides in PBS accordingly to remove excessive staining from the dye.
9. Mount the slides in DPX.

Microscopy

The sections can be visualized under a brightfield microscope (Fig. 1G).

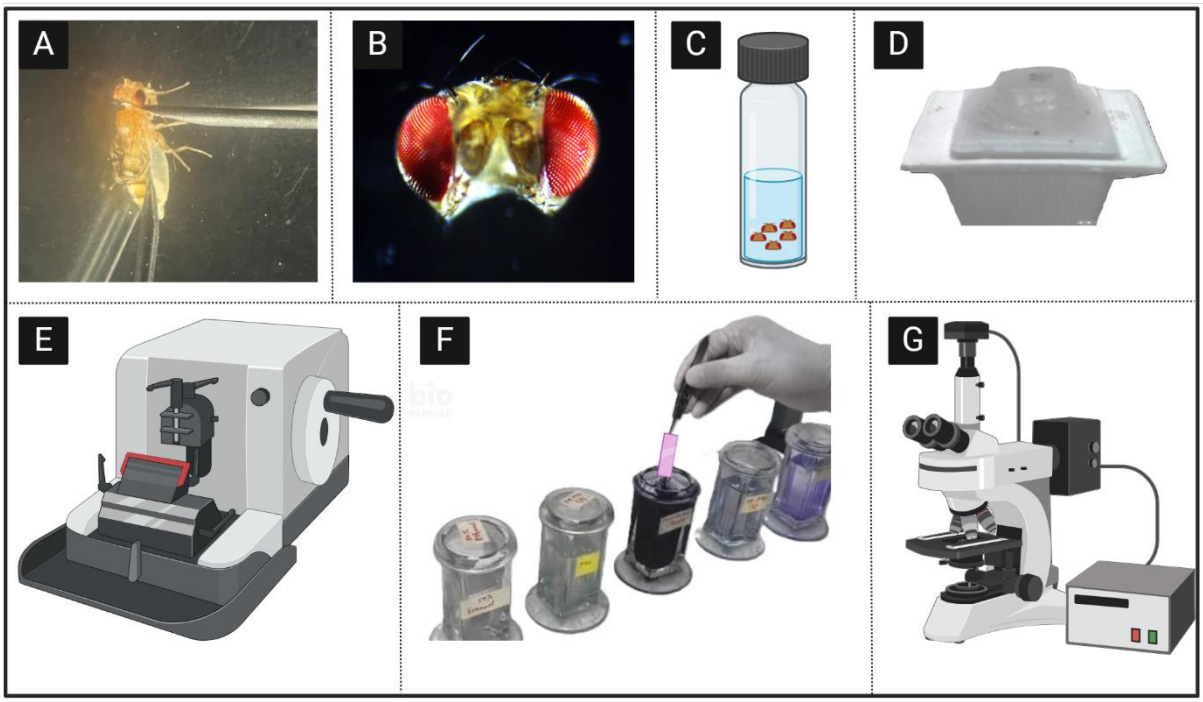

Figure 1. Steps for paraffin sections of *Drosophila* adult head. (A) Decapitation of 2-day-old adult *Drosophila* flies using dissecting needles. (B) After removing the proboscis, the head is now ready for fixation. (C) Culture vial containing decapitated heads are dehydrated and suspended in wax. (D) *Drosophila* heads embedded in Paraffin wax for sectioning. (E) Microtome equipment is employed in the sectioning process. (F) Slides containing *Drosophila* head sections rehydrated and stained with toluidine blue. (G) The stained slides are then visualized under the microscope. Figure created with BioRender.com.

OBSERVATIONS

In this protocol, the *Drosophila* heads were decapitated, and the proboscis was removed (Fig. 1 A-B). The head tissue was fixed and dehydrated in an alcohol gradient series and embedded in wax. (Fig. 1C-D). The head sections are prepared using a microtome at 10 µm thickness, rehydrated in an alcohol gradient series, stained with toluidine blue, and observed under a brightfield microscope (Fig. 1E-G). *Drosophila* head was sectioned in the frontal plane in this protocol (Fig. 2A). The sectioning of the brain allows us to visualize the different regions of the brain as represented in the illustration (Fig. 2B). The prepared frontal sections of *Drosophila* heads provided clear visualization of the brain and retinal architecture. The following observations are made:

I. **Brain Architecture**: The central body, located centrally within the brain, can be distinctly visualized along with the optic lobes situated on either side of it (Fig. 2C).

II. **Retinal Structure**: The photoreceptors are attached to the optic lobe, where they send information for visual processing. The section reveals part of the retina and the neuropil layers of the optic lobe of the adult *Drosophila* brain. The retina contains all photoreceptors, which are connected to the lamina neuropil. The lamina neuropil is linked to the medulla neuropil. Centrally located between the lamina and medulla neuropils are the lobula and lobula plate neuropils (Fig. 2C).

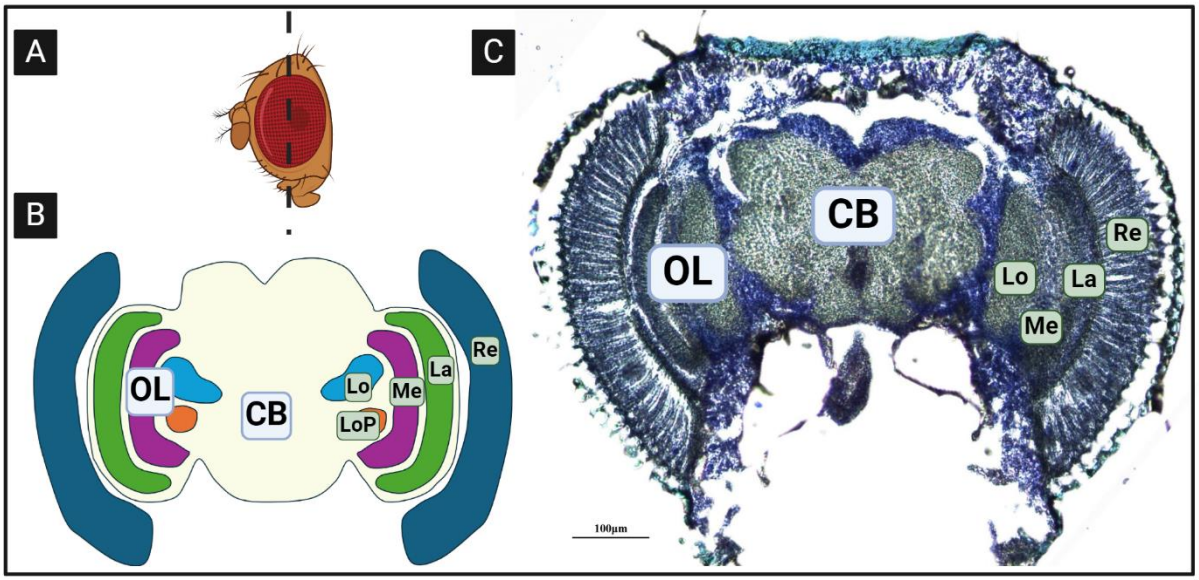

Figure. 2: *Drosophila* adult brain section: A) Frontal sections of the *Drosophila* head sliced from the anterior to the posterior portion. B) Graphic illustration depicts a section of the adult *Drosophila* brain, highlighting the central brain (CB) present at the center and the optic lobes (OL) on its either side. The retina (Re) displays a uniform arrangement of photoreceptors connected to the lamina (La) neuropil. The lamina neuropil is linked to the medulla (Me) neuropil. Centrally positioned between the lamina and medulla neuropils are the lobula (Lo) and lobula plate (LoP) neuropils. (C) Toluidine-stained frontal section of an adult *Drosophila* head, with all mentioned parts. Scale Bar: 100 µm.

QUESTIONS FOR FURTHER STUDIES

1. What is the rationale behind initially immersing *Drosophila* heads in ethanol rather than directly immersing the heads into wax after fixation?
2. Why gelatin-coated slides is used in the sectioning experiment?
3. What are other histological stains used for staining tissue sections?
4. What is the use of DPX?

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Chapter 37: ROUTINE HISTOLOGICAL STAINING TECHNIQUE USING HAEMATOXYLIN AND EOSIN

Recommended Level: UG, PG

INTRODUCTION

Haematoxylin and Eosin stain (commonly known as H&E stain or HE stain) was introduced in 1877 by chemist Nicolaus Wissozky at Kazan Imperial University in Russia. This method was modified by Luna, in 1968. It is the first and foremost primary step and most important staining technique used in histology or histopathology studies. It is the most commonly employed stain in medical diagnostics and is often considered the gold standard. It is also called double staining or differential staining technique as it involves two specific steps in utilizing two stains: hematoxylin and eosin. Haematoxylin helps in staining nuclei of cells giving a purplish-blue appearance, while eosin helps to stain the extracellular matrix and cytoplasm pink or reddish pink colour, as well as the other structures adopting various shades and combinations of these colors as a background stain. Thus, it is called the differential staining technique as it differentiates the nucleus from the cytoplasm with two different colours. The resulting colour patterns also reveal the general layout and distribution of cells, providing a comprehensive overview of the tissue's structural architecture.

It is also called progressive and regressive staining technique.

Progessive staining – It involves leaving the tissue in the stain just long enough to achieve the proper or desired endpoint. The slides must be examined at various intervals to determine when optimal staining is reached.

Regressive staining – Regressive staining involves initially overstaining the tissue and then destaining the excess of stain (differentiation) until the proper or desired endpoint is achieved.

In this staining method, Delafield or Harris hematoxylin is an example of a regressive stain. The excess stain is removed by using acid-alcohol, a process known as **differentiation.** Hematoxylin alum initially imparts a reddish blue (colour) to tissues due to its acidic pH. To convert this to the final blue colour, an alkaline pH is required. This process, called **"blueing," or "laking,"** can be achieved using tap water or ammonium hydroxide or ammonium solution (NH_3).

Principle

Haematoxylin- a cationic (positively charged) and basic stain binds with negatively charged phosphate (acidic) groups in the DNA backbone of the nucleus of a cell to give blue or purplish blue colour based on the dye-binding capacity of metal ions as a mordant. On the other hand, eosin – an acidic stain (negatively charged ion) having an anionic group, binds with the positively charged component, viz., proteins or amino groups, in the cytoplasm of a cell, thereby pertaining these components as pinkish red or pink colour. In this way, the nucleus can be differentiated from the cytoplasm by the intensity of the two-colour stain.

Haematoxylin Staining

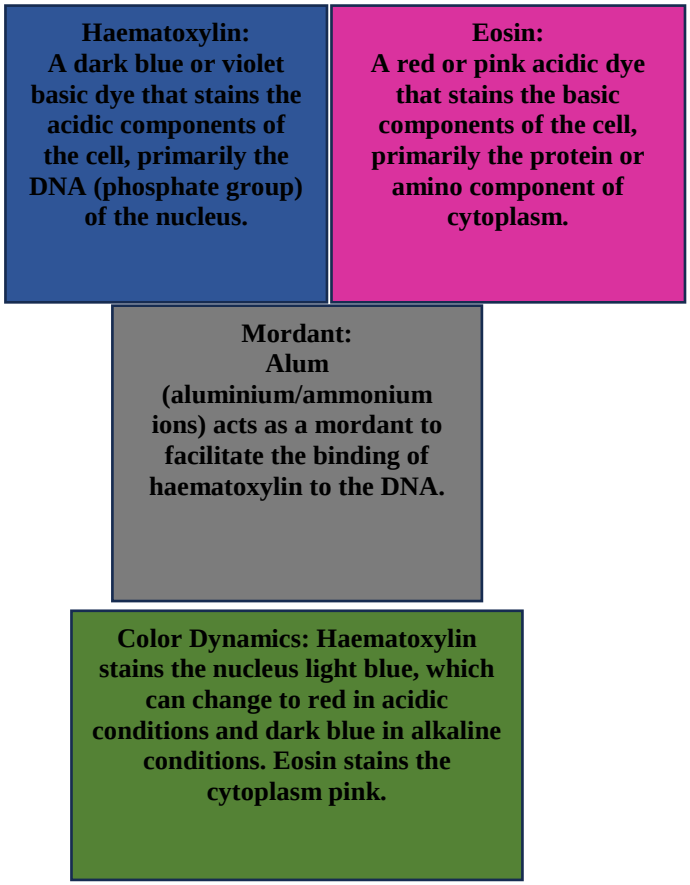
1. **Oxidation:** Haematoxylin is initially colourless and becomes a dye only after oxidation to hematein.
2. **Complex Formation**: Hematein forms intense colour complexes with metal ions, such as aluminum (Al^{3+}), which act as mordants. Hematin with mordants such as ammonium or

potassium alum forms a lake that functions as a cationic dye and stains anionic tissue components.

3. **Binding to Nuclei**: Hematein, when combined with aluminum ions (forming hemalum), stains the DNA in the cell nuclei. This binding occurs due to the interaction with the negatively charged phosphate groups in the DNA backbone. The dye-metal complex gives a blue colour in neutral to basic conditions.
4. **Colour Variation:** In the presence of acid, the stain can turn red, while in alkaline conditions, it remains dark blue.

Eosin Staining

1. **Nature of Eosin:** Eosin is an anionic (negatively charged) and acidic stain.
2. **Binding to Cytoplasm:** Eosin binds to positively charged groups on proteins, such as amino groups, in the cytoplasm, staining these components red or pink.
3. **Counterstaining:** Following haematoxylin staining, eosin is used as a counterstain to impart a contrasting pink colour to the cytoplasm, enhancing the visibility of cellular structures.



OBJECTIVE

Cytological differentiation of two important cellular components viz., nucleus and cytoplasm based on the intensity of the colour reaction of acidic (Haematoxylin) and basic (Eosin) stain.

MATERIALS REQUIRED

Stretched paraffin embedded tissue sections 4-5 µm (any animal tissue), haematoxylin and eosin stains, series of graded ethanol in coplin jars (30%, 50%, 70%, 90%, absolute ethanol), distilled water,

1% HCl (1 ml conc. HCl in 100 ml distilled water), 1% NH_3 water (1 ml liquid NH_3 in 100 ml distilled water), Xylene, slides, coverslips, DPX (Dibutylphthalate Polystyrene Xylene) mountant

Preparation of hematoxylin stain (Carleton, 1947)

a.	Hematoxylin	4 gm
b.	Absolute ethanol	25 ml
c.	Ammonium alum saturated (aqueous)	400 ml
d.	Glycerin	100 ml
e.	Absolute methanol	100 ml

To prepare the hematoxylin stain, first dissolve hematoxylin in ethanol and mix it gradually in ammonia alum solution. Expose the mixture to light in a flask with a cotton plug for 5 days and then filter. To filtrate, add glycerin and methanol. Leave the solution to ripen for at least 6 weeks. The ripened solution will keep for years.

Preparation of Eosin-Phloxine B solution

a. For Eosin Stock solution –

Eosin Y	1 gm
Distilled water	100 ml

b. For Phloxine Stock solution –

Phloxine B	1 gm
Distilled water	100 ml

c. Eosin-Phloxine B working Solution –

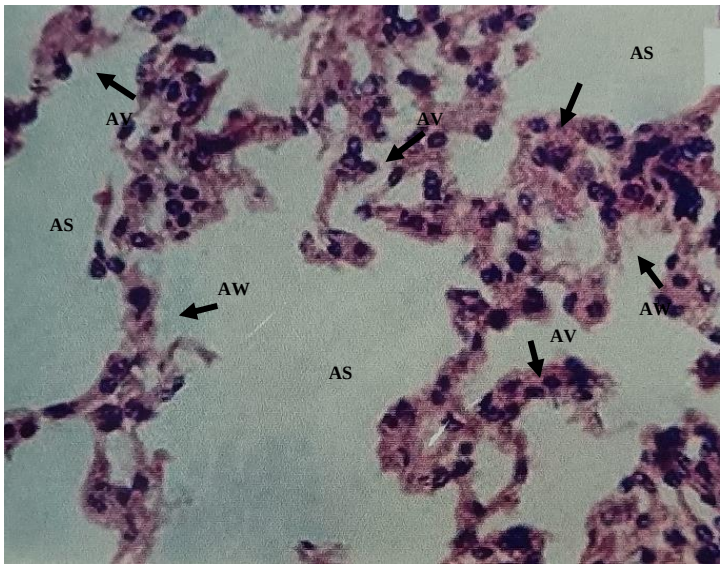
Eosin stock solution	100 ml
Phloxine stock solution	10 ml
Ethanol (95%)	780 ml
Glacial acetic acid	4 ml

PROCEDURE

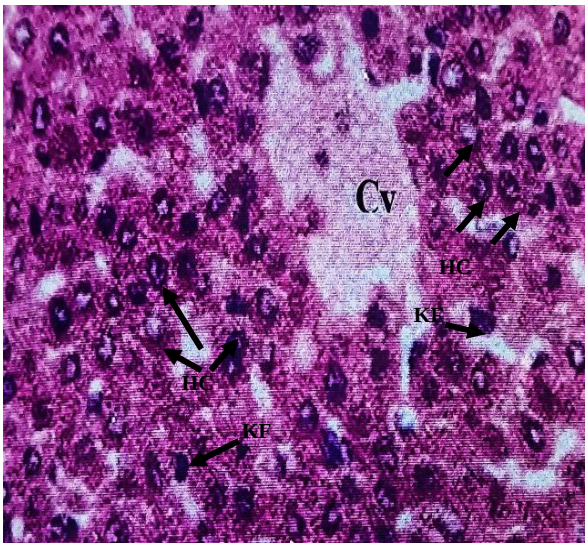
1. Deparaffinization: The stretched paraffin tissue sections (4-5 µm range) are deparaffinized in xylene for 2-3 changes, each of which must be for 30-45 min duration.
2. Tissues are proceeded to downgrade series of ethanol as from absolute ethanol to 30% alcohol, each of which must be maintained for 3-5 min, then passed to distilled water.
3. The tissue sections are stained in Haematoxylin for 5-10 min.
4. Rinse in distilled water, the tissues are rinsed in dilute HCl (1%) one or two times followed by rinsing in distilled water then in 1% NH_3 water, then again rinsed in distilled water (Blueing or Histological Laking step; Regressive and differentiation).
5. Proceed to upgrade series of ethanol from 30% to 70% by maintaining the tissue sections in each change for 3-5 min.
6. After 70% alcohol, the tissue sections are kept into cytoplasmic stain Eosin for 5-10 min, followed by 2 changes in 90% ethanol, 3-5 min each.
7. Kept in absolute ethanol for 3 min, cleared in xylene and mounted on DPX. Slides are now ready for observation.

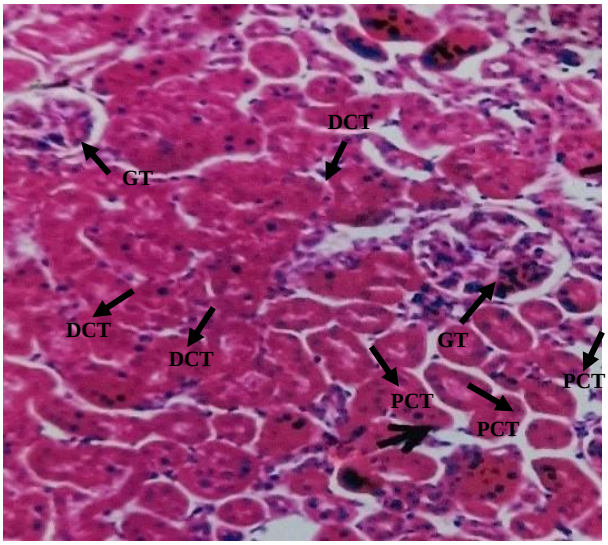
OBSERVATION

Nuclei appear purplish-blue, Cytoplasm shows pink to red colour (Fig. 1).



(A)





(B) (C)

Figure 1: Photo micrographic image showing histological structure (H&E stain) of mammalian (Albino rat) tissues. (A) Lungs (AV – Alveolar cell; AW – Alveolar wall; AS – Alveolar space); (B) Liver (Cv – Central venule; HC – Hepatocyte; KF – Kuffer cell) (C) Kidney (GT – Glomerulus Tuft; Tubular epithelium: PCT – Proximal convoluted tubule, DCT – Distal convoluted tubule)

Significance of the study

- The primary method for staining tissue sections.
- Enables clear differentiation between various tissue components, highlighting cellular and extracellular structures for detailed analysis.
- Essential for diagnosing a wide range of diseases, including cancers, infections, and inflammatory conditions, by providing clear visual distinctions in tissue architecture.
- Widely used in biomedical research to study tissue morphology and pathology.
- Provides a fundamental tool for teaching histology and pathology, helping students and trainees to learn about tissue structure and function through practical experience.

- Offers a standardized and reliable method for staining tissue sections, ensuring consistency and reproducibility in histopathological examinations and research studies.

Note: Before H&E staining, a brief knowledge of tissue processing and microtomy method needs to be known: Pieces of the required tissues are fixed in Zenker's fluid or Carnoy's fixative overnight. It can also be fixed in Bouin's fixative or 4-6% formaldehyde. Then, the next day, in case of Zenker's fluid as well as for Bouin's fixative and 4-6% formaldehyde, tissue should be washed thoroughly in water to remove the fixative and passed through graded series of ethanol for dehydration (from 30% → 50% → 70% → 90% → absolute ethanol, each for 30-40 min, depending on the size of the tissue). Passing through ethanol grades, is also a process of washing to remove the fixative. It also ensures gradual dehydration and clearing of the tissue, preparing it for subsequent staining procedures. In case of Carnoy's fixative, after overnight fixation, tissue should be washed from absolute ethanol → 90% → 70% (30-40 min each) and kept it overnight in 70% ethanol. Keeping tissues in 70% ethanol stabilizes them and prevents damage. When tissue suspended in a higher concentration of fixative is transferred into a lower concentration of 70% alcohol, it causes gradual dehydration and hardening. This process helps in the preservation and fixation of the tissue by preventing excessive shrinkage or swelling, thus maintaining the tissue's structure and making it suitable for further histological examination. Tissues are then cleared in xylene until the tissues become transparent. Tissues are then embedded in equal proportion of xylene and paraffin (1:1) in the water bath and finally embedded in pure paraffin (3 changes). Duration of paraffin embedding is 5-8 h or overnight, depending on the size of the tissue. In all the cases, the temperature of the paraffin should be between 55°C to 58°C otherwise tissue will become charred in higher temperatures of paraffin. Paraffin blocks are then prepared, trimmed, sections should be made in microtome machine in thickness of 4-5 µm, fixed on the slide by giving a thin layer of Mayer's albumin or dipped in 70% ethanol and stretched in a water bath. The tissue sections, fixed on the slide, are now ready for staining but it is preferable to stain after keeping them for 5-6 h or overnight.

- The tissues sections are rinsed in distilled water after passing through downgrade series of alcohol, then to haematoxylin stain as haematoxylin is prepared in aqueous condition (to maintain a uniform concentration gradient) and then again washed in distilled water.
- When ethanol is not obtained from suitable and authentic sources, it is advised to put the tissue sections in acetone before clearing them in xylene. This ensures complete dehydration and removal of water from the tissues.
- It is not advisable to observe slides immediately after mounting in DPX because the sticky nature of the DPX could adhere to the microscope's objective lens before it has dried, risking damage to the lens.

General DO's –

- ❖ Do ensure proper fixation using appropriate fixatives like Carnoy's fixative or Zenker's fluid Bouin's fixative or 4-6% formalin to preserve tissue morphology.
- ❖ If Bouin's fixative is used, the tissue sections should be washed thoroughly under running tap water for 3-5 h depending upon the size of the tissue as traces of picric acid in the tissue may interfere with the required acidic staining procedure.
- ❖ Do deparaffinize thoroughly to ensure complete removal of paraffin by immersing sections in xylene.
- ❖ Do rehydrate tissue sections by gradually passing through a series of decreasing alcohol concentrations to distilled water.

- ❖ Do rinse adequately in distilled water between staining steps to remove excess stain and prevent cross-contamination.
- ❖ Do dehydrate properly through increasing alcohol concentrations before clearing in xylene.
- ❖ Do clear with xylene to ensure complete removal of alcohol by immersing slides in xylene or a similar clearing agent.

General DON'TS –

- ❖ Do not rush deparaffinization, as incomplete deparaffinization can lead to poor staining results.
- ❖ Do not skip rehydration as it can cause uneven staining.
- ❖ Do not over or under-stain as it can result in too dark or too light staining.
- ❖ Do not forget the blueing step which can result in nuclei appearing red instead of blue.
- ❖ Before mounting, the tissue sections should not be exposed to air to prevent moisture capturing which can deteriorate the desired result and may develop air bubbles.
- ❖ Do not forget to rinse the tissue sections in distilled water after rinsing in acid water and ammonia water. It is mandatory, if not done, then it might cause interference during eosin staining.

QUESTIONS FOR SELF STUDY

1. Why is deparaffinization necessary before staining tissue sections with H&E?
2. Explain the chemical basis behind how hematoxylin and eosin interact with cellular components to produce distinct staining patterns.
3. Explain why hematoxylin is chosen as the nuclear stain in the H&E method.
4. What is the basic principle of H&E staining?
5. What is the role of acid water and ammonia water in H&E staining method?
6. How does eosin selectively stain cytoplasmic and extracellular components?
7. What is the role of the mordant (e.g., aluminum ions) in hematoxylin staining? Why is it necessary?
8. Describe the mechanism of action of acid alcohol in differentiating overstained sections.

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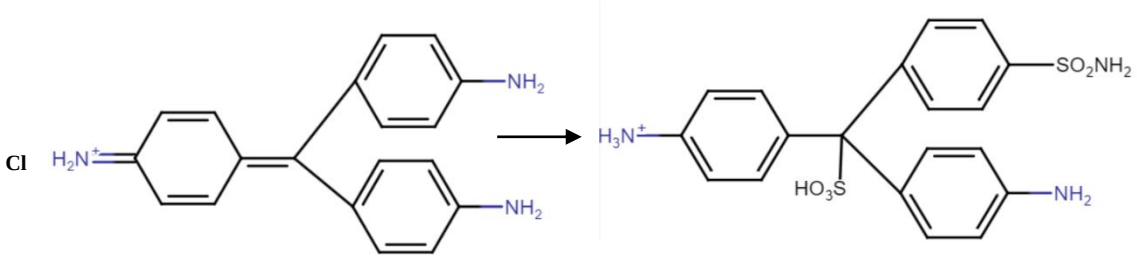
Chapter 38: CYTOCHEMICAL DETECTION OF DNA BY FEULGEN STAINING

Recommended Level: UG, PG, Research

INTRODUCTION

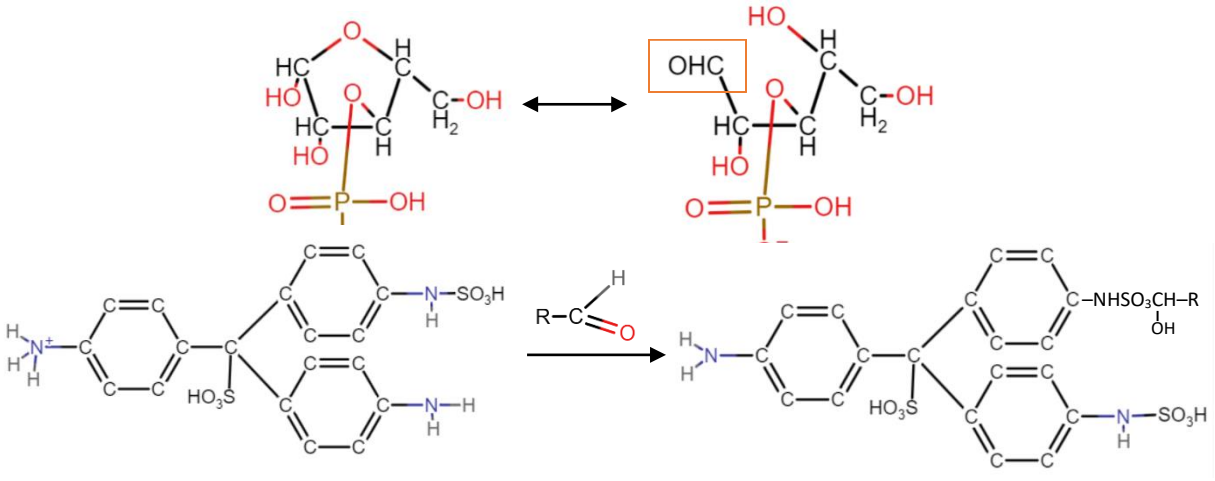
The Feulgen reaction was introduced by Feulgen and Rossenbeck in 1924. Feulgen, or Schiff's reagent, is made by using basic fuchsin stain, which is a mixture of pararosanilin, rosanilin, and occasionally, magenta II. Basic fuchsin is a parent substance (Pararosaniline) of range of dyes of various colour from red, purple, violent, blue, depending upon CH3 or CH5 groups added to the molecule of original dye. Sulphurous acid, produced from SO_2 at a low pH, interacts with pararosanilin and rosanilin to create a nearly colorless pale-yellow product known as **Schiff's reagent**, which is a fuchsin sulphurous acid, leuco basic fuchsin, or Feulgen stain.



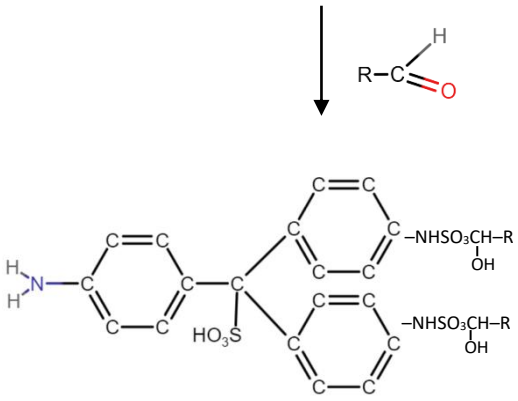
Pararosanilin Feulgen

Principle

- The reaction of Feulgen technique is based on the acid hydrolysis of deoxyribose furanose (carbohydrate component of DNA) to release -CHO group which reacts with Schiff's reagent (basic fuchsin) and in turn produces a purplish-reddish or magenta coloured product, in the form of Fuchsin-aldehyde complex.
- Basic fuchsin shows the following Feulgen hydrolysis which is specific for DNA. During acid hydrolysis, purine base of DNA is split off or broken down to expose stainable aldehyde (-CHO) group.
- Within the stipulated period of acid hydrolysis, it first breaks down the sugar linkages that are engaged in polymeric bonding and secondly ruptures the glycosidic linkages between sugars and purine bases.
- Now, the deoxyribose compound (apurinic acid) revealed as attachment through phosphate linkages in the main nucleic acid chain, where they are firmly held as furanose form with exposure of aldehyde residues.
- These arrangements at C-atom of furanose sugar, after the removal of nitrogenous bases, become a potential aldehyde, and that this OH-CHO would react as an aldehyde component with Schiff's reagent to give the purplish-pinkish or magenta coloured product, i.e., the aldehyde-fuchsin complex.



Feulgen First Product



Purplish-pinkish or magenta coloured product (aldehyde-fuchsin complex)

- When DNA reacts with Schiff's reagent, it shows graded response of colour intensity of the Feulgen reaction, which indicates the amount of DNA present in tissues (high intensity of colour = high amount of DNA, moderate intensity of colour = moderate amount of DNA, low or no intensity = low amount of DNA or absence of DNA. This is purely dependent on the prescribed duration of hydrolysis in the hot 1N HCl at 60°C.

OBJECTIVE

Cytochemical detection of DNA based on the colour intensity of Feulgen reaction.

MATERIALS REQUIRED

Stretched paraffin embedded tissue sections 4-5 µm [preferably any animal tissue sections or non-mammalian blood or buffycoat preparation of mammalian blood smear]

Reagent A: Schiff's reagent –

Distilled water	100 ml
1N HCl	15 ml

Basic fuchsin	0.5 g
Potassium metabisulphite	1.5 g
Vegetable Charcoal	1 g

The Schiff reagent (Basic fuchsin) is prepared by the following method:
Boil 100 ml distilled water, cool down to 50°C in a volumetric flask, then add 0.5 g basic fuchsin, dissolve by shaking and stirring well. Next, add 15 ml of 1N HCl and 1.5 g of potassium metabisulfite ($K_2S_2O_5$) and the solution is cooled further to approximately 25°C. The neck of the flask is plugged with cotton wool and allowed to stand for several hours or overnight. During this time, the bisulphite decolorizes the fuchsin, i.e., it forms the colorless SO_2-pararosaniline complex (leuco-basic fuchsin). Add 1 g of a decolorizing activated charcoal (commercial vegetable charcoal), shake well and the preparation is allowed to stand for about half an hour, before filtering. Impurities and other colored matter are then removed by adsorption on to activated charcoal. Filter rapidly (to avoid re-colorization the leuco-basic fuchsin) through a coarse filter paper or glass-wool. The solution should be clear and colorless; it can be stored for more than 6 months in a well-stoppered dark-colored bottle in cold temperature or fridge.

Reagent B: Potassium metabisulphite solution –

Potassium metabisulphite	15 g
1N HCl	10 ml
Distilled water	90 ml

Reagent C: Fast green 0.5% aqueous or Eosin
1N HCl, distilled water, graded ethanol (absolute ethanol, 90%, 70%, 50%, 30% ethanol), Xylene, DPX

PROCEDURE

1. The paraffin embedded stretched tissue sections on the slide are deparaffinized or dewaxed 2-3 changes in xylene 30-45 min each till complete paraffin is removed.
2. Pass through the graded ethanol (downgrade: absolute ethanol → 90% → 70% → 50% → 30% ethanol, 3-5 min in each → and then down to distilled water.
3. The tissue sections were washed or rinsed in cold 1N HCl (4°C) swiftly (2-3 dips).
4. Dip the slide in hot 1N HCl (at 60°C) for 5 min. Before dipping the slide, temperature should be noted using thermometer and the temperature accuracy must be maintained. This step is the most important and crucial step of the whole process because in this step, hydrolysis occurs and aldehyde (-CHO) group is released. The time of hydrolysis should be 5 min and should not exceed or stay below it.
5. It is then rinsed swiftly in cold 1N HCl (4°C, 2-3 dips).
6. Rinsed in distilled water.
7. Immersed the slide in Reagent A (Schiff's reagent) for 0.5 to 1 h in a staining jar kept in the refrigerator.
8. Excess fluid (stain) is drained off the slides, then rinsed well with 3 changes of Reagent B (Potassium metabisulphite solution) (3-5 dips for each change) [The Reagent B should be kept in 3 coplin jars separately].
9. Rinsed well in distilled water.

10. Counterstained in Reagent C (aqueous Eosin or fast green) for 3-5 min.

11. Rinsed well in distilled water (3-5 dips).

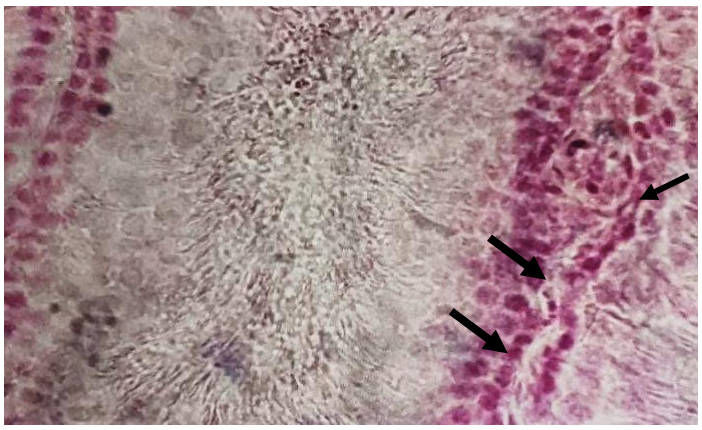
12. Dehydrated in upgrade ethanol: 30% → 50% → 70% → 90% → absolute ethanol, in (3-5 min each).

13.It is cleared in xylene and mounted in DPX.

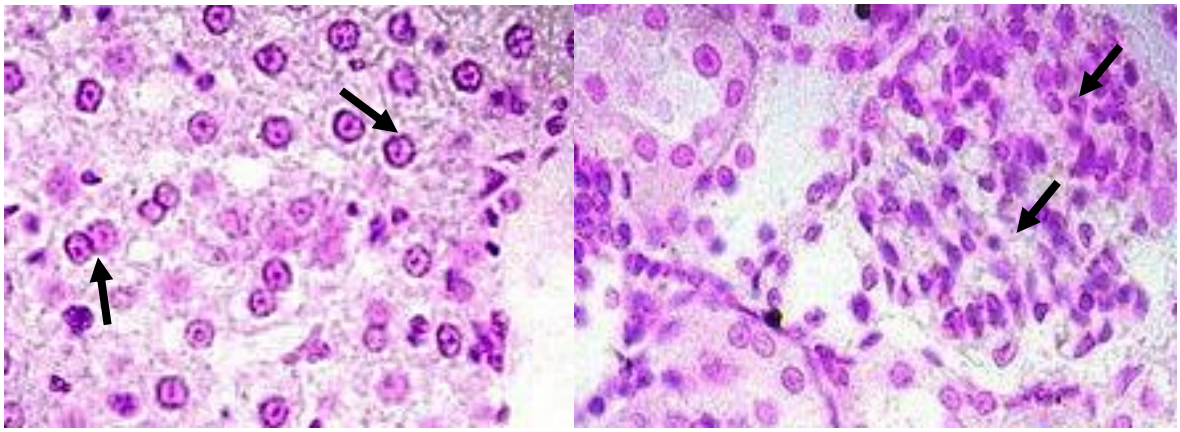
14. Observed under microscope (preferably after drying within 2-3 h)

OBSERVATIONS

DNA is seen in the tissue sections as purplish reddish or magenta against faint green (fast green) or reddish orange (eosin) background (Fig. 1).



(A)



(B) (C)

Figure 1: Photo Micrograph showing intensity of Feulgen reaction in DNA. (A) Intestinal DNA, (B) Liver DNA, (C) Kidney DNA.

Note: After distilled water, if the slide containing tissue sections is directly placed in hot 1N HCl, it might remove the tissue sections from the slide due to sudden fluctuation of temperature and medium. Hence, prior to dipping in hot 1N HCl, the sections should be immersed in cold 1N HCl swiftly before and after the hot HCl to stabilize them and balance the change in temperature, preventing any deterioration or removal of tissue sections from the slide.

Mild acid hydrolysis unmasks aldehyde groups in DNA but not in RNA. This is because the ribose sugar in RNA, which has a hydroxyl group at the 2-position instead of a hydrogen atom as in deoxyribose, does not get hydrolyzed by 1N HCl and thus does not react in the Feulgen test. Consequently, RNA does not interact with Feulgen or Schiff's reagent. During this process (1N HCl at 60°C for 5 min), the acid also removes purines from DNA, which is crucial for exposing the aldehyde group in the deoxyribose moiety. A balance must be maintained: hydrolysis should remove purines from DNA to provide more aldehyde groups for staining, but prolonged hydrolysis can break inter-nucleotide bonds, making DNA soluble and causing its gradual loss from the section. Fixation affects the binding of DNA to nuclear proteins, thereby slowing the loss of purines and the vulnerability of inter-nucleotide bonds to hydrolysis.

Many studies reported that hydrolysis should not be performed for longer durations than the prescribed period (10 min, 12 min, etc., instead of 5 min) as it will remove all histones as well as the phosphorus groups from the tissue sections (Di Stefano, 1948; Stedman, 1950). In some cases, it is also reported that the loss of the intensity of Feulgen reaction by overtime hydrolysis could be attributed to the changing or lability of the sugar attachments and to instability of the furanose to itself (Li and Stacey, 1949).

Time of hydrolysis depends on type of different fluids used for fixation.

Before staining procedure, tissues should be fixed in an appropriate fixative on following the microtomy method mentioned briefly in earlier H&E staining method. For the study of DNA, the preferable fixatives are Zenker's fluid and Carnoy's fluid. Both the fixatives have rapid penetration power, causing rapid fixation within 8-12 hours in case of Carnoy's and 4-6 hours in Zenker's fluid. They are good fixatives for preserving cyto-components such as nucleus, glycogen, connective tissues, etc. Although both fixatives are very good fixatives, the tissue sections should not be kept for longer durations as the tissues will become brittle and the glacial acetic acid present in both fixatives will change the colour of the chrome salt and as such desired result will not be obtained.

Zenker's fluid – a microanatomical and cytological fixative

Mercuric chloride	5 g
Potassium dichromate	2.5 g
Distilled water	100 ml
Glacial acetic acid	5 ml

(Add glacial acetic acid immediately before use)

Carnoy's fluid – a cytological fixative

Absolute ethanol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

(Chloroform is said to accelerate the action of the fixative)

Formaldehyde is not a preferred fixative for the cytochemical detection of DNA by the Feulgen reaction because Formaldehyde forms covalent cross-links between DNA and proteins, as well as within the DNA itself. This cross-linking can inhibit the acid hydrolysis step that is critical for the Feulgen reaction, that essentially requires the generation of apurinic sites as well as the exposure of aldehyde group from the sugar moiety of DNA to react with Schiff's reagent.

General DO's –

- Do use an appropriate fixative (Zenker's fluid or Carnoy's fixative) to preserve tissue morphology and DNA integrity.

- ❖ Do prepare hydrochloric acid solution freshly as per protocol to ensure accurate staining. Schiff's reagent should be prepared and matured 15 days to 1 month prior to starting of the experiment for obtaining better results.
- ❖ If commercial Schiff's reagent is used, ensure it should be from a genuine source.
  The tissue sections should be kept in hot 1N HCl essentially for 5 min duration and not exceed it.
- ❖ The tissue sections should be swiftly passed in cold 1N HCl prior and after hot 1N HCl to maintain the balanced medium.
- ❖ Do ensure thorough washing after staining to remove excess reagent and reduce non-specific background staining.
- ❖ Do store Schiff's reagent and other chemicals in dark and cool conditions (in fridge) to maintain their effectiveness.

General DONT'S –

- ❖ Do not perform hydrolysis for longer durations.
- ❖ Do not expose Schiff's reagent to light to prevent premature colour development.
- ❖ Do not use sections with folds, tears, or improper thickness, as these can affect staining uniformity.
- ❖ Do not let tissue sections dry out and ensure tissue sections remain hydrated throughout the staining process to prevent artifacts.
- ❖ Before mounting, the tissue sections should not be exposed to air to prevent moisture capturing that can deteriorate the desired result and may develop air bubbles.

QUESTIONS FOR SELF STUDY

1. State the chemical basis of the Feulgen reaction. Why is it specific for DNA?
2. What is the role of hydrochloric acid (HCl) in the Feulgen method?
3. Explain how Schiff's reagent is prepared and how it reacts with aldehyde groups to produce a magenta color.
4. What are the expected visual results when observing a sample stained using the Feulgen method under a microscope?
5. How does the intensity of staining correlate with the amount of DNA present in the sample?
6. State the reason why tissues need to be swiftly passed through 1N cold HCl and then in hot 1N HCl.
7. If sections are kept in hot 1N HCl for prolonged period, what would happen?
8. Why RNA cannot be stained with Feulgen stain although it is a nucleic acid?
9. What is the crucial and most important step of Feulgen reaction? State reason.
10. Why the optimum duration i.e., 5 minutes is required for keeping the tissue sections in hot 1N HCl?
11. What are the preferable fixatives for detection of DNA by Feulgen method? Give reason.
12. Why an aqueous counterstain (Eosin or Fast green) is used instead of alcoholic Eosin or Fast green after washing the tissue sections in distilled water?
13. Why the tissue sections are passed through graded alcohol series (dehydration or hydration) instead of directly placing the tissue sections in 90% to absolute alcohol?

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Chapter 39: CYTOCHEMICAL DETECTION OF LIPID GRANULES BY SUDAN BLACK B STAINING METHOD

Recommended Level: UG, PG, Research

INTRODUCTION

Histochemically, lipids can be defined as fatty matter that has some of the chemical characteristics of lipid molecules, e.g., they contain choline, unsaturated fatty acids, or they are phosphatides.

The lipid detection can be done by two methods –

a) Physical methods, such as the Sudan Black method, depend on the readiness of lipophilic molecules to partition between their solvent and the lipid in the section. Therefore, these are the tests that depend upon the degree of 'fattiness' of the material.

b) Chemical methods, like the acid haematein test or the use of the Nile blue method.

Amongst the two methods, the physical method is generally more convenient as lipids to a large extent are characterized histochemically by their ability to concentrate Sudan Black or benzpyrene from semi-aqueous solutions in which these compounds are not very soluble.

The dye, Sudan Black B, was first introduced by Lison in 1934. It is a non-fluorescent, thermally stable, fat-soluble (lysochrome), lipophilic diazo dye used to stain a variety of lipids, including phospholipids, sterols, neutral triglycerides, and lipoproteins, etc., either in frozen tissue sections (through cryotome) or paraffin-embedded tissue sections (fixed with formalin solution or Zenker's fluid or Carnoy's fixative). It has also been employed in the study of mitochondria, the Golgi apparatus, myelin sheaths of nerve fibers, and the lipids of endocrine glands such as the adrenal cortex, testis, and corpus luteum because all of them have membranes made up of lipids.

Principle

The dye is dark brown to black powder and it is dissolved in a lipid solvent (e.g., 70% ethanol, 100% ethanol or propylene glycol). When tissue sections are treated with the dye-solvent solution, the dye leaves the solvent due to its higher solubility in lipids. The dye is more soluble in the lipid in the tissue sections than in the original solvent, so the dye will move out of the solvent and thereby, do the coloration of the tissue lipid as black. The staining process involves boundary-surface adsorption, a physical process rather than a chemical reaction. Under a microscope, lipid granules stained with Sudan Black B appear as deep blue-black/ brownish-black pigments or black pigments.

In the below-mentioned process, when tissues are fixed in a formaldehyde-ethanol solution, on one hand, ethanol dissolves the lipid granules in tissue or cell membrane (blood), thereupon, is fixed by formaldehyde. When these sections or samples are stained with Sudan Black, the dye will move out from the original solvent due to its higher affinity to the solvent containing lipids (as fixed by formaldehyde) and thereby, impart the colour of the tissue lipid as black. Besides, the localization of intensity of the black color reaction (such as high intensity= "3-5+", /moderate intensity = "2-3+" / low intensity = "1+" /no intensity = "-") is suggestive or gives the first site of clue or indication for quantification of lipid content simply in the desired tissue material. Based on this, further process of estimation of different varieties of lipids can be done.

OBJECTIVE

Cytochemical detection of lipid granules in the tissue or cellular materials.

MATERIALS REQUIRED

Any paraffin-embedded animal tissue section (microtome) or frozen tissue section (cryotome) or mammalian or vertebrate blood samples
Coplin jars, glass slides, small beakers, blotting and filter paper, lancet/prickling needle (sterilized), cotton, rectified spirit (90% ethanol for sterilization)
Sudan Black B Solution, Safranin 1% (in distilled water), Fixative (9:1 of 95% ethanol and 40% formaldehyde), Xylene and DPX

Preparation of Sudan Black B staining solution

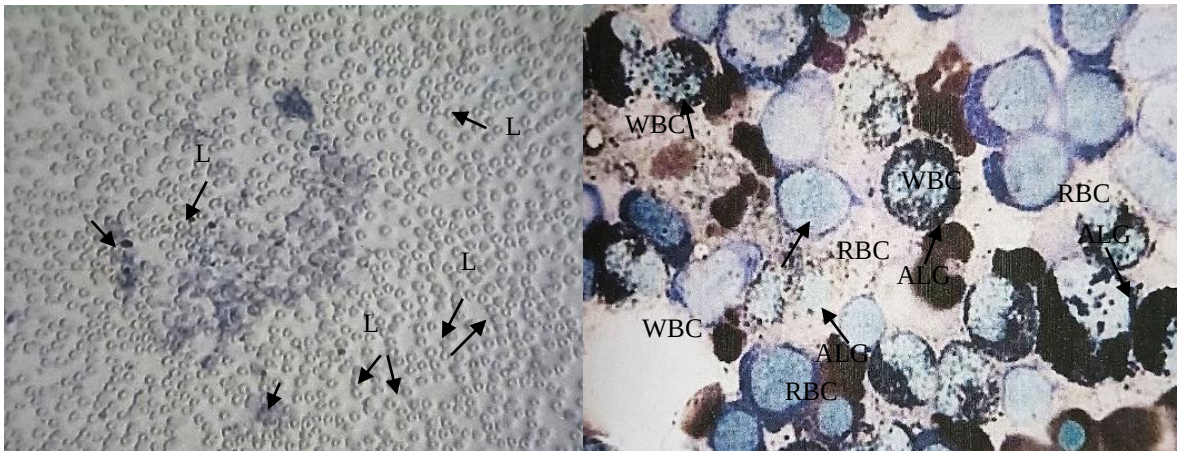
A saturated solution of Sudan Black B (16 mg/ml) is prepared in 70% ethanol which should be allowed to stand for 24 h before use. Then, it should be used after filtering it.

PROCEDURE

1. Blood smear is to be taken on a clean dry slide and then air dried.
2. The air-dried blood film is to be fixed in fixative for 5 min in a Coplin jar. Paraffin-embedded 4-5 µm tissue sections can also be taken, they are deparaffinized in xylene, brought in absolute ethanol for 1-2 min, after that, the tissue sections should be immediately fixed in the fixative for 15-20 min. Cryosections can also be processed from this fixation step.
3. The slide is then dipped in Sudan Black B solution in a Coplin jar for 45-60 min at 37°C in an incubator.
4. The staining solution is allowed to drain to remove excess stain from the blood film or tissue sections without washing.
5. Counterstained with 1% Safranin solution in another Coplin jar (2-3 min).
6. The slide containing blood film or tissue sections is air-dried.
7. Cleared in Xylene and mounted in DPX.

OBSERVATIONS

- Lipid granules/droplets are observed in aggregated form or sparingly distributed as intense black or blue/black – dark granulated structures (or droplets) as shown in Fig. 1 and 2.
- Nucleus observed red and distinct (stained by safranin).



A B

Figure 1: Photomicrographic images showing lipid granules/ droplets in A) LD – lipid droplets in RBC. 10X B) ALG – aggregated lipid granules in RBC and WBC.

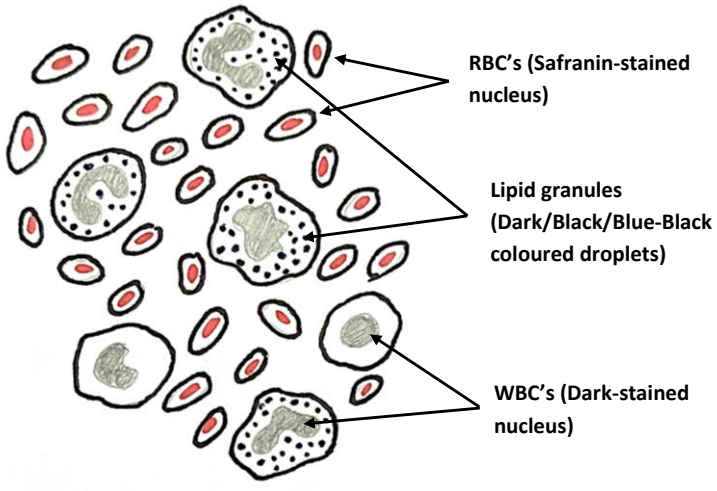


Figure 2: Diagrammatic representation of lipid granules in WBCs in blood

Significance of the study:

- Provides detailed histopathological evaluation of tissues, aiding in the assessment of disease progression and severity.
- Facilitates the study of lipid metabolism and storage at the cellular level. Researchers can investigate lipid-related cellular processes, contributing to a better understanding of cell biology.
- The alterations in binding caused by physiological changes, trauma, drugs, and hormones offer crucial and sensitive indications into the tissue's function.

General DO's

- Do fix tissue sections or blood smears with appropriate fixatives to preserve cellular structures and prevent degradation of lipids.
- Do use clean slides, pipettes, and staining jars to avoid contamination that could interfere with the staining process.
- Do allow adequate time for the staining process to ensure proper adsorption of the dye onto the lipid granules.

- Do handle stained slides carefully to avoid smudging or damaging the smear, which could affect the quality of the observations.
- In the case of steroidal lipids (hormones), it is advisable to stain only at higher temperatures, i.e., temperatures closer to the melting point of steroids or steroidal hormones present in the tissues.
- Besides, for steroidal lipids, the procedure is the same as above but when the solution of Sudan Black B has covered the section containing the steroidal lipids, the alcoholic solution is burned out. When the alcohol has finished burning of the tissue section, now, the tissue section is covered with a fresh solution of Sudan Black and the process is repeated 4-5 times.
- In case of heavy black deposition of Sudan Black solution in tissue sections, it must be removed by rinsing in 70% alcohol (4-6 dips). This process is called the **"unmasking"** of the lipid.
- It is advisable to use Oil Red O for staining triglycerides but it is very poor for phospholipids.

General DON'T's

- Do not use solvents that are not compatible with Sudan Black B such as 70% alcohol or that may dissolve the lipid granules.
- Do not allow slides to dry out in between the steps to ensure that the slides remain hydrated throughout the staining process to prevent the formation of artifacts.
- Before clearing in xylol, the tissue sections are required to quickly air dry or oven dry at low temperatures 27°C after staining with safranin stain as safranin solution is prepared in an aqueous medium. If not so, it will develop air bubbles during mounting.
- After safranin staining, the tissue sections are required to air dry because
- Do not expose the tissue sections to 70% alcohol because it itself may affect the lipids. That's why, in the above-mentioned process, we are not proceeding with the tissue sections through the downgrade series of alcohol.

QUESTIONS FOR FURTHER STUDIES

1. What are the key differences between lipophilic and hydrophilic substances?
2. Why is Sudan Black B specifically used for staining lipophilic substances?
3. What is the basic mechanism of staining of lipid granules by Sudan Black B solution?
4. Why does Sudan Black B leave the solvent during the staining process?
5. What is the purpose of fixing tissue sections or blood smears with a fixative before staining with Sudan Black B?
6. What are the expected microscopic features of lipid granules stained with Sudan Black B in a blood smear?
7. Why you should not downgrade to 70% alcohol after deparaffinization of tissues?

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Chapter 40: CYTOCHEMICAL LOCALIZATION OF GOLGI COMPLEX

INTRODUCTION

Golgi complex is a secretory organelle of eukaryotic cells. Its organization and location differ from cell to cell. In muscle cells, it is small but in nephrons and secretory cells, it is large, well-developed, and reticulated. Its location may be circumnuclear (neurons) or polar, between the nucleus and periphery. It may be selectively stained by osmium tetroxide and silver salts.

Golgi was first stained by silver staining technique by Camillo Golgi in 1873 in nervous tissue, which was also called as Black reaction method or Golgi method. The technique was improved afterward by Ramón Y Cajal, which was known as the Double Impregnation method. After that, this technique was modified by several scientists and accordingly, the methods were known as –

i. Da Fano Silver Nitrate-Gold Chloride Method (1921)
ii. Kolatchew-Nassonov Method (Nassonov 1923-1924)
iii. Sudan Black Method (Baker 1944)
iv. Direct Silver Method (Elftman 1952)
v. Ludford Osmium Tetraoxide Method (Lillie 1965)

In almost all the methods, silver salts or lustrous heavy or soft silvery metals (e.g., osmium tetroxide, silver nitrate, calcium chloride, mercuric chloride) are used for impregnation of the tissue that ultimately develops the black colour indication for the presence of Golgi complexes.

Principle: As Golgi is secretory in function, it contains secretory enzymes, which means it contains several proteins. The Golgi staining method is based on soft or heavy silvery metal ions. When the metal ions mentioned above react with tissue section containing Golgi complexes, the metal ions help in the precipitation of the protein or enzyme from their respective impregnated metal-based solution. In the process of precipitation, the protein dissociates as a protein anion (Pr^-) which combines with a positive metal ion (cation) to form an insoluble precipitate of a specific metal proteinate (which shows black colour) that is based on the name of the protein or enzyme found in the tissue specific golgi bodies. Then, formaldehyde or formalin solution cross links with this protein precipitate in turn to fix it.

OBJECTIVES

Cytochemical or histological localization of Golgi complex in the tissues or epithelial cells based on the intensity of black colour precipitation**.**

Several methods are followed to perform this staining

Kolatchew-Nassonov Method (Nassonov 1923, 1924)

REQUIREMENTS

Fixative

Potassium dichromate 3% aqueous 10 ml
Chromic acid 1% aqueous 10 ml
Osmic acid 2% aqueous 5 ml

PROCEDURE

1. The fixative is made by adding freshly prepared reagents in given proportion mentioned. Then, 2-4 mm tissue pieces are fixed in the above fixative and kept for overnight or 24 h.
2. Wash the tissue pieces in running tap water for 18-24 h.
3. Place the tissue pieces in 2% aqueous Osmic acid at 40°C for 8 h or at 34°C for 1-2 days
4. Wash the tissue pieces thoroughly in running tap water overnight (this can be done by keeping the tissue pieces in porcelain bag or net cloth bag and binding it with help of a thread to a tap where water will be released from the tap in a slow motion overnight).
5. Dehydrate the tissue pieces by passing through upgrade series of ethanol (30%, 50%, 70%) 10-15 min each and then in 90% and absolute ethanol 30-45 min, 2 changes each.
6. Clear the tissue pieces in xylene and embed in paraffin [½ xylene: ½ melted paraffin 4-5 h (1 change, overnight), 2 changes in melted paraffin (each change 5-8 h, depending on the size and nature of the tissue), prepare block, trim the block to make appropriately for fixing to the block holder, tissue sectioning 3-5 µm, tissue section stretching and mounting on the slide.
7. If tissue sections appear too dark, it can be treated with turpentine (old and oxidized better) to remove excess colour.
8. Deparaffinize tissue sections in 2-3 changes of xylene and mount in DPX.

OBSERVATION

Golgi complex appears as black aggregation against a yellow background.

Note: Mitochondria will be stained black if the mounted sections are proceeded in Step 3 for prolonged period.

<u>Ludford Osmium Tetroxide Method (Lillie 1965)</u>

Fixative

Osmic acid 1% aqueous 50 ml
Aqueous saturated Mercuric chloride + 0.37 g sodium chloride 50 ml

PROCEDURE

1. The fixative is made by adding freshly prepared reagents in the proportion mentioned above. Then, 2-4 mm tissue pieces are fixed in the above fixative and kept for 15-18 h or overnight
2. Wash thoroughly in distilled water for 30 min.
3. Impregnate the tissue pieces with osmic acid solution in a sequential manner as indicated:
   2% Osmic acid at 30°C for 3 days → 2% Osmic acid at 35°C for 1 day →1% Osmic acid at 35°C for 1 day → 0.5 % Osmic acid at 35°C for 1 day.
4. Wash in distilled water for 18-24 h (washing as mentioned in earlier procedure).
5. Dehydrate the tissue pieces in the same way as mentioned in Step 5 of earlier Method.
6. Clear the tissues in xylene, embed in paraffin, section, mount and dry in the same way mentioned in Step 6 of earlier Method (in this case, the tissue sections should be a little bit thick in the range of 5-6 µm).

7. Deparaffinization of tissue sections in 2-3 changes of xylene and mounting in DPX.

OBSERVATION

A. Golgi complex appears black
B. Yolk and fat: black (these maybe bleached with turpentine)

Note: Mitochondria may also take black stain. But after Step 6 on hydration to water and treating with 0.125% potassium permanganate followed by Altmann method, mitochondria appear as crimson.

Direct Silver Method (Elftman 1952)

PROCEDURE

In this process, the fixation is not solely done at the beginning but it is impregnated both with silver nitrate and formalin fixative simultaneously.

1. Place the small pieces (3-5 mm) of tissue in 2% silver nitrate in 15% formalin solution* for 2 h. (*2 g silver nitrate in 100 ml of 15% formalin solution)
2. Rinse rapidly in distilled water and then dip the tissue pieces into the 2% hydroquinone in 15% formalin solution* for 2 h. (2 g hydroquinone in 100 ml of 15% formalin solution)
3. Again, fix the tissue pieces in 10% formalin solution overnight for complete fixation.
4. Wash thoroughly the tissue pieces for complete removal of the fixative with running tap water for 15-20 min (same as mentioned in earlier protocol in Step 4).
5. Dehydrate the tissue pieces in the same way as mentioned in Step 5 of earlier protocol.
6. Clear the tissues in xylene, embed in paraffin, section, mount and dry in the same way as mentioned in Step 6 of earlier protocol.
7. Deparaffinize tissue sections in 2-3 changes of xylene and mount in DPX.

OBSERVATION

The tissue sections show the appearance of Golgi complex as black colour aggregates or precipitates.
Note: If the silver stain is too dark black colour, it should be bleached with 0.7% iron alum. Differentiation can be done under microscope and the reaction can be stopped by washing thoroughly in running tap water.

Da Fano Silver Nitrate-Gold Chloride Method (1921)

PROCEDURE

1. Fix the 3-5 mm fresh tissue pieces in 1% cobalt nitrate in 15% aqueous formalin* for 2-18 h (depending upon the size and nature of the tissue). (*1 g cobalt nitrate in 100 ml 15% aquous formalin solution)
2. Wash the tissue pieces quickly in distilled water.
3. Place the tissue pieces in 1.5% aqueous silver nitrate in dark for 36-48 h.
4. Quickly wash the tissue pieces in large quantity of distilled water.
5. Immerse the tissue pieces in Cajal reducer for 4 h.

(Cajal reducer is prepared by mixing the reagents in the following-mentioned proportion:
2% aqueous Hydroquinone 100 ml
Neutral Formalin 15 ml
Anhydrous sodium sulphite 0.5 g

6. Wash the tissue pieces thoroughly in several changes of distilled water.
7. Dehydrate the tissue pieces in the same way as mentioned in Step 5 of earlier protocol.
8. Clear the tissues in xylene, embed in paraffin, section, mount and dry in the same way as mentioned in Step 6 of earlier protocol (in this case, the tissue sections should be a little thicker in the range of 5-8 μm).
9. Deparaffinize tissue sections in 2-3 changes of xylene (45-60 min each).
10. Hydrate the tissue sections by passing through downgrade series of ethanol (absolute ethanol → 90% ethanol → 70% ethanol → 50% ethanol → 30% ethanol → distilled water, each change 2-3 min).
11. Tone the tissue sections by placing them in 0.2% gold chloride for 5-10 min.
12. Wash the tissue sections quickly in distilled water and fix them in 5% sodium thiosulphate for 10-15 min.
13. Wash the tissue sections again in distilled water and counterstain with haematoxylin, if desired.
14. Rinse the tissue sections in distilled water and quickly pass through upgrade series of ethanol, each change 1-2 min, then 95% and in absolute ethanol.
15. Clear the tissue sections in xylene and mount in DPX.

OBSERVATION

The Golgi complex appears as black in cytoplasm against the background appearing pinkish-violet in the nucleus.

Sudan Black Method (Baker 1944)

PROCEDURE

1. Fix the 3-5 mm fresh tissue pieces in formalin-calcium for 2-3 days.
2. Wash the tissue pieces in running tap water (15-20 min) for removal of formalin
3. Infiltrate the tissue pieces in gelatin solution at 37°C in a glass container (in an incubator) for 1 h

   Gelatin solution can be prepared by mixing the reagents in the following proportion:
   Gelatin 15 g
   Glycerin 15 ml
   Distilled water 70 ml
   A few crystals of thymol
4. Bring the tissue pieces to room temperature (20-24°C) and harden the gelatin-containing tissue block by putting them in the hardening solution.

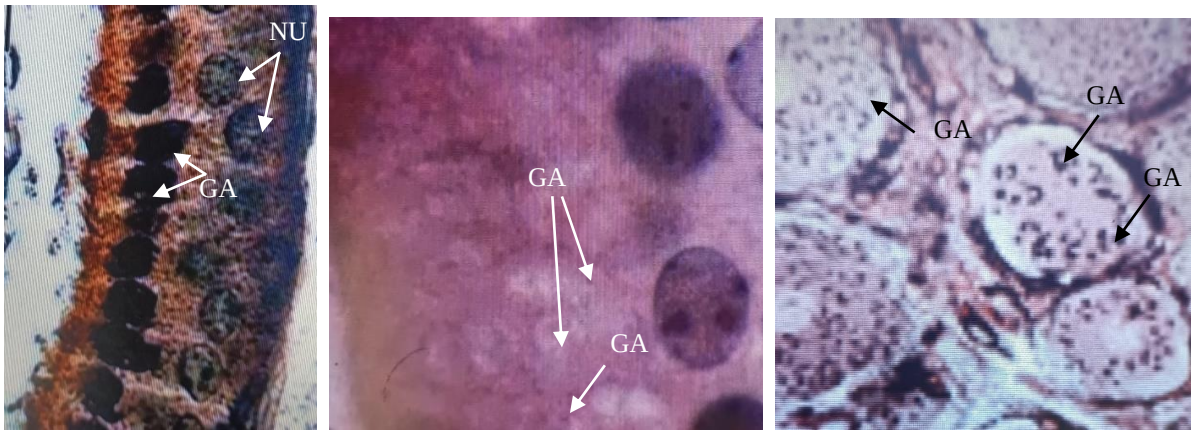
   The hardening solution can be prepared by mixing the reagents as follows:
   Formalin 10 ml
   Aqueous 10% Calcium chloride 10 ml

Aqueous 10% Cadmium chloride	10 ml
Distilled water	70 ml

5. Wash the tissue pieces in running tap water, cool at 4°C, and cut the frozen tissue sections of 10-15 µm size.
6. Pass the tissue section through 30%, 50%, and 70% ethanol (1-2 min each).
7. Dip the tissue sections in saturated Sudan Black for 7 min
   (Sudan Black prepared in 70% alcohol)
8. The excess stain is removed by dipping the slides in 3 changes of 50% ethanol (2 min for each change)
9. Wash in running tap water and rinse in distilled water.
10. Mount in glycerin.

OBSERVATIONS

Golgi vesicles appear as black in colour (see Fig. 1).



A **B** **C**

Figure 1: Photo micrographic image showing the localization of Golgi bodies; A) Golgi apparatus (GA) stains black and nuclei (NU) in epididymis; B) Golgi apparatus (GA) (H&E stain); C) Golgi apparatus (GA) in seminiferous tubule.

Significance of the study

- Helps in understanding and visualizing the localization and organization of the Golgi complex.
- Give an insight or clue about knowledge of protein trafficking, glycosylation, and secretion processes of a cell in terms of the presence of Golgi complexes in them.
- Identifies abnormalities in the Golgi complex related to diseases.
- Aids in the study of neurodegenerative disorders, cancer, and congenital glycosylation disorders.
- Provides insights into cellular responses to drugs.
- Helps in assessing the impact of therapeutic agents on Golgi function and structure.
- Based on the intensity of the black colour precipitate, it is indicative or suggestive of the functionality of the cells in terms of Golgi complexes.

General DO's –

- ❖ Precaution should be taken while using osmium tetroxide in the laboratory as it is corrosive and highly toxic and can cause burning to the skin, eyes, and respiratory tract. In severe cases, shortness of breath, pulmonary edema, and, even death can also occur.
- ❖ Essential care should be taken when using silver nitrate solution as it is very corrosive and can cause gastrointestinal problems if ingested.
- ❖ It is essentially required to wash the tissue materials with a large quantity of water when any metal-based fixative or formalin is used. It is a most important and essential step in all of the methods.
- ❖ Time duration must be maintained while the tissue sections are immersed in metal-based solutions.
- ❖ It is advisable to take such tissues whose cells are physiologically more active and are secretory in function such as testis, liver, pancreas, glandular tissue, etc.

General DON'Ts –

- ❖ Do not use sections with folds, tears, or improper thickness, as these can affect staining uniformity.
- ❖ Before mounting, the tissue sections should not be exposed to air to prevent moisture capturing that can deteriorate the desired result and may develop air bubbles.
- ❖ Do not section tissues beyond the size range mentioned for every method.

QUESTIONS FOR FURTHER-STUDIES

1. What do you mean by impregnation of tissue section while performing Golgi staining?
2. Why silvery metal-based solutions are used for the localization of Golgi complex?
3. What is the chemical basis of the reaction of metal-based solutions while identifying Golgi complex in the tissue?
4. Why thorough washing in distilled water is essential in every method of Golgi staining?
5. What is the role of osmic acid?
6. Is it possible to stain mitochondria and Golgi complex of a same tissue section? If so, mention the method?
7. Mention the cell or tissue in which Golgi body can be easily localized by cytochemical staining.

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Chapter 41: OIL RED O STAINING FOR DETECTION OF LIPIDS DURING *DROSOPHILA* OOGENESIS

Recommended level: PG, Research

INTRODUCTION

Drosophila melanogaster, commonly known as the fruit fly, is a widely used model organism for studying development and metabolism. It is sexually dimorphic and contains approximately 14,000 genes, a number comparable to that of humans. Importantly, more than 65% of human disease-associated genes have identifiable homologues in *Drosophila*. The organism is easy to maintain in the laboratory, can be cultured in bottles or vials at 25°C, has a short generation time of 10-14 days, and a lifespan of about 50-60 days, making it highly suitable for experimental studies.

Each female fly possesses a pair of ovaries, with each ovary consisting of an average of 15-20 ovarioles. Within each ovariole, egg chambers develop in a sequential and highly ordered manner (Figure 1). This developmental process, known as oogenesis, progresses through 14 distinct stages, beginning in the germarium, which contains germline stem cells (GSCs), and culminating in the formation of a fully grown oocyte (Hudson & Cooley, 2014). As oogenesis proceeds, cells undergo extensive differentiation and functional specialization. Developing egg chambers require a continuous supply of nutrients, both to support cell growth and to ensure adequate storage for the future embryo. Lipid droplets (LDs) play a central role in this process. LDs are cytoplasmic organelles surrounded by a phospholipid monolayer and contain neutral lipids such as triacylglycerols and sterol esters. During oogenesis, lipid droplets progressively accumulate within the nurse cells and are ultimately transferred to the mature oocyte. At stage 10B, when the oocyte occupies approximately half of the egg chamber, a rapid process known as nurse cell dumping occurs, during which cellular contents including lipid droplets, mRNAs, and organelles are transferred from the nurse cells into the oocyte.

Lipid droplets are metabolically active structures that serve as transient energy reserves. They are formed when neutral lipids synthesized within the endoplasmic reticulum accumulate between the membrane leaflets and subsequently bud off to form independent organelles (Murphy, 2001). Lipid biogenesis and lipid utilization occur simultaneously, allowing cells to dynamically regulate energy storage and consumption (Olzmann & Carvalho, 2019). Proper regulation of lipid metabolism is therefore essential for normal development. Impaired storage or mobilization of maternally supplied lipids leads to developmental arrest and embryonic lethality (Kühnlein, 2012). Beyond development, dysregulation of lipid metabolism is associated with metabolic disorders such as obesity, type II diabetes, non-alcoholic fatty liver disease, and metabolic syndrome (Hagberg et al., 2012; Samuel et al., 2010; Unger, 2002).

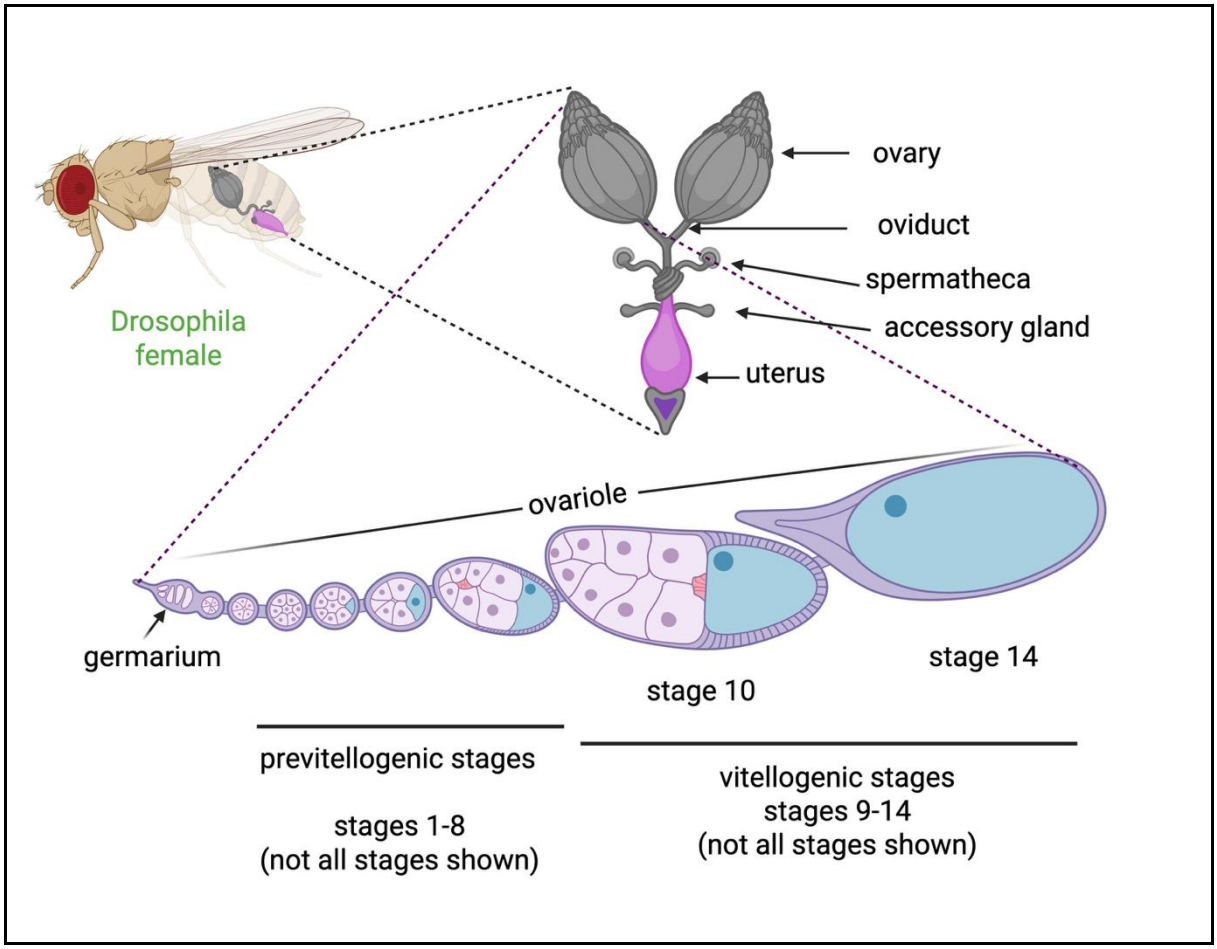

Figure 1. *Drosophila* female reproductive system highlighting nonvitellogenic and vitellogenic stages

Together, these features make *Drosophila* a powerful and versatile model for studying the cellular and developmental roles of lipid metabolism.

OBJECTIVE

To visualize lipid droplets in *Drosophila* ovaries using Oil Red O staining, enabling the study of lipid distribution during different stages of oogenesis.

Principle:

Oil Red O (1Z)-1-[[4-[(2,5-dimethylphenyl)diazinyl]-2,5 dimethylphenyl]-hydrazinylidene]-naphthalen-2-one) is a lysochrome dye used for staining neutral lipids, including triglycerides, cholesteryl esters, and lipoproteins (Figure 2). It exhibits a characteristic red coloration with a maximum absorbance at approximately 518 nm (Du et al., 2023). It (C.I. 26125) is a fat-soluble diazo dye ($C_{26}H_{24}N_4O$) whose structure contains diazo (R–N=N–R′) groups attached to aromatic rings, rendering the molecule non-ionizable and highly hydrophobic. These chemical properties facilitate its preferential solubility in neutral lipids (Mehlem et al., 2013).

Lysochrome dyes are insoluble in water but dissolve in organic solvents due to the absence of charged groups. When diluted in an aqueous buffer, the solubility of Oil Red O decreases, promoting its migration from the solvent phase into lipid-rich regions of tissue sections. The staining mechanism relies on the hydrophobic nature of the dye, resulting in selective association with lipid droplets rather than biological membranes (Prentø, 2001).

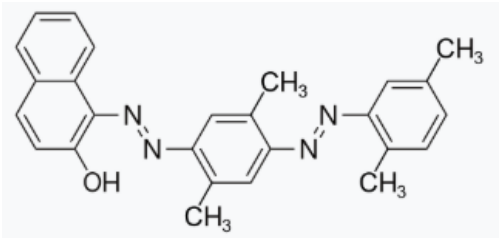

Figure 2. Chemical structure of Oil Red O

MATERIALS REQUIRED

Equipment

Stereozoom microscope, fine paint brush, blunt and fine-tipped pairs of forceps (preferably Dumont No.5, FST, Switzerland), fine dissection needles or pins, cavity slides, microscope slides and coverslips (Borosil, India), micropipettes - 2 µl, 20 µl, 200 µl, 1000 µl and corresponding micropipette tips, 1.5 ml microfuge tubes, transparent nail polish, Bright field microscope [optional: differential interference contrast microscope (DIC)]

Chemicals:

Anesthetizer- Diethyl ether

Dissecting media- 1X PBS or 1X Grace's medium (Invitrogen, USA)

1X Phosphate Buffer Saline (PBS pH 7.4)

Dissolve 8 gm of NaCl, 2 gm of KCl, 1.44 gm of Na_2 HPO_4 , 2.4 gm of KH_2 PO_4 sequentially in 800 ml of distilled water. After all the components are completely dissolved, adjust the pH to 7.4. Then, make up the final volume to 1000 ml by adding required amount of distilled water. Sterilize the prepared solution by autoclaving, and store it at room temperature or 4°C for future use.

10% BSA- To coat the micropipette tip

Fixative- 4% Paraformaldehyde in 1xPBS pH-7.4

Add 4 gm PFA to 80 ml 1X PBS. Heat to 60ºC and allow it to dissolve overnight. . Allow to cool and adjust pH to 7.0 with NaOH. Finally make up the volume to 100 ml with 1X PBS pH 7.4. Can be used upto 30 days if stored at 4ºC.

0.1% PBTx- 0.1% Triton-X 100 in 1X PBS

Lipid stain Oil Red O

Oil Red O Stock: Sigma (Cat# O-0625), FW 408.5. Weigh 1 mg Oil Red O and dissolve in 1 ml of isopropanol. Stir overnight, filter (0.2 µ) and store in fridge for up to one year.

Mounting media- 80% Glycerol

Mix 8 ml glycerol in 2 ml 1X PBS

PROCEDURE

Dissection:

1. Fly culture should be maintained at 25°C and yeast should be added to the fly vial two days before dissection. Transfer the flies to another fresh vial containing yeast 1 day before dissection.
2. Anaesthetise ~10 flies using diethyl ether.
3. Add a few drops of 1X PBS to the cavity slide and place a single female inside the cavity.
4. Use blunt forceps to hold the fly near the thorax and using the fine forceps, pull the last segment of the abdomen to expose the internal organs. Using the forceps separate the ovaries and remove the carcass.
5. Gently comb through the ovary to tease the ovarioles apart using dissection pins/needles. Care should be taken to ensure that the ovarioles are still connected at the posterior end to the common oviduct.
6. Repeat the above steps for each ovary. Once the required number of females are dissected, the ovaries should be transferred to a 1.5 ml microfuge tube.
7. Cut a 200 µl micropipette tip to increase the width of the tip. Aspirate the cut tips in 10% BSA to coat internal walls of the tip with BSA. This helps avoid the ovaries sticking to the inner tip surface.
8. Transfer the tissue present in the glass cavity slide to a fresh 1.5 ml tube using this tip.
9. Remove excess 1X PBS and add 500 µl of 4% PFA and keep it for 20 min at room temperature on shaker to fix the tissue.
10. Remove fixative and wash the tissue in 500 µl of 0.1% PBTx for 5 min. Repeat this step two times.
11. Prepare working solution (1 ml) of Oil Red O stain by adding 600 µl of 1 mg/ml Oil Red O stock solution to 400 µl of 0.1% PBTx.
12. Incubate the ovaries in 1 ml of the freshly made Oil Red O solution from step 11 for 30 min.
13. Remove stain solution and wash the tissue in 500 µl of 0.1% PBTx for 5 min. Repeat this step two times.
14. Add 50 µl of 80% glycerol using a cut 200 µl tip and mount the tissue on a glass slide for easy visualisation of the ovaries

Mounting

1. Clean the slides, coverslip and needles using 70% ethanol.
2. Use the Stereozoom microscope for further steps.
3. Using a cut 200 µl tip, transfer the ovaries in 80% glycerol on the slide and remove any debris/unwanted fibres from the slide.
4. Adjust the contrast of the light to better visualise the different stages of the ovariole and separate the ovarioles from each other.
5. It is important to remove the muscle fibres surrounding the ovarioles.

6. Once all the ovarioles are separate, gently place the coverslip over the specimen. Ensure that air bubbles are not trapped between the coverslip and slide.
7. Image this slide under the Brightfield/Differential Interference Contrast (DIC) microscope.
8. Observe under different magnifications available. Use 10X objective to observe the entire ovariole. Use 63X Oil objective to observe developing eggs under high magnification.

OBSERVATION

Oil Red O staining of *Drosophila* ovaries allows clear visualization of lipid droplets at different stages of oogenesis. When clusters of ovarioles are observed under the microscope, a gradual increase in red staining intensity is seen as egg chambers mature (Figure 3A–D). This increase in staining reflects the progressive accumulation of lipid droplets during oocyte development.

In the germarium, where germline stem cells and early cysts are present, no detectable Oil Red O staining was observed (Figure 3B). Similarly, early pre-vitellogenic egg chambers (stages 1–7) show little to no staining, indicating that significant lipid storage has not yet begun (Figure 3C). From stage 8 onwards, which marks the onset of vitellogenesis, faint Oil Red O staining becomes visible (Figure 3A and F). This suggests that lipid droplets begin to accumulate as the egg chamber prepares for rapid growth and yolk deposition.

As oogenesis progresses, lipid accumulation increases markedly in both the nurse cells and the developing oocyte. During stage 10B, intense Oil Red O staining is observed in the nurse cells as well as in the anterior region of the oocyte (Figure 3E). This stage corresponds to the onset of nurse cell dumping, a rapid process during which cytoplasmic contents, including lipid droplets, are transferred from the nurse cells into the oocyte.

In later stages (stages 11–13), strong Oil Red O staining is predominantly observed in the oocyte (Figure 3D and F). This indicates successful transfer and storage of lipids within the mature oocyte. The most mature egg chambers show the highest staining intensity, reflecting maximal lipid accumulation required to support early embryonic development after fertilization.

Overall, these observations demonstrate that lipid droplets accumulate in a stage-specific manner during *Drosophila* oogenesis. Oil Red O staining therefore provides a simple and effective method for visualizing lipid dynamics and understanding the relationship between oocyte development and energy storage.

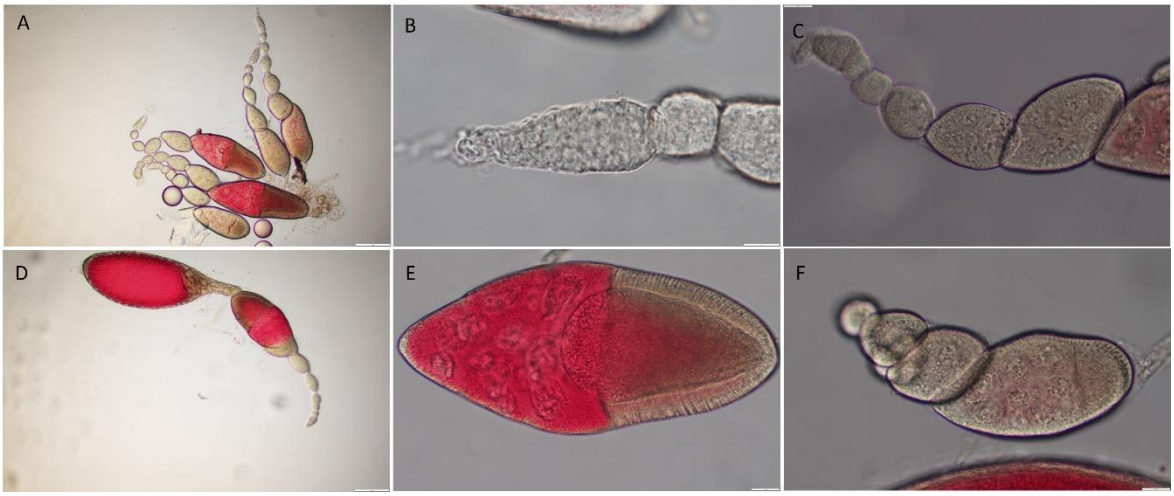

Figure 3. Oil red O staining in the ovary. A. Partial ovary showing different stages of oogenesis stained using Oil red. B. Germarium does not show any detectable Oil Red O staining. C. Pre vitellogenic stages (stage 1-stage 8) do not show any Oil Red O staining. D. A single ovariole with germarium at right bottom corner and Stage 13 egg chamber left up corner shows Oil Red O staining in the developing oocyte. Stage 10A egg chamber also shows Oil Red O staining in the oocyte as well as nurse cells. E. Stage 10 B egg chamber shows intense Oil Red O accumulation in nurse cells and anterior part of the developing oocyte. F. Stage 8 egg chamber shows faint Oil red O staining indicating the beginning of LD accumulation

Conclusions

1. Lipid droplets are important energy storage organelles required for normal oocyte development and early embryogenesis in Drosophila.
2. Lipid accumulation during oogenesis is stage-specific: In early egg chambers lipids were not detected using Oil Red O staining. (However, lipid droplets have been observed using alternate stains such as BODIPY). Mature egg chambers contain high levels of lipids.
3. Oil Red O selectively stains neutral lipids, allowing lipid droplets to be visualized as red-stained structures under a bright-field microscope.
4. Lipid droplet accumulation begins at the vitellogenic stages (from stage 8 onwards) and increases progressively as oogenesis proceeds.
5. During nurse cell dumping, lipids are transferred from nurse cells into the developing oocyte, leading to strong staining in later stages.
6. The strongest Oil Red O staining is observed in mature oocytes, indicating maximal lipid storage to support embryonic development.
7. Oil Red O staining is a simple, reliable, and effective method for studying lipid distribution during development and is well suited for undergraduate laboratory experiments.

QUESTIONS FOR FURTHER STUDIES

1. What are lipid droplets, and what is their primary function in the cell?
2. Why is *Drosophila melanogaster* considered a useful model organism for studying development and metabolism?
3. What is Oil Red O, and which class of biomolecules does it specifically stain?
4. Why are lipid droplets difficult to visualize in unstained tissues?

5. How many stages are there in *Drosophila* oogenesis, and where does the process begin?
6. At which stage of oogenesis does vitellogenesis begin, and what major changes occur during this stage?
7. Why do early egg chambers (stages 1–7) show little or no Oil Red O staining?
8. Describe the pattern of Oil Red O staining observed as egg chambers mature.
9. What is nurse cell dumping, and at which stage does it occur?
10. How does nurse cell dumping contribute to lipid accumulation in the oocyte?
11. What differences in Oil Red O staining would you expect between the germarium and a stage 13 egg chamber?
12. If strong Oil Red O staining is observed in nurse cells but weaker staining in the oocyte, which stage of oogenesis is most likely being observed? Explain your answer.
13. How does lipid storage in the oocyte support early embryonic development?
14. What developmental defects might arise if lipid accumulation during oogenesis is impaired?
15. Why is Oil Red O considered as a selective stain for neutral lipids rather than membrane lipids?
16. Why is proper fixation important for accurate Oil Red O staining?
17. How would poor tissue handling during dissection affect staining and interpretation of results?
18. Why is it important to separate individual ovarioles during mounting before imaging?
19. Why are bright-field or differential interference contrast (DIC) microscopes suitable for observing Oil Red O-stained ovaries?
20. What are the advantages of using Oil Red O staining in an undergraduate teaching laboratory?
21. What are the limitations of Oil Red O staining for studying lipid droplets?
22. Why is studying lipid metabolism in *Drosophila* relevant to understanding human metabolic diseases?

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Chapter 42: FLUORESCENCE ACTIVATED CELL SORTING: A BASIC IDEA OF GRADING, SORTING OF CELLS AND DATA ANALYSIS

Recommended Level: PG, Research

INTRODUCTION

A flow cytometer equipped with a sorting mechanism, known as Fluorescence Activated Cell Sorter (FACS) is an extremely powerful technology that allows the individual measurement of physical and chemical characteristics of particles as they pass one by one through a light source. The cells can be sorted in a viable and sterile condition so that they can be used further for tissue culture experiments. Sorted cells with uniform characteristics have been used in many critical experiments in immunology, cell biology and molecular biology.

FACS of live cells is capable of segregating the population of cells into subpopulations based on various parameters such as size, granularity, DNA/RNA composition, protein/enzyme levels and surface markers, i.e., fluorescent labeling. Fluorochromes are attached to the surface molecules or intracellular macromolecules of cells under investigation either directly or via specific monoclonal antibodies. Signals are generated when the laser beam hits each cell. These signals are captured and amplified by photomultiplier tubes (PMT) assigned for each parameter (Figure 1) (Macey 2007)**.** The signals are computed and analyzed by the processing unit and displayed on the monitor. Besides fluorochrome signals, forward scatter and side scatter of the beam measure the cell size and granularity, respectively. Fluorochromes can be attached to the monoclonal antibodies (MAbs) which are directed to different cell surface markers or intracellular macromolecules. Thus, cell size and granularity can be analyzed simultaneously. Sorting involves a complex mechanism in the flow cytometer. To sort particles, the cells are ejected into air in a stream of sheath fluid. Any fluid stream ejected into air will break up into droplet due to vibration which is produced by a transducer (a piezo-electric crystal acoustically coupled to the nozzle). A 15 kHz vibrating transducer is implemented to break the flow of cells into droplets (Break-off-point) (Figure 1). The flow rate is modified to ensure that each droplet contains only one cell. Subsequently, the droplets containing cells are electrically charged based on the fluorochrome using a charging collar. Typically, flow cytometers employ a methodology based on the electrostatic deflection of charged droplets. Additionally, the apparatus is equipped with charged deflection plates to divert cells with an opposing charge into collection containers. Subsets of cells within a specific cell group can be measured utilizing the gating feature. For example, if we want to separate the CD4 positive helper T cells labelled with FITC from the negatively charged CD8 positive cytotoxic T cells labelled with Phycoerythrin from human Peripheral Blood Mononuclear Cells, the positively charged CD4 cells get deflected towards the negatively charged deflecting plate and get collected in the receptor tube A. This is in contrast to the negatively charged CD8 positive cells which get deflected towards the positively charged deflecting plate and get collected in the receptacle B. Finally, the uncharged cells (B cells, NK cells and monocytes) and empty fluid droplets get collected in the waste receptacle C placed in the middle (Gangal 2010).

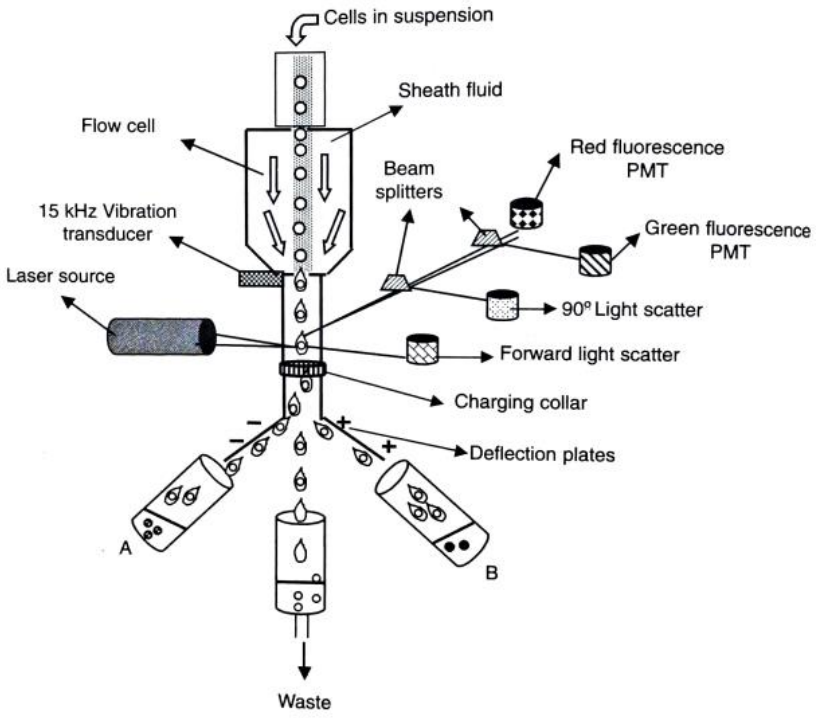

Figure 1. A diagram of generalized cell sorter

(Figure from Gangal S (2010) Principle and practice of animal tissue culture, University Press II edn, Chennai, pp.154-161)

Principle: Flow cytometry is a technique comprise of three major systems, Fluidics, optics and electronics. The cells are transported in stream (fluidics system), illuminated by laser and directed to detectors by filters (optics system) followed by conversion of light signal to electronic signal which can be processed by computer. To sort particles or cells, the flow cytometer first needs to identify the cells of interest, then separate out the individual cells (Macey 2007). The fundamental principle of flow cytometry is the separation of cells stained with fluorophore-conjugated antibodies from one another. Their separation depends upon which fluorophore they have been stained with. For example, a cell expressing one cell marker can be detected using an FITC-conjugated antibody that recognizes the marker, while another cell type expressing a different marker could be detected using a PE-conjugated antibody specific to that marker. The process of cell sorting enables the physical separation of a particular cell or particle from a diverse population. For the purpose of sorting particles through this technique, cells are propelled into the air within a sheath fluid stream. Subsequently, the cells are hydrodynamically focused to sequentially pass through a light source, usually emanating from lasers. Consequently, scattered light and fluorescence signals are produced, identified, and quantified. Following this step, cells are extracted under vacuum conditions and directed towards a waste container.

OBJECTIVES

To differentiate the population of lymphocytes in a given sample of whole blood. After performing this exercise, the students will be able to

- Analyze SSC Vs FSC dot plot
- Distinguish different populations of leucocytes.
- Analyze the distribution of CD3+ cells (T lymphocytes) and CD19+ cells (B lymphocytes by gating.

MATERIALS REQUIRED

RBC Lysing Solution (10X concentration):

NH_4Cl	8.02 g
$NaHCO_3$	0.84 g
EDTA (disodium)	0.37 g

(make volume up to 100 ml with RO water. Store at 4°C for six months [Brown et al, 2016])

Flow Cytometry Staining Buffer

Add 10 g of bovine serum albumin to 700 ml of amphibian phosphate-buffered saline (APBS). Stir well until dissolved (5).

1XPBS

70% ethanol

Fluorophore-labeled primary antibodies (*Thermo Fisher, BD Biosciences, Abcam)*

- FITC (Fluorescein isothiocyanate) or PE (Phycoerythrin) conjugated anti-CD19 antibody,
- APC (Allophycocyanin) or PerCP (Peridinin-Chlorophyll Protein) conjugated anti-CD3 antibody

12 x 75 mm round-bottom test tubes
50 ml conical tubes
Surgical gloves
Dispensing bottles
Centrifuge tubes
Micropipettes (10 µl-100 µl)
Micropipette tips

PROCEDURE

T and B lymphocytes are differentiated from whole blood using distinct cell surface markers. Specific antibodies conjugated to fluorescent molecules (fluorophores) are used to target these markers. Common surface markers of T cells include CD3, CD4, and CD8 while for B cells it is CD19. Lysed blood is incubated with these fluorescently labeled antibodies which bind to their specific markers on the cell surface. The stained cell suspension is injected into a flow cytometer which by using lasers to excite the fluorophores, causing them to emit light at specific wavelengths. Based on the light intensity (fluorescence) and scatter properties, the FACS machine can differentiate T and B cells. The software allows visualization of the data as a scatter plot, where different cell populations are identified based on their unique fluorescence and scatter profiles.

A stepwise list of actions and their timeline that one needs to follow

I. Sample preparation is a prerequisite for flow cytometry. Cells or particles must be in a monodispersed suspension. e.g., blood cells:

- Add 10 ml of 1X RBC Lysis Buffer per 1 ml of blood (previously collected in EDTA /heparin coated tube).
- Incubate for 10-15 min at room temperature.
- Observe turbidity to evaluate red blood cell lysis. Once the sample becomes clear, lysis is complete.
- Centrifuge at 500 x *g* for 5 min at room temperature and decant supernatant.

- Resuspend the pellet in the appropriate volume of Flow Cytometry Staining Buffer or ready to use buffer*.
- Perform a cell count and proceed with cell staining with fluorochromes conjugated antibodies.

II. **Fluorochrome Selection:** Choose fluorochromes that have minimal spectral overlap for clear signal detection.

B cells: FITC (Fluorescein isothiocyanate) or PE (Phycoerythrin) conjugated anti-CD19 antibody

T cells: APC (Allophycocyanin) or PerCP (Peridinin-Chlorophyll Protein) conjugated anti-CD3 antibody

III. **Staining and Washing:** Incubate the lysed whole blood sample with the chosen fluorochrome-conjugated antibodies for 15-30 min at room temperature **(as per manufacturer's protocol)** in the dark. Wash the cells afterwards to remove unbound antibodies as per manufacturer's protocol.

IV. **Flow cytometry Run:** Analyze samples on a flow cytometer equipped with appropriate filters, corresponding to the selected fluorophore labels. A minimum of 10^7–10^5 cell events must be acquired to find an event that occurs at a frequency of 0.1% in the cell population. The acquisition rate should be set to slowly collect events (e.g., 200–500 µl/min) (6).

Dos and don'ts

- 1X RBC lysis buffer should be used at room temperature.
- Do not incubate sample with RBC lysis buffer for more than 15 min.
- Prior to use, quick spin the antibody vial to recover the maximum volume.
- For optimal performance of fluorochrome conjugated antibodies, store vials at temperature 2–8°C in the dark. Do not freeze it.
- Always *follow manufacturer's instructions when working with antibody as well as flow cytometry staining buffer.

OBSERVATIONS

1. Observe SSC Vs FSC dot plot and differentiate all populations of leucocytes.
2. Analyzes the distribution of CD3+ cells (T lymphocytes) and CD19+ cells (B lymphocytes by gating.
3. Record the number of events of each cell population.

Data analysis

During FACS acquisition, the flow cytometer excites the fluorochromes on the antibodies. B cells will show fluorescence in the channel detecting the fluorochrome used for the anti-CD19 antibody (e.g., FITC or PE). T cells will show fluorescence in the channel detecting the fluorochrome used for the anti-CD3 antibody (e.g., APC or PerCP). Data analysis software allows creating gates based on the fluorescence intensity. Cells positive for CD19 will be identified as B cells, while those positive for CD3 will be identified as T cells.

Interpretation of Results

Each dot plot corresponds to a cell or particle, with data positioning along the scales reflecting the strength of the signal. Along the X-axis is the FSC (Forward SCatter) parameter. This parameter is a measurement of the amount of the laser beam that passes around the cell and thus gives a relative size

for the cell. Smaller value of FSC depicts smaller size of cell e.g. lymphocytes. Along the Y-axis is the SSC (Side SCatter) parameter, is a measurement of the amount of the laser beam that bounces off of particulates inside of the cell and gives the relative granularity of the cell. Higher the value of SSC shows highly granular cells e.g. granulocytes. The FSC vs SSC plot allow for differentiation of cell types in a heterogeneous cell population. Major leucocyte subpopulations can be differentiated using FSC and SSC (Fig 2)

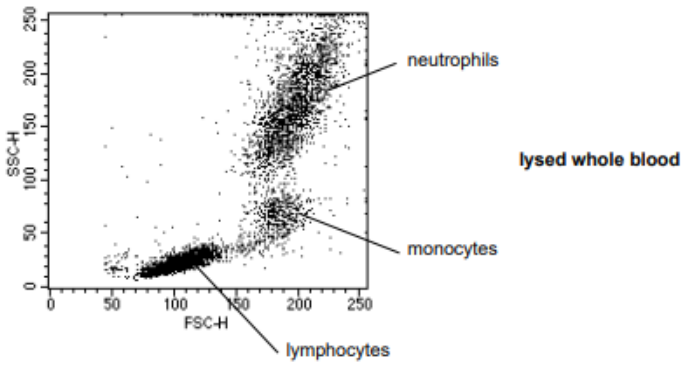

Figure 2. FSC vs SSC plot of leucocytes population

Ref figure: Introduction to Flow Cytometry: A Learning Guide, BD Biosciences, USA, pp. 1-58 2002.

Gating means the capacity to separate individual populations of interest within a diverse sample and facilitates the limitation of analysis to a specific subset of cells e.g. a gate can be set on the FSC vs SSC plot to allow analysis only of lymphocytes (Fig 3).

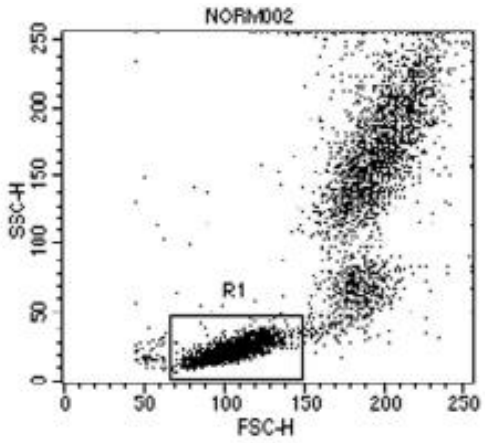

Figure 3. Dot plot with a gate encompassing the lymphocyte population

Ref figure: Introduction to Flow Cytometry: A Learning Guide, BD Biosciences, USA, pp. 1-58 2002.

QUESTIONS FOR FURTHER STUDIES

1. What properties of a cell or particle can be measured by a flow cytometer?
   Relative size, relative granularity or internal complexity, and relative fluorescence intensity
2. What light source is used in most flow cytometers?
   Laser
3. What are the three main systems in a flow cytometer?

The fluidics, the optics, and the electronics

4. What type of biological sample is best suited for flow cytometric analysis?
   A single cell suspension
5. What is the purpose of the fluidics system in a flow cytometer?
   To position the sample in the center of the laser beam
6. How many cells or particles should pass through the laser beam at a given time?
   One
7. What is Forward-scattered light (FSC)?
   Light scattered in the same direction as the laser beam is called FSC.
8. What is Side-scattered light (SSC)?
   Light scatter collected at 90 degrees to the laser beam is called as (SSC).
9. Name two fluorescent dyes commonly used in flow cytometry.
   FITC (Fluorescein isothiocyanate) and PE (Phycoerythrin)
10. What does the horizontal axis in a histogram represent?
    The parameter's signal value in channel numbers

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Chapter 43: CELL CYCLE ANALYSIS IN MAMMALIAN CELLS

Recommended Level: PG/Research

OBJECTIVE

To assess the duration of different phases of cell cycle

INTRODUCTION

Cell cycle analysis can be performed by measuring the DNA content of cells using flow cytometry. This technique involves staining the DNA of mammalian cells with various DNA-binding dyes. These dyes are stoichiometric, meaning they bind proportionally to the DNA present within each cell. Cells in the S phase, which are synthesizing DNA, will contain more DNA than cells in the G1 phase. Consequently, S phase cells will absorb more dye and exhibit higher fluorescence intensity until they have doubled their DNA content. Cells in the G2 phase will display approximately twice the fluorescence intensity of those in the G1 phase, reflecting their doubled DNA content. DNA-binding dyes such as propidium Iodide (PI), 7-aminoactinomycin-D (7-AAD), Hoechst 33342, Hoechst 33258, Hoechst S769121, and 4',6-diamidino-2-phenylindole (DAPI) intercalate into the major groove of double-stranded DNA, producing a highly fluorescent signal when excited at 488 nm with a broad emission centered around 600 nm. Since PI can also bind to double-stranded RNA, it is necessary to treat the cells with RNase for optimal DNA resolution. The excitation of PI at 488 nm facilitates its use on benchtop cytometers. Typically, cells are fixed or permeabilized to allow the entry of these dyes, as living cells actively pump them out.

MATERIALS REQUIRED

70% Ethanol
Propidium Iodide (stock solution 50 µg/ml)
Ribonuclease I (stock 100 µg/ml)

PROCEDURE

b. Harvest the cells in the appropriate manner and wash in PBS.

c. Fix in cold 70% ethanol. Add drop wise to the pellet while vortexing. This should ensure fixation of all cells and minimize clumping.

d. Fix for 30 min at 4°C.

e. Wash 2 times with 1X PBS. Spin at 850 g in a centrifuge and be careful to avoid cell loss when discarding the supernatant especially after spinning out of ethanol.

f. Treat the cells with ribonuclease. Add 50 µl of a 100 µg/ml stock of RNase and incubate for 30 min at 37°C. This will ensure only DNA, not RNA, is stained. Wash the cells once with PBS.

g. Add 200 µl PI (from 50 µg/ml stock solution), incubate at room temperature for 30 min.

Data Acquisition and Analysis Using a Beckman Flow Cytometer

1. Measure the forward scatter (FS) and side scatter (SS) to identify single cells.
2. Pulse processing is used to exclude cell doublets from the analysis. This can be achieved either by using pulse area vs. pulse width or pulse area vs. pulse height depending on the type of cytometer.

3. PI has a maximum emission of 605 nm so can be measured with a suitable bandpass filter.
4. While running the cytometer, the following plots should be displayed:
   Forward and side scatter to identify the cells
   Pulse shape analysis to identify clumps and doublets (this can be pulse area vs. pulse width or pulse area vs. pulse height depending on cytometer)
   Forward scatter vs. PI signal; PI histogram.

OBSERVATION

The figure below illustrates how flow cytometry can be used o study the distribution of cells across different stages of cell cycle. By measuring DNA content, the data reveals distinct peaks representing cells in G1, S and G2/M phases. This helps us understand how treatments or conditions affect cell division/growth.

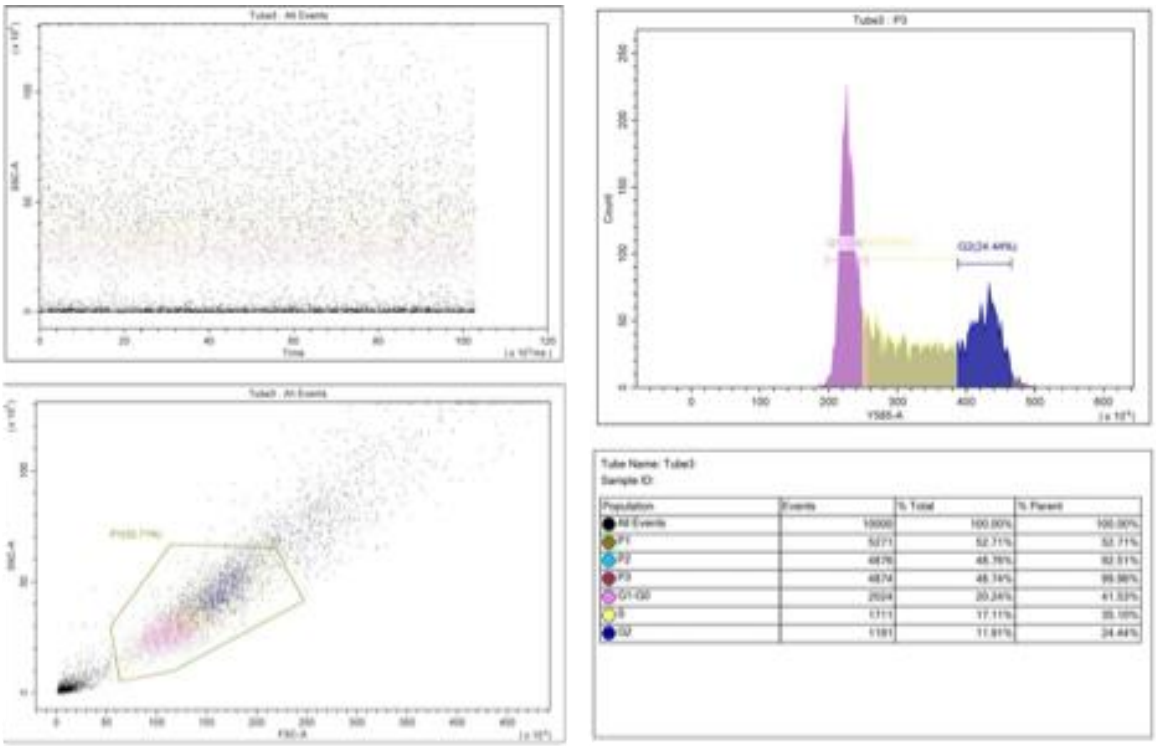

Figure1. Gating cell population- A. First gate on the single cell population using side scatter vs forward scatter area. Then apply this gate to the scatter plot and gate out obvious debris. Black : debris (gate out)
B. Demarcating the stages of cell division- stages are demarcating using count PI (emission plot). Three peaks are obtained. The right taper of the 1st peak signifies the Gaussian area to be G1-G0. The immediate second region signifies S phase, and the third peak signifies G2 phase. This is a senescence study where the cells were given cues that would lead them to senescence hence most of the cells are in the G1-G0 phase.

QUESTIONS FOR FURTHER STUDIES

1. How can synchronization methods be optimized to accurately assess different phases of cell cycle?
2. What controls are essential to ensure reliability and reproducibility in cell cycle analysis using flow cytometry?

3. How do DNA content and cell size parameters together help distinguish between G0/G1, S and G2/M phases?

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Chapter 44: ANALYSIS OF CELL KINETICS BY DIFFERENTIAL STAINING TECHNIQUE

Recommended level: UG, PG, Research

INTRODUCTION

Cellular division is a crucial process in cellular growth, involving DNA replication in the S phase and mitosis, which is directly linked to cellular growth. Research teams often analyze cell cycle progression through different phases, using various approaches to characterize this process. This chapter is focused exclusively on the basic techniques of cell cycle analysis in asynchronous cell populations. Here, the analysis of cell cycle kinetics can be studied by two simple methods. They are mitotic index studies and differential staining methods.

Mitotic index is a classical method for measuring mitotic activity, achieved through manual counting of mitotic cells among a given cell population of interest. It is calculated by dividing the total cells undergoing mitosis by the total cells visible. An elevated MI indicates more cells are dividing. It is simply a measurement to determine the percentage of cells undergoing mitosis. With the advent of 5-bromo-2-deoxyuridine (BrdU) labeling and the fluorescence plus Giemsa staining technique (Perry and Wolff 1974), the lymphocyte and other cell culture systems have become more suitable for radiation and chemical dose-response and dose-modification studies (Bianchi et al 1982). This technique facilitates the scoring of first, second and subsequent cycle metaphases based on their differential staining patterns and thus made possible the scoring of radiation and chemical mutagen-induced cell cycle delay in terms of reduction in the frequency of second and subsequent division metaphases following treatment (Lloyd et al 1977).

OBJECTIVE

To assess the pattern of cell cycle kinetics in cultured cells.

MATERIALS REQUIRED

Bromodeoxyuridine 100 μg/ml in water, Hoechst 33258 50 μg/ml,
2X Saline sodium citrate (SSC) – 0.3 M NaCl + 0.03 M Sodium citrate

Giemsa stain

Stock solution -	Giemsa Powder	380 mg
	Methanol	25 ml
	Glycerol	25 ml

Leave overnight at 37ºC. Filter the stain and store.

Working solution -	Stock solution	2.5 ml
	Methanol	1.5 ml
	Giemsa water	50.0 ml
Giemsa water -	0.2 M Na_2HPO_4	80 ml
	Distilled water	800 ml

Adjust pH to 6.8 with 0.1 M Citric acid (roughly 24 ml) and then make up the volume to l000 ml

PROCEDURE

Setting the culture

1. About 5 ml of blood is collected in a sterile, heparinised syringe in suitably clean environment. Blood is kept in refrigerator until used.

2. Arrange TC medium, FCS, PHA, L-glutamine, pipettes, beakers and flasks etc. on the alcohol-swabbed work bench before setting up the culture.

3. Prepare the TC media by adding antibiotics and L-glutamine and 10% fetal bovine serum (10 ml serum to 100 ml medium - serum can be added separately to the culture). Since commercial media have phenol red as indicator, colour of the medium indicates its pH which must be 7.2 (light pink to orangish). If the pH is alkaline (indigo), use CO_2 or a few drops of 1N HCl to bring it to the proper range.

4. Prepare a working solution of PHA (5 ml in sterile distilled water). This can be stored under sterile conditions for 1-2 months at 4°C.

5. Before using pipettes, container, culture vial, etc., flame them gently but use only after cooling.

6. For each culture, add the following in the order given:

TC medium	5 ml
Fetal bovine serum	1 ml (if not already added)
Blood	0.3 ml
PHA	0.1 ml

h. In order to buffer the pH, blow CO_2 from a CO_2 cylinder or bubble exhaled air orally through a cotton-plugged-pipette.

While setting the culture, one may consider one vial as untreated control and another vial as treated (with either radiation or with chemicals for 2 h or more as per requirement). This will allow comparing the cell cycle kinetics in two conditions.

8. Add 5 μg/ml BrdU to the culture and keep in an incubator at 37°C for 48 to 72 h.

9. Culture must be inspected every morning and evening for change in pH and infection and shaken to break the clumps of RBCs.

10. About 2-3 h prior to harvesting the culture for chromosome preparation, colcemid (working conc. 0.02 μg/ml) is added.

Chromosome Preparation

1. Transfer the culture to a centrifuge tube and centrifuge at 1000 to 1200 rpm for 5 min.

2. Decant the supernatant and make a fresh suspension of cells in prewarmed 0.56% KCl (hypotonic). Initially add a small volume and agitate the sediment. Once the cells come in suspension, make up the volume to 8-10 ml. Keep in incubator (37°C) for 18-20 min.

3. Immediately before centrifugation, add 3-4 drops of fixative to the tube and mix. Spin (1000 - 1200 rpm) the tubes and decant the hypotonic completely. Add the fixative drop-by-drop to fix the cells and to keep them in suspension. Make up the volume to ~8-10 ml. Keep for 15 min.

4. Re-centrifuge (1000-1200 rpm) for 5 min, decant the supernatant and resuspend the pellet in fresh fixative (~ 8-10 ml). Keep for 10 min.

5. Re-centrifuge (as in #4) and discard the supernatant. Add only about 0.2-0.4 ml of fixative. Resuspend the cells well by gentle agitation.

6. Take out a slide from 70% alcohol and wipe it with a clean piece of cloth. Add 2-3 drops of the cell suspension on the slide and either blow it dry (air-drying) or expose to a flame for instant drying (flame drying).

Differential staining

11. Stain the prepared slides with Hoechst 33258 (50 µg/ml) for 15 min in dark.

12. Rinse with distilled water, mount with 2XSSC and expose to bright sunlight for 30 to 60 min (standardization is required because sunlight intensity and day temperature varies in different months).

13. Rise the slides in water and stain with 5% Giemsa stain for 10 min (rinsing the slides through ascending ethanol grade prior to staining makes the slide more clean and thus gives better staining with very little background).

14. Dry the slides, mount with DPX and observe under the microscope.

OBSERVATIONS

Study the slides with 40x or 100x objective lens of the microscope and score the number of metaphases in 1st, 2nd and 3rd cycles based on their staining pattern (Fig. 1). For scoring cell cycle kinetics from peripheral blood lymphocytes, metaphases will be categorized in different division cycles based on their differential staining pattern. At least 200 to 300 metaphases should be scored from 2 to 3 slides for each sample. The cell cycle data can be presented as Average Generation Time which is the ratio of BrdU duration (h) and Replicative Index (RI), where RI= (1xM1 + 2xM2 +3xM3) / number of cells.

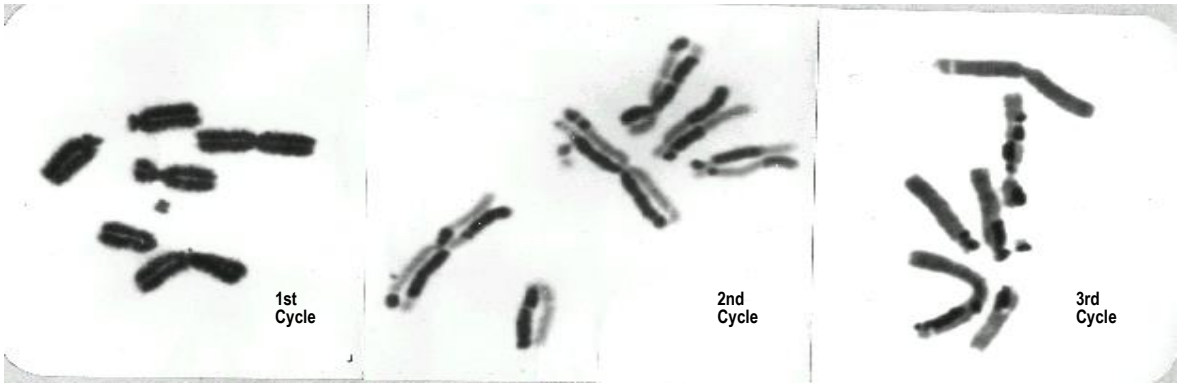

Figure 1. Three metaphase plates from peripheral blood lymphocytes of barking deer (*Muntiacus muntjac*). 1st cycle metaphase shows both the chromatids of a chromosome are dark stained. 2nd cycle metaphase shows one chromatid is lighter stained and the other chromatid is dark stained. Patches of dark and light-stained exchanges are visible in two chromatids of a chromosome which are known as Sister chromatid exchanges. 3rd cycle metaphases show mostly light stained chromatids.

For scoring cell cycle kinetics from peripheral blood lymphocytes, metaphases will be categorized in different division cycles based on their differential staining pattern. At least 200 to 300 metaphases should be scored from 2 to 3 slides for each sample. The cell cycle data can be presented as Average

Generation Time which is the ratio of BrdU duration (h) and Replicative Index (RI), where RI= (1xM1 + 2xM2 +3xM3) / number of cells.
The data can be tabulated in the following manner:

Sample	Scoring metaphase number				Mitotic index (%)	Average Generation Time (h)
	M1	M2	M3	Total		
Untreated	94	150	24	268	36	41.4
Treated	134	106	12	252	53	47.4

From the table, the data indicate a higher frequency of Mitotic index and Average generation time in the treated samples compared to the untreated control. Therefore, the treatment with the chemical/radiation induced a significant delay in cell kinetics.

QUESTIONS FOR FURTHER STUDIES

1. What is the mechanism of this differential staining?
2. How exactly the first, second or third cycle metaphases could be identified?
3. Compare the methods available for cell cycle analysis. Can you design experiments utilizing the various available methods for specific needs?

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Chapter 45: ISOLATION OF DNA FROM MOUSE/RAT LIVER CELLS

Recommended level: UG and PG

INTRODUCTION

This method is designed for a class-room experiment for teaching purposes only. Here the tissue is grinded in water or saline to get a cell suspension. The cells are then lysed by SDS and therefore after addition of SDS the solution becomes viscous. The DNA can be precipitated by adding chilled ethanol. The DNA prepared in this way is crude. It will still contain some proteins and RNA that can be removed by further treating with RNase (to remove RNA), and Protease (to remove the residual proteins) followed by phenol extraction and ethanol precipitation. The DNA obtained by this method can be quantified either by spectrophotometric or colorimetric measurements. It can be used for X-ray diffraction, restriction endonuclease digestion, transformation, gene-cloning, and other genetic-engineering techniques.

The EDTA in homogenization buffer inhibits DNases that are activated after lysosomal breakage. NaCl helps in deproteinization during extraction steps, and is essential while precipitating DNA by ethanol. The anionic detergent, SDS, solublizes the membranes, and denatures the proteins, thus helps in the release and deproteinization of DNA. The phenol, an aromatic alcohol, selectively solublizes the lipids, proteins and other cellular components while leaving the nucleic acids in aqueous phase. The interphase constitutes mainly the denatured proteins and polysaccharides. The other organic solvents like phenol or phenol: chloroform: isoamyl alcohol (PCI) mixture works better than chloroform: isoamyl alcohol (C:I) mixture. But phenol being highly corrosive requires great precaution and care in handling; consequently, PCI is avoided for general class-room at school level experiments.

OBJECTIVE

To isolate genomic DNA from liver cells of a mouse or rat.

MATERIALS REQUIRED

Mouse or rat (instead the chicken-liver can also be collected in ice-box from slaughterhouse; Goat-liver has lot of fats, hence creates some problems during DNA isolation), water-bath, ice-box, beakers, measuring cylinders, pipettes, conical flasks, glass-rod, blotting paper, cheese-cloth, balance, centrifuge, centrifuge tubes (should be made-up of polypropylene), homogenizer (motor-driven homogenizer is preferred, otherwise ordinary mortar and pestle would also be all right).

Normal saline- 0.9% NaCl in distilled water.

SDS (10%) solution- Weigh out 5 g sodium dodecyl sulfate (SDS) and dissolve in 40 ml distilled H_20. The solution may be warmed a little for proper dissolution. Make-up the volume to 50 ml with distilled water, and store at room-temp. SDS is a detergent, hence avoid excessive shaking or mixing of SDS or SDS-containing solutions, otherwise it will cause frothing of the solution.

NaCl (5 M) solution- Weigh out 29.22 g NaCl and dissolve in 80 ml distilled water. Make-up the volume to 100 ml, and store at room-temp.

Chloroform:Isoamyl alcohol (CI) mixture (24:1) – For 100 ml , mix 96 ml of chloroform with 4 ml of isoamyl alcohol. Store at room-temperature in a tight-capped brown bottle

Absolute ethanol-The ethanol should be cooled to 4^{o}C before use.

PROCEDURE

1. Sacrifice a mouse/rat by cervical-dislocation, and quickly remove the liver. (Alternatively, freshly excised liver of any animal (chicken preferred) can be taken from a slaughter house.
2. Transfer the liver to ice-cold normal saline and wash to remove the adhering blood. Blot the liver dry with the help of a blotting paper.
3. Weigh the liver and keep in a watch-glass in an ice-box.
4. Mince the liver and make a 5% homogenate (W/V) in ice-cold water or normal saline (for example if the weight of the liver is 1g, use 19 ml of water or saline).

   DNA content in rat liver is approximately 2 mg/g wet weight of tissue. Hence a 5% homogenate gives a concentration of approximately 100 μg/ml DNA. Concentrated homogenate (more than 10%) would have higher DNA concentration, and give problem in pipetting aqueous phase at step # 14. For easy pipetting of aqueous phase at step # 13, the DNA content should be between 50-100 μg/ml. Very low DNA concentration (less than 10 μg/ml) may create problem in spooling the DNA at step # 18.
5. Filter the homogenate through four layers of cheese-cloth.
6. Measure the volume of the filtered homogenate and transfer it to a conical flask.
7. Add 10% SDS to dilute it to 1%. The volume of SDS added will approximately be the $1/9^{th}$ volume of the filtered homogenate. Mix the contents of the flask gently by swirling motion (excessive shaking will cause frothing of SDS). The homogenate should start becoming viscous due to the lysis of nuclei, and release of the DNA from histones.
8. Add 5 M NaCl to dilute it to 1 M. The volume of NaCl will approximately be equal to the $1/5^{th}$ volume of the filtered homogenate. Mix gently the content of the flask.
9. Add equal volume of chloroform: isoamyl alcohol (C:I) mixture (the volume of C:I mixture will be equal to the volume of homogenate + the volumes of SDS and NaCl added).
10. Mix gently but thoroughly to form an emulsion. The mixing should be done for 10 min for the proper separation of DNA.
11. Transfer the emulsion to the centrifuge tubes (which have either screw-caps or snap-on caps) and centrifuge for 10-15 min at 10,000 rpm at room-temp. Low-speed centrifugation will also work, but the time of centrifugation has to be increased.
12. The emulsion, after centrifugation, will be separated in to three layers; upper-aqueous phase, middle- inter phase, and lower- C:I (organic) phase. Carefully take out the centrifuge tubes without disturbing the separated phases.
13. With the help of a wide-bore pipette (a 10 ml glass or polypropylene pipette) fitted with a rubber-bulb, remove the aqueous phase in to a measuring cylinder. The aqueous phase will be highly viscous, hence, care should be taken not to suck and mix the interphase along with it.
14. Pool all the aqueous phases together and measure the volume.
15. If the aqueous phase is very cloudy and by mistake some interphase has been sucked out, re-extract by mixing with equal volume of CI mixture and repeat from steps 11 to 15.
16. Measure volume of the clear aqueous phase and transfer to a beaker.
17. Gently layer two and a half volumes of cold ethanol over the aqueous phase.

18. With the help of a glass rod, start mixing the aqueous and ethanol phase. Keep mixing only in one direction. Slowly the fibrous DNA will start spooling around the glass rod. When total DNA has been spooled-out, the solution will become clear.
19. With the help of clean forceps, remove the spooled DNA from glass-rod and transfer to a fresh tube containing ethanol. The DNA can be stored in ethanol at -20^{O}C for years together. If desired, the ethanol may be dried-out and the fibrous DNA may be dissolved in distilled water after drying a little-bit.
20. Total time required for above operation is approximately 1 h.

OBSERVATION

White fibrous DNA can be seen as precipitate which can be spooled on a glass rod.

QUESTIONS FOR FURTHER STUDIES

1. Why do we not observe the double stranded helical DNA strands?
2. Why do we extract DNA with phenol-chloroform?
3. How ethanol precipitates DNA and what is the role of temperature in DNA precipitation?

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Chapter 46: STUDY OF GROWTH PATTERN OF BACTERIA, *E coli DH5α*

Recommended level: UG and PG

INTRODUCTION

When a culture of bacteria is initiated, it may take some time to get acclimatized before growing exponentially. This period is known as lag phase. Under ideal conditions *Escherichia coli* grows exponentially (log phase). The rate of growth is dependent on the medium, the genotype of the strain, the temperature and the degree of aeration. As the density of culture increases, the rate of division decreases until the bacteria reach a concentration (saturation density) at which they no longer divide but are viable (stationary phase). At a later stage when nutrient supply of the medium falls limiting and toxic materials (excretions) accumulate, the cells start dying (decline phase).

The rate of growth of a bacterial culture is most conveniently monitored by withdrawing aliquots at various times and reading the optical density (OD), i.e., scattering of light by suspended bacterial cells in the medium at a wavelength of 550 or 600 nm. Number of cells present in a culture at a given time in the medium can be calculated by using the following:

1 OD_{600nm} = 8 X 10^8 cells/ml.

Because the exact relationship between the optical density and the number of bacteria varies a little from strain to strain of *E coli*, it is advisable to construct a calibration curve that relates the number of viable bacteria (counted in Hemocytometer) to the OD_{600nm} of the culture using a normal graph paper.

Though *E coli* lives in anaerobic condition in the intestine, it grows well under aerobic condition. Aeration of cultures is best achieved by attaching a flask or tube to a rotary shaking platform. The volume of the vessel should be at least four times larger than the volume of medium it contains so as to allow vigorous shaking (250 rpm) for proper aeration.

Storage of bacteria

Colonies of most strains of bacteria can be maintained for 4-5 weeks on the surface of agar media if the plates are tightly wrapped in parafilm and stored inverted at 4°C.

Most strains of bacteria can be maintained for a year or two in stab cultures. Such cultures are usually prepared in small screw-capped vials containing 2-3ml of agar medium. The culture is inoculated using a sterile, straight platinum wire that is dipped into a dense liquid culture of bacteria and then stabbed deep into the agar medium. The vial is incubated overnight at appropriate temperature keeping the cap loose. The cap is then tightened and the vial is stored at room temperature.

Bacteria can be stored for many years in media containing 15% glycerol (0.85 ml overnight culture + 0.15 ml of glycerol) at –20 or –70°C without significant loss of viability.

Characteristics of *E coli DH5α*

Genotype: *supE*44 Δ*lac*U169(ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1

Properties: A recombination-deficient suppressing strain used for plating and growth of plasmids and cosmids. The ϕ80*lacZ*ΔM15 permits α-complementaion with the amino terminus of β-galactosidase encoded in pUC vectors.

OBJECTIVE

To study growth pattern of *E coli DH5α.*

MATERIALS REQUIRED

1. *E. coli DH5α* strain cells, a clean table with a spirit lamp or a Laminar flow hood
2. Shaker incubator set at 37°C
3. Autoclaved test tubes containing 5 ml LB medium and autoclaved 250 ml conical flasks containing 50 ml LB medium
4. Pipettes, micropipettes with sterile tips

LB (Luria-Bertani) Medium

Bacto-tryptone	10 gm
Bacto-yeast	5 gm
Sodium chloride	10 gm
Distilled water to make the volume to	1000 ml

Adjust pH to 7.5 and autoclave for 20 min at 15 lb pressure.

PROCEDURE

1. Inoculate 5 ml of LB medium in aseptic condition (by flaming the rim of the tube containing LB and by making the tungsten wire loop red hot) with the required bacterial strain using a sterile tungsten wire loop. Incubate at 37°C overnight with vigorous shaking (at ~ 150 rpm).
2. Inoculate 50 ml of fresh LB in a 250 ml conical flask in aseptic condition with 1 ml of the overnight grown culture. Incubate at 37°C with vigorous shaking. Take OD at 550 or 600 nm wavelength in a spectrophotometer by taking out 2 ml of the inoculated medium under aseptic condition in a cuvette immediately after inoculation (0 time) and then at every 30 min till the OD shows an increasing trend.
3. Plot a graph on semi-log graph paper taking time on X-axis and OD on Y-axis as shown in Fig. 1.

OBSERVATION

A sigmoid growth curve is obtained as shown in Fig.

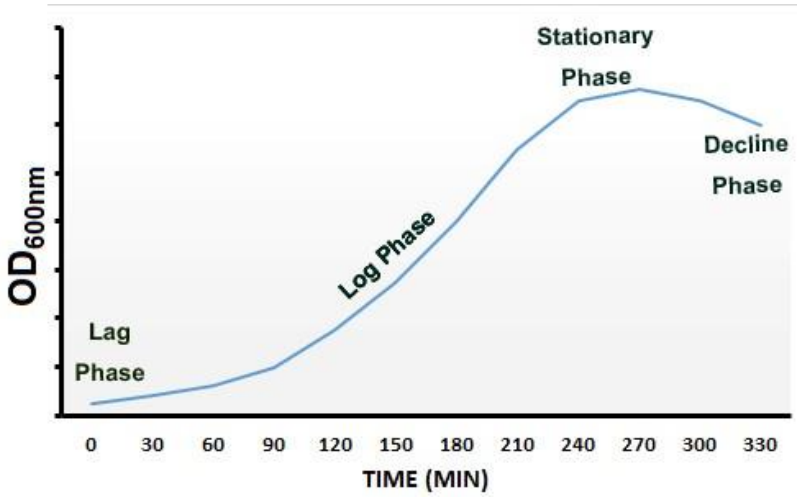

Figure 1. Growth curve of *E. coli DH5α*

QUESTIONS FOR FURTHER STUDIES

1. Why did you see a lag phase, a log phase, a stationary phase and a decline phase in growth curve?
2. Why Optical density for assessing bacterial growth in LB or turbidity of LB is taken between 550 to 600 nm wave length of light?
3. What will happen if a LB tube is opened once in the lab without using a flame or Laminar hood, closed and incubated at 37^{o}C?
4. Why shaking of culture is done in a shaker incubator at 37^{o}C?

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Chapter 47: TRANSFORMATION OF *E. coli* WITH RECOMBINANT PLASMID VECTOR

Recommended level: UG, PG and Research

INTRODUCTION

Transformation is a process in which free DNA is taken up by a cell resulting in a genotypic and phenotypic change in the recipient. Bacterial cells capable of incorporating free DNA are said to be competent. Some of the bacterial species are highly competent during specific phases of growth and can take up DNA without any pre-treatment. Their competency is due to secretion of a small protein called competence factor which provides special DNA binding and uptake properties. Other bacteria, like *E. coli*, do not produce competence factor and are thus normally not competent. However, they can be made competent artificially by treating with calcium chloride and subjecting them to heat shock (Mandel and Higa, 1970; Sambrook and Russell, 2001, 2006). The mechanism by which calcium chloride or heat shock promotes competence is not well understood.

Transformation of *E. coli* is of immense importance in gene cloning techniques. The desired constructs of genes to be cloned are first ligated into a vector and then inserted into bacterial cells by transformation for rapid multiplication (cloning). A vector is the vehicle to clone other pieces of DNA. The vectors in common use with *E. coli* are genetically engineered derivatives either of bacteriophages (phage vectors) or of naturally-occurring drug-resistance plasmids (plasmid vectors). The phage vectors do not require a transformation process since the mature phage particles can by themselves infect an appropriate bacterial host. It is the plasmid vectors that need to be inserted into *E. coli* host by the process of transformation. Since the plasmid vector carries its own origin of replication, it can be stably maintained after it is introduced (by transformation) into an *E. coli* host cell.

A fundamental principle of all cloning is selective growth of a clone of cells containing identical copies of a single vector molecule. Plasmid-containing clones can be selected as bacterial colonies that are resistant to a particular antibiotic due to expression of the plasmid-coded resistance gene. In addition to the basic requirements of a replication origin and selectable phenotype/s (e.g., antibiotic resistance), the commonly used vectors have been engineered to have other desirable features like unique sites for several different restriction enzymes to allow insertion of foreign DNA at specific sites for cloning. Most of the plasmid vectors in current use have all these cloning sites in a single region (the polylinker or multiple cloning sites or MCS): the MCS is a synthesized oligonucleotide with the necessary recognition sequences for a variety of restriction enzymes and is inserted at a suitable position in the vector.

In a typical cloning experiment, not all vector molecules will get joined to the "target" molecule and therefore, not all transformed clones will contain the desired insert. Therefore, it is advantageous to have a ready method of distinguishing clones of *E. coli* containing recombinant DNA molecules (plasmid with the desired foreign DNA insert) from those that contain only the vector DNA (note that both types survive on anti-biotic containing medium due to the antibiotic resistance conferred by the plasmid). A simple colour test is now commonly used for the above distinction. This is based on the fact that *E. coli* enzyme β-galactosidase (the product of *lacZ* gene from *lac* operon) converts the colorless chromogenic substrate 5-bormo-4-chloro-3-indolyl-β-D-galactoside (X-gal) into a blue product. An efficient transcription of the *lacZ* gene is ensured by inclusion of its specific inducer, isopropylthiogalactoside (IPTG) in the growth medium. The *lacZ* gene itself is too big to be included

as a whole in the plasmid vector. Fortunately, the phenomenon of α-complementation of β-galactosidase enzyme activity (Sambrook and Rusell, 2001) was known and this has been very diligently used to design host strains and plasmid vectors to allow the above colour detection. A short 146 amino acid long containing only the N-terminus of the enzyme (the α-peptide) can interact with a mutant *lacZ* derived protein lacking this region (but normal in the C-terminal side) to make a functional enzyme. Thus, many of the plasmid vectors (e.g., the *pUC* series) carry a short segment of *E. coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase (α-peptide). The MCS is located within the coding region of this truncated *lacZ* gene. These types of vectors are used to transform host cells (e.g., DH5α) that code for β-galactosidase defective in the N-terminal region. Thus, neither the host encoded nor the plasmid encoded enzyme is active, but they can associate (complement at protein level) to form an enzymatically active protein if simultaneously present in the cell. Colonies of *E. coli* (DH5α) transformed with such plasmid vectors in which this α-complementation has taken place appear blue when grown on IPTG-X-gal plates. However, insertion of a foreign DNA at any site in MCS of the plasmid vector results in production of an aberrant N-terminal fragment that is incapable of α-complementation. Therefore, these bacterial cells can not convert X-gal into a blue product so that only white (colourless) colonies are formed. This allows a simple distinction between transformed colonies containing recombinant (white or colourless) or original plasmid vector (blue).

As bacterial cells multiply in number, plasmids present in them also multiply using the bacterial replication machinery. Most of the commonly used plasmid vectors are maintained at a high copy number in bacterial cells. Since bacterial life cycle is short and isolation of plasmids from bacterial cells is very easy, several million copies of the given gene can be obtained within a brief period of time.

MATERIALS REQUIRED

1. *E. coli DH5α* strain cells
2. Shaker incubator set at 37°C
3. Autoclaved bacterial culture tubes containing 5 ml LB medium and autoclaved 250 ml conical flasks containing 50 ml LB medium
4. 200 mM and 80 mM $CaCl_2$ (sterile Millipore filtered)
5. 50 ml autoclaved polypropylene centrifuge tubes
6. Pipettes, micropipettes with sterile tips, microfuge tubes and tube stand
7. Bacterial plates with appropriate antibiotic/without any antibiotic or appropriate antibiotic + X-gal + IPTG.

LB (Luria-Bertani) Medium

Bacto-tryptone	10 gm
Bacto-yeast	5 gm
Sodium chloride	10 gm
Distilled water to make the volume to	1000 ml

Adjust pH to 7.5 and autoclave for 20 min at 15 lb pressure

Bacterial Plates

Autoclave l.5% Bacto-agar in fresh LB medium for 20 min at 15 lb pressure. Leave at room temperature till temperature of the medium is around 50°C. Add antibiotic/s as desired and pour on autoclaved bacterial plates. Let the plates cool down so that medium solidifies. Keep these plates at

37°C overnight to make sure there is no bacterial growth. Plates are always kept in inverted position to avoid moisture condensation on the surface of the solidified medium.

Plates for testing complementation

Add 40 µl of X-gal stock (20 mg/ml in dimethylformamide) and 4 µl of isopropylthio-β-D-galactoside (IPTG) stock (200 mg/ml) to a prepared bacterial plate containing appropriate antibiotic; spread the solution over entire surface of the plate with the help of a glass spreader. Incubate the plate at 37°C until the added liquid dries.

Ampicillin stock solution

5 mg/ml in sterilized water (store at -20°C). Add to the medium to a final concentration of 50 µg/ml

OBJECTIVE

To transform *E. coli DH5α* cells with the given plasmid.

PROCEDURE

Preparation of competent cells

1. Inoculate 5 ml of LB medium with the required bacterial strain under aseptic condition. Incubate at 37°C overnight with vigorous shaking (at ~ 150 rpm)
2. Inoculate 50 ml of fresh LB in a 250 ml conical flask with 1ml of the overnight grown culture. Incubate at 37°C with vigorous shaking for 2 h (approx.) till O D_{550} comes to 0.5. When ready, cool the culture on ice and transfer to a 50 ml polypropylene tube. Spin cells into loose pellets (at 6500 rpm for 5 min at 4°C)
3. Add 5 ml cold 200 mM $CaCl_2$ to the tube on ice. Resuspend the pellet with pipette till the solution becomes smooth and silky. Add 15 ml more of 200 mM $CaCl_2$ and leave it on ice for 20 min. Spin at 6500 rpm for 5 min at 4°C
   (N. B. the pellet should be spread out)
4. Resuspend in 4 ml of 80 mM $CaCl_2$ and leave on ice for 1 h. The cells are now competent and can be stored for one week on ice.

Transformation

1. Take 100 µl of competent cells in 1.5 ml microfuge tube kept on ice. Add 25 ng of the desired plasmid DNA (1-2 µl). Mix and leave on ice for 40 min. Transformation can be done with 10 to 20 pg DNA also.
2. Give heat shock at 42°C for 2 min.
3. Add 0.5 ml LB and incubate cells at 37°C for 60 min without shaking.
4. Spread 100 µl of the transformed cells on plate with or without appropriate antibiotic or on appropriate antibiotic + X-gal + IPTG. Incubate overnight at 37°C to grow transformed bacterial cell colonies.

OBSERVATION

A large number of bacterial colonies (a lawn of bacterial cells) appear on plates without any antibiotic; relatively fewer colonies appear on plates with appropriate antibiotic while no colonies grow on plates with inappropriate antibiotic.

Distinct blue and white colonies appear on X-gal+IPTG plates as seen in Fig.

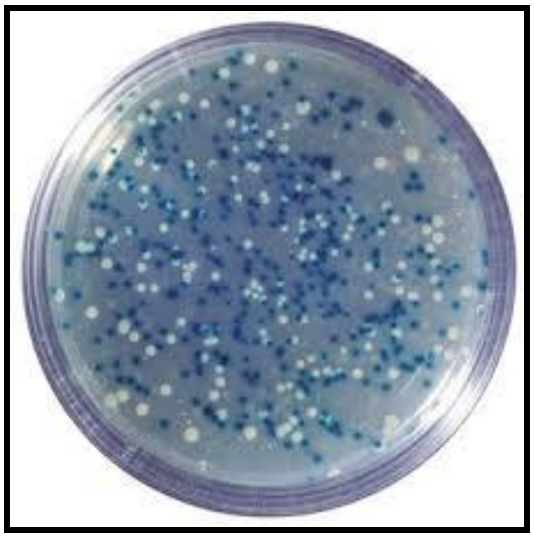

Figure. Blue and white colonies as seen on LB agar-Ampicillin-X-gal-IPTG plate

QUESTIONS FOR FURTHER STUDIES

1. Why bacterial plates without any antibiotic showed a lawn of cells?
2. What kind of colonies you expect when non transformed E. coli DH5-α cells are plated on bacterial plate containing ampicillin?
3. Can the efficiency of transformation be calculated in terms of colonies/µg of DNA and also in terms of % efficiency?

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Chapter 48: RAPID ISOLATION OF PLASMID DNA

Recommended level: UG and PG

INTRODUCTION

Plasmids are extrachromosomal, double stranded, circular DNA molecules with their own origin of replication. However, they use bacterial enzymes for their replication and/or expression. Since they multiply rapidly in the bacterial hosts, they are widely used as vectors in recombinant DNA technology. A desired gene can be cloned using these vectors and the chimeric construct can be inserted into host bacteria by transformation for its multiplication.

Most of the plasmids used as cloning vectors are not naturally occurring ones, but have been extensively modified (genetically engineered) so that they have properties useful for cloning, e.g., markers to aid their detection in transformed cells, unique restriction sites for inserting the target DNA molecule, etc.

Unlike the host chromosome, plasmids are small and can be easily isolated as intact molecule from the complex large molecule of bacterial DNA. A variety of effective methods are available for isolation of plasmid DNA and one of them is the "alkaline lysis method", in which bacterial cells are lysed by alkali treatment and then bacterial DNA (high molecular weight DNA) and plasmid DNA (low molecular weight DNA) are differentially precipitated and purified. However, for many purposes, it may not be necessary to prepare large quantity of highly purified plasmid DNA - a method which is relatively lengthy and costly. Hence, methods have been developed which permit a rapid isolation of plasmid DNA from a few cells (Holmes and Quigley, 1981; Sambrook and Russell, 2001). The small amount of resulting plasmid DNA can be used to screen for the presence of desired clones in a population by using restriction endonuclease digestion pattern. This DNA can be used for further transformation.

OBJECTIVE

To isolate plasmid DNA from the given *E. coli* culture.

MATERIALS REQUIRED

Transformed *E. coli* colonies containing the desired plasmid
Autoclaved bacterial culture tubes with 5 ml LB medium
Desired antibiotic solution
Shaker incubator set at 37^{o}C; Water bath with boiling water
Microfuge tubes, microfuge tube stand, microfuge, micropipettes and tips
Lysis buffer

1M Tris-HCl (pH 8.0)	100 µl
0.5M EDTA	2 µl
Sucrose	15 mg
Lysozyme	2 mg
Pancreatic RNase	0.2mg
Bovine Serum Albumin	0.1mg
Distilled Water	to 1 ml

LB Medium

Bacto tryptone	10 gm
Bacto yeast	5 gm
Sodium chloride	10 gm
Distilled water	1000 ml

Adjust pH to 7.5 and autoclave for 20 min at 15 lb/inch^2 pressure

PROCEDURE

1. Inoculate 5 ml of LB medium containing the appropriate antibiotic with a single bacterial colony in aseptic condition. Incubate overnight at 37^{o}C with vigorous shaking (at ~ 150 rpm).
2. Pour 1.5 ml of overnight culture into a microfuge tube. Centrifuge in a microfuge (~10000 rpm) for 5 min at 4^{o}C (Store the remainder of the overnight culture at 4^{o}C). Remove the supernatant medium completely by inverting the tube and tapping the inverted tube on a clean blotting paper, leaving bacterial pellet as dry as possible.
4. Resuspend the pellet in 30 µl of Lysis buffer and gently tap the tube to allow cells to be uniformly suspended and incubate at room temperature (~25^{o}C) for 5 min
6. Place the tube in a boiling water bath exactly for 1 min and immediately chill on ice for at least 1 min
7. Spin in microfuge (10,000 to 12,000 rpm) for 15 min at room temperature
8. Carefully collect the supernatant and directly use for restriction endonuclease digestion, etc.

OBSERVATION

Approximate yield of plasmid DNA is about 2 µg/1.5 ml of bacterial culture. The quality of DNA can be seen after doing agarose gel electrophoresis.

QUESTIONS FOR FURTHER STUDIES

1. What is the function of each component of Lysis buffer?
2. How does boiling help in isolation of the plasmid DNA?
3. Why did plasmid DNA stay in supernatant?
4. There are three forms of plasmid DNA – supercoiled (majority), nicked circular and linear. In which order we see them after running the isolated plasmid DNA in agarose gel?

REFERENCE

Holmes D S, Quigley M (1981). A rapid boiling method for the preparation of bacterial plasmids. Analytical Biochemistry 114, 193-197

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Chapter 49: RESTRICTION ENDONUCLEASE DIGESTION OF GENOMIC AND PLASMID DNA

Recommended level: UG and PG

INTRODUCTION

Reactions typically contain 0.2-1 µg of DNA in a volume of 20 µl. However, reaction volumes may be increased or decreased according to need. Each restriction endonuclease has a set of optimal reaction conditions which are given on the information sheets of various catalogues. The major variables are incubation temperature and the composition of reaction buffer (Sambrook and Russell, 2001). Broadly buffers are of three types: high ionic strength, medium ionic strength and low ionic strength. Usually, buffers are made up as 10x stock and then stored at -20ºC.

MATERIALS REQUIRED

Desired DNA

Digestion buffer

Restriction endonuclease

Water bath at 37ºC

PROCEDURE

Setting up of digestion

1. Mix the following in a clean 1.5 ml microfuge tube kept on ice in the order given:

DNA (0.2 to 1.0 mg)	2 µl
10X Restriction enzyme buffer	2 µl
Restriction enzyme (3 units/µg DNA)	1 µl
Distilled Water to make	20 µl

Mix the contents after each addition by tapping the tube

(The actual amounts of each solution may be adjusted in relation to the amount of DNA being digested in the given case)

(Note: A. The tube containing Restriction enzyme should be taken out of the -20ºC Freezer after the DNA and enzyme buffer have already been added, i.e., just before you are ready to add the enzyme; the enzyme should be replaced in the freezer immediately after addition of the enzyme to the reaction mix; B. Volume of enzyme should not exceed one-tenth the total reaction volume because glycerol present in the storage buffer of enzyme may inhibit or alter the enzyme activity)

2. Incubate the tube at appropriate temperature for the required period of time (one to a few h)

3. Stop the reaction by heating the tube for 5 min at 75ºC or by addition of 0.5 M EDTA (pH 8) to give a final concentration of 10 mM (i.e., 0.4 µl of 0.5 M EDTA to a 20 µl reaction vol)

4. Analyze the digested DNA on a gel (see Fig. 1 in chapter 50)

When digesting bulk genomic DNA for Southern hybridization (~10 µg DNA per lane), restriction enzyme digestion is performed in a total reaction volume of 200 µl. After checking a small aliquot for

completion of digestion by running a test gel, the remainder **of the DNA is extracted with phenol-chloroform, ethanol precipitated, dried and redissolved in 15 to 20 µl of TE, mixed with sample buffer and fractionated on agarose gel (Green and Sambrook, 2016).**

OBSERVATION

An agarose gel is run to observe the digested DNA (see Fig. in page 130).

QUESTIONS FOR FURTHER STUDIES

1. What is meant by star activity of a restriction endonuclease?
2. Is this better to set digestion for overnight?

REFERENCE

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Chapter 50: AGAROSE GEL ELECTROPHORESIS FOR DNA

Recommended level: UG, PG and Research

INTRODUCTION

The standard method used to separate and identify DNA fragments is electrophoresis through agarose gels. The location of DNA within the gel can be determined directly by visualizing ethidium bromide stained fluorescent bands in ultraviolet light (254 nm or 310 nm or 354 nm) using a Transluminator (Sambrook et al, 1989).

The electrophoretic mobility of DNA through agarose gel is dependent on the following 4 parameters:

a. Molecular size of DNA
b. Agarose concentration
c. Conformation of the DNA
d. Applied current

OBJECTIVE

To run the isolated plasmid DNA digested with given restriction endonucleases and also the genomic DNA digested with given restriction endonucleases in an agarose gel.

MATERIA LS REQUIRED

1. Tris-Acetate-EDTA (TAE) stock solution (5X)

Tris base	54 g
0.5M EDTA (pH 8.0)	20 ml
Distilled water to make	1000 ml

Working Buffer: 1X TAE

2. Loading Buffer (l0X)

0.25% Bromophenol blue
0.25% Xylene cyanol
25% Ficoll (Type 400) in distilled water
Store at room temp.

Preparation of Agarose gel

For analyzing restriction endonuclease digested plasmid or genomic DNA, usually a 0.8% or 1% agarose gel is made in 1X TBE (a higher or lower % gel can be used depending upon the size of DNA molecules to be analyzed)

1. Prepare the gel mold by sealing the two free ends of gel platform with plastic tape and positioning the comb on the gel platform (at least 0.5-0.1 mm gap should be left between the platform and base of the comb, otherwise wells may get damaged).

2. Add the appropriate amount of agarose to a measured quantity of the electrophoresis buffer (e.g., for a medium size gel, take 400 mg of agarose in 40 ml of 1XTAE)

3. Boil till the agarose becomes sol (total of about 5-10 min heating on a heater)

4. Cool solution to ~50^{o}C and add ethidium bromide to a final concentration of 0.5 μg/ml. (a stock solution of 10 mg/ml ethidium bromide can be made and stored in a coloured bottle/tube at 4^{o}C) and mix.

5. Pour the gel solution into the prepared mold; let it cool at room temperature (avoid any vibrations of the table or the gel mold while polymerization is in progress).

6. After the gel is completely set (30-45 min at room temperature), carefully remove the comb (preferably add electrophoretic buffer beforehand so that the comb comes off easily.

7. Remove the plastic tape from both ends of gel mold and put the gel with its platform in a submarine electrophoresis. Add enough electrophoresis buffer (1X TAE) to let the gel just submerge

8. Prepare DNA samples for electrophoresis as follows

DNA (at least 150-200 ng) + distilled water	18 µl
10x loading Buffer	2 µl

(N.B. Volume of water can be adjusted according to the volume of DNA sample)

Vortex the samples and centrifuge briefly before loading in the wells. After loading the samples with the help of a 100 µl pipette. Switch on the power supply at 50-150 volts depending upon the gel size (5 volts/cm being the optimal). After the run is over, examine the gel in ultra violet light using a transluminator (UV light is harmful to eyes: use protective glasses or the perspex shield. Ethidium bromide is a potent carcinogen, so the vial of ethidium bromide should be touched after wearing gloves and gel with ethidium bromide also should be handled after wearing gloves. Now a days non carcinogenic GelRed has been introduced by Life technologies (India) Pvt Ltd as a substitute of ethidium bromide, this stain also gives red fluorescence like ethidium bromide when viewed in UV transilluminator. Non harmful GreenView system is another staining system recently introduced by Chromous Biotech which uses visible light for viewing).

OBSERVATIONS

Lambda DNA digested with *Hind*III restriction endonuclease is usually run as molecular size marker in DNA-agarose gel electrophoresis. This facilitates finding the molecular weight of experimental DNA. Lambda DNA digested with *Hind*III shows 8 bands of the following sizes:

23.130 Kb
9.416 Kb
6.557 Kb
4.361 Kb
2.322 Kb
2.027 Kb
0.564 Kb
0.124 Kb

Restriction endonuclease digested plasmid or phage DNA gives sharp band/s of expected molecular size/s. However, similarly digested genomic DNA shows a smear.

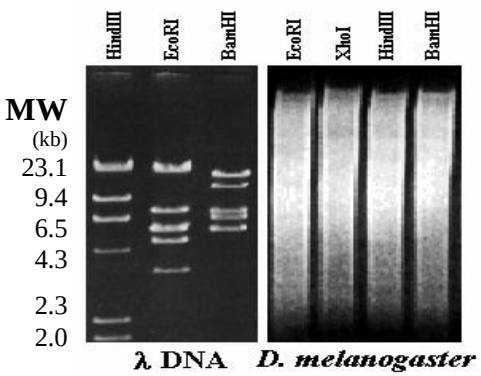

Figure. Lambda phage and *D. melanogaster* genomic DNA digested with the different restriction enzymes (as noted on the lanes), separated on agarose gel, stained with ethidium bromide and viewed on UV transilluminator

QUESTIONS FOR FURTHER STUDIES

1. Why do we select higher concentration of agarose gel when the size of DNA samples to be separated are small and vice versa?
2. What is the function of loading buffer?
3. What will happen if we increase the voltage for running the is more than the recommended?

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Chapter 51: ISOLATION OF RNA AND ITS QUANTIFICATION USING RT-PCR

Recommended Level: UG/PG/Research

INTRODUCTION

It is widely accepted that the information present in DNA is first copied into messenger RNA (mRNA) through the process of transcription which further undergoes the process of translation to make the proteins. This flow of information is classically termed as the **Central Dogma of Molecular Biology (**Figure-1). While every cell in an organism has similar amount of DNA, the cells express distinct levels of RNA transcripts and proteins in response to various environmental stimuli. The process of "turning on" a gene (the functional segment of DNA) to produce RNA and protein is known as **gene expression**.

In the 1970s, virologists David Baltimore (MIT, Cambridge, USA) and Howard Temin (UW, Madison, USA) while studying the RNA-tumor virus (later known as the Human Immunodeficiency virus, or HIV) independently and simultaneously observed that the virions obtained their DNA from reverse transcription of the viral RNA with the help of a unique **RNA dependent DNA polymerase**, subsequently renamed as the **reverse transcriptase (RT)**. The discovery although contradicted the theory of Central Dogma of Molecular Biology, gained widespread appreciation and became the primary driving force behind identification of **RNA viruses** as well as **retroviruses**.

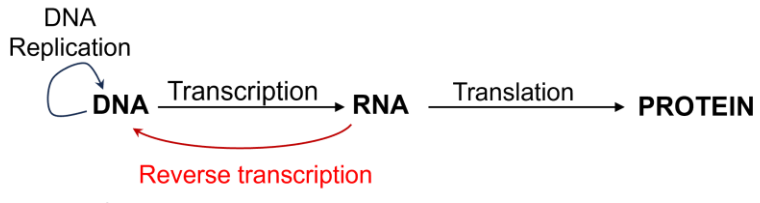


Figure 1. Central dogma

The classical polymerase chain reaction (PCR) technique, extensively used for enzyme (DNA polymerase, a DNA dependent DNA polymerase) mediated synthesis of multiple copies of a DNA segment cannot be applied directly to RNA, but requires an initial reverse transcription step, which copies the information present in the RNA to complementary DNA (cDNA). This cDNA then serves as the template for the PCR. The discovery of the RT thus paved way for conceptualization of the "**reverse transcription coupled polymerase chain reaction"** technique, commonly abbreviated as the **RT PCR.** The development of the RT coupled PCR (or RT PCR) has led to major advancements in understanding biology at RNA level. This technique not only allows the detection of trace amounts of RNA present in cells (high sensitivity) but also enables the detection of an RNA molecule of interest in a sequence specific manner (high specificity).

The RT PCR is a versatile molecular biology technique. It is widely used in basic research wherein gene expression analysis is an integral part of understanding biological processes. The RT PCR is also an important component of nucleic acid amplification (NAA) based molecular diagnostic assays which are currently the most efficient tool for diagnosis of RNA viruses. This chapter will cover basic principles governing the basic steps involved in extraction of RNA from cells, performing the RT PCR as well as analysis of data.

OBJECTIVES

1. Extraction of RNA from cells
2. Preparation of cDNA
3. Quantitative Polymerase Chain Reaction (qPCR)
4. Analysis of data

MATERIALS REQUIRED

While working with RNA it is important to maintain a nuclease free environment as the RNA structure is inherently weaker than that of DNA and is very susceptible to degradation in the presence of nucleases. To this end, all the essential apparatus required for working including the work desk, pipettes (pipettes of volumes: 100-1000 µl, 20-200 µl, 0.5-10 µl), etc., need to be wiped well with 70% ethanol before use. It is important to use autoclaved, nuclease-free micro centrifuge tubes and PCR tubes. The objective wise requirements are listed below:

Extraction of RNA: The most popular method for extraction of RNA is based on acid guanidinium thiocyanate-phenol-chloroform triphasic separation described by Chomczynski and Sachhi in the research article "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction", published in the journal *Analytical Biochem*istry, 1987. Since then, several ready-to-use reagents such as TRIzol (Invitrogen, CA, USA), Qiazol (Qiagen, GmbH, Germany) or Tri reagent (SIGMA-Aldrich, St. Louis, MO, USA) and several kits have been commercially developed. The requirements for extraction of RNA include primarily a biological source material such as cells or tissues, TRIzol reagent, chloroform, isopropanol, ethanol, nuclease free water and a cooling microcentrifuge.

cDNA preparation: A typical cDNA synthesis reaction includes the RNA, the reverse transcriptase (RT), RT Buffer, primer (either oligo dT primer or random hexamer primer), dNTPs, RNase inhibitor, Dithiothreitol (DTT), NF water and a thermal cycler.

Quantitative Polymerase Chain Reaction (qPCR): A typical qPCR reaction includes the cDNA, gene specific PCR primers (forward and reverse primer), dNTPs, DNA polymerase fluorescent dye (e.g., SyBr Green or TB Green) or dual-labelled fluorescent probe, NF water and a real-time thermal cycler.

PROCEDURE

Extraction of total RNA (Approximate timeline: ~ 2 h):

1. In a 1.5 ml micro-centrifuge tube take the mammalian cells (10^3 to 10^6 cells) or a small piece of tissue.
2. To this add 1 ml TRIzol reagent and let the tube stand at room temperature for 5 min.
3. Add 200 µl chloroform, close the tube cap and shake the tube by inverting nearly 10 times and then let the tubes stand at room temperature for 5 min.
4. Meanwhile, pre-set the microcentrifuge at 4ºC. Centrifuge the tube at 13000 rpm for 15 min at 4ºC.
5. Carefully take out the tubes from the centrifuge without disturbing the tri-phasic separation. The three phases include
6. the uppermost transparent aqueous phase containing the RNA

7. the white thin layer (also called the interphase) containing the DNA and cell debris
8. while the third and lowermost layer containing mostly the cellular proteins.
9. Carefully aspirate out the aqueous phase containing the RNA using the 200 µl tip of the pipette without disturbing the interphase, and transfer it to a fresh 1.5 ml microcentrifuge tube.
10. To the aqueous phase add 400 µl of isopropanol (for precipitation of nucleic acid) and incubate at -20^{o}C for overnight (to achieve maximum precipitation). Centrifuge the tubes at 13000 rpm for 15 min at 4^{o}C. A small white pellet of the RNA precipitate is formed at the bottom of the tube. Carefully discard all the supernatant with help of a pipette.
11. To the pellet add 500 µl of pre-chilled 75% ethanol and again centrifuge the tubes at 13000 rpm for 15 min at 4^{o}C. This step washes off the slats co-precipitated with the RNA. A small white pellet must be visible.
12. Carefully discard all the supernatant with help of a pipette. Let the pellet to air dry for nearly 10 min. Resuspend the pellet in pre-warmed (~60^{o}C) 50 µl nuclease free water. Store the RNA in a -20^{o}C refrigerator until use or store in a -80^{o}C refrigerator for long term storage.
13. The quality of the extracted RNA can be assessed by running nearly 5 µl of the RNA extract on 2% agarose gel followed by visualization of the gel on a UV transilluminator or gel doc system. In case of human RNA, the 28S and 18S rRNA are visible as intact bands in the ratio 2:1. In case of bacterial RNA, the 23S and 16S rRNA are visible as intact bands in the ratio 2:1. This indicates good quality RNA. Any smearing of bands would indicate degradation of the RNA. The concentration of the RNA may be measured using a spectrophotometer based nanodrop instrument.

OBSERVATIONS

The quality of the extracted RNA is assessed by running nearly 5 µl of the RNA extract on 2% agarose gel followed by visualization of the gel on a UV transilluminator or gel doc system. In human RNA, the 28S and 18S rRNA are visible as intact bands in the ratio 2:1 (see Fig. 2).

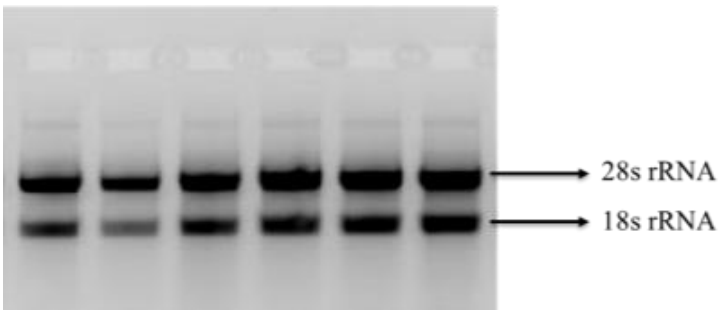


Figure 2. Representative image of isolated RNA from human cell line run on a 2% agarose gel showing that two bands of 28s rRNA and 18 rRNA are observed in ~2:1 ratio. Also, a ratio of quantitation value of 2.0 in 260 and 280 nm wave length of light in a spectrophotometer signifying that the RNA isolated was pure

cDNA preparation (Approximate timeline: ~ 1 h)

With the use of oligo dT primers, the mRNA fraction of the total RNA can be reverse transcribed to cDNA, while, with the use of random hexamer primers, all of the RNA, irrespective of the origin of RNA species, can be reverse transcribed to cDNA.

1. The calculation for setting up of a single reaction based on the protocol recommended by the kit manufacturer (Bio Bharti super reverse transcriptase kit) is given below:

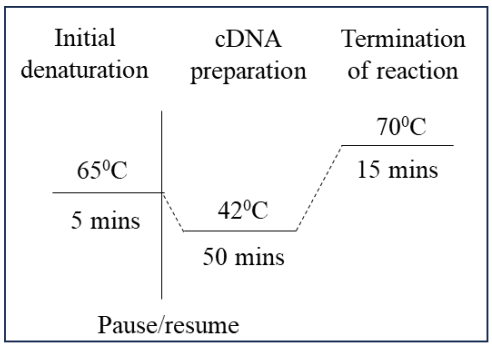
   Preparation of the half reaction mix (R1) in a 0.2 ml PCR tubes

Oligo dT/Random hexamer primer	1 µl
100 ng RNA	X µl
dNTP mix (10mM each)	1 µl
Nuclease free water	X µl
Total	Up to 12 µl

2. Heat the mixture at 65ºC for 5 min in the thermal cycler for denaturation of possible RNA secondary structures.
3. Meanwhile, prepare the remaining half of the reaction mixture (R2) in a 0.2 ml PCR tube as mentioned below:

5X RT Buffer	4 µl
0.1M DTT	2 µl
RNase Inhibitor	1 µl
Reverse Transcriptase	1 µl
Total	8 µl

4. Keep the R1 on ice for 2 min, gently vortex and then spin the tube in a mini-spin to collect the possible vapors on the inside of the cap. Add the components in R2 to R1 using the pipette.
5. Place the reaction tube (R1= 20 µl) in the thermal cycler and incubate the reaction at 42ºC, 50 min followed by termination of the reaction at 70ºC, 15 min. The overall thermal cycling conditions are described below:



The cDNA can be stored at -20ºC until use

Targeted PCR amplification of the cDNA using either fluorescent dye or dual labelled probe for real time monitoring of the reaction kinetics:

The RNA can be quantified in two ways:

a) Absolute quantitation using standard curve analysis

b) Relative quantitation (Fold change: $2^{-\Delta\Delta Ct}$)

First, we will understand the basic concepts underlining the quantitative PCR (qPCR) reaction. The primary component of the PCR includes the targeted amplification of the gene of interest, which may ether be of microbial origin or host derived. For example, when evaluating the presence of a microbe

such as the SARS CoV-2 virus in the oral sample, a small region of the SARS CoV-2 genome will be amplified using SARS CoV-2 gene specific primers. When the expression level of a host gene such as the cell surface receptor, angiotensin converting enzyme-2 (ACE2) is to be compared in different cell types, a region of the ACE2 transcript will be amplified using ACE2 specific primers from the cDNA derived from RNA extracts derived from the different cell types. The "q"PCR reaction includes a fluorescent reporter system such as a DNA intercalating fluorescent dye (e.g., SyBr Green) or a dual-labeled probe in addition to the usual PCR components. The reaction mix for both types of qPCR reactions is demonstrated below:

SyBr Green based qPCR mix:	
cDNA	1.0µL
Forward primers (10µM)	0.4µL
Reverse primers (10µM)	0.4µL
Taq DNA Pol, 10x Taq Pol Buffer, dNTPs, SyBr Green	2X SyBr green Master mix: 5 µL
Nuclease free H_2O	3.2µL
Total	10.0µL

Probe based qPCR mix:	
cDNA	1.0µL
Forward primers (10µM)	0.4µL
Reverse primers (10µM)	0.4µL
Probe (10µM)	0.2µL
Taq DNA Pol, 10x Taq Pol Buffer, dNTPs	2X PCR Master mix: 5 µL
Nuclease free H_2O	3.0µL
Total	10.0µL

The thermal cycling conditions for the PCR reaction are demonstrated below:

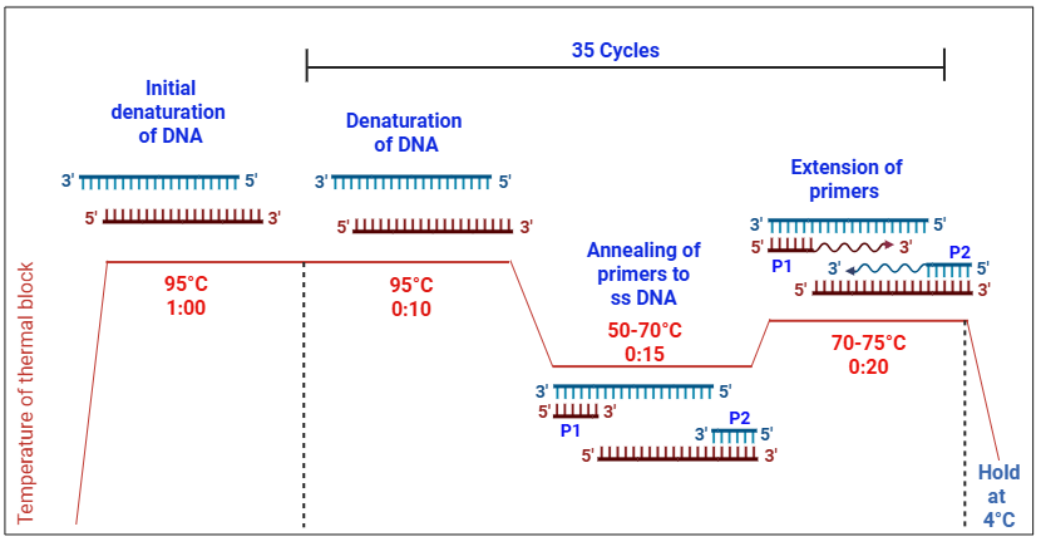


Figure 3. Thermal cycles and DNA amplification

The idea of incorporating a fluorescent reporter system (fluorescent DNA intercalating dye or fluorescent-dual labelled probe) in the PCR was first described by Higuchi and coworkers in 1992. The **SyBr Green DNA intercalating dye** binds to the minor groove of the double stranded DNA and fluoresces 1000-fold more after intercalating into the DNA. Here, as the PCR cycles proceed and the DNA (or amplicons) are synthesized (2^n per cycle, where n=cycle number), simultaneously the intercalation of the fluorescent dye occurs and fluorescence emission from the reaction tube increases. A **dual-labeled probe** is an oligonucleotide (~20 nt) which is labelled at its 5' end with a fluorescent molecule (e.g., Fluorescein amidite or FAM) and a quencher molecule at its 3' end. This probe is so designed that its binds to the cDNA slightly ahead of the forward primer. In the PCR reaction, every time the forward primer binds to its target site and proceeds in the 5' to 3' direction, it causes a hydrolytic cleavage of the probe thereby separating the fluorescent molecule from the quencher. The fluorescent molecule is released in every PCR cycle and the increase in fluorescence corresponds to

the increase in number of dsDNA copies (or amplicons). This increase in fluorescence after every PCR cycle is recorded in the real time thermal cycler. Both the fluorescent detection systems are described pictorially below (Figure 4):

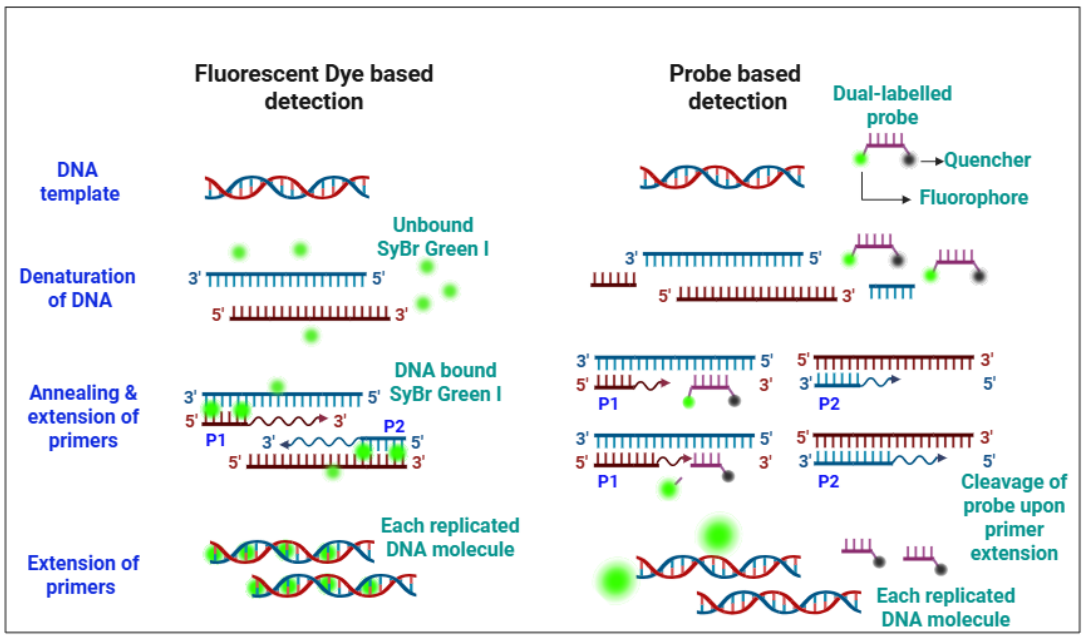


Figure 4. Pictorial presentation of Real time PCR

The PCR which allows both, the amplification of DNA as well as quantification of the amplified PCR products simultaneously is known as "real-time" PCR. This eliminates the need for post-PCR processing involved in conventional methods such as running the PCR products on an agarose gel electrophoresis assembly followed by its visualization on a UV transilluminator system.

The real time PCR machine comprises of a standard "PCR thermal cycling block" coupled with an "excitation source" (such as a laser or tungsten lamp), "camera" for detection of fluorescence, a computer and software for data analysis. The thermal-block elevates and lowers the temperature of the block in cycles in predefined fashion (the PCR is set to run for 35-45 cycles) which allows cDNA to be amplified exponentially (similar to replication of DNA). The camera (or optical detection unit) records the increase in fluorescent signals after each cycle of the DNA amplification.

Data analysis

The instrument software allows the visualization of the reaction kinetics by providing a graphical representation of the increase in fluorescent signal generated at each PCR cycle. The increase in fluorescent signal at each PCR cycle when plotted on graph, resembles **a sigmoidal pattern of amplification**. The y axis represents the relative fluorescence units (RFU), and the x axis represents the PCR cycle number.

The amplification curve: The amplification curve can be divided in three phases as shown in the Figure 5a:

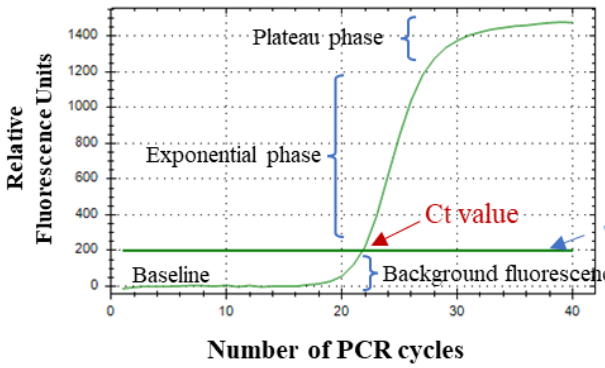
i. Background (or baseline): At the beginning of the run (usually between cycles 0 and 15), when the amount of PCR product is low, little fluorescence is emitted and this fluorescence is indistinguishable from the background signals. This section of cycles is known as the background signal or noise. The background fluoresce is denoted by the instrument as signal "threshold".

ii. Exponential phase: After initiation of the PCR, once the fluorescent signal exceeds the background fluorescence or in other words, exceeds the signal threshold set by the instrument, the reaction is said to have entered the exponential phase.

iii. Plateau: Once all of the reaction components (including nucleotides) have been used up in the PCR reaction, the amplification slows down and is represented in the amplification as plateau phase. This is the region where no more PCR products can be produced. This can be seen in cycles 30 to 40 in the amplification plot.

Ct Value: The C_t value also known as "Cycle threshold" is defined as the number of PCR cycles after which the fluorescent signal of the PCR reaction crosses the background signal or the threshold. The background signal may be due to any signal inherently produced by the reaction components. The Ct value and the amount of input DNA present in the beginning of the PCR are inversely correlated. This means that a PCR assay starting with abundant amount of input DNA tends to demonstrate an early Ct value and a PCR assay starting with low amount of input DNA tends to demonstrate late Ct value.

(a) Amplification curve :

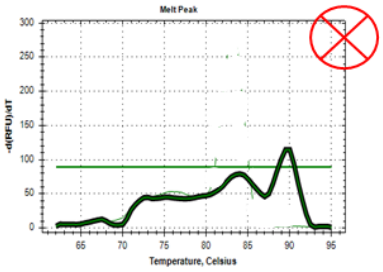


The PCR Cycle number at which the fluorescence generated by each amplified DNA sample crosses the threshold is referred to as the "Ct" value

Threshold set by the instrument

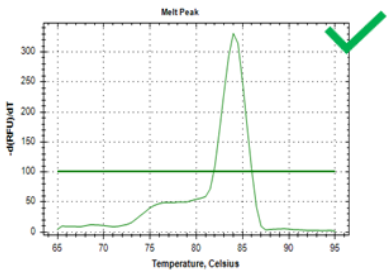
(b) Melting curve : Each amplification curve has a corresponding melting curve

(a)



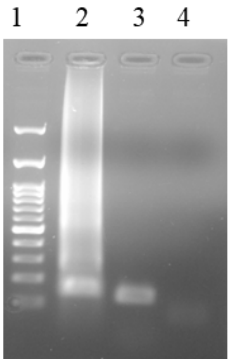
Multiple peaks indicates multiple (non-specific) amplicons in the PCR product.

(b)



Single peak indicates single (specific) amplicon in the PCR product

(c) Below is the agarose gel electrophoresis picture of the RT PCR products corresponding to the above mentioned melt peaks (a, b).



1: 100bp DNA ladder

2: The smear of the PCR product corresponds to the multiple peaks as shown in (a), this indicates non specific amplification because of inefficient primers.

3: The sharp band of the PCR product corresponds to the single sharp peak at 84^0C as shown in (b), this indicates specific amplification by primers.

4: No amplification, only primer dimer visible, this is usually represented by a small peak near 75^0C in the melting curve.

Figure 5: Visualization of amplification curve, melt peak and resolution of the PCR products on agarose gel.

Absolute quantitation using standard curve analysis: This method is used to precisely measure the amount of DNA or RNA present in a biological sample. Firstly, the qRT PCR is performed with ten-fold serially diluted molecules (or copies) of RNA/DNA of known (or standard) concentration ranging from 10^5 to 10^1 DNA copies/µL, using gene specific primers. The resulting qPCR signals (Ct values) are plotted as a linear regression of Ct values versus Log_{10} of DNA copies/µL on a graph, as shown in Fig. 5a-b and the corresponding equation on the standard curve (supposedly a straight line) is obtained. Subsequently, the qRT PCR is performed with DNA/RNA samples of unknown concentration (the test sample). The Ct values of these samples are then compared with the standard curve.

An ideal standard curve represents a linear correlation and is mathematically defined by the equation: $y = mx + c$, wherein y is the Ct value, m is the slope of the straight line and x describes the concentration of DNA, c is the y intercept value. The Ct value obtained for the test sample, can be evaluated using the equation ($y = mx + c$) of the standard curve prepared for the particular gene being analyzed. The slope (m) of the standard curve indicates the efficiency of the PCR. A disadvantage of absolute quantification includes the increased effort to generate standard curves. Example: this method is often used to evaluate the viral load (or microbial load) in oral sample of patients suffering from flu like symptoms. As one cell of a microorganism (or virus) comprises of one copy of its whole genome and the genome comprises of only one copy of each gene. Determining the number of copies of the gene of interest amplified via qPCR provides an estimation of the number of microbial cells (or viral load in case of viral infection) present in a biological sample of the infected individual.

Relative quantitation (Fold change: $2^{-\Delta\Delta Ct}$): It is also known as comparative quantitation. Here the change in expression level of the gene of interest is measured by measuring the level of its mRNA relative to the mRNA level of the reference gene (also known as house-keeping gene) via q RT PCR. is The house keeping genes are abundantly expressed in cells and their expression level is unaffected by the condition/stimuli being investigated in the study. For example, this method is widely employed during quantitative analysis of gene expression in given test conditions and at given timepoint. After the qPCR run is complete, the data is obtained in the form of Ct values as shown in Fig. 5d.

The change in expression of gene of interest is calculated using the formula $\mathbf{2^{-\Delta\Delta Ct}}$ as described below:

$\Delta Ct = Ct$ (Gene of interest) – Ct (internal control).

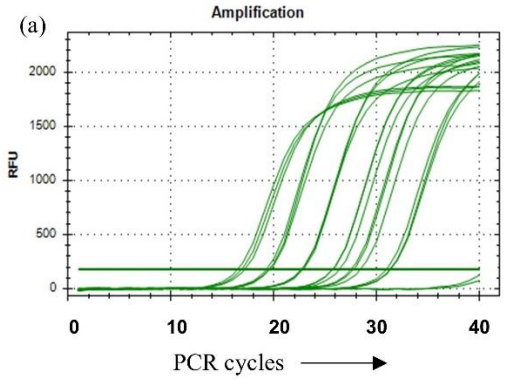
$\Delta\Delta Ct = \Delta Ct$ (Condition) – ΔCt (control).

Fold change = $\mathbf{2^{-\Delta\Delta Ct}}$

Examples of internal control genes include GAPDH, β-actin, 18s rRNA, etc for eukaryotic organism, 16s rRNA for prokaryotic organism, RdRp for certain viruses. The RT PCR tests are referred to as "quantitative" as this method allows quantification of the specific RNA/DNA present in a sample.

Absolute quantitation :

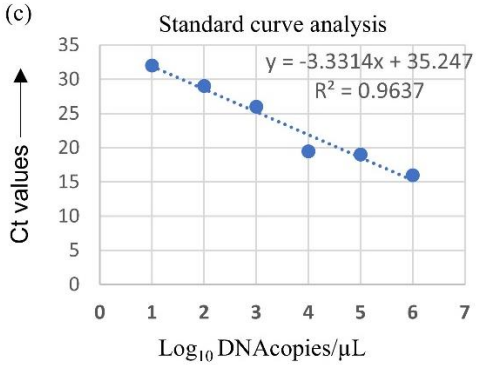
(a)



(b)

No. of copies of DNA/µL	Log_{10} (No. of copies DNA/µL)	Ct Value (mean of 3 replicates)
0	0	0
10	1	32.01
100	2	29.02
1000	3	25.99
10000	4	19.50
100000	5	19.00
1000000	6	16.00

(c)



y = mx+c (equation defining the linear correlation)

$\boldsymbol{y = -3.3314x + 35.247}$

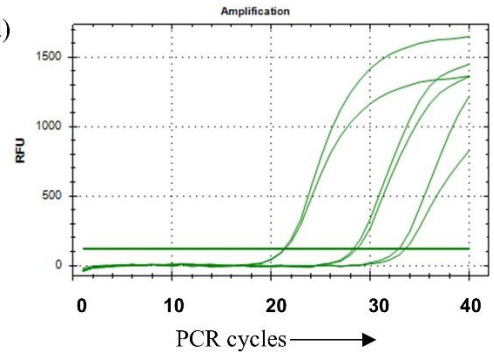
Here: y = Ct Value;

$x = Log_{10}$ (DNA/RNA copies)

DNA copies/µL = 10^x

Relative quantitation:

(d)



For example:
Gene of interest (GOI) is ACE2
Internal control (IC): 18s rRNA

	Ct (GOI: ACE2)	Ct (IC: 18s rRNA)	Δ Ct: Ct(GOI) - Ct(IC)	ΔΔ Ct: Δ Ct (Treated)- Δ Ct (Untreated)	Fold change $2^{-\Delta\Delta Ct}$
Lung epithelial cells	29.1	21.5	7.6	-3.3	9.84
Red blood cells	32.2	21.3	10.9		

Figure-6

OBSERVATIONS

The results of the RT PCR reaction are denoted in terms of the Ct values. The Ct values are further tabulated using either the absolute quantification or the relative quantification method as shown in Fig. 5 and 6.

QUESTIONS FOR FURTHER STUDIES

1. In what ways does the plant derived compound curcumin effect the expression of pro inflammatory of apoptosis related genes. Are the genes up or downregulated?

2. By how many fold did the p53 gene expression change after treatment with curcumin?

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Chapter 52: SLOT BLOT PROTOCOL TO STUDY R LOOPS (DNA-RNA HYBRIDS)

Recommended Level: PG, Research

INTRODUCTION

Slot blot is a molecular biology technique used to detect and quantify nucleic acids (DNA or RNA) or proteins in a sample. It is like Southern blotting (for DNA), Northern blotting (for RNA) and Western blotting (for proteins), but it doesn't involve electrophoresis. Instead, samples are directly applied onto a membrane in discrete spots or slots.

A slot blot can be used to study R-loops, which are three-stranded nucleic acid structures consisting of an RNA-DNA hybrid and the displaced single-stranded DNA.

The function of the R-loop, a three-stranded nucleic acid structure, in regulating gene expression is becoming more widely acknowledged. R-loops were originally believed to be transcriptional byproducts, but more recent research showing less R-loops in diseased cells has demonstrated that R-loops have functional roles in different human cells (Paul et al, 2025). Next, it is crucial to comprehend how cells regulate their abundance and the functions of R-loops. This test can be used to measure R-loop abundance and evaluate the impact of gene alterations, such as senataxin, on R-loop abundance in both research and clinical situations (Dowling et al, 2024; Ramirez et al, 2021).

OBJECTIVES

To compare the expression levels of a particular gene or protein across multiple samples.

To screen many samples for the presence of a specific nucleic acid or protein.

To perform quantitative analysis when the amount of sample is limited.

MATERIALS REQUIRED

Desired cells
1X Phosphate buffered saline
Trypsin
Haemocytometer
Cell lysis buffer
Nuclear lysis buffer
Elution buffer
RNase H
Ethanol
Phenol-Chloroform-Isoamyl alcohol (25:24:1)

PROCEDURE

Cell seeding

Cells were seeded in 6 well cell culture plate and put in incubator overnight until 70-80% confluency is reached.

Cell lysis

Next day wash cells with 1x phosphate-buffered saline (PBS) twice. Trypsin (0.25% in PBS) was used to dissociate cell from surface. Count cells with hemocytometer. Approx 2 × 106 cells were taken in experiment for each sample.

1. Transfer the cell suspension to a 1.5 ml tube
2. Pellet the cells using centrifuge at 300×g for 5 min at 4°C. Remove the media
3. Wash twice with ice-cold 1x PBS and centrifuge each time like previous step
4. Add cold cell lysis buffer to the cell pellet (300 µl per 2 × 106 cells). Resuspend the pellet Incubate on ice for 10 min

Cell lysis buffer	For 10 ml
Water, nuclease-free	9 ml
10% NP-40	0.5 ml
2M KCl	0.4 ml
0.5M PIPES (pH 8.0)	100 µl

5. Spin at 500×g for 5 min to pellet the nuclei
6. Discard supernatant and re-suspend the nuclear pellet in 400 µl of cold nuclear lysis buffer Incubate on ice for 10 min

Nuclear lysis buffer	For 10 ml
Water, nuclease-free	8.65 ml
10% SDS	1 ml
1M Tris-HCl (pH 8.0)	0.25 ml
0.5M EDTA	100 µl

7. Add 3 µl of 20 mg/ml proteinase K and incubate for 3–5 h at 55°C

Purification of Nucleic acid sample

[Sonicate the samples depending on its viscosity]

1. Add 400 µl of elution buffer and 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1 pH 8.0). Vortex for 10 sec

Elution Buffer	10 ml
1M Tris-HCl, pH 8.5	0.1 ml
Water, nuclease-free	9.9 ml

2. Centrifuge at 12,000×g for 5 min at 4°C
2. Transfer the aqueous phase (approximately 350 µl) to a new tube
3. Extract once using 1 volume of chloroform, vortex for 10 sec, then spin down at 12,000×g for 5 min at 4°C. Transfer the aqueous phase to a new tube (approximately 300 µl)

4. Add 35 µl of 3 M sodium acetate (pH 5.2), 1 µl glycogen/glycoblue and 700 µl of ice-cold 100% ethanol
5. Vortex for 10 sec and spin down at 12,000×g for 30 min at 4°C
6. Wash the pellet with 1 ml of 70% ethanol
7. Vortex for 10 s and spin down at 12,000 × g for 15 min at 4°C
8. Discard the supernatant and let the pellet air dry
9. Add 20 µl of elution buffer and vortex for 10 sec to resuspend. Incubate the sample at 4°C overnight to re-suspend the pellet
10. DNA concentration was measured using Nanodrop

Ribonuclease H treatment to assure signal specificity:

One set of samples was treated with RNase H that should show reduction in intensity of s9.6 signal (an antibody widely used to detect global DNA-RNA hybrids). Incubate samples at 37°C for 15 min in 20 µl volumes

Blotting on membrane

1. Prepare different concentrations (dilutions) of eluted nucleic acid sample. (e.g., 12.5 ng, 25 ng, 50 ng, 100 ng).
2. Two Nitrocellulose membrane (positively charged) of appropriate size was taken to blot.
3. Spot 2 µl of each sample onto 2 membranes - One for RNase H untreated and one for RNase H treated sample.
4. Allow the samples to saturate into the membrane. Wait at least 2 min before crosslinking the membrane with UV light.
5. Place the membrane into the center of the UV device and crosslink the membrane using a UV crosslinker (1200 µJ × 100).

Incubation with s9.6 antibody

1. Incubate the membrane in blocking solution (skimmed milk) for 1 h in room temperature on a shaker.
2. Give 1x PBS, PBST (0.1% Tween 20), PBS wash each for 5 min putting on shaker.
3. Incubate samples overnight with primary s9.6 antibody (1:1000 dilution) at 4°C.
4. Next day give 1x PBS, PBST (0.1% Tween 20), PBS wash each for 5 min putting on shaker.
5. Incubate samples in secondary antibody for 2 h.
6. Give 1x PBS, PBST (0.1% Tween 20), PBS wash each for 5 min putting on shaker.
7. Check the blot in an image acquisition equipment.

Image quantification

Image quantification can be done using ImageJ software.

OBSERVATION

The image below (Fig. 1) displays a slot blot that was used to detect RNA-DNA hybrid. A reduction in signal upon RNase H treatment confirms that the detected signal is specific to RNA-DNA hybrid, validating the effectiveness of the assay.

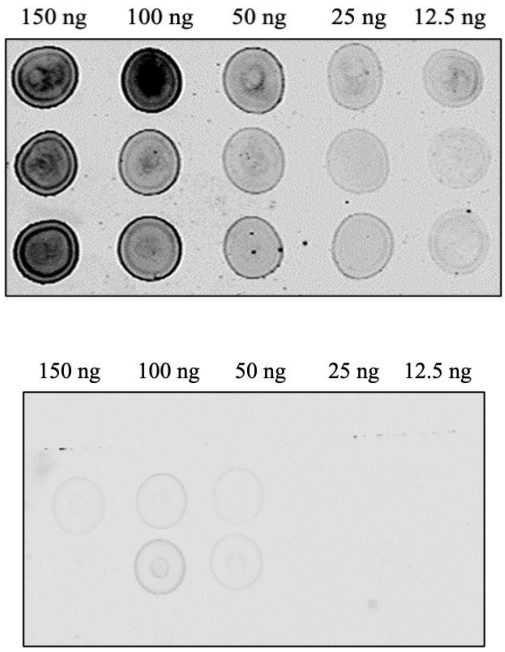

Figure 1. Representative image of slot blot with s9.6 antibody showing that RNA-DNA hybrid formation is more without RNase H treatment whereas treatment with RNase H decreases hybrid formation (Upper panel: -RNase H, lower panel: +RNase H)

QUESTIONS FOR FURTHER STUDIES

1. Why is UV-crosslinking preferred for immobilizing nucleic acids onto nitrocellulose membrane prior to antibody incubation?
2. How do variations in nucleic acid concentration and sample denaturation influence the linearity and sensitivity of R-loop detection in slot blot assay?

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Chapter 53: PROTEIN EXTRACTION FROM CELLS AND QUANTIFICATION

Recommended Level: UG, PG, Research

INTRODUCTION

Protein extraction: Protein extraction is the first step for many biochemical and analytical techniques (SDS-PAGE, Western blotting, mass spectrometry). Efficient disruption and homogenization of animal tissues and cultured cells are required to ensure high yields of proteins. There are physical, chemical and biological methods of protein extraction depending on the nature of the cells and the purpose of the extraction. In recent years, 80% formic acid and 100% acetonitrile are used for quick and efficient protein extraction. Sonication is also applied to enhance cell disruption and to extract membrane bound proteins.

Protein quantification: The Bradford Protein Assay (*Bradford*, 1976) is one of the methods used to measure protein concentration in a sample. Coomassie dye binds to protein molecules under acidic conditions resulted in a color change from brown to blue. The level of blue can then be measured using a spectrophotometer to determine the concentration of protein in the sample. Binding of Coomassie Blue with proteins causes red shift in its absorption spectrum and the absorption maximum shifts from 590 to 620 nm. Spectrometry measurements were recorded at 595 nm to avoid any contribution from the green form of the dye. Bradford Protein Assay measures the basic amino acids, arginine, lysine, and histidine; however, most proteins have a fairly balanced level of these amino acids with all other amino acids. Therefore, Bradford can be used to determine the total protein concentration of a sample. **Note:** Coomassie dyes work by binding to proteins through ionic interactions between the sulfonic acid groups in the dye and the positive amine groups in the protein, and through Van der Waals attractions. There are two kinds of the Coomassie dyes: R-250 and G-250. Coomassie R-250 is sensitive and used variant for protein detection since it can be used to detect as little as 0.1 ug of protein. Coomassie G-250 (also known as colloidal Coomassie dye) is used for gel staining and eliminates the need for destaining the gel.

OBJECTIVES

To extract the whole cell proteins using a single step procedure

To determine the protein concentration using Bradford's method.

MATERIALS REQUIRED

Cell lysis and protein extraction

The choice of buffer depends on the purpose of the experiments. The lysis buffer should be tested before starting the experiment. The following lists the general composition of the lysis buffers (Walker JM 2009):

Lysis buffer (10 ml) – This buffer usually includes the following Buffers and salts (such as Tris-HCl, Bicarbonate buffer, HEPES) that provides pH, ionic strength.

Detergents disrupt the membrane and enhance protein solubility. Detergents may be of non-disruptive - non-ionic such as Triton X100, Tween-20, zwitterionic such as CHAPS (3-[(3-cholamidopropyl) dimethylammonio] -1-propanesulfonate)) and can also denature proteins such as anionic surfactant: Sodium dodecyl sulfate (SDS) or sodium lauryl sulfate (SLS) and cationic such as ethyl trimethyl ammonium bromide.

Chelators (EDTA) – chelates the metallic divalent cations such as magnesium, zinc, manganese, nickel, copper ions required for the DNAase and protease activities.

Protease inhibitors – to block or inactivate endogenous proteolytic and phospholytic enzymes. This helps to minimize the target protein degradation and to preserve the proteins of interest.

The following cell lysis buffer is routinely used. Add the following (% to final volume) and make up to 20 ml

10 ml of 20 mM Tris-HCl pH.7.5
0.1%-Benzonase
1 %-Protease inhibitor
1 mM EDTA
1 % triton X 100
10 % glycerol

Rapid method: In recent years, a single step protocol for cell lysis and protein extraction, for which the requirements are Formic acid (80% v/v) and Acetonitrile.

Protein quantification:

Bovine serum albumin standard stock standard solution: 2 mg/ml of BSA
Working standard solution

Pipetted in a series of tubes 0.2 µl, 0.4 µl, 0.6 µl, 0.4 µl, 0.8 µl, 1.0 µl and 1.2 µl of stock solution and made up to 2.0 ml with distilled water. The working standard contains 0.2, 0.4, 0.6, 0.4, 0.8, 1.0 and 1.2 µg/ml of protein, respectively.

Bradford reagent (**RC DC kit** - reagent compatible and detergent compatible)-)

PROCEDURE

General sample preparation

The sample preparation for protein analysis should be made in fairly large quantities (up to 2.0 ml) and always stored frozen at −18°C in small aliquots. Various samples require different methods or sample preparation, and one must optimize the method of sample preparation according to the need/nature of sample. The methods described here are the ones we use in our laboratory for routine purposes.

W**hole cell protein extraction**

To extract the protein out of cells, several methods are commonly used that includes mechanical disruption, liquid homogenization, high frequency sound waves, freeze/thaw cycles and manual grinding. Here we use chemicals and sonication for lyses our cells. **Note:** Traditionally, various cell lysis buffers were used for protein extraction and in the following rapid extraction using acetonitrile and formic acid, while the sonication step remains the same between the methods.

1. Resuspend the cell pellet in equal volume of acetonitrile (100 %) and 80% formic acid. The volume should be decided by observing the size/volume of the cell pellet, e.g., for a size of 10 µl of cell pellet, 100 µl of acetonitrile and 100 µl of 80% formic acid can be used. Whatsoever the case, the volume of acetonitrile and formic acid should be equal.
2. Vortexed the content thoroughly and sonicate on ice (1 duty cycle, 100% amplitude for 1 min). If sonication is not possible, mix the content for 1 min or mix thoroughly with glass beads.
3. Following sonication, incubate the cells on ice for 20 min with occasional shaking.

4. Centrifuge the sample at 13000 rpm (2271 g) for 10 minutes at room temperature.
5. Transfer the clear supernatant into another set of Eppendorf tube.
6. The extracted proteins can be stored at -20°C until further use.

Protein quantification

Protein estimation is one of the most crucial experiments in proteomics experiments while dealing with quantitative stuffs. Most protein estimation is based either on the Lowry or Bradford method. We use a kid from Bio-rad which utilizes the principle of Bradford method known as RC DC kit (reagent compatible and detergent compatible) since the samples usually contain triton X100/ glycerol /EDTA.

1. In a series of microfuge tubes, pipette out working protein stock solution as detailed in the table. As a black, 20 µl of distilled water was also pipetted in Eppendorf tube.

   Working standard solution

S. No.	Volume of working stock (µl)	Concentration in µg /ml	Volume of distilled water / buffer (µl)	Volume of Bradford's reagent (µl)	OD at 595 nm
1	Blank	0.0	2.0	498	
2	0.2	0.2	1.8	498	
3	0.4	0.4	1.6	498	
4	0.6	0.6	1.4	498	
5	0.8	0.8	1.2	498	
6	1.0	1.0	1.0	498	
7	1.2	1.2	0.8	498	

2. Add 498 µl of Bradford's reagent to all the tubes.
3. Incubate for 2 min in dark condition at room temperature.
4. Measure OD at 595 nm in the spectrometer within 2 min.
5. A standard graph was drawn by taking the protein concentration on x-axis and OD at y-axis. The sample protein concentration was calculated from the graph.

OBSERVATIONS

Whole cell protein extraction: Often a white mass is observed after centrifugation. It is better to store at - 20°C for long-term usage.
Protein quantification: At the end of the experiment, we will be able to determine the concentration (µg/µl) and amount (µg) of the proteins in the extract.

QUESTIONS FOR FURTHER STUDIES

1. The cells undergo cell division through cell cycle, should we consider the cell cycle for effective cell harvesting for protein isolation?
2. Usually, cell culture is carried out at a standard temperature, while the cell harvesting is carried out at 4°C. Will these exposure of different temperature influence the protein expression. If this is the case, flash freezing of cells are recommended?
3. While cell fixation is an advantage for quick inactivation of proteases, will the denaturation of proteins lead to the loss of low abundant or sensitive proteins?

4. What might the exact role of formic acid and acetonitrile in protein extraction?
5. There are several protein quantitative methods that include spectrophotometry (Bradford, Lowry) and fluorometry (Qubit). Should we check the protein quantification constituency by running SDS-PAGE?

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Chapter 54: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS FOR PROTEINS

Recommended level: UG, PG, Research

INTRODUCTION

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most commonly used method to fractionate polypeptides on the basis of their molecular sizes in an electrical field. The gel matrix in polyacrylamide gel provides small pore sizes (actual size depends upon the concentration of polyacrylamide and the ratio of acrylamide to bis-acrylamide) which allows a clear separation of polypeptides of varying lengths along the length of the gel. The use of ionic detergent like sodium-dodecyl-sulphate (SDS) helps to eliminate secondary structures of the polypeptides and also provides a net negative charge to each polypeptide so that all of them move towards the anode (positive pole) when a voltage gradient is applied to the gel. Due to these features, the mobility of a polypeptide in SDS-PAGE is directly proportional to its molecular size (polypeptide length). The chart below shows the linear range of mobility of polypeptides of different sizes (in kilodaltons, kD) in polyacrylamide gels of varying acrylamide concentration (acrylamide to bis-acrylamide ratio being 29:1 in all cases):

% Acrylamide Concentration	Size (in kd) in Linear Range of Separation
15%	12 to 43 kD
10%	16 to 68 kD
7.5	36 to 94 kD
5.0	57 to 212 kD

Thus, depending upon the size of polypeptides that are desired to be resolved, a gel with corresponding concentration of acrylamide may be prepared.

After the electrophoresis, the proteins can be conveniently visualized by staining the gel with stains like Coommassie Blue or Silver. The electrophoresed proteins may also be transferred to nitrocellulose or nylon membranes for immunological detection of specific proteins/polypeptides (Western Blotting) or if these are labeled with ^{35}S-methionine (or other radioactively labeled amino acids), these may be detected by autoradiography (or fluorography) of the dried gel.

OBJECTIVE

To separate the proteins in the given tissue samples according to their molecular sizes in a polyacrylamide gel.

MATERIALS REQUIRED

1. Acrylamide Stock Solution (acrylamide: bis-acrylamide:: 29: 1)
   Acrylamide 29 gm
   N,N'-methylene-bis-acrylamide 1 gm
   Deionised Water 100 ml
   Warm the water to assist dissolution of Acrylamide. Store the stock solution in dark bottle at 10^{o}C.
2. Sodium-dodecyl sulfate (SDS) stock solution
   SDS 10 g

Deionized Water to make 100 ml
Store at room temperature

3. Tris Buffers (1M Stock) - pH 8.8 and 6.8
   Tris base 12.1 g
   Dist. Water 100 ml
   Adjust to pH 8.8 or to 6.8 with 1N-HCl

4. Ammonium persulfate (APS) stock solution
   Ammonium persulfate 10 g
   Dist. Water to make 100 ml

5. N,N,N',N'-tetramethylene diamine (TEMED) - stock stored at 4^{o}C in dark bottle

6. Tris-Glycine Electrophoresis Buffer (5x)
   Tris Base 15.1 g
   Glycine 94 g
   Dist. Water to make 100 ml
   (let the contents dissolve fully before adding SDS solution and water)

   10% SDS 50 ml
   Dist. Water to make 1000 ml
   pH should be 8.3

7. 3 M Sodium Acetate (pH 5.2)
   Sodium acetate 40.81 g
   Dist. Water 80 ml
   dissolve and adjust pH to 5.2 with Glacial Acetic Acid and make the volume to 100 ml

8. 10 mM Sodium Acetate (pH 5.2)
   3 M Sodium Acetate (pH 5.2) 3.3 µl
   Distilled Water to make 1 ml

9. 1M Dithiothreitol (DTT)
   DTT150 mg
   10 mM Sodium Acetate (pH 5.2) 1 ml

10. 100 mM Phenyl methyl sulfonyl fluoride (PMSF)
    PMSF 17.4 mg
    Abs. Ethanol 1 ml

11. Sample buffer
    1 M Tris (pH 6.8) 50 µl
    1 M DTT 100 µl
    10% SDS 200 µl
    1% Bromophenol Blue 100 µl
    Glycerol 100 µl
    100 mM PMSF 20 µl
    Distilled water to make 1 ml

12. Coomassie Brilliant Blue (CBB) Staining Solution

This is a modified Neuhoff procedure (Candiano et al 2004).
To 100 ml water, add phosphoric acid (to obtain 10% in the final 1 l).
Add 100 g ammonium sulphate.
Add 1.2 g Coomassie Blue G-250 (as powder and shake well).
Add distilled water to fill up to 800 ml
Add 200 ml of 100% methanol.
Store this 1 l staining solution at Room temperature in dark. The solution will look dark to light green but will turn to dark blue upon addition to the gel.

13. Gel casting glass plates, spacers and combs (for 15cm x 15cm x 0.6mm gel)

PROCEDURE

A. Protein sample preparation

Take the tissues/cells which are to be used as the source of protein and put them in the sample buffer (volume depends on the amount of protein in the tissue) in a 1.5 ml microfuge tube, close the tube tightly and immediately keep the tube in a boiling water bath for 5-10 min. Make sure that the tube cap does not open in between. After 10 min, briefly spin the tube and load the sample in the gel or store the sample at -70ºC till use.

B**. Casting of vertical slab gel**

Separating the lower gel

1. Assemble the cleaned glass plates with spacers of required thickness
2. For a 12.5% separating polyacrylamide gel, prepare the following solution by adding in the order

Distilled water	3.46 ml
1M Tris (pH 8.8)	7.50 ml
Acrylamide Stock solution	8.36 ml
50% Glycerol	0.40 ml
10% SDS Stock solution	0.20 ml

Mix, filter and degas for 5 min under vacuum (presence of dissolved oxygen inhibits polymerization of Acrylamide)
Add the following polymerizing catalysts

10% Ammonium persulfate	100 µl
TEMED	10 µl

Swirl the solution rapidly, taking care not to create any bubbles, and quickly proceed to the next step

3. Pour the gel solution rapidly into the gap between the glass plates without trapping any bubbles. If any bubbles are trapped, they must be immediately removed either by tapping or by inserting a thin strip of plastic or X-ray film to dislodge the bubble. Leave sufficient space at the top for stacking gel (to be poured later).
4. Carefully overlay the separating gel Acrylamide solution with water saturated isobutanol (to prevent contact of the Acrylamide solution with atmospheric oxygen).
5. Leave the gel mold undisturbed for 45-60 min to let the Acrylamide polymerize. Polymerization is complete when the interface between the gel and the overlaid water becomes distinct.

6. When the polymerization is complete, drain off the overlaid water, wash the top layer several times with distilled water to remove any unpolymerized Acrylamide. Drain the excess water completely.

Stacking of the upper gel

1. Prepare the following stacking gel (5% Acrylamide) solution

Distilled water	3.46 ml
1M Tris (pH 6.8)	0.50 ml
Acrylamide stock solution	0.66 ml
10% SDS	40 µl

Mix and degas under vacuum for 5min and add the TEMED and APS as follows

10% APS	40 µl
TEMED	4 µl

2. Quickly mix by swirling, without creating any air bubbles. Quickly pour the solution between the glass plates on top of the polymerized separating gel. Immediately insert the teflon comb into the stacking gel solution without trapping any air bubbles. The height of the stacking gel (between the teeth of the comb and the separating gel) should not generally exceed 1cm. Leave the gel assembly undisturbed for 45-60 min to let the stacking gel polymerize.

3. Carefully remove the comb and immediately wash the wells by flushing with distilled water using a syringe fitted with a fine hypodermic needle. After a thorough cleaning of the wells, the extending teeth of polymerized Acrylamide are straightened, if required, with a blunt needle.
(Leaving the comb in stacking gel for a longer period, results in polymerization of Acrylamide in the capillary space between the comb teeth and glass plates: this may lead to "streaks" in the fractionated protein bands. Therefore, the comb should be removed as soon as the gel has polymerized)

Running of gel

1. Mount the gel assembly on the vertical gel tank and add the Tris-Glycine buffer to the upper and lower tanks. Care is taken to ensure that no bubbles are trapped in the gel wells.

2. Connect the wire leads from the gel tank to the power supply (the lower tank lead (red) is connected to the positive pole (anode) while the lead from the upper tank (black or blue) is connected to the cathode of the power supply).

3. Load the protein samples to be electrophoresed into the bottom of the well using a micro-syringe. (The actual volume of the sample loaded depends upon the dimensions of the well: it should not make a layer more than a few millimeters in height: for a 5 mm x 0.6 mm well, 20 µl of sample containing about 40 µg protein is adequate).

4. Apply current (20 mA constant for a 15 cm x 15 cm x 0.6 mm slab gel) till the dye enters the separating gel. At that time increase the current to 25 mA (constant) and run till the bromophenol dye front has reached bottom of the gel.
(During the run, it is desirable that the temperature of the gel is maintained between 4-8^{o}C either by keeping the gel assembly in a cold room or, preferably, by circulating cold water in the gel assembly) (Gel tanks have special built-in water circulating channels)

5. After the run is over, the gel assembly is disassembled and one of the glass plates is carefully removed. The orientation of the gel is marked by cutting one of the corners of the gel.

D. Staining of the gel

1. Remove the gel from the supporting glass plate and carefully transfer it to a suitable size tray containing distilled water. Wash once, decant water and add 100 to 120 ml of Coomassie staining solution and kept shaken overnight.

2. The gel is destained by washing several times with distilled water.

E. Protein scanning / image documentation: Several commercial instruments are readily available in the market; however, we recommend ordinary paper scanners such as Canon Scanner LiDE 300 Flat Bed, which allows image documentation at 300 dpi (recommended for publication purpose).

F. Protein MW determination:

a) Calculate the R_f of each of the protein bands of the ladder whose MW is already known by measuring the migration distance of the protein through the gel divided by the migration distance of the dye front. This migration distance is calculated from the top of the resolving /separating gel.
b) A standard curve of the Rf (x -axis) versus log MW (y axis) is drawn using Excel.
c) Draw the straight line and match the R_f value of the unknown protein to determine the MW.
d) Gel analysis using Image J software: We recommend freely available Image J software tool, downloadable at https://imagej.net/ij/ (accessed on 30.06.2024).

MW can be automatically determined using Image J software. Lane to lane comparison and quantification can also be performed using this software. The software manual provides the operational procedures.

OBSERVATION

A Coomassie Briliant Blue stained gel is shown in Fig. 1.

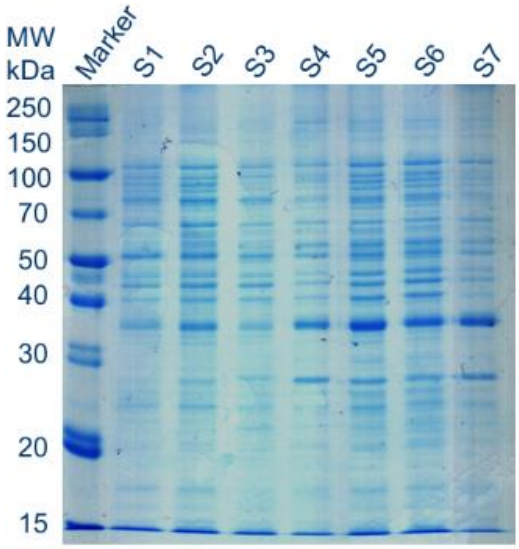


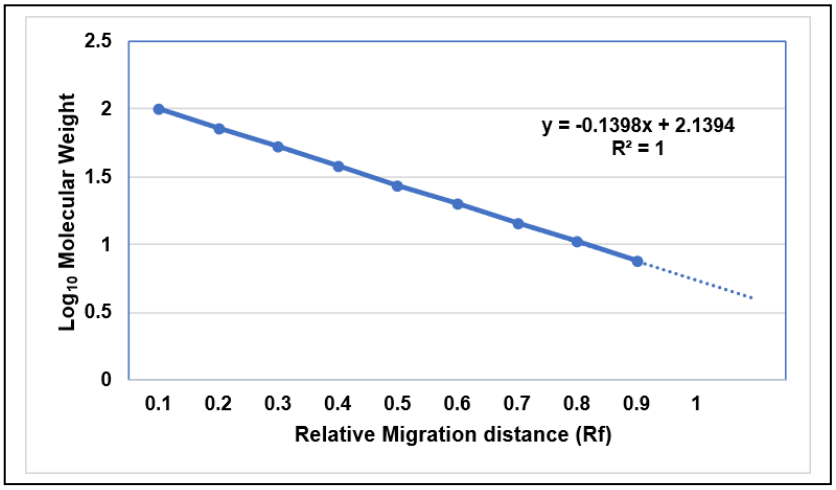
Figure 1. The image of a 12% SDS-Polyacrylamide gel stained with Coomassie Briliant Blue. Lane 1 contains Marker and lane 2 to 8 exracted proteins from *E. coli* (5 µg in each lane).

The R_f of each of the protein bands of a ladder whose MW is already known by measuring the migration distance of the protein through the gel divided by the migration distance of the dye front are

tabulated as shown in (Fig. 2A) and a standard curve of the Rf (x -axis) versus log MW (y axis) is drawn (Fig. 2B).

S. No	RF Value	Log MW	MW in Da
1	0		
2	0.1	2	100
3	0.2	1.860	72.4
4	0.3	1.720	52.5
5	0.4	1.580	38
6	0.5	1.439	27.5
7	0.6	1.301	20
8	0.7	1.161	14.5
9	0.8	1.021	10.5
10	0.9	0.881	7.6
	1		

A



B

Figure 2. (A) R_f of each of the protein bands of a given ladder, their molecular weight and log MW are shown in the table while (B) shows the standard curve drawn from the data.

QUESTIONS FOR FURTHER STUDIES

1. Why it is advisable to keep the table vibration free while making the gel?
2. Why the gel must not get heated during run?
3. Why is it recommended to clean the wells thoroughly before loading?
4. What will happen if an air bubble is trapped in the well while loading the sample?

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Chapter 55: SILVER STAINING OF PROTEINS SEPARATED ON SDS-PAGE GEL

Recommended Level: PG, Research

INTRODUCTION

Silver staining is an excellent method for visualizing nanogram amounts of proteins run on SDS-PAGE. Proteins bind silver ions, which can be reduced under appropriate conditions. Silver staining protocol starts with the fixation step which helps in the immobilization of proteins in the gel and removal of interfering compounds. Then the gel is treated with compounds that either make the proteins more reactive to silver or with reagents that could accelerate the reduction of silver ions. This step increases the sensitivity of the detection of proteins. The third step is the silver impregnation by either plain silver nitrate or ammoniacal silver and the final step is the rinsing and image development to build up the silver metal image. The reaction is stopped before excessive background formation occurs and therefore rinsing removes excess silver ions and other chemicals that could interfere in the image development.

MATERIALS REQUIRED

Fixative solution: 50% Methanol + 10% Glacial Acetic Acid + 10% Formaldehyde. Make up the volume to 100 ml with distilled water

20% Methanol

Silver staining solution: 0.2 g Silver Nitrate + 35 µl Formaldehyde. Make up the volume to 100 ml with distilled water.

Sensitizing solution: 0.02% Sodium thiosulphate + 100 ml distilled water

Developing solution: 6 g sodium carbonate + 2 ml 0.02% Sodium thiosulphate (sensitizing solution) + 50 µl Formaldehyde. Make up the volume to 100 ml with distilled water.

Terminating solution: 12 ml glacial acetic acid + 88 ml distilled water

PROCEDURE

1. Clean hands with 70% iso-propanol. Wear gloves.
2. After completion of SDS-PAGE, remove the gel and place it in a clean container with lid.
3. Pour the fixing solution and place the container on the shaker at room temperature overnight.
4. The next day, discard the fixative and rinse the gel using 20% methanol for 7 mins. Repeat this step 3 times.
5. Discard the 20% methanol and add sensitizing solution for 2 min.
6. Rinse with distilled water 2 times, for 1 min each.
7. Discard the water and add cold silver staining solution and incubate the gel in the dark for 30 mins.
8. Discard the silver staining solution and rinse the gel with distilled water 2 times, for 1 min each.
9. Discard the distilled water and pour the developing solution on the gel.
10. Once the desired level of staining is achieved, terminate the reaction by pouring the terminating solution till the effervescence disappears.

11. Discard the terminating solution and rinse the gel 2 times with distilled water, for 5 mins each.

12. Capture the image and label it appropriately.

OBSERVATIONS

Sharp brown silver-stained bands of the proteins will appear on the gel as shown in the Figure.

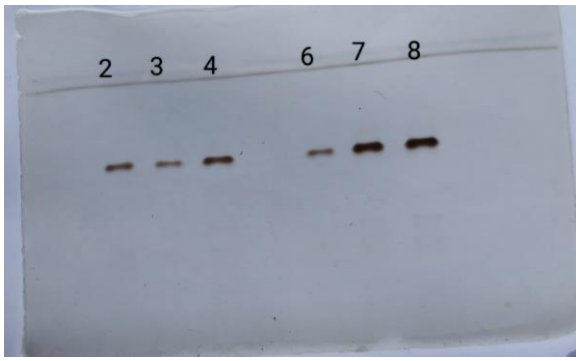

Figure 1. A silver-stained gel showing 1 µg (lanes 2,6), 2 µg (lanes 3,7) and 3 µg (lanes 4,8) BSA run on a SDS-PAGE gel

QUESTIONS FOR FURTHER STUDIES

1. What are the chemical reactions of each step from fixation onwards?
2. Is there any advantage of silver staining over Coomasie Brilliant Blue staining?
3. After silver staining, can the gel be destained and used for Western blotting?

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Chapter 56: WESTERN BLOTTING

Recommended Level: PG, Research

OBJECTIVE

To identify specific protein with molecular size in a mixture of a large number of proteins.

INTRODUCTION

Western blotting is a technique that uses specific antibodies to identify proteins that have been separated based on size by gel electrophoresis. The immunoassay uses a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane and the application of an electrical current induces the proteins to migrate from the gel to the membrane. The membrane can then be further processed with antibodies specific for the target of interest and visualized using secondary antibodies and detection reagents.

MATERIALS REQUIRED

0.5M Tris-HCl (pH- 6.8)

12.114 gm Tris in 200 ml dH_2O; adjust pH to 6.8

1.5M Tris-HCl (pH- 8.8)

36.342 gm Tris in 200 ml dH_2O; adjust pH to 8.8

20% SDS

10% APS (Ammonium persulfate):

1 gm APS in 10 ml dH_2O

10X PBS

Na_2HPO_4 (Sodium phosphate dibasic)	17.8 gm
KH_2PO4 (Potassium phosphate monobasic)	2.4 gm
NaCl	80 gm
KCl	2 gm
dH_2O	1 liter
pH: 6.8	

TEMED

10. Sample Loading buffer

1 M Tris (pH 6.8)	50 µl
1 M DTT	100 µl
10% SDS	200 µl
1% Bromophenol Blue	100 µl
Glycerol	100 µl
100 mM PMSF	20 µl
Distilled water to make	1 ml

Running buffer

Glycine	14.4 gm
Tris	3.03 gm
SDS	1 gm

dH_2O	1 liter (maintain)

Transfer Buffer

Glycine	14.4 gm
Tris	3.03 gm
dH_2O	900 ml
Methanol	100 ml

Staining Solution (100 ml)

Methanol	50 ml
Glacial acetic acid	10 ml
dH_2O	40 ml
Coomassie brilliant blue	50 mg

De-staining Solution (100 ml)

Methanol	45 ml
Glacial acetic acid	10 ml
dH_2O	45 ml

Lysis buffer

RIPA Lysis Buffer

20 mM Tris, pH:8.0
1 mM EDTA
0.5 mM EGTA
0.1% sodium deoxycholate
150 mM NaCl
1% IGEPAL(Sigma)
10% glycerol

NP-40 Lysis Buffer

Thermo Scientific: Cell Lysis buffer II (Cat No:FNN0021)

PROCEDURE

Cell Seeding

Cell trypsinization

1. Take out T-25 flasks containing cells in 4 ml media
2. Check for 70-80% cell confluency under microscope
3. (Inside Laminar Hood) Discard the old media in the sink and wash the cells with 2 ml 1X PBS and 1 ml 0.01M EDTA
4. Add 250 µl (750 µl for T-75 flask) of 1X Trypsin-EDTA (0.05%) and incubate inside the CO_2 incubator for 3-5 mins
5. After Trypsin incubation, add 1-2 ml (4-6 ml for T-75 flask) media to the cells and transfer the cell suspension to a 15 ml screw-capped tube
6. Centrifuge the tube (with weight balance) at 2500 RPM for 3 mins
7. Resuspend the pellet in 1ml media (6ml media if the pellet is large).

Cell Counting (Hemocytometer) and Seeding

1. Add 20 µl of trypan blue on a parafilm and add 20 µl of resuspended cells in 3 ml media.
2. Add 10 µl from this mixture to each side of the hemocytometer.
3. Take reading from the hemocytometer.
4. After calculating the volume based on number of cells required, resuspend cells in a sub stock and add 1.5 ml of cell suspension to each well in a 6 well plate.

Lysate collection

At this point, one should have treated the cells of interest and have verified transfection success under fluorescence microscope. 3 images of each well should be clicked. After 24 h of treatment, the following lysate collection step is performed.

1. Prepare Lysis buffer in the ratio of 1:1000 (Protease inhibitor cocktail, PIC: NP-40 lysis buffer)
2. Remove media from the wells
3. Add ice-cold 1X PBS to the wells
4. Completely remove the 1X PBS after wash
5. Add 70 µl of Lysis buffer (volume of lysis buffer depending on the well size and cell confluency).
6. Scrape the cells and collect the suspension in microfuge tubes.
7. Store the samples in -80°C until further use.

BCA Protein estimation assay

1. Make reagent A and B in the ratio of 49:1 (A:B) for each sample.
2. For each sample, prepare 400 µl of reagent so that 80µl of the analyte could be added to each well in a 96 well plate.
3. Further, add 1 µl of protein sample to each tube (1 µl is added corresponding to 1 µl BSA's concentration that was estimated)
4. Vortex the samples.
5. Add 80 µl of each sample in quadruplicate in a 96 well plate.
6. Take absorbance reading at 560 nm.
7. The resulting absorbance should be divided by 0.01 (The concentration of 1µl BSA is 0.01 µg) to estimate the concentration of the protein sample.

Casting SDS gel

1. Wash the plates with water and keep it in the cassette and pour DDW to check for any leakage by waiting for 10 mins.

2. Make resolving gel with the following composition:

S. No.	Components	6%	8%	10%	12%	15%
1	H_2O	5.25 ml	4.65	3.85	3.25	2.25
2	Acrylamide/Bis-acrylamide (29:1)	2.0 ml	2.6	3.4	4.0	5.0
3	1.5 M Tris (pH 8.8)	2.6 ml	2.6	2.6	2.6	2.6
4	20% SDS	50 µl	50 µl	50 µl	50 µl	50 µl
5	10% APS	100 µl	100 µl	100 µl	100 µl	100 µl
6	TEMED	10 µl	10 µl	10 µl	10 µl	10 µl

3. Meanwhile, the test water in the plate should have been discarded and the plate should be dry.
4. Immediately after adding TEMED, the tube should be vortexed, and the resolving gel should be casted.
5. A layer of Isopropanol should be added on top of resolving gel for uniform levelling of the gel.
6. Once the gel gets solidified, 2% stacking gel with the following composition should be prepared:

S. No.	Components	Volume (5 ml)
1	H_2O	3.075 ml
2	30% Acrylamide:Bisacrylamide (29:1)	670 µl
3	0.5M (6.8 pH) Tris HCl Buffer	1.25 ml
4	20% SDS (0.1% final)	25 µl
5	10% APS	25 µl
6	TEMED	

7. Stacking gel should be vortexed and added using a pipette.
8. Comb should be immediately fixed, and the gel should be let undisturbed for polymerization.

Sample preparation and loading

1. Prepare your samples by the time the gel polymerizes.
2. Samples should be prepared in the ratio of 3:1 (Sample in 1X PBS: 4X dye).
3. Once the samples are ready, they should be heated in thermomixer at 95°C for 5 mins.
4. Samples should be cooled down and meanwhile the comb should be removed inside the gel tank with electrode buffer.
5. Perform a pre-run of the gel for 5 mins to be assured of any buffer leakage.
6. Samples should be loaded in the wells and the gel should be run at 80-100 V (Voltage can change depending on the size of the protein) for ~90-180 min or till the desired protein is resolved.

Blotting

1. Cut Nitrocellulose membrane to the required size.
2. In a glass tray, add ice-cold transfer buffer add setup the transfer cassette as shown in Fig. 1.
3. Once the setup is ready, run it at 100 V for 90 mins for the transfer to happen.

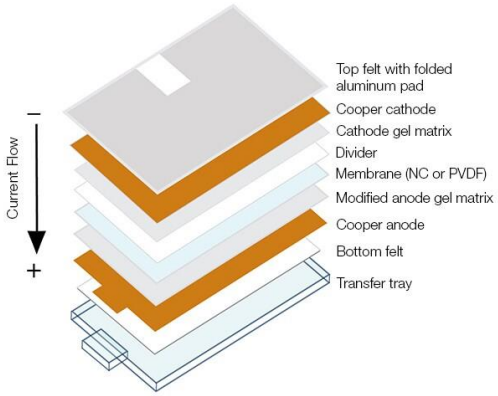

Figure 1. Representative image showing the sequence of filter paper, gel and membrane, image courtsey (https://www.thermofisher.com)

Blocking and antibody incubation

11. After 90 mins, remove the setup and transfer the membrane to a boat and incubate it in blocking solution (5% skim milk in 1X PBS) for 1 h in a rocker.
12. After 1 h, remove the blocking solution and add 1X PBS and incubate on rocker for 5 mins.

OBSERVATION

The image below (Fig. 2) shows a Western blot detecting LINE-ORF1p tagged with GFP and p53 tagged with mCherry in different samples. β-actin was used as a loading control to assess equal protein loading across lanes. Clear bands for ORF1p-GFP and p53-mCherry indicate successful expression of the tagged protein in this case while equal intensity of β-actin bands validate the reliability of comparison.

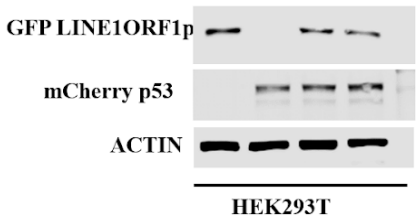

Figure 2. β-actin was used as control and blot against GFP and mCherry to check for our overexpressed protein of interest.

QUESTIONS FOR FURTHER READING

1. How does protein transfer efficiency vary with membrane type and electroblotting conditions?
2. What parameters must be optimized to minimize non-specific binding of the antibody?
3. How do blocking agents and detergent composition influence signal clarity and background reduction?

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Chapter 57: CO-IMMUNOPRECIPITATION

Recommended Level: PG and Research

OBJECTIVE

To identify the interacting partners of a given protein.

INTRODUCTION

The word ‘immuno’ stands for antibody and the word immunoprecipitation implies that we are pulling down a protein using an antibody. It is a technique which can be used to concentrate a particular protein and study its interacting partners. By pulling down a known protein, we can pull down and identify the unknown proteins interacting with it. This works when the proteins involved in the complex bind to each other tightly, making it possible to pull multiple members of the complex out of the solution by latching onto one member with an antibody. This concept of pulling protein complexes out of solution is sometimes referred to as a "pull-down". Co-IP is a powerful technique that is used regularly by molecular biologists to analyze protein–protein interactions.

MATERIALS REQUIRED

HEK-293T, culture discs, Transfection kit

Phosphate buffered saline (PBS), NP-40 lysis buffer

Desired antibody, Immobilized Protein A/G, Bovine serum albumin

Components of SDS-PAGE

PROCEDURE

Seeding

Seed the cells HEK-293T cells in 10 cm disc (About 50-60% confluent next day) and incubate overnight at 37℃ at 5% CO_2.

Transfection

Transfect the cells with desired plasmids through calcium phosphate transfection kit (For HEK-293T cells only) and incubate the plate for 18-24 h. For endogenous IP cells, should be 80-90% confluent for adherent cells.

Cell Lysate preparation

Remove the medium and wash the cells with 5 ml of ice-cold PBS twice. Lyse the cell in NP-40 lysis buffer (Protease inhibitor + PMSF + Phosphate) and make whole cell lysate. (450 µl lysis buffer for 10 cm 2 µ disc). Collect 50 µl of lysate for input sample and use the rest of the lysate for IP.

Antibody

In 400 µl lysate add desired antibody (2-3 µg) and keep in the tube rotator for 12 h at 4^{O}C cold room. (seal the MCT cap with parafilm to avoid spillage) [Endogenous IP-Maximum incubation period: 12-14 h]

Immunoprecipitation uses 50 µl of settled Immobilized Protein A/G (100 µl resin slurry). This amount of resin is sufficient to bind 25-250 µg of antibody. Depending on the amount of antibody needed to immunoprecipitated the desired amount of antigen, we need to optimize the volume of resin and

suggested wash and elution volumes accordingly. It must be noted that there is proper mixing at each step.

Add 100 µl of Immobilized Protein A/G resin slurry to a new microcentrifuge tube and briefly centrifuge at 1200 g for 1 min to pellet resin. Discard the supernatant. Wash the beads twice with an NP40 lysis buffer and twice with 1X PBS. Volume is dependent on the volume of the bead's slurry. For example, if we take 100 µl of beads slurry we wash with twice the volume, i.e., 200 µl of NP-40/1X PBS. Centrifuge briefly each time at 1200g for 1min.

Blocking

Block the Protein A/G beads with 3% of 2 mg/ml of BSA overnight. This will be done simultaneously during antibody incubation with the lysate. Blocking is done to prevent nonspecific binding of antibodies to proteins present in the cell lysate.

Washing

The blocked Protein A/G beads are washed two times with NP40 lysis buffer and two times with 1X PBS. This ensures specific antibody binding to the protein of interest. After each step of washing, beads are centrifuged at 1200 g for 1min

Incubation

The lysate + antibody conjugate is incubated with blocked protein A/G beads for 4-5 hours.

Washing

Beads complexed with antibody and conjugated protein are washed twice with NP-40 lysis buffer and twice with 1X PBS to remove unspecific binding of the protein complexed antibody to the beads.

NOTE: The number of washes must be standardized according to the experiment performed.

Elution

After the last wash the beads complex is centrifuged and 40 µl of 2X Laemmli sample loading dye (β-mercaptoethanol added) and heated at 95˚C for 5-8 mins.

SDS-PAGE gel runs to check for the protein of interest. Input must be run first to check for equal transfection and equal loading of the sample. Then IP samples must be run on SDS-PAGE to check for any interaction.

OBSERVATION

The image below (Fig. 1) shows the result of a co-immunoprecipitation experiment. Cells were transfected with constructs expressing LINE1 ORF1p tagged with GFP and p53 tagged with mCherry. A GFP antibody was used to pull down ORF1p and associated proteins. mCherry antibody was used to detect the presence of p53 protein on western blot. The presence of a band in the IP lane confirms that p53-mCherry was pulled down along with ORF1p-GFP.

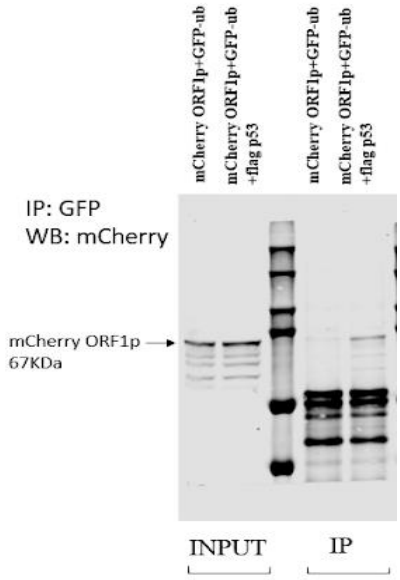

Figure 1. Sample run of input and pulldown samples (Pulldown with GFP antibody). Western blot was performed using mCherry antibody.

QUESTIONS FOR FURTHER READING

1. How do lysis buffer composition and salt concentration affect the preservation of native protein-protein interactions during co-immunoprecipitation?
2. What controls are essential to distinguish specific protein interactions from non-specific antibody binding?
3. How do antibody immobilization methods influence pull-down efficiency and complex stability in co-immunoprecipation assays?

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Chapter 58: PROXIMITY LIGATION ASSAY (PLA): A POWERFUL AND VERSATILE TOOL TO STUDY PROTEIN-PROTEIN INTERACTIONS *IN SITU*

Recommended Level: PG, Research

INTRODUCTION

Protein-protein interactions (PPIs) govern many biological processes including gene expression, signal transduction, metabolism, immune responses, etc. The sub-cellular localization, stability and activity of a protein mostly depend on its interaction with other proteins, and disrupted/altered PPIs may be implied in disease conditions. Therefore, PPI studies help identify the mechanisms governing cellular homeostasis, disease development, etc. Co-immunoprecipitation, yeast two hybrid assays, phage display, etc., are some techniques used to study PPIs.

Proximity Ligation Assay (PLA) is a technique that employs the principles of immunofluorescence (IF) and polymerase chain reaction (PCR) to identify and/or quantitate interaction between two proteins, *in situ* (within cells/tissues). Briefly, interaction between the two desired proteins is detected by primary antibodies raised in two different hosts (Fig. 1A). These in turn are recognized by oligonucleotide-labelled, host-specific secondary antibodies (Duolink®In Situ PLA® Probes; Figure 1B). The oligos attached to the secondary antibodies MUST be of opposing polarity (one PLUS and one MINUS) (Fig. 1B). In a consequent ligation step, oligos of opposite polarity in close proximity are linked by two connector oligos thereby forming a circular-DNA template after the ligation reaction (Figure 1C). This circular-DNA undergoes rolling circle replication in an amplification step (Figure 1D). Fluorescently labelled oligos in the amplification buffer bind to complementary sequences in the resulting amplicon (Figure 1E) allowing for a 1000-fold amplification of the interaction signal. Consequently, PPIs in a cell maybe visualized as discrete spots/PLA-foci using fluorescence microscopy (Reviewed in 1-3).

The usage of primary antibodies enables the user to monitor interactions between a diverse range of protein partners, with high specificity. Furthermore, the connector oligos link the PLUS and MINUS probes only when the two target proteins are less than 30 nm apart, thereby enabling *in situ* detection of PPIs with a low false positive rate. Additionally, PLA may also be used to study protein modifications, e.g., Ubiquitination, SUMOylation, etc. Moreover, PLA maybe used to assess PPIs in a variety of sample types, e.g., adherent cells, suspension cultures, cytospin-preparations, tissue samples, etc. Modified versions of the technique enable flow-cytometric or brightfield PPI detection [4,5]. Thus, PLA is a highly versatile, specific and sensitive assay to identify and monitor PPIs to study cellular homeostasis, disease progression, drug therapy, etc. (Reviewed in 1, 2).

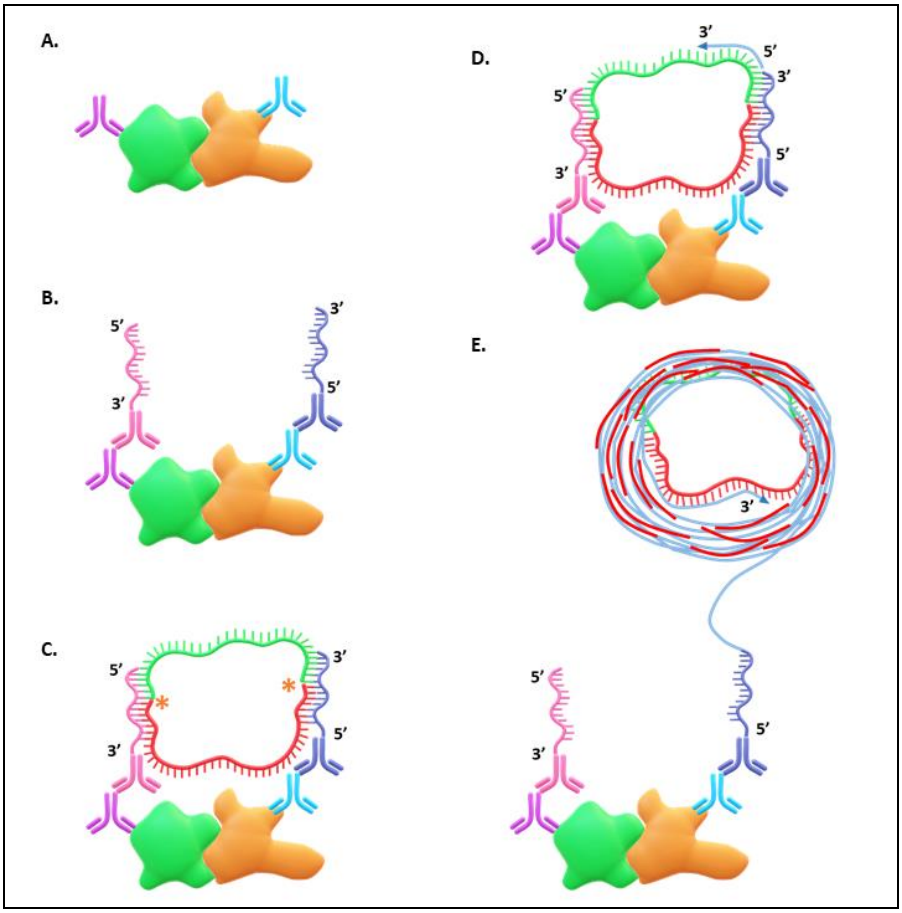


Figure 1. Principle of Proximity Ligation Assay (Adapted from [1]. (A) Primary antibodies bind specifically to the epitopes on the proteins of interest. (B) Duolink® In Situ PLA® Probes are secondary antibodies conjugated with oligos (pink and dark blue). These PLA probes bind to their respective primary antibodies. (C) In the ligation step, single stranded connector oligos (green and red) with partial complementarity at their 5' and 3' ends to the PLA probe oligos, bind to complementary sequences on the PLA probes (pink and dark blue). When the two proteins of interest are in close proximity (<30nm), the connector oligos are ligated together forming a circular DNA template (In this figure the red oligo would be ligated to the green oligo at the ligation junction indicated by the orange asterisk). When the proteins of interest are farther apart (>30nm), the oligos form a linear DNA strand, and are not ligated into a circular DNA. (D) In the amplification step, Polymerase mediated amplification (Blue arrow) of the circular DNA occurs based on rolling circle replication model. (E) PLA foci are formed when fluorescent oligos (red) hybridize with complementary sequences in the amplicon (blue).

OBJECTIVES

To determine if protein X and Protein Y interact with each other
To determine if interaction between protein X and protein Y changes under given conditions (diseased state, infections, drug treatments, etc.)

MATERIALS REQUIRED

Desired cell sample cultured on a glass coverslip [diameter = 12 mm, Blue Star]
PLA stage
1X PBS
4% Paraformaldehyde (PFA) [Sigma, P6148] or chilled 100% methanol [HPLC grade]
0.5 % TritonX-100 [USB, 22686]
Wash Buffers for fluorescence (A and B) (provided by Sigma)

Desired primary antibodies raised in different hosts (Host Range: Rabbit, Mouse, Goat)
Duolink® In Situ PLA® Probe Anti-Rabbit (/Mouse/Goat) Plus* [Sigma, DUO92002]
(Kit contains Duolink® Blocking Solution (1X), Duolink® Antibody Diluent and 5X PLA Probe Anti-Rabbit PLUS)
Duolink® In Situ PLA® Probe Anti-Mouse (/Rabbit/Goat) Minus* [Sigma, DUO92004]
(Kit contains Duolink® Blocking Solution (1X), Duolink® Antibody Diluent and 5X PLA Probe Anti-Mouse MINUS)
Duolink® In Situ Detection Reagents (Red/Green/Orange) # [Sigma, DUO92002 or DUO92014]
(Kit contains 5X Ligation Buffer, 1X Ligase (1 U/µl), Duolink Amplification 5X Buffer and 1X polymerase (10 U/µl)
Mounting Medium [Vectashield, H-1000]
Glass slides
Humidity chamber
37°C incubator
Fluorescence Confocal Microscope

*Similar to the principle of immunofluorescence, the antibody against the two proteins of interest should be raised in separate host species. Consequently, the Duolink®In Situ PLA® Probes Minus/Plus should each target one of the primary antibodies. For example, to detect interaction between proteins X and Y, one could use anti-X raised in rabbit and Duolink®In Situ PLA® Probe Anti-Rabbit Plus to detect it, while anti-Y could be raised in mouse and Duolink®In Situ PLA® Probe Anti-Mouse Minus maybe used to detect it. # Duolink® In Situ Detection Reagents may be used in either colour. However, in case the samples are expressing GFP- and/or RFP-tagged proteins, the colour of the PLA detection reagents should be selected accordingly.

PROCEDURE

Cell culture and treatments

1. Seed the desired cells on glass coverslips and culture them.
2. The necessary treatments (drug treatment, protein overexpression, etc.), the effects of which on protein-protein interaction are to be assessed, can be performed.
   Note: For best results, the cell confluency at the time of fixation should be 80-90%. Alternatively, tissue sections, suspension cells, etc. may also be processed for PLA post appropriate sample preparation

Preparing for PLA

1. PLA Stage: Prepare a PLA stage (same stage used for immunostaining of coverslips) to process the coverslips by attaching a sheet of parafilm to a sturdy plastic box as shown in Figure 2A. Ensure that the stage fits well into the humidity chamber (Figure 2C). Additionally, keep ready another box that can sit atop this stage, like a lid, and cover the coverslips (Figure 2A).
2. Humidity Chamber: Take a breadbox, large enough to accommodate the PLA stage and its lid. Wrap the box and its lid externally, with Black paper or Aluminium foil (As the 'Amplification' step of the PLA is light sensitive, this will prevent stray light from interfering with the assay). Prepare a humidity chamber by layering the base of the bread-box with tissue/blotting paper (Figure 2B). Thoroughly soak the tissue/blotting paper in RO water. (One may require to resoak the paper between steps to prevent drying of the humidity chamber during incubation steps)
3. Set an incubator to 37°C. Preferably place a bowl filled with water in the incubator, to increase the moisture content within the incubator.

Fixation

1. Make 100 µl 1X PBS spots on the PLA stage to transfer the coverslips. Using forceps, transfer the coverslips to the PLA stage, into the 1X PBS, cell-side facing upwards.
2. Wash the coverslips thrice with 500 µl 1X PBS.
3. Add 100 µl of 4 % PFA to each coverslip, and incubate at room temperature (RT) for 20 min. Alternatively, the coverslips maybe fixed by incubating the coverslips in 100 µl chilled methanol, for 5 min, at RT.
4. Post incubation, remove the fixative and wash the coverslips thrice with 100 µl 1X PBS.

Permeabilization

(To be done ONLY when fixation is performed with 4 % PFA)

1. Add 100 µl 0.5 % Triton-X 100 to each coverslip and incubate at RT, for 15-20 min.
2. Wash the coverslips thrice with 100 µl 1X PBS.

Note: As methanol fixes and permeabilizes the cell, the permeabilization must be skipped if fixation is done with methanol. However, one can give a 0.1 % Triton-X 100 wash to the coverslips to ensure the sample is fully permeabilized.

Blocking

1. Each Duolink® In Situ PLA® Probes Minus/Plus kit contains a Duolink® Blocking Solution (1X). Thoroughly vortex the blocking solution.
2. Ensure the PBS has been completely removed from the coverslips. Immediately add 1 drop (~40 µl) of the Duolink® Blocking Solution to each coverslip. Ensure that the solution is uniformly spread across the coverslip.
3. Cover the stage with the lid, and carefully place the set-up inside the humidity chamber. Place the humidity chamber inside a 37°C incubator. Incubate for 1 h. DO NOT wash the coverslips post incubation.

Primary Antibody Incubation

1. Vortex the Duolink® Antibody Diluent, provided in the Duolink® In Situ PLA® Probes MINUS/PLUS kit, thoroughly. Dilute the primary antibodies in the Duolink® Antibody Diluent, to the desired concentration (50 µl of primary antibody solution per coverslip; See note on how to determine primary antibody concentration below).
2. Remove the blocking solution from the coverslips, completely. Do not wash the coverslips. Immediately add the primary antibody solution to the coverslips.
3. Incubate in the humidity chamber at 37°C for 45 mins or at RT for 1 h.
4. Post incubation, remove the antibody solution and wash the coverslips twice for 5 min with 1X Wash Buffer A.

Duolink® In Situ PLA® Probe Incubation

1. Dilute the 5X PLA probe PLUS and 5X PLA probe MINUS, each specific to one of the primary antibody hosts, in the Duolink® Antibody Diluent as indicated in Table 1.

Table 1: Preparation of Secondary Probe solution

Component	Volume for 1 reaction
Duolink® Antibody Diluent	24 µl
5X PLA probe PLUS	8 µl
5X PLA probe MINUS	8 µl
Total Volume	40 µl

2. Remove the wash buffer completely. Immediately add the 1X PLA probe solution to the coverslips.
3. Incubate in the humidity chamber at 37 °C for 1 h.
4. Post incubation, remove the solution and wash the coverslips twice for 5 min with 1X Wash Buffer A.

Ligation

1. Dilute the 5X Ligation Buffer to 1X with nuclease free water as indicated in Table 2. Add the Ligase enzyme to this ligation solution at a final concentration of 1 U per reaction.

Table 2: Preparation of Ligation Solution

Component	Volume for 1 reaction
Nuclease free water	31 µl
5X Ligation Buffer	8 µl
1X Ligase (1U/µL)	1 µl
Total Volume	40 µl

2. Give the coverslips one wash with 100 µl nuclease free water. Remove the nuclease free water completely.
3. Immediately add the Ligation solution to the coverslips.
4. Incubate in the humidity chamber at 37°C for 45 min.
5. Post incubation, remove the solution and wash the coverslips twice for 5 min with 1X Wash Buffer A.

Amplification

Note: The amplification buffer is light sensitive so prevent excessive exposure of the buffer or coverslips, from this step on to light.

1. Dilute the Duolink Amplification 5X Buffer to 1X in nuclease free water as indicated in Table 3. Add the Polymerase enzyme at a final concentration of 5 U per reaction.

Table 3: Preparation of Amplification Solution

Component	Volume for 1 reaction
Nuclease free water	32 µl
Duolink Amplification 5X Buffer	8 µl
1X Polymerase (10 U/µl)	0.5 µl
Total Volume	40.5 µl

2. Give the coverslips one wash with 100 µl 1X nuclease free water. Remove the nuclease free water completely.
3. Immediately add the Amplification solution to the coverslips.
4. Incubate in the humidity chamber at 37 °C for 1 h 30 min.
5. Post incubation, remove the solution and wash the coverslips twice for 10 min with 1X Wash Buffer B and then for 1 min in 0.01 % Wash Buffer B.

Nuclear staining

1. Dilute Hoechst or DAPI stain appropriately in 1X PBS (100 µl per coverslip).
2. Add this solution to the coverslips and incubate for 5-10 min.
3. Wash the coverslips thrice with 500 µl 1X PBS.

Mounting

1. Take 1 drop (per coverslip) of mounting medium on a glass slide.
2. Using forceps pick up the coverslips from the stage. Gently remove any excess PBS solution from the coverslip by dabbing the rim of the coverslip on a tissue. Place the coverslip face down in the drop of mounting medium, ensuring no air bubbles form during the process.
3. Once all the coverslips are mounted, dab out any excess mounting medium and seal the coverslips with a transparent nail-polish.

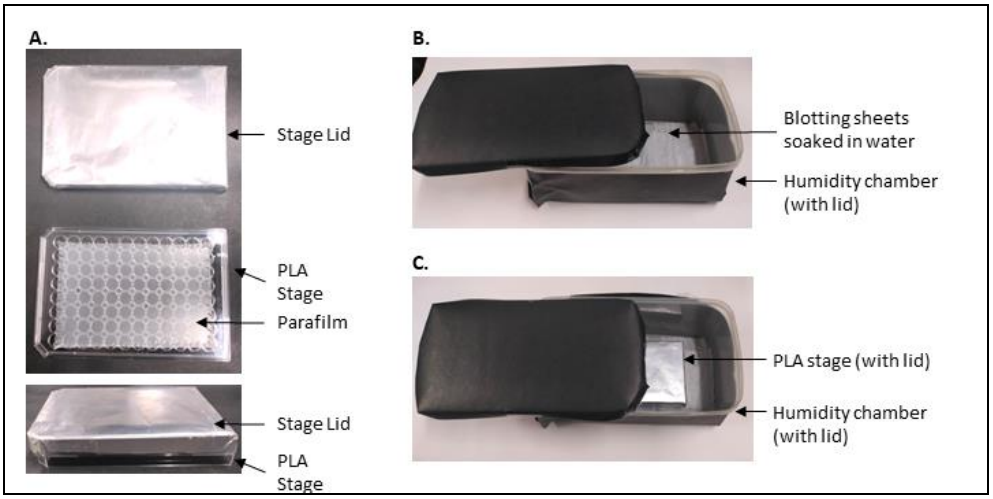


Figure 2: PLA Stage and Humidity Chamber set-up. (A) Top: PLA stage was prepared by putting a parafilm sheet on a plastic platform. Here, we have used the lid of a 96-welled plate as a stage (middle). Another 96-welled plate lid was wrapped with aluminum foil and used as a lid for the PLA stage. The lid must sit sturdily atop the PLA stage (bottom). (B) A bread box wrapped in black paper was layered with tissue paper to prepare a humidity chamber. The tissue paper must be thoroughly soaked with water. (C) The PLA stage is placed inside the humidity chamber.

OBSERVATION

The coverslips may be viewed / images may be obtained using the 63X or 100X lens of a confocal microscope.

Quantitation

The number of PLA dots per cell may be quantitated manually or using NIH ImageJ, CellProfiler or other software.

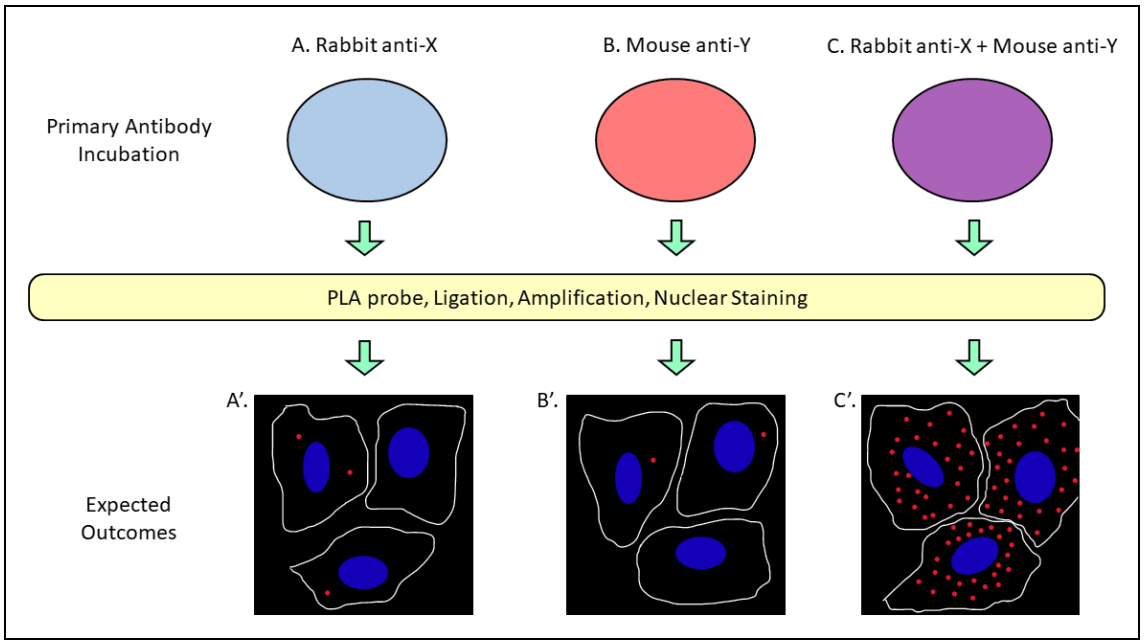
If required, use appropriate statistical tests (t-tests, One-way ANOVA) to compare between

a) single primary antibody versus a combination of primary antibodies to determine if two proteins interact
b) two or more treatment groups, to determine if the specific conditions/treatments affect PPI

Dos and Donts/Tips

- Prepare the solutions for any step during the last 10 min of the incubation time of the previous step. (Note: all reaction volumes are for one 12 mm diameter coverslip)
- After adding the primary/PLA probe/ligation/amplification solution to the coverslips, one can carefully pipette the solution back and forth slowly to ensure that the concentration of the solution is uniform across the coverslip.
- Ensure that the coverslips do not dry between steps or during washing. Moreover, while the commercial protocol suggests using a 40 µl 1X reaction mix per coverslip for the PLA probe incubation, ligation and amplification steps, we recommend using 50 µl of a 0.75X dilute reaction mix in order to prevent drying of the coverslips during incubation periods.
- PBS may be used for washing the coverslips between steps. Give 3 washes of 500 µl to each coverslip. Incubate the coverslips in the wash buffer for 30 sec to 1 min during the washes to reduce background.
- The ligation and amplification buffers need to be stored at -20°C. As a result, the buffer components tend to precipitate and need to be thoroughly resuspended before use. It is therefore recommended to properly thaw, thoroughly resuspend (by pipetting back and forth) and aliquot (to suitable volumes) the ligation and amplification buffers during/before the first use, and store the aliquots at -20°C. This will prevent repeated freezing and thawing of the entire buffer, and in the case of the amplification buffer will prevent repeated exposure of the buffer to light. Nevertheless, ensure that all precipitated components (either in the main vial or aliquot vial) have been solubilized completely before diluting.
- Preferably add the ligase or the polymerase enzymes to their respective buffers just prior to adding the solutions to the coverslips to prevent enzyme inactivation.
- The amplification buffer is light sensitive. Prevent exposing it to light. All treatments of coverslips post amplification step should preferably be done in low light conditions. Store the coverslips in dark conditions.
- It is recommended to view or take images of the PLA samples in 48 h of completion of assay. Slides can be stored at -20°C.
- Determining the concentration of primary antibody to be used: In general, antibodies compatible for IF or immunoprecipitation are compatible for PLA as well. Nevertheless, if the concentration of the antibody used is too high, non-specific PLA foci can develop. To determine the concentration of the antibody to be used for PLA, one must first determine the concentration of

the antibody that works for IF (and gives minimal background staining / noise). Generally, a 1:2 or 1:3 further dilution (of the concentration that works for IF) works for PLA. Next, the ability of the antibodies to generate specific PLA signals devoid self-PLA puncta may be assessed as in Figure 3, using single primary antibody controls. Briefly, using only a single primary antibody, should give no PLA puncta (however sometimes a few puncta maybe observed), while PLA foci indicative of PPI should be seen only when both primary antibodies are used. (Note: The number of PLA foci per cell may vary for different protein partners, and may range between as few as 8-10 dots to 100 or more dots per cell) Alternatively, a similar experimental set-up may be used to determine if two proteins can interact.



- **Figure 3: Experimental Set-up for verifying primary antibody concentration.** Seed cells on three coverslips, and proceed with the fixation, permeabilization and blocking steps as discussed. Next, incubate one coverslip each with the following primary antibody solutions: (A.) Single antibody control for protein X: Anti-X diluted appropriately based on the concentration determined by immunofluorescence. (B) Single antibody control for protein Y: Anti-Y diluted appropriately based on the concentration determined by immunofluorescence. (C) PLA for X and Y: Anti-X + Anti-Y solution containing the antibodies in the same concentration as that in (A) and (B), respectively. Post primary antibody incubation, process the coverslips for PLA probe incubation to nuclear staining as discussed in the protocol. The results should be as indicated in the expected outcomes section below. Both single antibody controls, (A') and (B'), should have zero to very few (negligible) dots per cell. However, when in combination (C'), specific PLA foci should be observed. If the number of dots in the single antibody controls, (A') and (B'), are too high, further dilutions of the antibodies should be assessed to determine the concentration that gives specific PLA foci with no foci in the single antibody controls. A similar experimental setup maybe used to determine if protein X and protein Y interact.

QUESTIONS FOR FURTHER STUDIES

1. The primary antibodies used to detect the two proteins for PLA can be raised in the same host. State TRUE or FALSE, and justify.
2. ER-Mitochondrial Contact sites (ERMCS) are subdomains of the ER that are tethered to the mitochondria by complex formation between ER-membrane and outer mitochondrial membrane proteins. E.g., VAPB is an ER protein that binds to the mitochondrial protein PTPIP51 thus bringing the ER and Mitochondria as close as 30 nm to each other. Professor X

is interested in understanding if ERMCS can change in the neurodegenerative disease ALS. Briefly, explain the experimental set-up to determine the same.

3. Discuss the advantages of using proximity ligation assay for studying protein-protein interaction as compared to other PPI assays such as co-immunoprecipitation, yeast-2-hybrid, and *in vitro* interaction studies.

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Chapter 59: GENOME ENGINEERING USING CRISPR/CAS9: DESIGN, DELIVERY AND EDIT CONFIRMATION

Recommended Level: PG, Research

INTRODUCTION

CRISPR/Cas9 has revolutionized the field of genetic engineering by providing a precise and efficient method for genome editing. Originating from the adaptive immune systems of bacteria and archaea, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) arrays, along with the Cas9 (CRISPR-associated protein 9) enzyme, offer a mechanism to target specific DNA sequences for modification (Jinek et al., 2012).

The CRISPR/Cas9 system consists of two primary components: guide RNA (gRNA) and Cas9 nuclease. The gRNA is designed to bind at the target DNA sequence, directing Cas9 to this specific site in the genome. Upon binding, Cas9 induces a double-strand break at the target location, following which cell's natural DNA repair processes such as non-homologous end joining (NHEJ) or homology-directed repair (HDR) repair the break, which can be exploited to introduce genetic changes such as insertions, deletions, or replacements (Hille & Charpentier, 2016; Jiang & Doudna, 2017).

CRISPR/Cas9 is widely applied in gene therapy to correct genetic disorders, agricultural biotechnology to develop disease-resistant crops, functional genomics to study gene functions, and synthetic biology to create novel biological systems. Its efficiency, simplicity, and versatility make it a crucial tool in contemporary biological research and biotechnology. Additionally, in basic research, it facilitates the creation of knock-out and knock-in models to study gene functions and interactions.

OBJECTIVES

1. Designing of precise guide RNA (gRNA) sequences for CRISPR-Cas9 to target specific genes or genomic regions
2. CRISPR/Cas9 delivery strategies for introducing CRISPR components into target cells
3. Confirming CRISPR-mediated edits through precise genomic assessment

MATERIALS REQUIRED

Equipment			
1	Thermal cycler for PCR	6	Water bath
2	Agarose electrophoresis setup	7	Haemocytometer
3	Gel documentation	8	Incubator (static and shaking)
4	Centrifuge	9	Refrigerators (4^{o}C, -20^{o}C and -80^{o}C)
5	Pipettes	10	Laminar air flow
Plasticwares			
1	Microtips with tip boxes	5	Sterile bacterial culture plates
2	PCR tubes	6	Sterile cell culture plates
3	1.5 ml microcentrifuge tubes	7	Cell spreader
4	15 ml Falcon tubes	8	Ice bucket

Reagents and chemicals			
1	gRNAs oligos	11	Lipofectamine3000 kit
2	PCR primers	12	FastAP (M0371S, NEB
3	CRISPR vector	13	Vector specific restriction enzyme (e.g., for PX458 vector BbsI-R0539S, NEB)
4	Agarose	14	T4 PNK (NEB)
5	Ethidium bromide	15	Cell line and culture media
6	Competent cells (Stbl3)	16	10X PlasmidSafe Buffer
7	Glycerol	17	2X Quickligation Buffer (B2200S, NEB)
8	Parafilm	18	10X FastDigest Buffer (B6002S, NEB)
9	Autoclave Milli-Q water	19	10X T4 Ligation Buffer (NEB)
10	Tris EDTA buffer	20	70% ethanol

PROCEDURE

Objective 1: Designing precise guide RNA (gRNA) sequences for CRISPR-Cas9 to target specific genes or genomic regions (1-2 h)

CRISPR designing starts with the target gene/region selection, followed by the design of 20 bp long guide RNA adjacent to the Protospacer Adjacent Motif (PAM) sequence (NGG for SpCas9). Potential off-target analysis of the designed gRNAs is necessary. There are numbers of CRISPR designing tools available such as https://chopchop.cbu.uib.no, www.benchling.com, www.e-crisp.org, www.idtdna.com, cctop.cos.uni-heidelberg.de, etc.

1. Obtain the desired genomic DNA sequence from the online available repositories (e.g., https://www.ncbi.nlm.nih.gov, https://genome.ucsc.edu, https://asia.ensembl.org/index.html).

2. Identify the 5′ coding sequence (~250 bp) of the gene of interest for the generation of knockout or knock-in lines. For knockout generation, select the starting N-terminal translated region as the target, as this typically results in early frameshift mutation and increases the likely hood of generating a truncated and non-functional protein.

3. Go to the CHOPCHOP website (https://chopchop.cbu.uib.no), you will see the 4 options there as ‘Target’ ‘In’ ‘Using’ and ‘For’.

4. In ‘Target’ column, you can add sequence simply by copy and paste FASTA sequence (>NNNNN format) by choosing paste and target option or write gene name, the software automatically retrieves the gene sequence from the gene databases.

5. In ‘In’ column select the species of interest with latest genome assembly, for human select *Homo sapiens* (hg38/GRCh38); in ‘Using’ column select type of editor for example CRISPR/Cas9 (SpCas9 which uses NGG PAM) and in ‘For’ column select the purpose of your study, for example knock out or knockdown.

6. Clicking on the ‘Find Target Sites’ option, the tool scans the provided sequence for potential PAM sites and generates a list of gRNAs adjacent to these PAM sites. Select the gRNA which has high GC content, minimum off-targets and self-complementarity, and high on target efficiency to avoid the unintended edits.

7. Once gRNAs selected, export the sequences and add restriction overhang sequences to the sense and antisense oligo for annealing into desired CRISPR vector.

8. Add G at the 5’ of designed sense strand and C at the 3’ of antisense strand of gRNA.

9. Based on choice of vector, check the restriction site and enzyme to anneal the gRNA into CRISPR vector, for example PX458 and PX459 CRISPR vectors have *BbsI* RE site and upon digestion it creates overhang sequence complementary to 5’CACC3’ and 3’CAAA5’sequences (sense gRNA sequence 5’–CACCG 20 bp gRNA sequence–3’ and antisense gRNA sequence 3’–C 20 bp gRNA sequence CAAA–5’).

10. Get the gRNAs oligonucleotides and resuspend it in double distilled water at 100 µM concentration for downstream experimentation.

Objective 2: CRISPR/Cas9 delivery strategies for introducing CRISPR components into target cells (~120-140 h)

Various delivery strategies, such as plasmid DNA delivery, ribonucleoprotein delivery (RNP), viral vector delivery, lipid nanoparticles, and microinjections, have been developed, each with its own advantages and limitations, depending on the target cell type and application. Here, we outline the plasmid DNA delivery approach.

1. Select an appropriate CRISPR plasmid, such as the PX458 vector (gRNA scaffold and GFP-tagged Cas9 sequence) or PX459 vector (gRNA scaffold, SpCas9 sequence, and puromycin selection marker) based on interest from Addgene (https://www.addgene.org) or any other commercial site.

2. Anneal the designed gRNA oligos by setting up the reaction mentioned below:

Reaction components	Volume (µl)
Sense gRNA (100 µM)	1 µl
Antisense gRNA (100 µM)	1 µl
10X T4 ligation buffer (NEB)	1 µl
Double distilled water	6.5 µl
T4 Polynucleotide kinase (NEB)	0.5 µl
Total	10 µl

3. The annealing reaction to be set in a thermocycler at 37°C for 30 min and 95°C for 5 min, followed by ramping down to 25°C at 5°C/min.

   Sense gRNA sequence 5’-CACCGNNNNNNNNNNNNNNNNNNN-3’

   Antisense gRNA sequence 3’-CNNNNNNNNNNNNNNNNNNNNCAAA-5’

4. Digest 1 µg of the isolated CRISPR plasmid with the appropriate restriction enzyme at 37°C for 30-60 min, for example, digest 1 µg of the PX458 plasmid with *Bbs*I, following the reaction below. Incubation time can be increased to get the completely digested plasmid.

5.

Reaction components	Volume (µl)
CRISPR Plasmid (e.g., PX458 vector)	X µl (1 µg)
FastDigest *Bbs*I enzyme (R0539S, NEB)	1 µl
Fast alkaline Phosphatase (M0371S, NEB)	1 µl
10X Fast digest buffer (B6002S, NEB)	2 µl
Double distilled water	X µl
Total	20 µl

6. Prepare 1% agarose gel and load the digested CRISPR vector. Do not forget to load 1 kb DNA ladder and undigested plasmid as control along with digested vector.
7. Run the gel electrophoresis unit at 100 V for 45-60 min, visualize under UV using gel documentation system. Save the raw image for the record.
8. After saving the image, cut the digested CRISPR vector band using 21 number surgical blade and collect it in prelabelled 1.5 ml microcentrifuge tube.
9. Purify the digested plasmid using available Gel Extraction Kit and elute in 15-20 µl of elution buffer or autoclaved Milli-Q water.
10. Quantify the isolated plasmid concentration using a spectrophotometer.
11. Ligate the annealed gRNA into digested CRISPR plasmid by setting the below mentioned reaction and incubate for 10 min at room temperature (RT).

Reaction Components	Volume (µl)
Enzyme digested plasmid (e.g., *Bbs*I digested PX458 plasmid DNA)	X µl (50 ng)
Phosphorylated and annealed oligo duplex (1:200 or 1:100 dilution)	1 µl
2X Quick ligation Buffer (B2200S, NEB)	5 µl
Double distilled water	X µl
Subtotal	10 µl
Quick Ligase (M2200S, NEB)	1 µl
Total	11 µl

12. Use PlasmidSafe exonuclease to treat the ligation reaction, preventing unwanted recombination products. This step is optional; however, it enhances the chances of obtaining desirable products.

Reaction components	Volume in µl
Ligation reaction mix	11 µl
10X PlasmidSafe Buffer	1.5 µl
10mM ATP	1.5 µl
Double distilled water	1 µl
Total	15 µl

13. Incubate the reaction for 30 min at 37°C.

14. Competent cells (such as Stbl2, Stbl3, DH5-alpha, etc.) can be directly used or prepared in the laboratory using various methods such as the Inoue method (Green & Sambrook, 2020), Calcium Chloride method, Rubidium Chloride method, Hanahan method or any other method based on preference.
15. To increase the copy number of gRNA cloned CRISPR vector, perform transformation by adding 1-5 µl (20-100 ng) of cloned CRISPR construct to the 1.5 ml microcentrifuge tube containing competent cells. Include a negative control (competent cells without plasmid DNA) to ensure that the antibiotic selection is working properly. One may also include a positive control (competent cells with a known good plasmid) to ensure that the transformation procedure is working. Gently flick the tube to mix. Do not pipette up and down to avoid shearing the cells.
16. Keep the 1.5 ml microcentrifuge tube on ice for 30 min.
17. Give 42°C heat shock to the cells for 30-35 sec by keeping the tubes in water bath. This step allows DNA to enter the cells.
18. Immediately, keep the tube back on wet ice for 2 min.
19. Add 750-850 µl of LB broth or SOC media (Super Optimal broth with Catabolite repression) to the tube for the recovery and incubate at 37°C in shaking incubator (200-250 rpm) for 45-60 mins. This allows the bacteria to recover and express antibiotic resistance gene.
20. Take out the tube and wipe with 70% ethanol to sterilize. Inoculate 50-100 µl of the transformation mix from the tube onto the prepared LB agar plate with appropriate antibiotic (e.g., ampicillin).
21. If a high transformation efficiency is expected, a dilution series (e.g., 1:10 and 1:100) can be plated to avoid overgrowth and obtain well-separated colonies.
22. Use a sterile spreader or sterile glass beads to evenly spread the cells.
23. Seal the plate with parafilm to avoid contamination.
24. Incubate the plates upside down at 37°C overnight (12-16 h).
25. The next day, check for the presence of colonies. Each colony represents a successful transformation event.
26. Add 3 ml of LB broth containing the appropriate antibiotic to each of the 15 ml tubes. Using sterile 200 µl tips, pick individual colonies from the agar plates and transfer them to the 15 ml tubes. Incubate these cultures in shaking incubator at 37°C overnight to grow the transformed plasmid for purification and further analysis.
27. Next day, prepare glycerol stocks from the culture broth by taking 500 µl of culture broth into a 1.5 ml tube, and add 500 µl of 50% glycerol and store the vials at −80°C till further use. Isolate the plasmid DNA from the remaining cultures by alkaline lysis method or plasmid isolation kit. If using kit, ensure ethanol has been added to the wash buffer before use as it should be added by the user.
28. Add 15-20 µl Tris-EDTA (TE) buffer or autoclaved Milli-Q water to dissolve the obtained plasmid DNA.
29. Quantify the plasmid concentration by any spectrophotometry method. Agarose gel electrophoresis can be done to check the quality of the isolated plasmid.

30. Store the plasmid DNA at -20°C for long-term storage or 4°C for short-term use.
31. Prepare dilution of each single colony raised plasmids, labelled properly and send for sanger sequencing with sequencing primer to confirm the ligation of gRNA into CRISPR vector.
32. Use the successfully ligated gRNA-CRISPR construct for downstream experimentation.
33. Take the desired cell type culture such as HEK293T cells or any other cells and seed the appropriate number of cells onto the wells of 24 well plate to get 60-70% confluency next day and incubate overnight (Jiang et al., 2021).
34. Next day, observe the cells under microscope and check the cells confluency and prepare the transfection reaction.
35. Take out the transfection reagent and OptiMEM media from the 4^{o}C, and thaw to room temperature.
36. Take 1.5 ml tube, label as 'A' and add 25 µl of Opti-MEM medium followed by adding 1.25 µl of Lipofectamine3000 reagent into tube A. Mix by gentle pipetting.
37. Take one more 1.5 ml tube, label as 'B' and add 25 µl of Opti-MEM medium followed by adding 0.5 µg of gRNA-PX458 plasmid DNA and 1 µl of P3000 Reagent. Mix by gentle pipetting.
38. Take 25 µl each of tube A and tube B into fresh tube and mix properly by pipetting.
39. Incubate the mixture for 15-20 min at room temperature.
40. Take out the culture plate from incubator and remove the media and add 1 ml complete media to labelled wells of 24 well plate.
41. Add the 50 µl of transfection mix to the well of 24 well plate dropwise.
42. Incubate the cells at 37^{o}C, 5% CO_2 for 24-72 h. Media can be changed after 24 h.
43. Transfection efficiency can be assessed by fluorescence microscopy or flow cytometry if the CRISPR vector has a fluorescence marker, such as GFP.

Objective 3: Confirmation of CRISPR-mediated edits through precise genomic assessment

After delivering CRISPR/Cas9 components and inducing genomic modifications, confirming that the desired modifications have occurred accurately and specifically is essential as shown in Figure 1. Single-cell expansion is must to get the pure edited population of cells which can be achieved by performing serial dilution of transfected cells in 96 well plates (Aouida et al., 2022; Giuliano et al., 2019; Hub et al., 2024).

1. After 24-72 h post transfection, trypsinise the transfected cells and do a cell count using haemocytometer and dilute the cells to 20 cells per 100 µl.
2. Using a 200 µl pipette, dispense 200 µl of diluted cells suspension into the first row (Row A to H) of a 96-well plate.
3. Using multichannel pipette, add 100 µl of complete culture medium to rest of the wells of 96 well plate.
4. Pipette-in 100 µl from the first row with cells and mix it with 100 µl of medium in the row below, resulting in a two-fold dilution for the second row.
5. Repeat this procedure down the rows of the 96 well plate to create a series of 2 fold dilutions.

6. Observe the plate under microscope and check for wells containing a single cell after 12-24 h and mark the wells. Change media every third day of each marked well. Higher serum concentration can be used to grow the single cell expanded populations.
7. Once the confluency reached 60-80%, trypsinise the cells and collect in 1.5 ml tube followed by centrifugation at 1000 RPM for 3 min at RT.
8. Resuspend the trypsinised cells in 1 ml complete media and seed each single cell expanded population in 1 well of 48 well plate with proper labelling. Continue the expanding the monoclonal population to 24 well plate, 12 well plate, 6 well plate and T25 flasks. After attaining the sufficient number harvested the cells for validation assays like DNA isolation, preservation, RNA isolation and Protein isolation.
9. Harvest the edited cell populations followed by their genomic DNA isolation and PCR amplification of the target region.
10. Label the PCR tubes and send the amplified PCR product for the sanger sequencing.
11. On receiving the Sanger sequencing data, using snap gene tool analyze the sequence and check any modification has occurred or not.

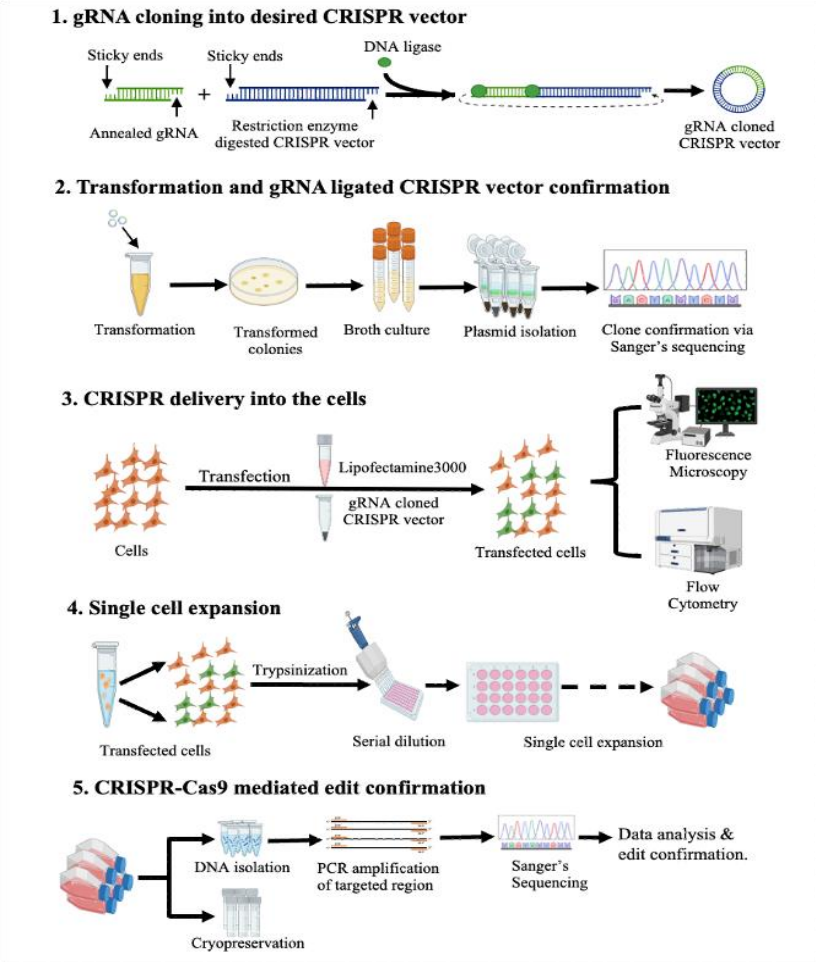

Figure 1. Schematic diagram of the CRISPR mediated modification method. Showing the process from 1). gRNA cloning to desired CRISPR vector, 2). Transformation and gRNA ligated CRISPR vector confirmation, 3). CRISPR delivery into cells; 4). Single-cell expansion, 5). CRISPR-Cas9 mediated edit confirmation.

OBSERVATIONS

When conducting CRISPR/Cas9 genome-editing experiments, students should observe and record various data points to ensure a comprehensive analysis and confirmation of the intended edits.

1. If construct has any fluorescence reporter gene, then transfection efficiency can be assessed by fluorescence microscopy or flow cytometry; for example, PX458 has a GFP-tagged Cas9 cassette.
2. While performing PCR, students should document the presence or absence of the target band in the PCR amplification and also record any additional bands indicating potential off-target effects.
3. Sequencing data of the edited cells should be compared with the control sequences and reference genomes**.** Note any insertions, deletions, or specific nucleotide changes at the target site.
4. Ensure that all observations are recorded across multiple biological replicates with proper positive and negative controls.

QUESTIONS FOR FURTHER STUDIES

1. How does the CRISPR/Cas9 system specifically target and cleave DNA at the desired location?
2. What are the roles of the guide RNA (gRNA) and the Cas9 protein in this process?
3. Why is it important to amplify and sequence the region surrounding the target site after CRISPR/Cas9 editing?
4. How can morphological changes in cells be linked to successful genome editing?
5. What are some potential applications of CRISPR/Cas9 in basic research or medical sciences?
6. Discuss the ethical considerations and potential risks associated with using CRISPR/Cas9 for therapeutic purposes in humans.

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Chapter 60: STUDYING INTERFERON-GAMMA RESPONSES IN MOUSE TUMOR CELL LINES: ESTIMATING NITRITE AND CELL NUMBERS

Recommended Level: UG, PG, Research

INTRODUCTION

IFN-gamma (IFN-γ) is an indispensable pleiotropic cytokine belonging to type II interferons. It activates genes crucial for immune responses, e.g., MHC molecule, via the JAK-STAT pathway. One of the prominent downstream effectors is *Nos2*, which synthesizes nitric oxide (NO) in response to IFN-γ stimulation (Ivashkiv, 2018).

Nitric oxide (NO) plays diverse roles in living organisms, functioning as a vasodilator, a neuromodulator, and a crucial regulatory and cytotoxic molecule within the immune system. It is generated by nitric oxide synthase (NOS), which can be either constitutively expressed (cNOS and nNOS) and an inducible one (iNOS or NOS2) (Dimmeler & Zeiher, 1997).

NO exerts diverse physiological effects in normal cells as well as plays a pathophysiological role in both macrophages and tumors via IFN-γ signaling. IFN-γ activation in certain cancer tumors via IFN-γ signalling. IFN-γ activation in certain cancer cells (e.g., H6 hepatoma cell line, Renca renal carcinoma cell line and RAW 264.7 macrophage cell line) promotes the transcription of Nos2, leading to increased levels of intracellular iNOS enzyme, which further aggravates the production of NO. However, this IFN-γ induced NO production is not seen in all cell lines (CT26 colon carcinoma cell line and B16F10 melanoma cell line) (Prasanna et al, 2007; Rakshit et al, 2014; Chandrasekar et al, 2015; Chattopadhyay et al, 2023).

NO has a short half-life and reacts with molecular oxygen to form nitrogen dioxide (NO_2). NO_2 is a reactive molecule that reacts with water to form nitrite and nitrate. Therefore, to estimate NO amounts, nitrite is other measure as a surrogate Griess diazotization reaction (Fig. 1). Initially, sulphanilamide reacts with nitrite in an acidic environment to form a diazonium salt. Subsequently, this diazonium salt couples with N-(1-naphthyl) ethylenediamine (NED) to produce an azo dye (pink colour), whose absorbance can be measured spectrophotometrically at 550 nm.

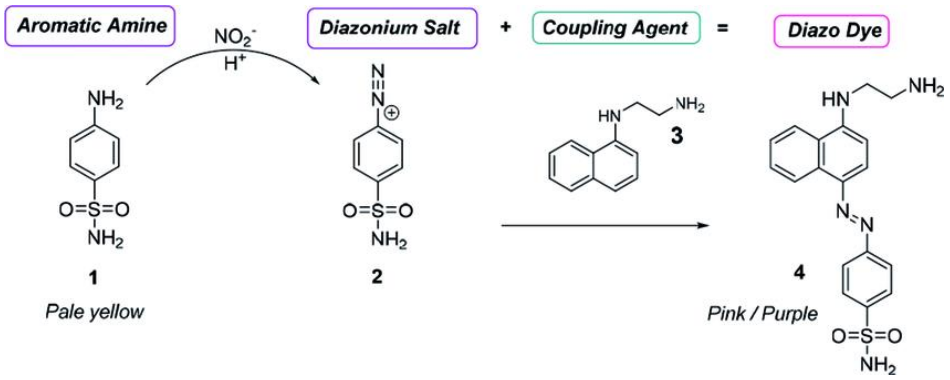


Figure 1. Griess reaction for the detection of nitrite in aqueous media (Source: RSC Advances. 9. 3994-4000. 10.1039/C8RA07656A)

OBJECTIVES

To estimate extracellular nitrite amount upon IFN-γ treatment
To quantify cell survival upon IFN-γ treatment

MATERIALS REQUIRED

H6 (hepatoma) and RAW 264.7 (monocyte/macrophage) cell lines, Dulbecco's Modified Eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), Dulbecco's Phosphate Buffered Saline (PBS), sulphanilamide, N-(1-naphthyl) ethylenediamine (NED), Orthophosphoric acid, double distilled water, 6 well plate (flat bottom), Interferon-γ, 0.5% Trypsin-EDTA, Trypan blue, Haemocytometer, Inverted microscope, CO_2 incubator, Microplate reader

PROCEDURE

Cell seeding

- Seed 5 X 10^6 cells per well in a flat bottom 6 well plate (cell culture grade) and maintain the cells overnight at 37°C in a humidified incubator with 5% CO_2 and 95% humidity.
- Discard the media, wash the cells with 1x PBS, and add fresh media to the cells.
- Add different concentrations of IFN-γ (10 u/ml and 25 u/ml) to the respective cells.
- Mix it gently in a circular motion.
- Incubate the cells for 24 h at 37°C in a humidified incubator with 5% CO_2 and 95% humidity
- Observe the cells under microscope (Fig. 2).

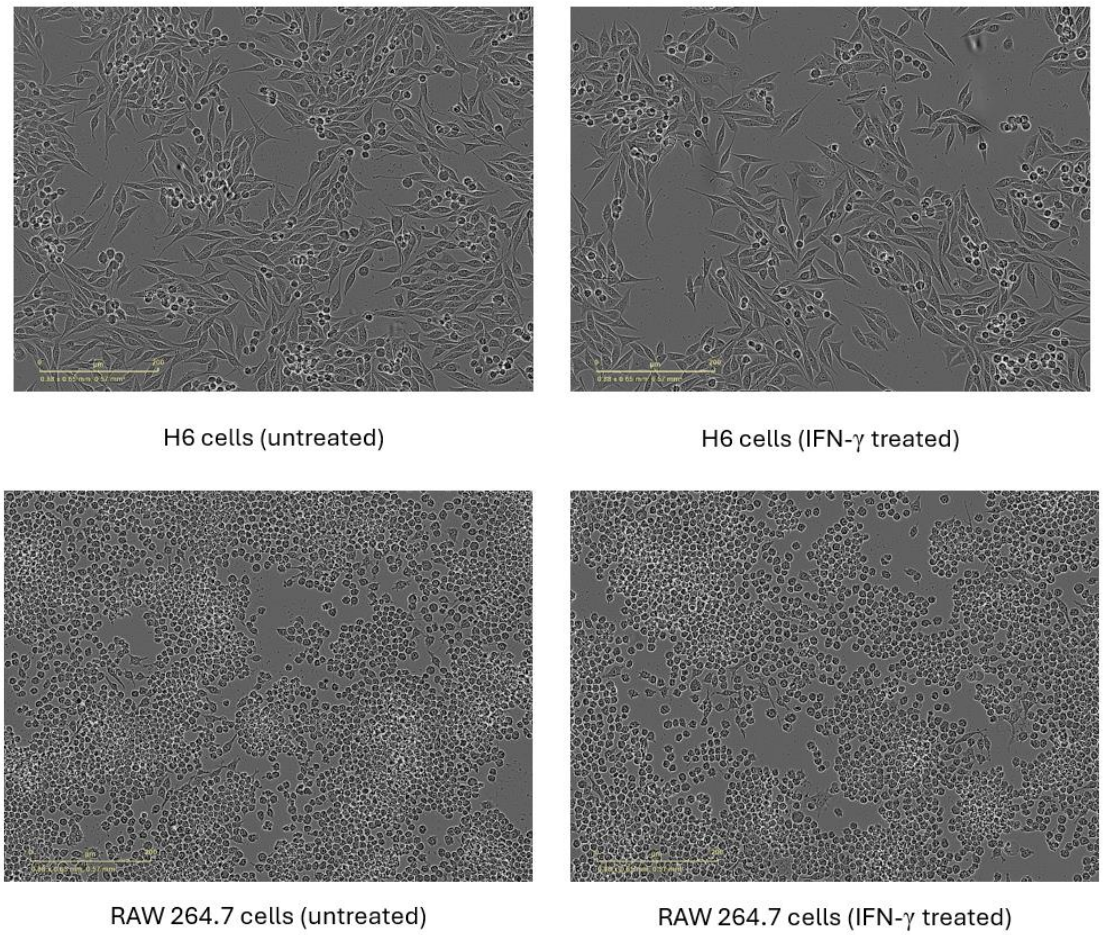


Figure 2. Cell morphology as observed under a microscope in low power in control (untreated) and IFN-γ treated

Preparation of Greiss Reagent

Sulfanilamide	200 mg
Napthyl ethylenediamine	20 mg
Orthophosphoric acid	500 µl
Make up the volume to	20 ml

The reagent is light sensitive, so keep the reagent in an Amber tube, or cover it with aluminium foil.

Standard curve

1. Make a stock of 1 mM sodium nitrite ($NaNO_2$).
2. Make standards of $NaNO_2$ in the final concentration of (100 µM > 50 µM > 25 µM > 12.5 µM > 6.25 µM > 3.125 µM > 1.56 µM) in the final volume of 150 µl (50 µl of standard or sample + 100 µl Griess reagent)
3. Pipette out 50 µl from the previous well to the next well and add 50 µl of autoclaved water to the very same well.
4. Make 1:2 serial dilution, as directed in step 2 except the last well set as blank.
5. Take the OD reading at 550 nm.
6. Plot slope of the standards and accordingly estimate the concentration of the test/unknown sample (see Fig. 3).

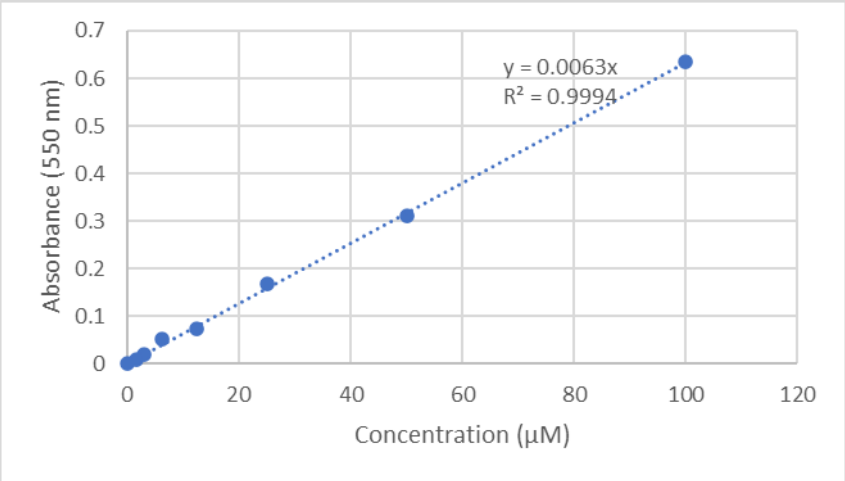


Figure 3. Standard curve for Griess reaction using sodium nitrite

Nitrite estimation

1. The levels of accumulated nitrite were measured to estimate NO production using the Greiss reagent.
2. Add 50 µl of test/unknown samples into the wells of a 96 well plate as duplicates or triplicates (samples can be pre-diluted accordingly with PBS).
3. Add 100 µl of Griess reagent to all wells containing samples.
4. Protect from light by covering with aluminium foil, incubate at room temperature for 5-10 min.
5. Measure the absorbance at 550 nm.

6. The amount of nitrite in the supernatants was calculated from the standard curve of sodium nitrite with the help of the following formula:

$$\text{Nitrite } (\mu M) = \frac{[\text{Absorbance (550 nm) of sample - Absorbance (550 nm) of blank}] \times \text{Dilution factor}}{\text{Slope of the standard curve}}$$

Cell numbers estimation

1. After discarding rest of the supernatant, add 1 ml of 0.5% trypsin-EDTA to each well. Incubate at room temperature for 1-2 min.
2. Flush the cells thoroughly from the bottom of the well to make a single cell suspension of the cells.
3. Mix equal volume of cell suspension with trypan blue.
4. Add 10 µl of the sample on the hemocytometer, cover with a cover glass and immediately observe under a microscope.
5. Live cells will not be stained while dead cells will be brightly stained. Count only the live cells for each sample.
6. Total number of live cells/ml = (Average of the number of live cells in all four quadrants × Dilution factor) × 10^4

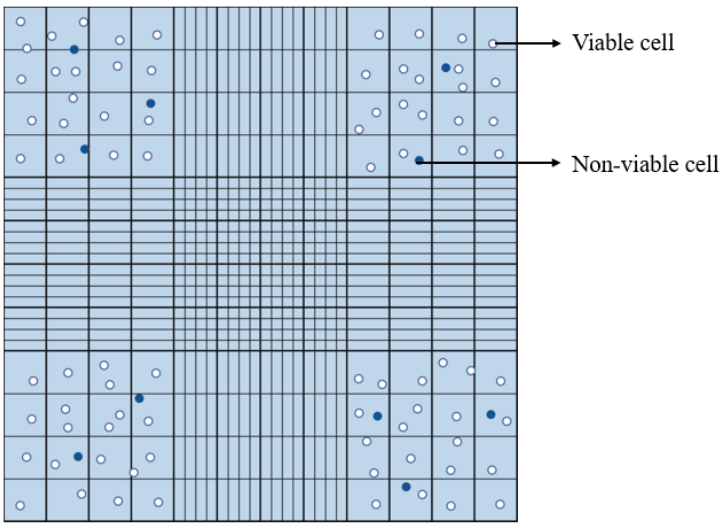
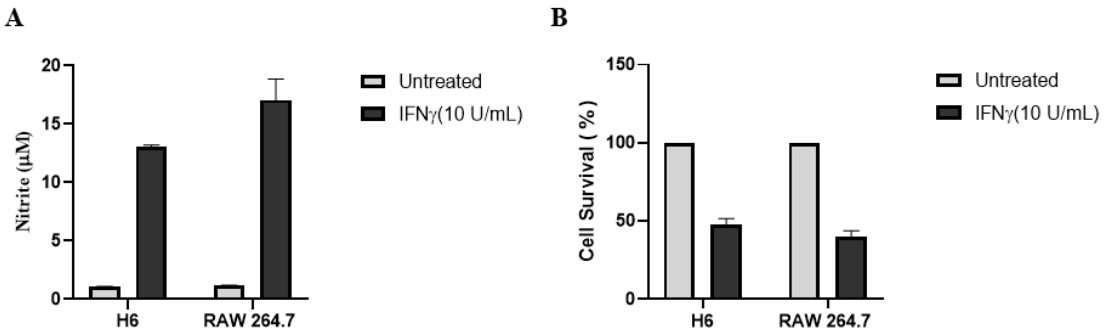


Figure 4. Cell counting on a hemocytometer in Trypan Blue Exclusion assay
(Source: Methods in Molecular Biology, https://doi.org/10.1007/978-1-0716-1020-6_11)

OBSERVATIONS

1. After calculating the nitrite, there is induction in nitrite level in IFN-γ treated compared to the untreated cells.
2. A significant reduction in cell survival can be seen in IFN-γ treated compared to the untreated cells.

	H6				RAW 264.7				Media Blank
	Cells alone		Cells + IFN-γ		Cells alone		Cells + IFN-γ		
OD (550 nm)	0.0629	0.0626	0.1379	0.1391	0.0635	0.0628	0.1555	0.1716	0.056
OD (550 nm) – Blank	0.0069	0.0066	0.0819	0.0831	0.0075	0.0068	0.0995	0.1156	0
Nitrite (µM)	1.095	1.047	13	13.190	1.190	1.079	15.793	18.349	0



A: Representative graph showing induction of nitrite level is cells treated with IFN-γ. B: representative graph showing lower cell numbers upon IFN-γ treatment

QUESTIONS FOR FURTHER STUDIES

1. Why is there cell death upon IFN-γ treatment?
2. Does this observation hold true for every cell line?

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Chapter 61: ISOLATION AND ACTIVATION OF PRIMARY PERITONEAL MACROPHAGES FROM MICE

Recommended level: UG, PG, Research

INTRODUCTION

During infection, neutrophils, monocytes, NK cells, and T cells dynamically respond and understanding these cells provides insights into immune responses (Yadav et al, 20181). Macrophages are crucial for immune defence and studying them provides insights into immune responses, tissue repair and clinical management. Peritoneal macrophages are fascinating players in our health, adapting dynamically to their environment (Cassada et al, 2015).

In the peritoneal cavity of mice, immune cells play critical roles. Under normal conditions, these peritoneal macrophages, exhibit heterogeneity and two main subsets exist: Large Peritoneal Macrophages (LPMs) and Small Peritoneal Macrophages (SPMs) (Yadav et al, 2018; Cassada et al, 2015; Ghosn et al, 2010; Chadrasekar et al, 2015). LPMs, abundant under normal conditions, express F4/80 (a cell surface marker, it is like a badge, helping us recognize who is who in our immune system). SPMs, derived from bone marrow, increase during inflammatory responses in the peritoneal cavity during infections (Vega-Perez et al, 2021).

Primary peritoneal macrophages isolated from mice are cells that are collected directly without prior stimulation from the peritoneal cavity of a mouse. These cells have more of the F4/80 marker on their surface, which suggests that they are LPM and are more mature (Ghosn et al, 2010). On the other hand, Thioglycollate (TG)-elicited peritoneal macrophages are special cells that infiltrate into the peritoneal cavity of a mouse when a substance called Brewer's TG broth is injected. These cells are more activated, but they express low F4/80 on their surface (Ghosn et al, 2010). They also have higher levels of phagocytic activity, which means they are better at engulfing and destroying harmful particles (Davis & Gordon, 2005). This protocol will discuss on how to isolate peritoneal macrophages from mice, study them using flow cytometry and perform activation experiments, in particular quantify cytokines secreted by these cells post activation using ELISA.

OBJECTIVES

To identify and characterize different subsets of peritoneal macrophages for understanding their roles in health and diseases.

To investigate functional properties of peritoneal macrophages to obtain an insight into developing therapeutic strategies for diseases

To study how macrophages react during infection or inflammation.

MATERIALS REQUIRED

Equipment:

CO_2 Incubator / Cell Culture Incubator
Microplate reader
General Purpose Centrifuge Machine
Harvesting of resident peritoneal cells
Dissecting board

70% ethanol
Scissors and forceps
Sterile phosphate-buffer saline – Ca^{2+}, Mg^{2+} free
5 ml syringe with 27-gauge needle
5 ml syringe with 20-gauge needle
15 ml polypropylene collection tubes.
Ice bucket with enough ice.
Tissue culture treated 24 well plate

Purification of resident peritoneal macrophages by adhesion

Dulbecco's Modified Eagle's Medium (DMEM)

Heat-inactivated fetal calf serum (HI-FCS). To heat inactivate fetal calf serum (FCS), place in a 56°C water bath for 30 min

Media supplements: penicillin, streptomycin, glutamine (Gibco, Invitrogen Ltd). Macrophage culture medium: Dulbecco's modified minimal essential medium-High Glucose (DMEM-HG). The media was supplemented with 5 μM β-mercaptoethanol, 100 μg/ml penicillin, 250 μg/ml streptomycin, 50 μg/ml gentamycin, and 2 mM glutamine. Filtered through a cellulose acetate membrane (pore size of 0.2 μm) and supplemented with 10% HI-FBS

24-Well tissue culture plates

Sterile PBS (37°C)

Brewer TG Broth Elicited Peritoneal Macrophages

Brewer TG Medium (M019-500G), 4.05 grams in 100 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Transfer in suitable container and sterilize by autoclaving at 15 lbs and 121°C for 15 min. Age the solutions for 2 weeks in a dark at room temperature before use. Aging will augment the yield of inflammatory cells, which is thought to be the result of an increase in glycation products. Ensure that the broth is clear, prior to use, any cloudiness indicates contamination and renders it unusable (Davis & Gordon, 2005).

Sandwich ELISA

1. ELISA plates (do not use cell culture plates)
2. Coating buffer (50 mM carbonate buffer, pH 9.4)
3. Blocking buffer (Assay buffer)
4. Capture antibody
5. Samples (target protein)
6. Wash buffer: PBS-T (1X PBS + 0.05 % v/v Tween 20 detergent)
7. Aluminium foil (to cover the plates)
8. Biotinylated detection antibody
9. Streptavidin-HRP conjugate
10. TMB (Tetramethylbenzidine) liquid solution as HRP substrate (for indirect detection of bound protein)
11. Stop solution (1 M H_2SO_4) – for TMB substate

Flow cytometry

1. Blocking buffer: PBS containing FBS (5% v/v) and Sodium Azide (0.09 % w/v).
2. Conjugated antibody: anti-F4/80-*Flurochrome* antibody (pre-titrated and dissolved in blocking buffer)

3. Fixative (for preserving cellular architecture): 1% paraformaldehyde (PFA).
4. PBS

Animal experiment

1. IAEC approval
2. Mice of appropriate weight (18-20 grams) and age (6-8 weeks).
3. Mice strain used C57BL/6 or BALB/c

PROCEDURE

Harvesting of Resident Peritoneal Cells

1. Sacrifice the mice by CO_2 asphyxiation (IAEC approval needed). Pin the mice to dissection board with abdomen up followed by sterilizing the mouse with 70% ethanol with a spray bottle.
2. Make a small of-center incision in the skin over the caudal half of the abdomen with sharp scissors and expose the underlying abdominal wall by retraction.
3. Inject 5 ml of ice-cold sterile PBS into the caudal half of peritoneal cavity using a 27-gauge needle (Note: keep the bevel up and try not to puncture the intestine or any other internal organ).
4. Remove the syringe gently and massage the abdominal cavity of 20 sec.
5. Slowly withdraw the lavage containing resident peritoneal cells by inserting a 20-gauge needle and collect in a 15 ml polypropylene tube. (Note: try to collect maximum possible volume to get a better yield of cells.
6. Store the tube on ice until required (Davis & Gordon, 2005).

Harvesting of TG elicited Peritoneal Cells

1. Lift the mouse performing the scruff hold. Secure the tail between the ring or small finger and the palm of the same hand. Use the other hand for the desired procedure.
2. Sterilize the abdominal cavity by spraying 70% ethanol using cotton.
3. Inject 2 ml aged Brewer TG medium using a syringe and 30G needle.
4. On fourth or fifth day, harvest the TG elicited peritoneal cells by following the protocol mentioned in the above section (Davis & Gordon, 2005).

Differentiation between resident and TG elicited peritoneal macrophages by flow cytometry

1. To perform flow cytometry, we must know the instrument and its configurations (for example, BD FACSVerse™ has a 2+2+4 laser configuration).
2. Based on laser configuration, we are supposed to select the appropriate fluorochrome which is conjugated to the antibody of our interest.
3. This antibody will bind to the surface maker of our interest, in this case, it is F4/80, which is present on macrophages.
4. To continue with the protocol for flow cytometry, first harvest the cells as mentioned in the above sections.
5. Centrifuge the tube at 400 g for 5 min at 4°C.
6. Discard the supernatant
7. Resuspend the cell pellet in 1 ml PBS

8. Dilute the cell suspension 20 times and proceed for counting using a Neubauer chamber.
9. Calculate the appropriate volume that should contain 1 x 10^5 cells.
10. Transfer 1 x 10^5 cells each in two different microcentrifuge tubes. One will be used as unstained control, while other one will be used to stain the cells using fluorochrome conjugated antibody.
11. Centrifuge the tubes at 400 g for 5 min at 4°C.
12. Discard the supernatant. Care should be taken while aspirating the volume using a micropipette. If the pellet is dislodged, centrifuge the tube again.
13. Resuspend the pellet in blocking buffer, followed by incubation for 20 mins at 4°C (use ice bucket).
14. Centrifuge the tubes at 400 g for 5 min at 4°C.
15. Discard the blocking buffer.
16. Resuspend the cell pellet in 100 µl anti-F4/80-BV421 antibody solution.
17. Incubate for 30 min at 4°C in dark. Tap to dissolve with 10 min intervals.
18. Centrifuge the tubes at 400 g for 5 min at 4°C.
19. Discard the supernatant.
20. Resuspend in fixative (1% PFA).
21. Incubate for not more than 5 min at 4°C in dark.
22. Centrifuge the tubes at 400 g for 5 min at 4°C.
23. Discard the supernatant.
24. Wash twice with PBS and resuspend in 200 µl PBS.
25. Proceed with data acquisition using flow cytometer. The F4/80 signal for the conjugated fluorochrome will be detected in one of the fluorescence channels, and this can be used to identify and quantify the macrophages in the sample (Ghosn et al, 2010; ThermoFisher).

OBSERVATIONS

FACS plot of Unelicited vs TG-elicited peritoneal cells for anti-F4/80-BV421 antibody are shown in Fig 1.

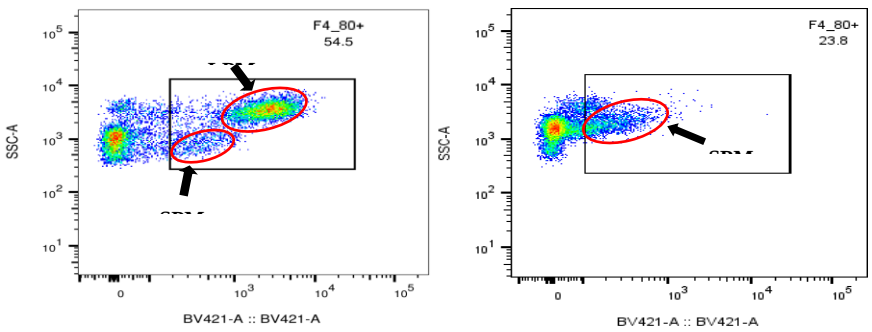

Figure 1: FACS plot of Unelicited vs TG-elicited peritoneal cells for anti-F4/80-BV421 antibody. The unstained cells comprise of population that is negative for F4/80 marker which includes B cells, T cells and neutrophils.

Purification of resident or TG elicited peritoneal macrophages by adhesion

1. Plate the resident peritoneal cells in DMEM at 1 x 10^6 cells or 0.2 x 10^6 cells per well in a 24-well or 96-well tissue culture plate respectively and incubate for 120 min at 37°C in a cell culture incubator.
2. Remove the non-adherent cells by washing three times with 500 µl warm PBS, using a gentle swirling action.
3. Using a 1000 µl pipette, discard the PBS containing non-adherent cells. Add 500 µl fresh media per well.
4. The population of adherent cell should consist of more than 90% macrophages.
5. At this stage, these cells will be called Adherent Peritoneal Exudate Cells (APECs).
6. Macrophages inherently have the property to adhere to polypropylene surfaces, provided the surface is treated for cell culture. This greatly enhances their adherence to the surface. In turn, this ensures the purification by adhesion (Ghosn et al, 2010, Chandrasekar et al, 2015).

Activation of primary macrophages or APECs

1. After purification of resident peritoneal macrophages by adhesion, incubate the cell in 500 µl or 100 µl DMEM containing 10 U/ml of mouse-interferon-gamma (IFN-γ) per 24-well or 96-well respectively. Keep a negative control where media alone is added.
2. Incubate for 24 h at 37°C in a cell culture incubator. Post incubation, collect the cell free supernatant for ELISA (store at -20°C for future use) (Chandrasekar et al, 2015).

OBSERVATION

Bright field images of APECs (magnification 10X); IFN-γ treatment leads to aggregation of cells.

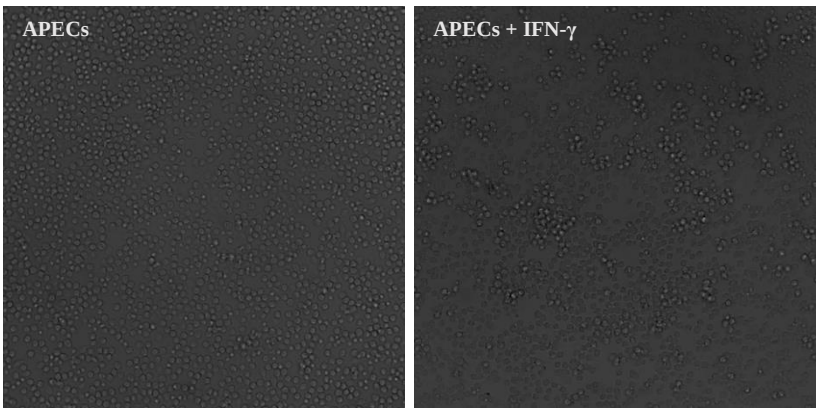

Figure 2. DIC image of APECs, isolated from a BALB/c strain of mice, (-/+) IFN-γ treatment.

PROCEDURE FOR SANDWICH-ELISA

Usually, manufacturer's instructions are followed. But, in this case we will elucidate a general protocol for the same as follows:

1. Coating with Capture Antibody: Start by coating the wells of a microtiter plate with the capture antibody. This is usually done at a concentration of 1–10 µg/ml in carbonate/bicarbonate buffer (pH 9.6). Cover the plate and incubate overnight at 4°C.

2. Blocking and Adding Samples: After removing the coating solution and washing the plate twice with PBS, block the remaining protein-binding sites in the coated wells by adding a blocking buffer. Then, add the samples to each well. Always compare the signal of unknown samples against those of a standard curve.
3. Incubation with Detection and Secondary Antibody: After incubating for 90 min at 37°C and washing the plate twice with PBS, add the detection antibody to each well. This antibody is specific to the target protein.
4. Detection: After washing away excess detection antibody, add an enzyme conjugate that binds to the detection antibody. Add a substrate (TMB) for this enzyme, which will produce a visible colour change in the presence of the enzyme.
5. Stop the reaction using 1M H_2SO_4
6. Measure the absorbance at 450 nm in a microplate reader.
7. Data Analysis: The intensity of this colour change can be measured and will be proportional to the amount of target protein in the original sample. Calculate the amount against a standard curve.

Note: This is a generalized protocol and may need to be optimized based on the specific antibodies and target protein being used (Yadav et al, 2018; ThermoFisher).

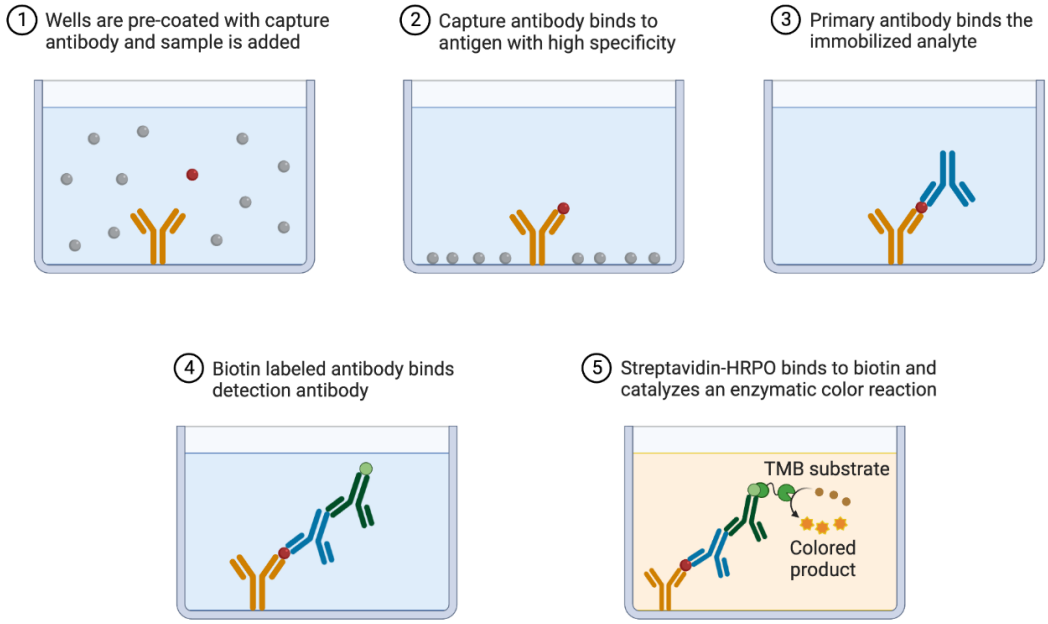

Figure 3. Schematic representation of sandwich ELISA (source: BioRender)

OBSERVATIONS

Cytokines level of C57BL/6 mice APECs -/+ IFN-γ treatment

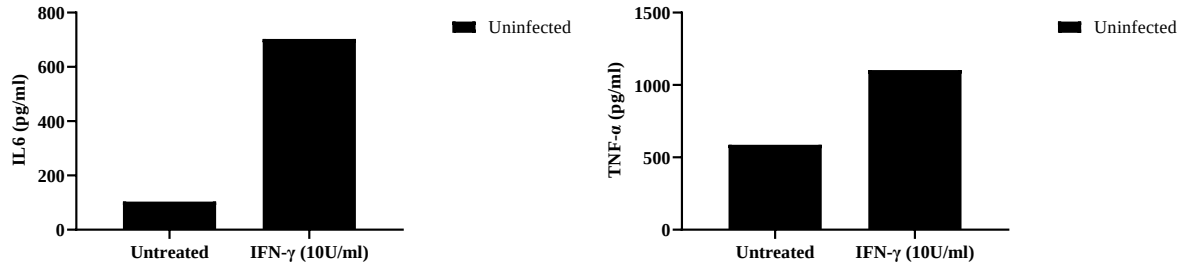

Figure 4. Levels of IL-6 and TNF-α increases upon IFN-gamma treatment in vitro

The above-mentioned experiments can also be done using TG-elicited macrophages. The injection of TG into the peritoneal cavity of mice leads to a sterile inflammatory response (protocol mentioned above). Similarly, TG elicited macrophages can be activated and the amounts of cytokines can be estimation using ELISA; in addition, nitrite can also be measured using the Griess assay.

QUESTIONS FOR FURTHER STUDIES

1. What are macrophages? What are the markers that can be used to identify macrophages?
2. Where do mouse macrophages originate?
3. What's the difference between pro-inflammatory and anti-inflammatory cytokines? Give examples.
4. What are the advantages and disadvantages of ELISA?
5. How does IFN-γ influence macrophage activation, and what downstream effects can it have on immune responses?

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Chapter 62: TMRM STAINING ASSAY TO DETERMINE MITOCHONDRIAL MEMBRANE POTENTIAL

Recommended Level: PG & Research

Background

Mitochondrial functioning is a vital parameter to assess cellular health. It produces energy as ATP molecules by oxidative phosphorylation through an electrochemical gradient between the mitochondrial membranes. Healthy mitochondrial membranes maintain a difference in electrical potential between the interior and exterior of the organelle, referred to as a membrane potential. The impairment of membrane potential during cell damage led to its depolarization and loss of cellular integrity which is widely implicated in the study of cell death and in certain diseases such as cancer, diabetes, Parkinson's, and stroke (Goyal, 2023; Garcia-Ruiz, 2002; Lee, 2023).

The measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) can be detected by staining cells with positively charged dyes such as Tetramethylrhodamine methyl ester (TMRM), Tetramethylrhodamine ethyl ester (TMRE) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1). However, TMRM is extensively used for labelling and measuring the $\Delta\Psi_m$ because of its optical and chemical properties. The lipophilic TMRM dye, bearing a delocalized positive charge, enters the negatively charged mitochondria where it accumulates and fluoresces orange/red upon excitation at 548 nm and exhibits emission maximum at 573 nm (Fakas, 1989).

When the mitochondrial $\Delta\Psi_m$ collapses in cells while apoptosis or by any damage, TMRM no longer accumulates inside the mitochondria, instead becoming more evenly distributed throughout the cytosol. When dispersed in this manner, overall cellular fluorescence levels drop dramatically. Cells with depolarized mitochondria exhibit reduced fluorescence when compared to cells with healthy mitochondria. This relative and differential fluorescence intensity in TMRM-stained cells can be read and analysed either by employing flow cytometry or fluorescent microscopy (Garcia-Ruiz, 2002).

MATERIALS REQUIRED

Cell culture and maintenance

Cell line**:** Any mammalian cell line can be used which should be free of contamination.

Reagents**:** Dulbecco's Modified Eagle's Medium (DMEM) cell culture media, Foetal Bovine Serum (FBS), 1X PBS (sterile), Trypsin-EDTA, Opti-MEM (reduced serum media), 100X Antibiotic antimycotic solution or Penicillin/Streptomycin solution,

Composition of Complete Media**:** DMEM + 10% FBS+ 1X Antibiotic solution

T-25 tissue culture flask, flow cytometry tubes (FACS tubes), 96 well tissue culture plate, 12 or 24 well tissue culture plate, culture based 15- and 50 ml sterile polypropylene falcon tubes.

Chemical reagents

TMRM (Tetramethylrhodamine methyl ester), Dimethyl Sulfoxide (DMSO), Etoposide (any drug or agent to be tested on cell line), FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone).

Major equipment

37°C humidified CO_2 incubator, Biosafety cabinet type II, Centrifuge with rotor for 15 mL tubes, Inverted phase contrast microscope, Inverted Epi-fluorescent microscope, Flow cytometry analyzer

PROCEDURE

Preparation of TMRM Stock Solution

Prepare 5 mM TMRM stock by dissolving 1 mg TMRM in 525 µl DMSO. Store at -20°C in the dark. Dilute the main stock 1:100 in DMSO to obtain 50 µM solution, aliquot into amber microfuge tubes, and store at -20°C.

Typically, TMRM working concentrations range from 20 nM to 200 nM, depending on the specific cell assay. In this experiment, we utilized a 50 nM working solution.

Note: TMRM is available in powder or liquid form by various companies. It is a light sensitive reagent and therefore, it shall be avoided from light exposure.

TMRM staining protocol for measuring the mitochondrial membrane potential ($\Delta\Psi_m$) by employing flow cytometry

1. Seed the cells in a 24 well plate with 40-50% of confluency.
   (Note: For cells with spindle or larger shape, it is better to seed in a 12 well plate)
2. After 48 h, visualise the cells under a phase-contrast microscope to monitor the cell condition and density (Cell confluency should not be more than 80%).
3. When cells are in optimal condition, add your test compound to the culture medium at the desired concentration. In this protocol, cells were treated with etoposide (50 µM) for 24 h. Maintain a well without test compound as a negative control.

   Optional: Add FCCP (a mitochondrial uncoupler) in a culture well as a positive control.

   (Note: with undergoing treatment, many apoptotic or dying cells may detach from the surface and float in the media)
4. Post-treatment, collect the culture medium in a FACS tube. Rinse the well with 1X PBS and add to the same tube. Immediately, harvest the remaining adherent cells by trypsinization. Neutralize trypsin by adding 1-2 ml complete culture medium. Gently mix the cell suspension and transfer it to respective labelled FACS tube to ensure the collection of both adherent and detached cell.
   (Note: Before collecting cells, label the FACS tube according to each cell sample)
5. Centrifuge the collected cell suspensions at 3000 rpm for 3 min at room temperature. Carefully discard the supernatant by inverting the tube, leaving the cell pellet intact.
   (Note: Gently tap mouth of the FACS tubes on a tissue paper to discard the media completely. Avoid pipetting to discard the media as it may have risk of losing cell pellet)
6. Resuspend the cell pellet in 100-150 µl of serum-free Opti-MEM containing 25 nM TMRM. Gently pipette 2-3 times to ensure cell singlets. Incubate the suspension for 15-20 min in a 5% CO_2 incubator with 37°C.
   (Note: TMRM stain is light sensitive and therefore, avoid it from light exposure)
7. Dilute the cell suspension with 200-250 µl of serum-free Opti-MEM (without TMRM). Analyze samples using flow cytometry, measuring TMRM fluorescence with FL-2 channel.
   (Note: acquire at least 10,000 cell events for each sample to read out the fluorescence)

TMRM staining protocol for analyzing mitochondrial membrane potential by employing fluorescent microscopy

1. Seed the cells in a 96 wells tissue culture plate with 40-50% of confluency.

2. After 48 h, assess cell morphology and confluency using phase-contrast microscopy. If cells appear healthy and at appropriate density, then proceed for cell treatment with a test compound as described in protocol 1.
3. Upon completion of treatment time, gently aspirate 75% of the culture media using a pipette, taking care not to disturb the cell monolayer.
   (*Note*: *Avoid complete removal of media as it may eliminate weakly adhered and floating apoptotic cells completely from the well. Therefore, it is better to leave approximately 25 µl of media in the well. It will also prevent drying of the cells if one is handling many wells.*)
4. Prepare the required volume of serum-free *Opti-MEM* media with TMRM (working concentration: 50 nM) in a sterile tube.
   (*Keep it ready before performing step iii*)
5. Immediately add 50 µl of TMRM containing media in the well and incubate the cells for 15-20 min in a 37°C incubator supplied with 5% CO_2.
   (Plate shall be wrapped with aluminium foil to avoid light exposure)
6. Post-incubation, aspirate 75% of TMRM-containing medium. Immediately add 25 µl of TMRM-free Opti-MEM media in each well.
7. Capture the fluorescent images using a fluorescence microscope equipped with a TRITC filter and 20X objective.
   (*Note: TMRM fluorescence is susceptible to rapid photobleaching. If quenching occurs in the focal area, promptly shift the field of view to an adjacent region and immediately capture images.)*
8. For the analysis, compare the relative fluorescent intensity between treated and untreated cell samples.

OBSERVATIONS

Flow cytometric histograms (Fig. 1a) shows the relative fluorescence intensity of TMRM as measured by the FL-2 (PE) channel in A549 cells un-treated (left plot) and treated (right plot) with 50 µM etoposide for 24 h. In treated samples, 43% cell population reflected reduced fluorescence intensity suggesting loss in $\Delta\Psi_m$.

Fluorescence images (Fig. 1b, 20X, scale bar: 50µm) of TMRM-stained cells. TMRM intensity decreased markedly after 24 h treatment with etoposide (50 µM), indicating a significant reduction in mitochondrial membrane potential.

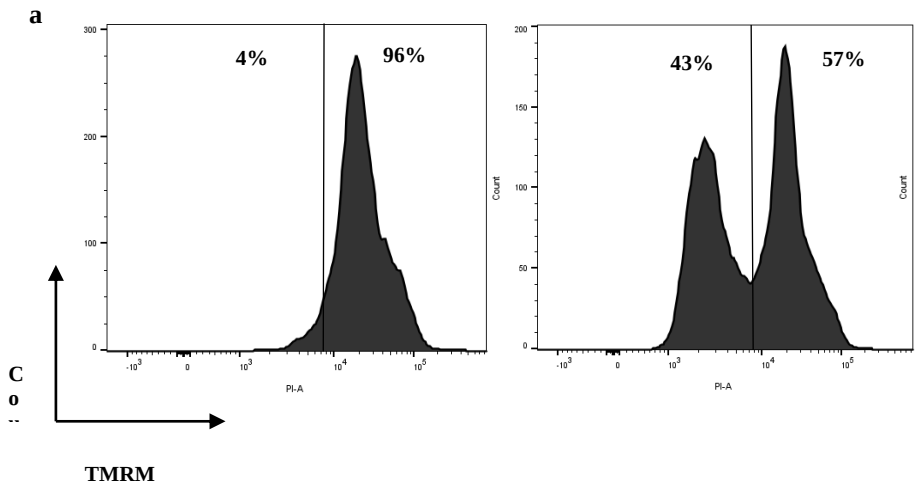

Figure 1. Analysis of fluorescence intensity in TMRM-stained cells by using flow cytometry and fluorescent microscopy

QUESTIONS FOR FURTHER STUDIES

1. What is the primary function of mitochondrial membrane potential ($\Delta\Psi_m$) in cellular energy production?
2. How does TMRM function as a reporter of mitochondrial membrane potential?
3. What are the precautions to be taken care while imaging of TMRM stained cells?
4. Why is Opti-MEM used for TMRM staining in both flow cytometry and microscopy analyses?

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Chapter 63: *DICTYOSTELIUM DISCOIDEUM* AS A MODEL SYSTEM FOR CELLULAR AND DEVELOPMENTAL STUDIES

Recommended level: UG, PG, Research

INTRODUCTION

Dictyostelium discoideum is a facultative multicellular organism with two mutually exclusive states in its life cycle: vegetative growth and development. During vegetative phase, it remains as a single cell (amoeba) that feeds on bacteria or in liquid medium and multiplies by mitotic divisions. Upon starvation, it initiates multicellularity and ultimately forms a fruiting body comprising two terminally differentiated cell types: the viable spores and the dead vacuolated stalk cells. There are multiple intermediate stages during development.

Neutral red is a vital dye, which accumulates in the lysosomes of viable, uninjured cells. Acidic vacuoles in the pre-stalk/stalk cells stain red when viewed under visible light.

OBJECTIVES

To study the development and pattern formation in *Dictyostelium.*

To study chemotaxis with different concentrations of cAMP.

MATERIALS REQUIRED

Dictyostelium discoideum—Ax2 can be grown in axenic medium (HL5) under shaken conditions at 22°C at 120 rpm.

Medium HL5 (1 litre)

Proteose peptone	14.3 g
Yeast extract	7.15 g
Glucose	16 g
$Na_2HPO_4.2H_2O$	0.626 g
KH_2PO_4	0.485 g

Adjust pH to 6.5 with dilute HCl and the volume make up with double distilled water

10X KK_2 buffer (for 1 litre)

KH_2PO_4	22.5 g
K_2HPO_4	6.2 g

Adjust pH to 6.2 and the volume make up to 1 litre. Autoclave and store at 4ºC

Neutral Red

Dissolve 50 mg of the dye in 1 ml of autoclaved water. Filter through washed glass fibre or well packed, wet cotton fibre.

Non-Nutrient agar plates

1.5% Agar in 1xKK_2 buffer

PROCEDURE

Cell culture

Ax2 cells are grown and maintained in petri-plates containing HL5 medium at 22°C. Add 10 ml of HL5 media/90 mm petri-plates and inoculate your cells/spores. Once the plates get confluent, take out

the medium and replenish with fresh HL5 medium. Usually, confluency is achieved by 10^7 cells in a 90 mm petri-plates. To obtain large cultures, cells are inoculated from petri-plates into flasks containing fresh medium and grown under shaken conditions at 120 rpm, 22°C till the log phase (3-5 x 10^6 cells/ml) is reached. Inoculate cells at about 3-5x10^5 cells/ml in a glass flask containing HL5 medium, shaking at 22°C at 120 rpm. Attachment of cell is weak and can be taken out by pipetting. Ax2 cells grown in axenic medium have slower growth rates: they have a doubling time of roughly 10 to 12 h depending on temperature, medium, the presence of selective drugs and most likely several unknown factors. Subculture after 40 h into fresh medium.

Precaution: It is generally not a good idea to split the cells so that the cell density is less than 1x10^5 cells/ml. Cultures of lower cell densities lag for longer time period and can be difficult to estimate. Optimal growth of *Dictyostelium* is observed at a temperature of 22°C.

Troubleshooting: Cells do not grow: Start a new culture from stocks; Check number of cells inoculated. If cell density is below 1x10^5 cells/ml, cells may go through a lag phase and have difficulties in growing in suspension. If cells are kept stationary, most of the cells would be dead. Check water quality; Check pH of the medium before and after autoclaving; Check peptone quality.

Do not maintain the same strain growing for more than a month since they may accumulate mutations. Try to grow the cells from the spores for reproducible results.

Cell counting

Cell density is determined by taking an aliquot of the culture and counting it in a hemocytometer. When the cells approach densities of greater than 1-5x10^6 it is usually a good idea to do a 1:10 dilution of the culture sample prior to putting it in the hemocytometer.

A 100 µl sample is sufficient to fill the hemocytometer chamber. The standard hemocytometer carries a grid that is 3 mm on a side (see Fig. 1).

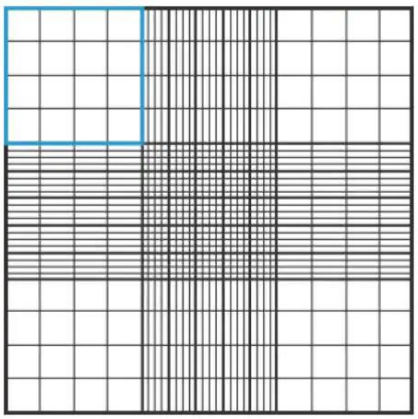

Figure 1. A hemocytometer with 9 grids. The 4 grids in 4 corners contain 16 squares each. These 4 grids are used for counting cell numbers.

Count cells in the 16 squares (2 diagonally opposite from each grid as one set). Take the average of all such 16 square counts.

Calculate as:

If the cell counts for each of the 16 squares were 50, 40, 45, 52, the average cell count would be: (50 + 40 + 45 +52) ÷ 4 = 46.75

46.75 x 10,000 (10^4) = 467,500

467,500 x 5 (dilution factor) = 2,337,500 live cells/ml in the original cell suspension

Determining cell density

To determine the cell density, the cells are counted in a haemocytometer after appropriate dilutions in duplicates and plotted as a function of time (Fig. 2). Doubling time is calculated using the formula given below:

$T_d = (t2- t1) \times \log2/\log(q2/q1)$

Where: t1- initial time
t2- final time
q1- growth at t1
q2- growth at t2

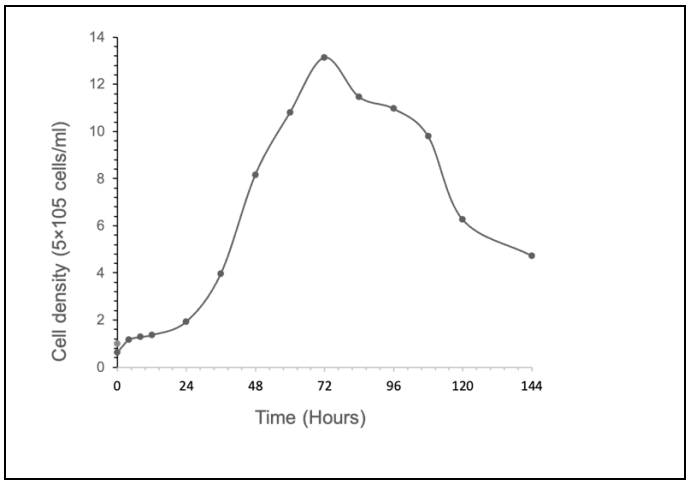

Figure 2. A graph plotted for cell density against time duration when the culture is growing.

Development

For development, cells are washed twice with ice-cold 1x KK_2 buffer and spotted on non-nutrient agar as 20 µl droplets at cell density of $5x10^7$ cells/ml. For synchronization of development, plates are kept at 22°C for 4-6 h followed by incubation at 22°C.

Development on a solid substratum

1. Take an aliquot of cells and determine cell density by counting them on a hemocytometer.
2. Collect $5x10^7$ cells by centrifugation at 1500-2000 rpm for 2-3 min.
3. Resuspend the pellet in 1 ml of 1 x KK2 buffer and pellet again.
4. Resuspend the pellet and spot them as 10-20 µl droplets on agar surface.
5. Let it settle for 5-10 mins and remove any excess liquid.
6. Incubate at 22°C.

OBSERVATION

The developmental stages that can be observed (Fig. 3) are shown below:

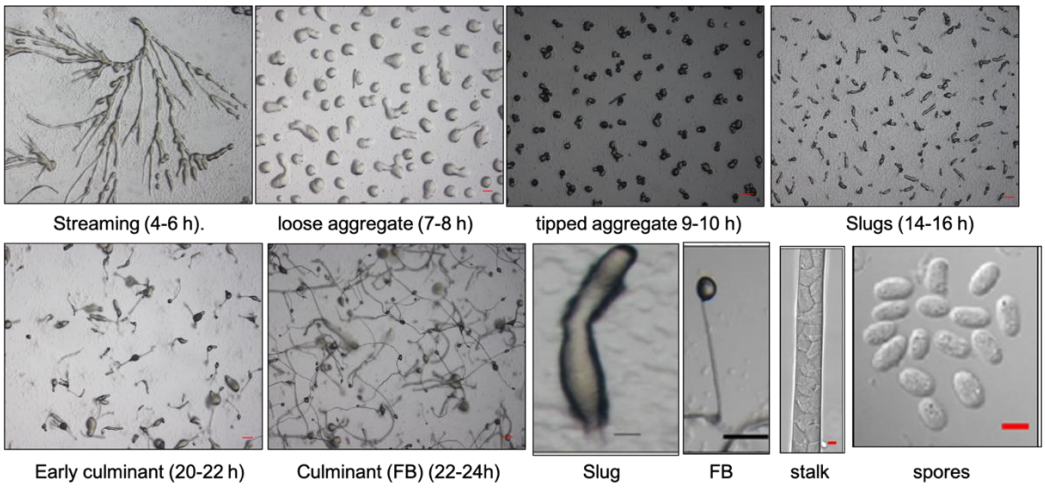

Figure 3. Ax2 cells when starved. All the stages of development from unicellular amoebae like cells to multicellular stalk and fruiting bodies are seen.

Pattern formation using neutral red stain (a vital dye)

Take *Dictyostelium* cells and add a drop or two of NR solution. Incubate cells at 22^0C for 30 mins under shaken conditions. Wash 3-4 times with chilled 1xKK2 buffer and plate for development.

The stages are observed as shown below to construct the life cycle (Fig. 4, 5).

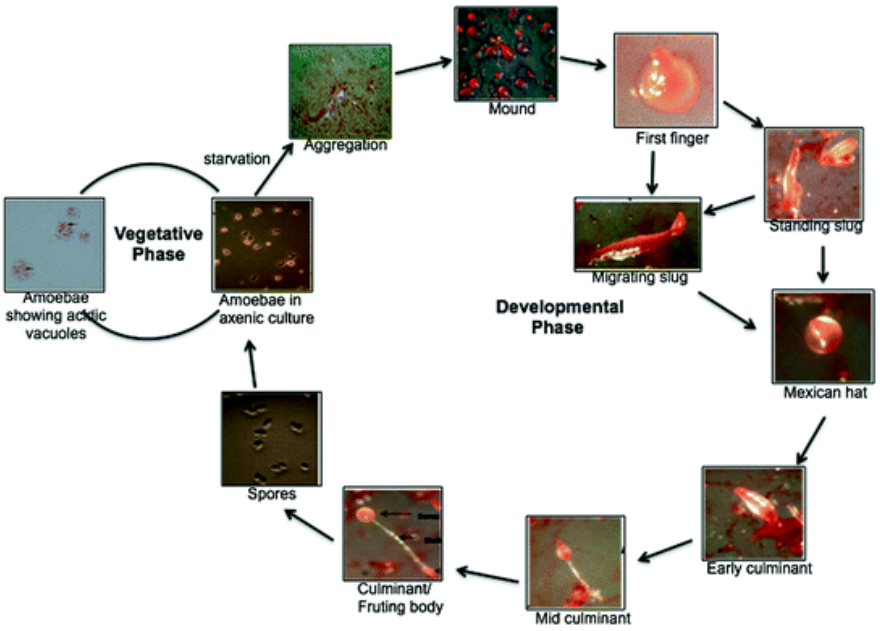

Figure 4. Life cycle of *Dictyostelium*

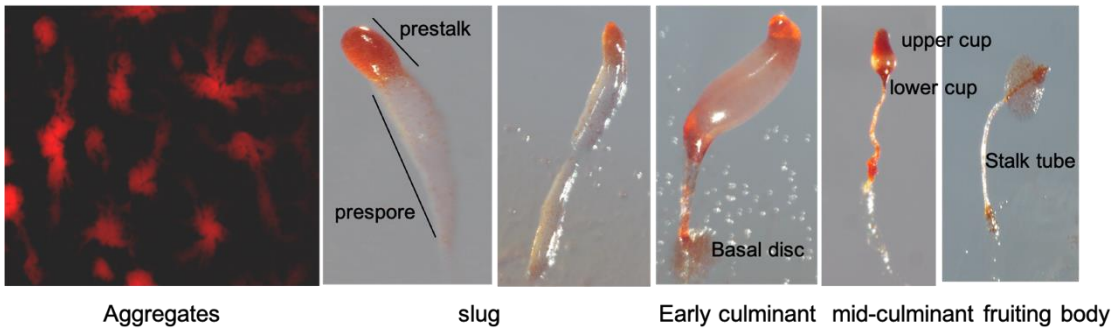

Figure 5. Enlarged pictures of the different stages of development

Chemotaxis experiment

1. Prepare vegetatively growing cells and starved cells. Harvest *Dictyostelium* cells via centrifugation, wash with KK2 buffer to fully remove medium (thereby inducing starvation), and resuspend at 10^7 cells/ml in KK2 buffer. Shake at 22°C for 6 to 8 h. Prior to assay, vegetatively growing cells and starved cells are harvested via centrifugation and resuspend at 2.5 x 10^8 cells/ml in KK2 buffer.
2. Prepare of chemoattractant stock solutions. cAMP stocks are prepared at 10 mM and kept cold in the dark.
3. Using the KK2 buffer, dilute the 10 mM cAMP stock to make the necessary solutions for the assay (10, 50 and 250 µM, in a serial dilution, max 500 µl of each solution). Place your diluted solutions in well-marked microfuge tubes. A tube of "buffer alone or KK2" containing a dye (NR) can be taken as a control.
4. The agar plates need to be fully hydrated before starting the experiment. Therefore, they are overlayed with KK2 buffer that now needs to be removed, thoroughly drained and wiped from the plate side.

Make 4 wells in the centre of each of the quadrant as shown in Fig. 6.

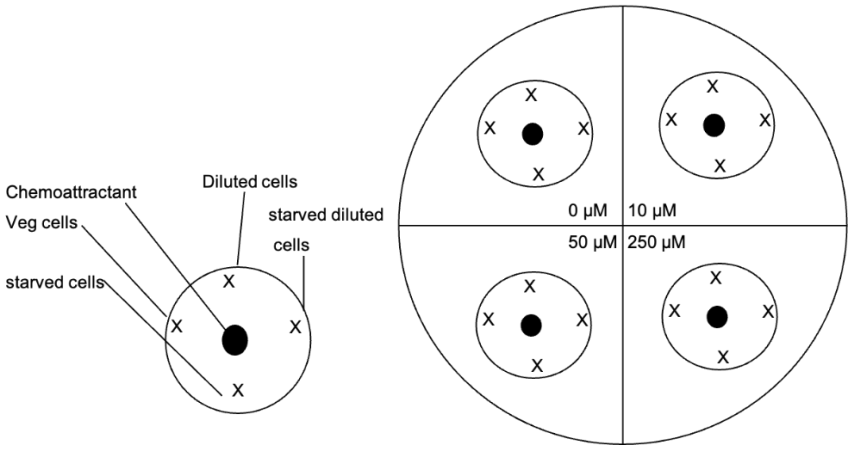

Figure 6. Schematic drawing of the Petri dish, with quadrants, wells and drops of cells

5. Put the lid back on the plate and turn the plate over. Using your marker and a ruler, make 4 small dots at 5 mm from the edge (not centre) of each well. Remember that the cell spots will occupy a larger area than the cross that you make.

6. Label the plates (in some way that will not get in the way of seeing cell movement using some sensible code to correspond to: buffer alone, 10, 50 and 250 µM cAMP. Each group of 4 cross will be used to spot Ax2 cells, both vegetatively growing and starved. Each group of dots should have the cells in the same configuration.
7. Carefully fill the wells in your chemotaxis assay plates. You may wish to practice this with some phosphate buffer on the practice plate. About 15– 30 µl is sufficient, but this will vary with the size of your wells. Whatever volume you have to use, do not overfill the wells, as spillage on the surface of the agar will disturb the assay. Leave the plates about 10-15 min to dry without lid.
8. Place your chemotaxis assay plates in a plastic box with a few humid tissues, close it and place it on a safe, levelled surface of the bench at 22^{o}C.
9. The images of the spot at time 0 and after 4 h are taken in stereo-zoom microscope and chemotaxis rate is calculated as given below:

   Migration rate (µm/minute) = difference in spot diameter (before and after) and divide by two to obtain the difference in spot radius, divide this value (µm) by time (min).

QUESTIONS FOR FURTHER STUDIES

1. Can you draw a life cycle of *Dictyostelium* showing pattern formation with the help of neutral red staining?
2. What is the smallest number of cells that can form a fruiting body. Check the ratio of stalk:spore in them.
3. What happens if you add cAMP to the entire plate and drop *Dictyostelium* cells? Do they show chemotaxis?

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Chapter 64: HYDRA AS A POWERFUL TEACHING AND RESEARCH TOOL: MAINTENANCE, BUDDING, CELL BIOLOGY AND REGENERATION

Recommended level: UG, PG, Research

INTRODUCTION

Hydra is a simple fresh water animal with a cylindrical body, radial symmetry and a well defined oral-aboral axis. The oral end bears a conical hypostome which is surrounded by a ring of tentacles. At the aboral end mucus-secreting basal disc which aids in attachment of the animal to substratum is present. Body column shows presence of peduncle or stalk in some species. In the Indian species (*Hydra vulgaris* Ind-Pune), polyps are light brown in well-fed conditions and appear pale on starvation. They are about 4-8 mm long, do not have a distinct stalk and have 5-6 tentacles (Fig. 1).

Kingdom: Animalia
Subkingdom: Eumetazoa
Phylum: Cnidaria
Subphylum: Medusozoa
Class: Hydrozoa
Subclass:Leptolinae
Order: Anthomedusae
Suborder:Capitata
Family: Hydridae
Genus: ***Hydra***

Figure 1. Morphological features of hydra. *H. vulgaris* Ind-Pune polyp with bud (Reddy et al., 2011).

Hydra is diploblastic and consists of two tissue layers, outer ectoderm and inner endoderm, separated by a thin layer of mesoglea. Hydra has around 30,000 to 200,000 cells (depending on the species) of about 20 different types distributed across its two tissue layers (Bode, 1996) . All cell types are derived from three basic stem cell lineages namely, ectodermal epithelial stem cells, endodermal epithelial stem cells and interstitial stem cells (Bode, 1996).

Modes of Reproduction

Hydra shows both asexual and sexual modes of reproduction. In normal, well-fed conditions asexual reproduction by budding occurs regularly, while sexual reproduction occurs in response to unfavourable conditions like starvation or change in temperature (Littlefield et al., 1991).

Asexual reproduction

Bud emerges as an outgrowth from a placode in the budding region, which is present just above the peduncle. In *Hydra vulgaris*, budding normally takes place at a position 2/3 distant from the apex of the animal. The process is initiated with the commitment of interstitial cells to the neural fate and occurs one day before any obvious macroscopic modification is observed (Berking, 1980). Subsequently, ~ 800 epithelial cells are recruited from both layers of the parental body and their number reaches 5000 cells within 24 hours (Graf and Gierer, 1980). Then the neuroblasts migrate within the evaginating bud tissue where they start proliferate and differentiate as neural and ganglion cells (Berking, 1980). Elongation of the bud occurs due to continuous cell division of ectodermal and

endodermal cells (Berking and Gierer, 1977). Hypostome and tentacles are formed eventually and the bud gets detached from the parent by contraction of the tissue near the point of attachment.

Sexual reproduction

Sexual reproduction occurs during unfavourable conditions like starvation or change in temperature (Littlefield et al., 1991). Gametes are formed within gonads and are exclusively ectodermal and develop between the ectoderm and mesoglea (Martin et al., 1977). Some hydra species are dioecious but several are hermaphrodite in which the sperms mature before the eggs, to prevent self-fertilization. *H. vulgaris* Ind-Pune polyps are dioecious with alternate, spirally arranged broadly triangular male gonads (Fig. 2A) on the body column from the sub-hypostomal region to budding zone. One or more round female gonads may occur along the body in the budding region (Fig. 2B).

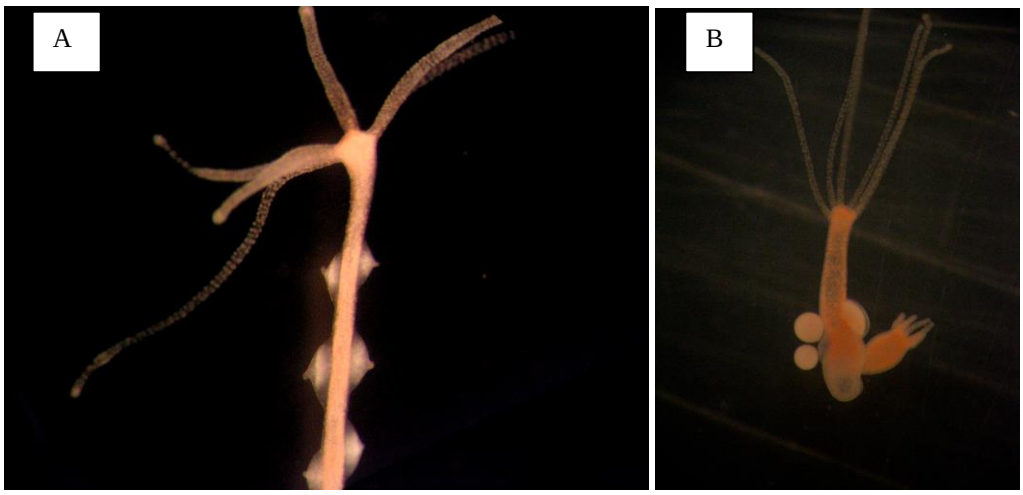

Figure 2. Appearance of male (A) and female (B) gonads in *Hydra vulgaris* AEP strain.

Hydra as a model system

1. Simple body plan
2. Primitive organisms displaying head-foot polarity
3. First tissue grade organization and primitive nerve network
4. Remarkable regeneration capacity
5. Ease of maintenance of culture and amenability to various experimental manipulations
6. Absence of organismal aging

Maceration preparation and identification of cell types (Jordan, 1965).

Epithelio-muscular cells: Epithelio-muscular cells in hydra show both epithelial and muscular function. There are two types of epithelio-muscular cells: ectodermal and endodermal. **Ectodermal epithelio-muscular cells:** These are cylindrical with their inner ends produced into 2-3 processes and have myonemes. These myonemes form longitudinal muscles which bring about contraction of the body. Ectodermal cells of basal disc are granular and secrete mucus for attachment of hydra. The basal ectodermal cells can also form pseudopodia by which the animal glides on its attachment. **Endodermal epithelio-muscular cells** are long and club shaped, their outer ends have two processes containing myonemes. Endodermal cells are larger than ectodermal cells in the body but in the basal disc they are much shorter.

Interstitial cells: Lying in the spaces between the inner end of the ectoderm cells and outer ends of endoderm cells are the interstitial cells. They are small oval and round in shape with a large nucleus. Interstitial cells renew all cells of the animal and hence are totipotent.

Gland cells: These are club shaped with slender bases reaching the mesogloea, they may be granular or vacuolated with a large nucleus and one flagellum. In the ectoderm, they are found only near the mouth and basal disc, while they are plenty in the endoderm. Mucous gland cells are found in the mouth and hypostome and secrete mucus which helps in swallowing solid food. Enzymatic gland cells are found in the gastric region where they secrete digestive enzymes.

Sensory cells: They are long and narrow cells with a large nucleus and one projecting flagellum or sensory hair. Their base may be produced into nodulated processes which join the nervous system. Sensory cells are found in both germinal layers. Each sensory cell acts both as a receptor and as a sensory neuron i.e., it both receives and transmits impulses.

Cnidoblasts: Some interstitial cells of the ectoderm give rise to highly specialized cells called cnidoblasts. A cnidoblast is round or oval cell with a nucleus lying to side. At one end is a projecting hair-like cnidocil and gives rise to a nematocyst or stinging cell. They are found only in the ectoderm.

Nematocytes: These are also called stinging cells, which help in catching the prey, are the characteristic feature of Cnidaria. The cells bear a large inclusion, the nematocyst which is a modified vacuole. Four types of nematocytes are observed: stenoteles, desmonemes, holotrichous isorhiza and atrichous isorhiza.

All nematocysts have a sensory hair, the cnidocil that causes discharge of the contents upon stimulation. Nematocytes are used in capturing the prey and also during locomotion. The nematocytes develop and form in one place and then move into battery cells in tentacles or body column and are unable to convert from one type to another.

Types of nematocysts

Stenoteles (penetrants). In stenoteles, one end of the vacuole is invaginated to form a coiled whip-like tube and the other end is bulbous and filled with toxins. The tube is open at the end and acts like a hypodermic needle to inject the toxins. Whips of the stenoteles flash out and inject the toxic substances. They have a large capsule, the butt is stout with three spiral rows of spines on its distal half, and the lowest spine is a large stylet. The thread has spirals of small spines and opens at the tip. Stenoteles are weapons of defense and offence, their thread penetrates the body of the prey and releases toxins.

Desmonemes (volvents). These are small structures with a thread tube that is coiled at the end. They have small oval capsule; the thread is thick with no spines and it is closed at the tip. it lies in a single loop inside the capsule. On being discharged, the volvents are thrown out of the body and the thread coils around the bristles of the prey. Desmonemes attach to any surface in their vicinity by coiling their thread tubes around projections on the surface.

Holotrichous isorhizae (large glutinants). They have a long and narrow thread tube with rows of tiny spines and 3-4 transverse coils at the end have an oval capsule. The butt is narrow and the thread is open at the tip, there are small spines on the butt and thread. They attach to any surface in their vicinity by coiling their thread tubes around projections on the surface.

Atrichous isorhizae (small glutinants). They bear a short, straight, open-ended thread tube. They have an elongate capsule, butt is absent, thread is open at the tip, and shows no spines. They attach to any surface in their vicinity by coiling their thread tubes around projections on the surface.

OBJECTIVES

To learn maintenance, to study general morphology and budding, cell biology and regeneration.

MATERIALS REQUIRED

1X Hydra medium

KCl	0.1 mM
NaCl	1.0 mM
$MgSO_4.7H_2O$	0.1 mM
$CaCl_2.2H_2O$	1.0 mM
Tris Base	1.0 mM (Use pH 8.0 Tris-Cl Solution)

(Dissolve all the components except $MgSO_4$, to avoid precipitation, in distilled water. Add separately dissolved $MgSO_4$ solution to this. Adjust pH to 8.0. Then make up the final volume and autoclave for 40 min. Prepare a stock of 100X hydra medium and dilute with distilled water to 1X for use).

ABTS solution

Citric Acid	65.5 mM
Tri-sodium citrate	34.5 mM
ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid))	0.1 %
Hydrogen peroxide	0.003%

***Artemia nauplii* hatching (to be used as food)**

Soak *Artemia* cysts in salt water in a beaker (3.2 g of rock salt dissolved in 1 l of tap water) under continuous aeration. Depending on ambient temperature, cysts will hatch within 24 to 48 h. Since these crustaceans are photo-attractive, freshly hatched larvae can be easily collected from the beaker facing towards light.

PROCEDURE

Hydra culturing and maintenance

Maintain clonal culture of hydra in glass crystallizing dishes containing 1X hydra medium at a constant temperature of 18 ± 1°C with 12 h day and night cycle. Feed the polyps with freshly hatched *Artemia salina* nauplii and change the medium after 6 h post feeding (Sugiyama and Fujisawa 1977). Before feeding, wash *Artemia* with tap water to remove excess salts and feed approximately 20 *Artemia* per hydra (Fig. 3).

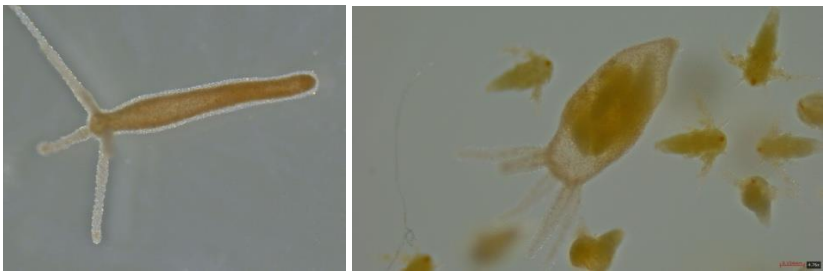

Figure 3: A live hydra polyp (left) and a hydra polyp post-feeding with several free swimming *Artemia* nauplii around it.

Tissue manipulations (Hydra bisection, trisection, longitudinal section and regeneration)

1. Place 24 h starved hydra on a clean glass slide for relaxing
2. Cut hydra with a sharp blade or needle into two/three pieces
3. Keep all these pieces (head, middle and base pieces separately) in a six well plate containing hydra medium
4. Observe every 24 h for head and foot regeneration for 72 h

OBSERVATION

Head pieces will regenerate a foot, middle pieces will regenerate both head and foot and the foot pieces will regenerate a head. Head regeneration can be visually observed with the appearance of tentacle rudiments. Foot regeneration can be observed by the presence a sticky secretion through which it starts adhering to the substratum. Foot specific staining can be done after 48-72 h on regenerating head and middle pieces to observe functional foot formation.

Foot specific staining (Hoffmeister and Chica, 1985)

Mucous cells in the basal disk of hydra contain a peroxidase-like enzyme which allows specific staining of these cells with substrates for peroxidases. Here, we use ABTS solution as a substrate for peroxidase. The peroxidase activity provides an excellent marker for foot mucous cell differentiation and is used to follow the appearance of foot specific cells during foot regeneration. After 48-72 h, foot regenerating pieces can be stained as follows to see whether they have formed a functional foot.

PROCEDURE

1. Take head pieces (foot regenerating pieces) in a six well plate, about 10 pieces per well.
2. Remove hydra medium completely and immediately add 2-3 ml of ABTS solution containing Hydrogen peroxide to the well.
3. Cover the plates with aluminum foil and incubate for 5-15 min at RT till the pink coloration develops.
4. Stop the reaction by adding 1X PBS (pH 5.0) or wash them with distilled water.
5. After 15-20 min wash these head pieces with fresh PBS and observe.

OBSERVATION

A purple coloured ring can be seen at the foot tip (Fig. 4).

Figure 4: A freshly bisected hydra polyp

OTHER PROTOCOLS THAT ARE ROUTINELY FOLLOWED FOR HYDRA

Polyp length measurements

Take adult polyps with a single bud in a glass Petri plate and let them relax and elongate. Place a graph paper under the Petri plate and measure the body column length of live polyps. Relax the polyps with 2% urethane for 1-2 min and fix them with 4% paraformaldehyde in 1X PBS overnight at 4°C or 1 h at room temperature. After fixation, wash the polyps thrice with 1X PBS and take images under a microscope. Measure the body column length using thread.

Cell maceration

Take 10 hydra polyps in a microcentrifuge tube. Dissociate the tissue in 200 µl of maceration medium containing glycerin: glacial acetic acid: 1X hydra medium (1:1:13) and incubate for 10 min at room temperature. Prepare a single cell suspension using a 2 ml syringe with 20 gauge needle followed by fixation with 20% formaldehyde and mount on microscopic slide with a cover glass in 50% glycerol. Observe under microscope for different cell types.

Nematocysts preparation

Decapitate live hydra and collect hypostomes along with tentacles. This will enrich the nematocysts population. For this, take adult non budding polyps (n=20) starved for 36 h and cut just below the hypostome. Dissociate the tissue in 200 µl of maceration medium containing glycerin: glacial acetic acid: 1X hydra medium (1:1:13) and incubate for 10 min at room temperature. Prepare a single cell suspension, fix and mount them on a microscopic slide. Observe under microscope and take images of different nematocysts (Fig. 5).

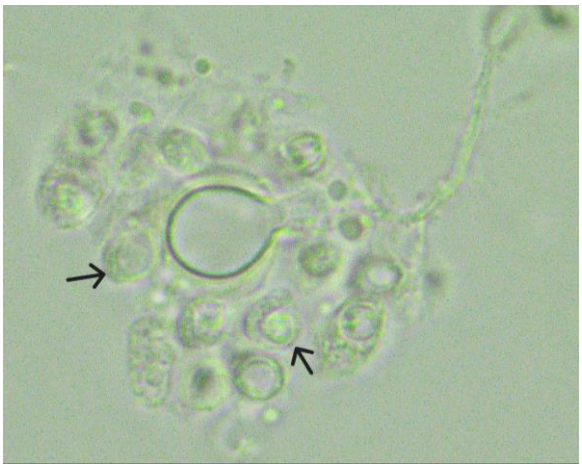

Figure 5. Nematocysts of Hydra

Histology

1. Relax the polyps in 2% Urethane and fix them in 4% paraformaldehyde overnight at 4°C
2. Wash with 1X PBS (3X5')
3. Dehydrate in 25%, 50%, 75%, 90% ethanol grades (10 min each)
4. Wash with butanol (3X5')
5. Wash with 1% celloidin dissolved in methyl salicylate
6. Remove excess solution with tissue paper

7. Wash with chloroform for (2X15')
8. Transfer in chloroform + wax (1:1) at 45°C (2 X 45')
9. Impregnate in molten wax at 60°C (2X10')
10. Prepare wax blocks in porcelain plate or L-blocks
11. Trim the block from all sides and mount the wax block on a wooden block. Keep the wooden block at 4°C
12. Take transverse sections of 10-12 µm thickness on microtome
13. Spread the wax ribbon with tissue-sections on a glass slide properly

Hematoxylin and Eosin staining of the sections (Fischer et. al., 2008)

1. Deparafinize the sections in xylene (2X 10 min)
2. Xylene + ethanol (1:1) (10 min)
3. Absolute ethanol (10 min)
4. Hydrate the sections in 90 % alcohol (10 min), 70% alcohol (20 min), 50% alcohol (10 min), 30% alcohol (10 min), 10 % alcohol (10 min) and distilled water (10 min)
5. Hematoxylin – 25 drops in a jar for---min (monitor till you observe dark purple coloration)
6. Wash in Acid water (5-6 drops of 0.1N HCl in 50 ml DDW in a Couplin jar)
7. Wash in alkaline water (5-6 drops of 1N NaOH in 50 ml DDW in a Couplin jar)
8. Wash in distilled water (5 min)
9. Dehydrate in 10% (10 min), 30%(10 min), 50% (10 min), 70% (20 min) ethanol grades.
10. 1% Eosin (Just dip and take out the slides)
11. 90% ethanol (5 min)
12. Absolute ethanol (2 min)
13. Ethanol + Xylene (1:1) (5 min)
14. Xylene (5 min)
15. Mount in DPX and observe under microscope

QUESTIONS FOR FURTHER STUDIES

1. What are the differences between regeneration in Hydra and regeneration of limbs and tail in vertebrates?
2. Why is Hydra a useful model system to study Evo-Devo (evolutionary developmental biology)?
3. What are the differences between the stem cells of Hydra and those of higher organisms including human?
4. Why is Hydra considered to be a potentially immortal organism?

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Chapter 65: LEARNING GENETICS, RNA INTERFERENCE, AND MICROSCOPY USING *CAENORHABDITIS ELEGANS*

Recommended Level: UG and PG

INTRODUCTION

Caenorhabditis elegans is a 1-mm-long nematode (roundworm) (Fig. 1). These worms thrive in temperate regions, where they feed on bacteria found abundantly in the mounds of decaying fruits, vegetables, and other organic matter. In the laboratory, they can be readily grown on *E. coli* lawns cultivated on agar plates. Many features of *C. elegans* anatomy, life cycle, and reproductive style have attracted a large number of scientists to use it as a model organism for studying a range of biological problems. This Introduction provides a brief description of these features; for a detailed description, visit www.wormatlas.org and www.wormbook.org.

The body of *C. elegans* consists of a defined number of cells

Caenorhabditis elegans exists in two sexual forms: hermaphrodites and males. Hermaphrodites are essentially females that make some sperm at the onset of gametogenesis and then switch to oogenesis. Males produce only sperm throughout adulthood and transfer the sperm to hermaphrodites during mating. Thus, hermaphrodites can use their own sperm or the male's sperm, if mated, to fertilize their oocytes. The adult body consists of a small, but a defined number of cells—959 and 1031 cells, respectively, in hermaphrodites and males. *C. elegans* has a simple anatomy with the bulk of its body made up of two tubes, the digestive system and the gonad, contained within a transparent cuticle made up of hypodermal cells. Transparency of the cuticle permits observation of internal organs during development in a live animal without the need for dissection or staining with dyes. Only 302 neurons make up the entire nervous system in hermaphrodites; it is slightly more, 385 neurons, in males. The male-specific neurons aid in seeking and mating with hermaphrodites. The small number of neurons facilitated the construction of *C. elegans*' entire neuronal circuitry, which enables scientists to study how the neural networks process information from various stimuli and convert it into appropriate animal behaviour. *C. elegans* has no dedicated circulatory system or a bony skeleton, but has a well-developed musculature involved in locomotion, feeding, mating, and egg-laying.

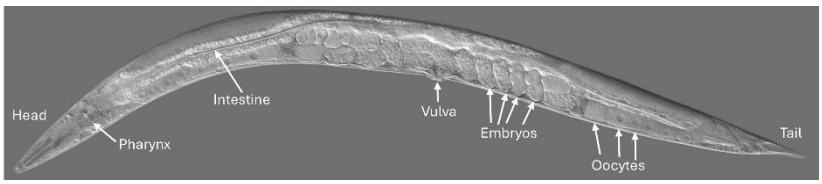


Figure 1. Differential Interference Contrast image of adult *C. elegans* hermaphrodite. The head-to-tail length is about 1 mm. The transparent cuticle permits direct visualization of internal structures.

***C. elegans* development** – invariant cell lineage and alternative developmental paths

The *C. elegans* embryo undergoes an invariant pattern of cleavage, which means that the cell division patterns, the number of cells formed, and the position and developmental fate of individual cells do not vary from one individual to another. This feature enabled scientists to determine the lineage, i.e., the developmental ancestry of cells—of all cells in *C. elegans* (Fig. 2).

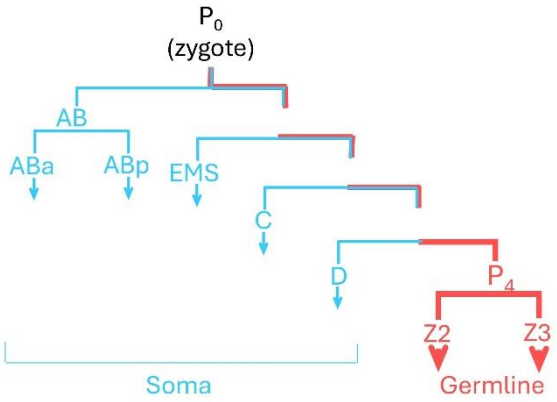


Figure 2. Schematic representation of cell lineage. As an example, a part of the early embryonic lineage is shown. Vertical lines represent the ancestry, and horizontal lines connect the siblings resulting from a cell division. Note: Subsequent lineages generated by the ABa, ABp, EMS, C, and D blastomeres are not shown.

In addition, the invariant lineage provided evidence for the existence of programmed cell death by pinpointing when and which cells would die during embryogenesis, and continues to aid researchers study developmental decisions at single-cell resolution. Embryogenesis takes about 18 hours and generates 558 cells. Some of these cells are blast cells that divide further and differentiate during larval development. When food is plentiful, the newly hatched larva, called L1 larva, continues growth and development and molts four times through the L2, L3, and L4 larval stages into a reproductively active adult (Fig. 3).

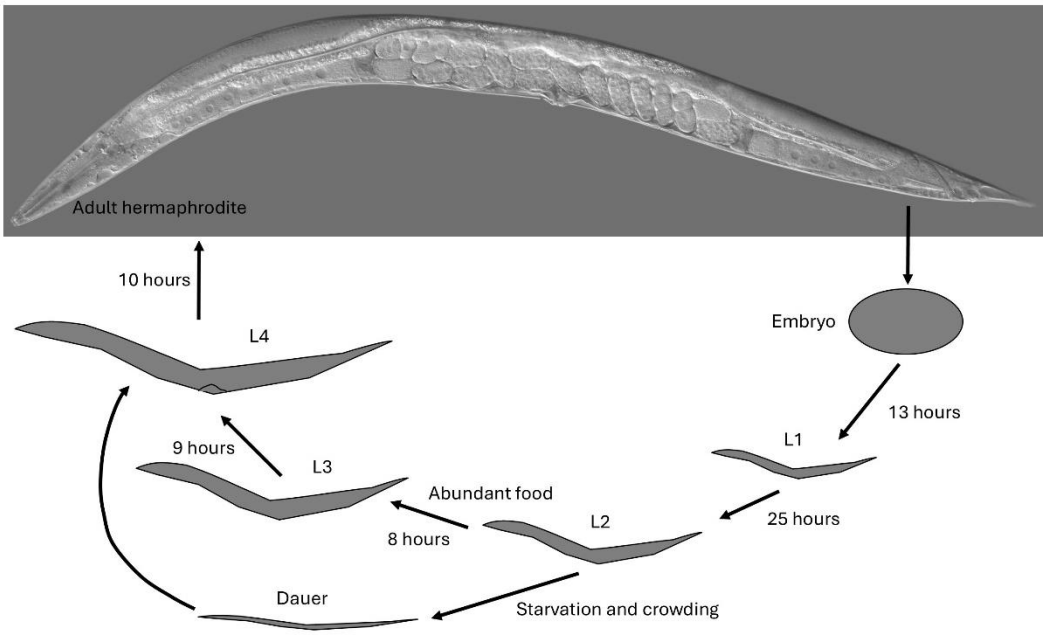


Figure 3. *C. elegans* life cycle. The durations indicated are at 25°C. The larva molts at the end of each larval transition.

When food is scarce, L1 larvae arrest their development and enter a dormant stage known as L1 diapause. Under high population density, coupled with limited food, L1 larvae enter an alternative developmental stage called the dauer, which can endure starvation for several months. Scientists are intensely studying how the entry, maintenance, and exit aspects of the dauer stage are regulated. Such

studies continue to generate valuable insights into the metabolic regulations associated with diabetes, obesity, and aging in diverse organisms, including humans.

C. elegans is genetically tractable

A newly hatched *C. elegans* embryo takes only 2.5 days to become a reproductively active adult. So, one can readily observe segregation patterns of traits over multiple generations within the period of a six-month dissertation project! As mentioned earlier, the *C. elegans* hermaphrodites can use their own sperm or the male's sperm, if mated, to fertilize their oocytes. Geneticists utilize self-fertilization to recover animals homozygous for recessive alleles quickly, and cross-fertilization to construct double-mutant strains. The relatively small genome size (100 million nucleotides) and a large brood size (300 embryos per hermaphrodite) of *C. elegans* reduce the number of animals required for examination in genetic screens. Furthermore, since they contain both sperm and egg, screening hermaphrodites doubles the number of mutagenized genomes screened.

C. elegans is well-suited for high-throughput RNAi screens

In classical genetics (a.k.a. forward genetics), a researcher first identifies a phenotype and then, based on the segregation patterns of the phenotype with respect to other known phenotypes, finds the DNA sequence that caused it. By contrast, in organisms whose genome sequence is known, one can utilize reverse genetics tools, such as RNA-mediated interference (RNAi), to define the phenotypes associated with specific DNA sequences. Genome-wide RNAi screens can be readily performed in *C. elegans*. In *C. elegans*, RNAi is performed by introducing double-stranded RNA (dsRNA) that corresponds to the target gene. dsRNA is introduced by microinjection, soaking, or feeding bacteria that produce dsRNA from a plasmid. The feeding method is especially suitable for high-throughput, genome-wide screens, as parts of all *C. elegans* genes have been cloned into plasmid vectors, and frozen stocks of *E. coli* strains carrying these plasmids, known as the RNAi library, are commercially available. So, simply feeding such *E. coli* strains to *C. elegans* worms is sufficient to deplete the gene products, which can be performed for a large number of genes at a time.

In addition to the above, robust methods for CRISPR/Cas9-based gene editing or endogenous tagging with reporters such as GFP, and stable integration of single-copy transgenes have been developed for *C. elegans*.

OBJECTIVES

1. To culture *C. elegans*, identify the different larval stages, and identify common mutant phenotypes
2. To set up genetic crosses and experimentally test Mendel's laws
3. To learn the concept of maternal-effect phenotypes
4. To test the effect of temperature on fertility
5. To determine gene function using RNAi

MATERIALS REQUIRED

***C. elegans* strains**: N2 (wildtype); CB187 – *rol-6(e187)* I; CB120 – *unc-4(e120)* II; CB128 - *dpy-10(e128)* II; DR103 – *dpy-10(e128) unc-4(e120)* II; IT2000 – *dpy-5(e61)* I*; unc-4(e120)* II; IT1547 – *age-1(kp96)* / mnC1] II

***E. coli* strains**: OP50 and HT115 carrying RNAi plasmid for *pos-1*, *mex-3* or *skn-1*

Components for culture media: Sodium chloride, Agar, Tryptone, Peptone, yeast extract, Tris base, Cholesterol, $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, Ampicillin, Tetracycline, and IPTG.

Other consumables include disposable, sterile 35 mm Petri dishes; conical flasks with 250 ml and 1 L capacities; micropipettes; glass pipettes; glass Pasteur pipettes; 0.3-mm-thick platinum wire; and scalpel blades with a holder.

Equipment: Stereo-zoom microscope with transmitted light base carrying an objective lens with 0.8x to 5.0x magnification range; flat nose plier with smooth jaw; a room with reasonably clean air maintained around 20 to 23°C [If microbial contamination is a recurrent issue, you may need a vibration-free laminar hood with vertical airflow]; Bunsen burner; low-temperature incubator that can maintain 20°C ±1°C; and autoclave; and incubator shaker. A peristaltic pump liquid dispenser with a foot pedal will facilitate the ease of preparing agar plates.

Preparation of media

LB liquid medium: Dissolve 5 gm NaCl, 5 gm tryptone, and 2.5 gm yeast extract in about 400 ml of water and make up the volume to 500 ml in a one liter conical flask, and autoclave: 15 psi, 20 min.

LB agar plates: In addition to the components in LB liquid media, add 7.5 g of agar, make up the volume to 500 ml in a one-liter conical flask, and autoclave at 15 psi for 20 min. Allow to cool for about 10 min, then gently swirl to mix. Dispense approximately 20-25 ml into 90 mm Petri dishes in a laminar hood. Leave the plates uncovered for approximately 15 min, allowing the agar to solidify. Cover the plates and leave them at room temperature for about 12-18 h before use.

Media for RNAi bacteria: Cool the above media to about 55°C and add ampicillin and tetracycline to a final concentration of 50 µg/ml and 12.5 µg/ml, respectively. Mix well.

Nematode growth medium (NGM)

Composition (per 800 ml): NaCl – 2.4 gm, agar – 20 gm, peptone – 2.0 gm, 1 M potassium phosphate buffer (K_2HPO_4 / KH_2PO_4) pH 6.0 – 20 ml, 1 M $CaCl_2$ 0.8 ml, 1 M $MgSO_4$ – 0.8 ml, and cholesterol (10 mg/ml stock) – 0.64 ml

Preparation:

1. Prepare the 1 M stock solutions of the buffer and salts separately by dissolving the required amounts in water and autoclaving. Dissolve cholesterol, 10 mg/ml, in ethanol.
2. In a 1-litre beaker, add NaCl, peptone, and 600 ml of water, and stir on a magnetic stirrer to dissolve.
3. Transfer to a 1-litre measuring jar, adjust the volume to 800 ml with water, and transfer to a 1-litre conical flask. Add the agar, cover the mouth of the flask with aluminium foil, and autoclave at 15 psi for 20 min. Allow to cool for about 10 min and gently swirl to mix the molten agar.
4. Cool to about 60°C and add $CaCl_2$, $MgSO_4$, and cholesterol. Mix well to spread the cholesterol uniformly. [For RNAi, in addition to the above components, add ampicillin and IPTG to a final concentration of 50 µg/ml and 0.8 mM, respectively. Mix well.]
5. In a laminar hood, dispense approximately 4 ml of medium/dish into 35 mm Petri dishes. Let the plates be at room temperature for one day. Plates can be stacked, covered with plastic wrap, and stored at 4°C for approximately two months. Do not store the RNAi plates for more than a month.

Preparation OP50 lawns

1. Streak out the OP50 strain from a frozen glycerol stock on an LB agar plate and incubate overnight (~15 h) at 37°C. Remove the plate, examine it for bacterial growth, wrap it in Parafilm, and store it at 4°C. The plate may be stored up to two weeks at 4°C.
2. Inoculate a single colony into 100 m of LB medium and incubate at 37°C at about 120 rpm for about 12-15 h. The culture may be stored at 4°C for up to two weeks.
3. Using a disposable, sterile transfer pipette, dispense two drops (~100 µ) of the OP50 culture at the center of the NGM agar plate and spread it into a circle using a sterile glass test tube. Cover the plate and leave at room temperature for two days. During this time, the bacteria will grow into a thin lawn.
4. Stack the plates, about 10 / stack, and cover with a plastic wrap. Store at 4°C for up to a month.

Preparation of HT115 lawns for RNAi

1. Streak-out HT115 bacteria carrying the appropriate RNAi plasmid on an LB agar plate containing ampicillin and tetracycline, and incubate overnight (~15 h) at 37°C. Remove the plate, examine for bacterial growth, and store at 4°C. Storing such plates for more than one week may reduce RNAi efficiency.
2. Inoculate 2 ml of LB medium containing ampicillin and tetracycline in a 15 ml sterile test tube with a colony of the HT115 bacteria and incubate overnight (~15 h) in a shaker incubator (~180 rpm) at 37°C.
3. Inoculate 40 ml of LB medium containing ampicillin (omit the tetracycline for this step) in a 250 ml sterile conical flask with 0.4 ml of the overnight culture. Incubate at 37°C with shaking ((~180 rpm) for 4 h.
4. Add 80 µl of 0.5 M IPTG [Prepare IPTG stock in water and store at -20°C.] and continue 37°C incubation with shaking for 4 h.
5. Centrifuge at 3000 g and 25°C for 5 min.
6. Discard the supernatant and resuspend the bacterial pellet in 1 ml LB medium.
7. Dispense 150 µl of the bacterial suspension in the centre of an NGM agar plate containing ampicillin and IPTG. Swirl gently to spread the culture evenly without reaching the edges. Let it dry for one day.

Making the "worm pick"

Using a scalpel blade, cut about a 2 cm-long platinum wire. Hold the wire piece with a forceps and insert a small amount (~0.5 cm) into the narrow mouth of a glass Pasteur pipette. Using a Bunsen burner's flame, heat and fuse the pipette's mouth so that the platinum wire is held firmly in place permanently. Using the flat-nose plier, flatten the free end of the platinum wire. Trim any sharp edges at the tip of the wire using a scalpel blade.

PROCEDURE

Objective 1: To culture *C. elegans*, identify the different larval stages, and identify common mutant phenotypes

Caution: All worm work must be performed in a temperature-controlled room kept in the range of 20-24°C. At temperatures of 27°C or higher, the worms will become sterile.

1. Working under a stereo-zoom microscope, using a worm pick, transfer 3-4 gravid N2 hermaphrodites onto the surface of a OP50 lawn. For this, flame the pick until it glows orange (takes less than 1 second), cool it briefly (again less than a second), and gently touch the OP50 bacteria at the edge of the lawn (the lawn is thicker at the edges) with the bottom surface of the flattened tip of the pick. This process will gather some bacteria on the pick, which will act as a glue to pick the worm. Gently touch a worm and lift the pick. The worm will stick to the bacteria on the bottom side of the pick. Place the worm on a fresh plate by gently touching the lawn with the pick and dragging it, allowing the worm to wriggle out of the pick. During this process, make sure not to gouge the agar. This will require a few days of practice for most beginners. Worms readily burrow into the agar if gouged.
2. Repeat the above for the other required strains.
3. Incubate at 20°C for 4 days. On the fourth day, there will be a lot of worms at different larval stages, including adults and embryos. Observe them under the microscope and learn to identify the various larval stages, adults, and embryos in the N2 plate. For recognizing the different larval stages, refer to Corsi et al (1). Observe the CB120, CB128, and CB187 plates and learn to identify uncoordinated (unc), dumpy (dpy), and roller (rol) phenotypes, respectively.
4. To maintain the culture, again transfer 3-4 gravid hermaphrodites to a fresh OP50 lawn and repeat this process every 4 days.

Objective 2: To set up genetic crosses and experimentally test Mendel's laws

A. Testing the law of segregation:

The required genetic cross is shown diagrammatically in Fig. 4.

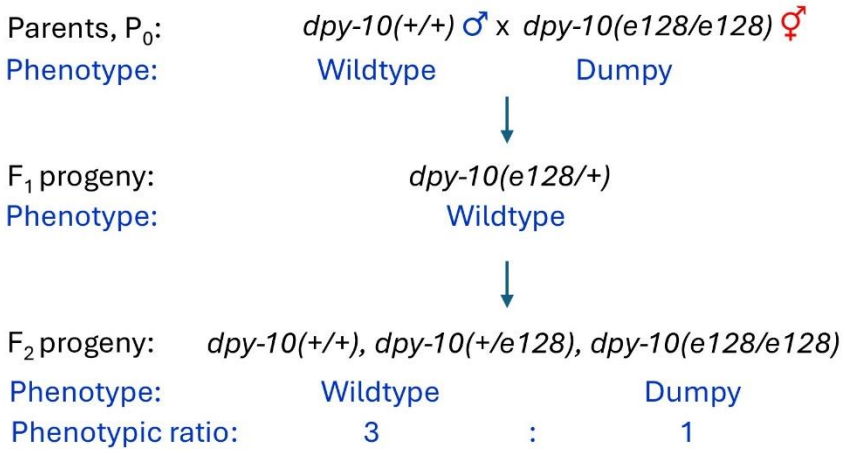


Figure 4. The mating scheme for testing the law of segregation.

1. To begin, borrow a plate containing both wildtype males and hermaphrodites from a worm laboratory. *[In the wildtype, males are produced only through mating. If you passage only unmated hermaphrodites, there will be no male progeny!]*

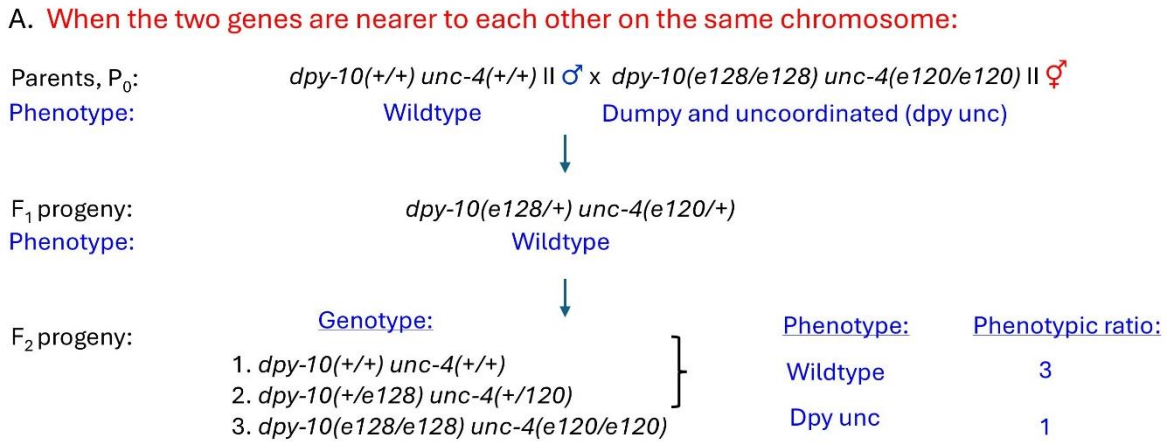
2. Day 1: Pick 9 young N2 males (the earliest stage at which you can readily identify males) and 3 CB128 (dpy) young hermaphrodites (past the L4 stage, but not yet gravid) to a fresh OP50 lawn, and incubate at 20°C. This is your Day-1 plate.
3. Day 2: Shift the males and hermaphrodites to a fresh OP50 lawn. This is your Day-2 plate. Avoid shifting any progeny embryos or larvae from the Day-1 plate.
4. Day 4: Remove the parental males and hermaphrodites.
5. Day 5: Progeny will now be at the L4 or adult stage. Males and hermaphrodites will roughly be in equal numbers. These are the F1 progeny. All these worms will be wildtype (non-dpy). Transfer 5 hermaphrodites, 1 per lawn, to 5 fresh OP50 lawns. This process, where one worm is placed per plate, is referred to as "cloning" within the worm community. Incubate the 5 plates at 20°C for 4 days.
6. Count the total number of progeny (F2) in each plate. Additionally, count the wildtype and dumpy worms separately.

OBSERVATION

1. All F1 progeny will display a wildtype phenotype.
2. About 25% (1/4th) of the F2 progeny will display a dumpy phenotype.

B. Testing the law of independent assortment:

The required genetic crosses are shown diagrammatically in Fig. 5.



Note: Occasional dpy non-unc and non-dpy uncs will be seen among F2 progeny due to recombination between the two loci.

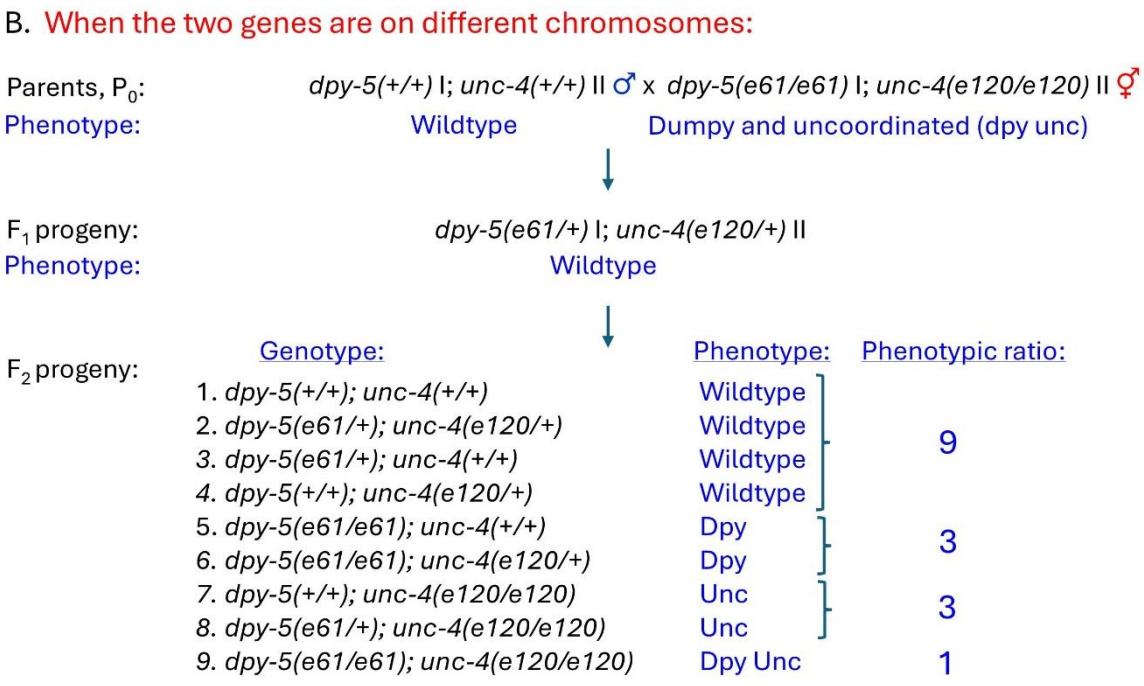


Figure 5. The mating schemes for testing the law of independent assortment

1. Repeat above steps 1-5 by setting the following two crosses:
    a) N2 males with DR103
    b) N2 males with IT2000
2. Count the total number of F2 progeny in each plate. Additionally, count the wildtype, dpy, unc, and dpy unc worms separately and tabulate the results.

OBSERVATION

1. In cross (a), since both dpy-10 and unc-4 are on the same chromosome (chromosome II), dpy non-unc and unc non-dpy animals will be rare. Wildtype and dpy unc phenotypes will show approximately a 3:1 ratio. [The few animals with dpy non-unc or unc non-dpy phenotypes are due to recombination between these two loci.]
2. In cross (b), since both dpy-5 and unc-4 are on different chromosomes (I and II, respectively), all four phenotypes—wildtype, dpy non-unc, unc non-dpy, and dpy unc—will show approximately a 9:3:3:1 ratio expected for independent assortment of the alleles of *dpy-5* and *unc-4* genes.

Objective 3: To learn the concept of maternal-effect phenotypes

Many genes that function during early embryogenesis are transcribed in the mother's germline and stored as either mRNA or protein in the oocyte. As a result, embryos from heterozygous mothers, even if they are homozygous for null alleles of such genes, develop normally and produce progeny. However, embryos of these progenies will display phenotypic defects (embryonic lethality, sterility, etc.) corresponding to the mutant allele. Such phenotypes, which appear only in "grandchildren," are known as maternal-effect phenotypes.

1. The strain, IT1547 [genotype: *age-1(kp96)* / mnC1], helps us test a maternal-effect defect in larval development. The maternally provided AGE-1 is essential for L2 larvae to develop into L3 larvae. In its absence, L2 larvae initiate an alternative life cycle and proceed to the dauer stage, a dormant, developmentally arrested state, rather than to the L3 stage. Thus, *age-1(kp96/96)* homozygous larvae produced by the *age-1(kp96)* / mnC1 heterozygous mother develop normally, but their progeny become dauer larvae.
2. Clone 16 gravid hermaphrodites of the IT1547 strain. Since heterozygous [*age-1(kp96)* / mnC1] and *age-1(kp96/kp96)* homozygous animals appear identical, they can't be distinguished. Hence, the need for cloning; about 4 of these ($1/4^{th}$) will be homozygous.
3. Incubate at 20°C for 4 days.

OBSERVATION

In about 4 of the 16 plates, there will be no adults. Instead, all progeny in these plates will become dauers. This result indicates that the genotype of the cloned worms in these plates is *age-1(kp96/kp96).* Despite lacking a functional copy of the *age-1* gene, these worms developed normally because of maternally derived AGE-1.

Objective 4: To test the effect of temperature on fertility

1. Transfer 3-4 N2 gravid hermaphrodites each to two fresh OP50 lawns.
2. Incubate one plate at 20°C and the other at 28°C for 4 days.

OBSERVATION

All adult animals on the 20°C plate will be gravid, and several embryos will be present on the lawn. By contrast, on the 28°C plate, no embryos will be present, and the adults will be sterile. The optimum temperature is crucial for reproduction in many species!

Objective 5: To determine gene function using RNAi

1. Prepare two sets of RNAi bacterial lawns, with 3 plates per set. For the first set, use HT115 bacteria transformed with the RNAi plasmid carrying a non-specific insert (RNAi control, e.g., the GFP coding sequence), and for the second set, use the RNAi plasmid carrying the target gene's sequence; targeting genes such as *mex-3*, *pos-1*, or *skn-1* results in highly penetrant embryonic lethality.
2. Transfer 5 wildtype L4 larvae each onto one RNAi control plate and one RNAi target plate. Incubate at 20°C for 1 day.
3. Shift the worms to a new set of RNAi control and RNAi target plates, and incubate at 20°C for 6 to 8 h. Remove the worms, but let the embryos laid be on the plate. Incubate both plates at 20°C for 1 day (24 h).

OBSERVATION

All embryos on the control plate will have hatched into larvae. By contrast, on the *mex-3*, *pos-1*, *or skn-1* target plates, most embryos (>90%) will not hatch. These results show that RNAi treatment causes a loss-of-function phenotype of the target gene.

REFERENCE FOR FURTHER READING

Corsi A K, Wightman B, Chalfie M A (2015). Transparent window into biology: A primer on *Caenorhabditis elegans*. WormBook, Ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.177.1.

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Chapter 66: ZEBRAFISH AS A SIMPLE MODEL SYSTEM FOR CELLULAR STUDIES

Recommended Level: PG, Research

INTRODUCTION

The use of model systems in biology provides simplified representations of biological processes, which are essential for understanding complex mechanisms and testing hypotheses. Model systems offer an elegant environment, where specific variables can be controlled and manipulated, making it easier to study biological processes without the confounding factors present in more complex organisms. Current disease research relies on a spectrum of models, from simple invertebrates like worms and flies to complex mammals. However, a gap exists between these extremes in biological complexity. Zebrafish, with their vertebrate features and rapid development, have emerged as a bridge, offering valuable insights in developmental biology.

The teleost zebrafish (*Danio rerio*), native to tropics, are small, freshwater surface-dwelling fish, commonly found in shallow ponds, canals, and streams, etc. Thus, the use of zebrafish as a model system has been documented since the 1960s, with George Streisinger (1981) pioneering its importance in the biomedical field. The features that make zebrafish a popular model for studying disease mechanisms include:

1. Zebrafish are easy and cheap to handle. Zebrafish larvae and embryos, both are transparent, and their eggs hatch quickly. This transparency allows for the real-time visualization and investigation of cells, tissues, and organs *in vivo* (Eisen, 1996; Fishman, 1999).
2. 70% of protein-coding genes in zebrafish have human counterparts, and 84% of genes linked to human diseases are also found in zebrafish (Howe et al. 2013).
3. Embryogenesis proceeds quickly, and zebrafish exhibit high fecundity, generating large numbers of embryos. In optimal conditions, females can spawn up to 200 eggs weekly, with rapid hatching following fertilization.
4. The zebrafish facilitates molecular and genetic analysis by helping determine when and where genes are expressed, study specific gene functions through the development of transgenic and antisense RNA knockdown techniques, and conduct large-scale mutagenesis experiments (Hsu et al., 2007). Moreover, different kind of phenotypes, which appear associated with specific organs in the zebrafish embryo and larvae, may serve as readouts, namely as, those changes to the lifespan, hatching of the chorion, the curvature of the tail, beating rate of the heart, etc. (Shang et al., 2020). The zebrafish is also an ideal vertebrate animal model for studying skeletal muscle development and disease progression *in vivo* (Tesoriero et al., 2023).

Zebrafish have proven to be an extraordinarily useful model system for toxicological research, among many others. The aquatic environment is crucial for ecosystem function and human health. Human activities, like population growth and industrialization, along with the boundless use of pesticides, introduce more and more pollutants entering our water-bodies. These pollutants often contain genotoxic (can alter DNA) and carcinogenic (can cause cancer) substances resulting in stunted growth, abnormal development, and reduced survival of embryos, larvae, and adults, further posing a risk to human health via bio-accumulation along the food chain (Al-Sabti & Metcalfe, 1995).

It has been previously shown how unchecked use of microplastics is affecting the aquatic life at the KRS Dam on the Cauvery River (Anifowoshe et al., 2022). Therefore, the following chapter will

explore the application of the zebrafish model in toxicological studies, and how various cellular techniques can help infer the possible risks posed by the chemical in question.

OBJECTIVES

To use zebrafish embryo/larvae to provide a comprehensive understanding of cellular functions and mechanisms, contributing to the broader fields of developmental biology, toxicology, and medical research.

MATERIALS REQUIRED

Fish strains: Wild type zebrafish

Equipment and other materials:
Stereo-binocular microscope and fluorescence microscope
No. 1 brush, 90 mm petri dish, E3 medium (Williams and Renquist, 2016), Alexa Fluor™ 488 Phalloidin, low-melting agarose, 1X phosphate buffered saline, Triton-X-100, Tween-20, H2DCFDA (Cat. No. D399, ThermoFischer), glass slides and cover slips, 1.5 ml microfuge tubes, Nanodrop machine, TRI reagent (Cat. No. T-9424, Sigma), chloroform, isopropanol, diethyl pyrocarbonate (DEPC), ethanol, RevertAid First Strand cDNA Synthesis Kit (Cat. No. K1622, ThermoFischer), SYBR green master mix, Real time PCR machine

PROCEDURE AND OBSERVTION

1. **Twitching ability:** Zebrafish embryos begin to develop the ability to move rapidly within the first few days of life, starting with twitching movements in their chorion (egg sac) as early as 18 h post fertilization (hpf). The twitching ability in zebrafish embryos refers to the spontaneous and rhythmic contractions of their muscles (Fig. 1). This phenomenon is crucial for studying neuromuscular development, motor function, and muscle physiology. Observing twitching in zebrafish embryos provides insights into how muscles and nerves interact during early development, and can be used to investigate the effects of genetic mutations or chemical treatments on motor behavior and muscle function. To assess this, we need embryos which are yet to hatch (<3-day post fertilization). With a basic stereo-microscope, we can observe the tail twitching within the chorion, which alters in response to different chemical treatments, revealing potential effects on embryos.

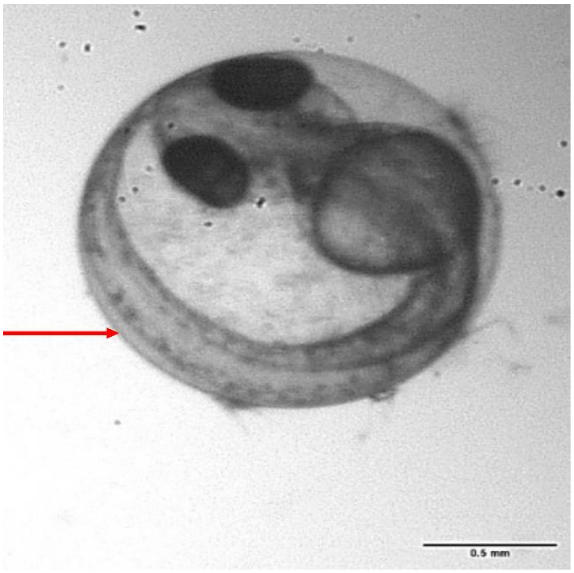

Figure 1. The twitching ability in zebrafish embryos, characterized by spontaneous rhythmic muscle contractions, is crucial for studying neuromuscular development, motor function and muscle physiology. Red line indicates region of interest.

2. **Hatching ability:** In nearly all animals, sexual reproduction involves releasing progeny from a maternal capsule at early embryonic stages. In fish, environmental factors influence hatching by affecting the secretion of the embryonic hatching enzyme (Helvik and Walther, 1993), an event which is tightly regulated in embryos. Proper timing of hatching is crucial for larval survival as premature hatching can cause deformities or expose larvae to hazardous conditions. The tail muscles also facilitate the hatching process. The enzyme degrades the eggshell in the perivitelline space, facilitating hatching. Therefore, factors impacting enzyme secretion significantly affect the reproductive success of the species. A delay in the emergence of zebrafish from the chorion, which occurs at 2 to 3 days post fertilization (dpf), can be considered developmentally fatal, and could therefore act as a potential readout.

3. **Heart rate:** Zebrafish embryos develop a functional heart (Fig. 2) by around 24 hpf (Hoage et al., 2012). The heart rate in zebrafish typically 120 to 180 beats per min post fertilization (De Luca et al., 2014), is a crucial parameter for assessing cardiac function and overall health. Influenced by temperature, oxygen levels, and exposure to drugs or toxins, monitoring this parameter aids in studying cardiovascular development, genetic mutations, and cardiotoxicity. This non-invasive measure provides valuable insights, making zebrafish an excellent model for cardiac research. To do this, a researcher can, with the help of a stereomicroscope, manually count the beats for 30 secs. To avoid any biases in the data, the researcher may request random volunteers to participate in a blind experiment to count the same. The data can be plotted in MS Excel or GraphPad PRISM to analyze for significance, if any.

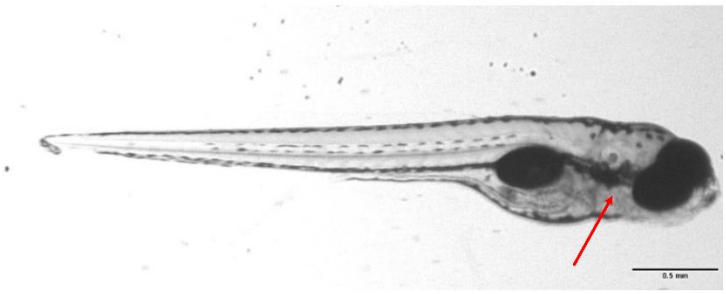

Figure 2. Zebrafish heart provides an excellent model in biomedical research. It shows regeneration property as well. The red arrow indicates the location of the heart in a 3 dpf embryo.

4. **Zebrafish gross phenotypes:** Exposure to chemicals can result in a wide range of phenotypic changes in zebrafish (Fig. 3). These phenotypes can provide important information about the toxicological effects and underlying mechanisms of the chemicals. Jeanray et al. (2015) have documented various phenotypic changes observed in zebrafish following chemical exposure.

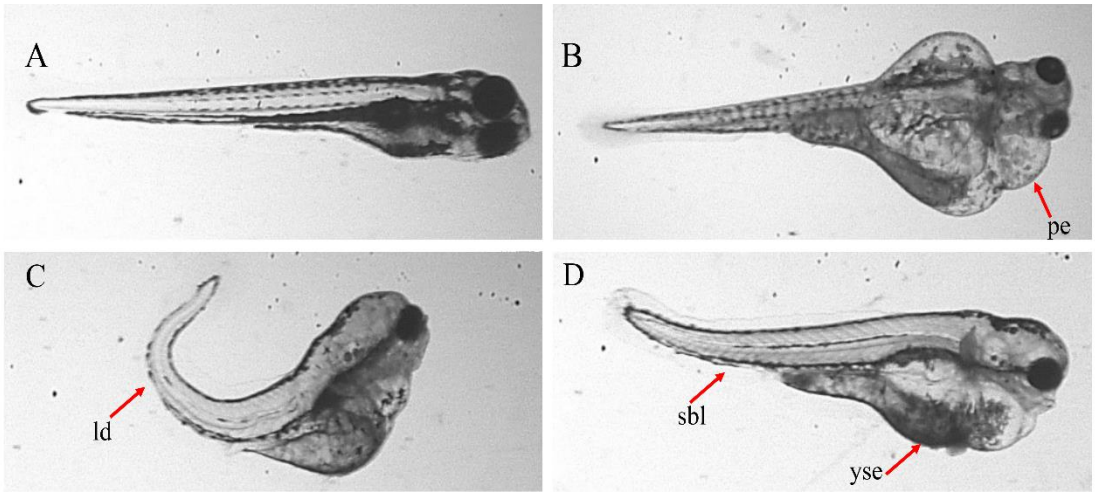

Figure 3. The different phenotypes that can be seen in larvae. A indicates vehicle control larvae whereas B-D indicate larvae with different phenotypes produced upon treatment with a chemical. pe: pericardial edema; ld; lordosis; sbl; short body length; yse: yolk sac edema. Scale bar = 0.5 mm

5. Escape response assay: The zebrafish escape response assay measures the reaction of larvae or adults to sudden perceived threats like a tap or a flash of light. Characterized by rapid swimming, this assay provides insights into neural function, muscle coordination, and overall health. This assay is valuable for studying the effects of genetic modifications, drug treatments, and environmental toxins on the nervous system and behavior. It also helps in understanding the mechanisms underlying stress responses and survival strategies in zebrafish, making it a crucial tool in neurobiology and toxicology research. At 2 dpf, a touch response assay can be performed by tapping the zebrafish on the back of the head with a no. 1 brush to stimulate their Mauthner cells (Kalueff et al., 2013). To do this, a simple swimming assay plate can be designed in the laboratory. Concentric circles with 0.5 cm intervals in their radii may be drawn at the bottom of 8 cm plastic petri dish and filled with 1X E3 medium (Fig. 4). Swimming assay was carried out by releasing the larvae at the center circle and touching it with a single-hair brush (a maximum of three times) to provoke an escape response. Zebrafish embryos have a natural tendency to move away from the center towards the periphery. All the swimming experiments can be recorded using a simple camera for further analysis. Total distance travelled by embryos along with the time taken to achieve, the task can be quantified automatically using ImageJ (FFmpeg plugin) (O'Connor, 2020) or manually and plotted using MS Excel or the GraphPad PRISM software for further analysis. The work from our laboratory utilizing this technique is detailed in our recent publication by Dutta et al. (2024).

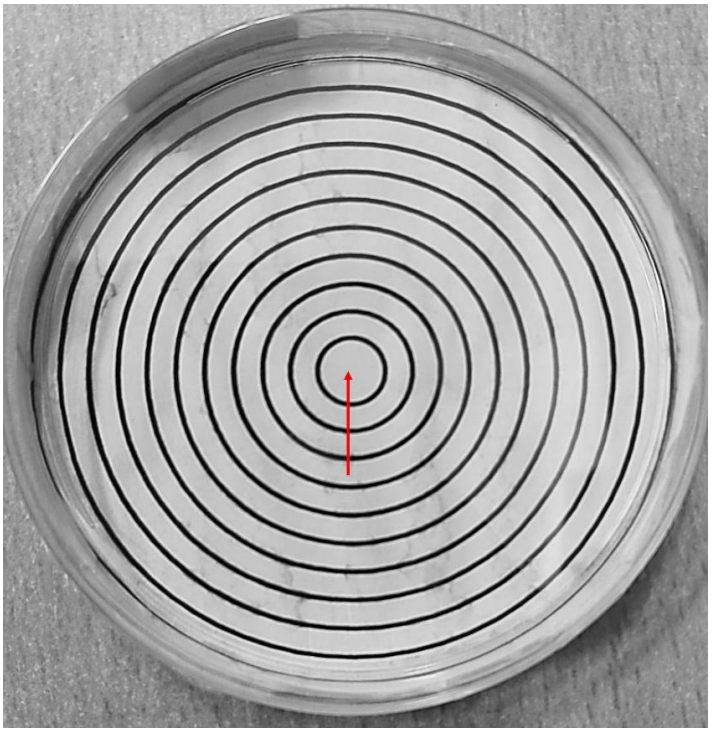

Figure 4. A 90 mm petri plate can be customized to be used for escape response assay. Larvae are released at the center and a no. 1 brush can be used to stimulate them. The movement and time to reach at the periphery can be recorded with a simple camera. Data can be analyzed automatically or manually.

6. **Immunohistochemistry for assessing muscle filamentous actin:** Phalloidin staining in zebrafish is a valuable technique for visualizing the actin cytoskeleton, crucial for cellular processes like shape, motility, and division. Derived from the *Amanita phalloides* mushroom, phalloidin binds specifically to F-actin, enabling detailed imaging of actin filaments. This method is particularly useful for:

    a. Observing muscle fibers and organization during development.

    b. Studying changes in cell shape and structure.

    c. Viewing actin structures during various developmental stages.

    d. Assessing the impact of toxicants on the actin cytoskeleton.

    e. Providing detailed images when combined with confocal microscopy.

    Overall, phalloidin staining is a powerful tool for investigating the role of actin cytoskeleton in development, cellular function, and response to environmental and genetic factors in zebrafish.

    To perform this experiment in a classroom, fix zebrafish embryos or larvae in 4% paraformaldehyde in a 1.5 ml microcentrifuge tube. Place the tubes at room temperature (RT) for 4 h or at 4˚C overnight on a rocker. After removing the fixative, rinse the embryos or larvae three times with 0.5 ml 1X phosphate-buffered saline (PBS) supplemented with 0.1% Tween-20 (PBS-T) for 5 min each on a rocker. After the final rinse, add 500 µl of PBS with 2% Triton X-100 (PBTX) to each tube to permeabilize the embryos for phalloidin staining. Place the tubes on a rocker for 1.5 h at RT. After this period, discard the solution and add 19 µl of PBTX and 1 µl of phalloidin Alexa Fluor 488 to each tube using a P20 pipette tip. Cover the tubes with aluminum foil and keep them at 4˚C overnight. The next day, remove the PBTX and wash the samples briefly

three times with PBS-T. Mount the embryos or larvae on a glass slide (Fig.5), taking care to avoid light exposure and considering de-yolking for better sample mounting (Goody and Henry, 2013).

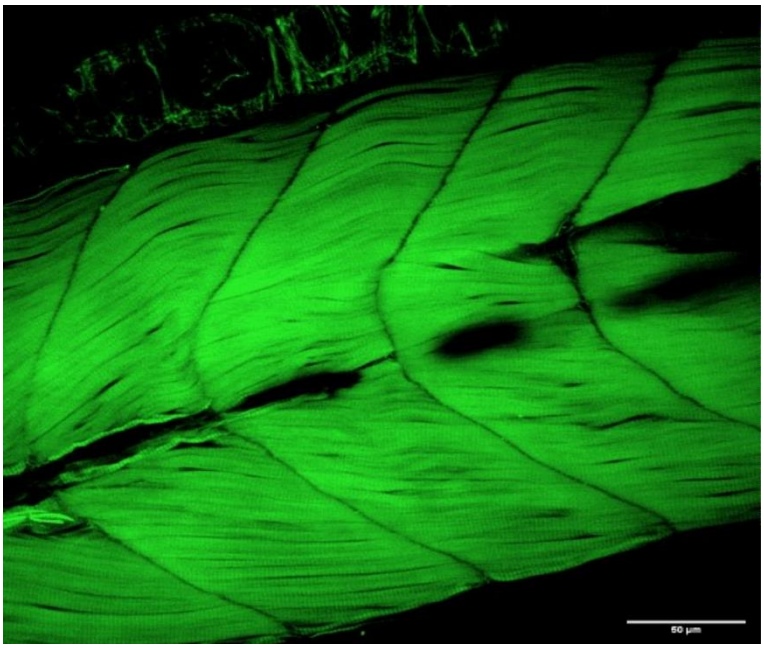

Figure 5. Arrangement of actin in the Zebrafish tail muscle stained with Phalloidin-488.

7. **Reactive oxygen species (ROS) quantification:** ROS in eukaryotes play significant roles in various physiological processes and pathological conditions. ROS, such as superoxide radicals ($O_2 \cdot -$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH), are natural byproducts of cellular metabolism and are involved in signaling pathways regulating growth, development, and immune responses. However, excessive ROS production can lead to oxidative stress, damaging cellular components like DNA, proteins, and lipids. Zebrafish serve as a valuable model to study ROS dynamics due to their transparent embryos and rapid development, facilitating real-time imaging of ROS generation and antioxidant defense mechanisms (Mugoni et al., 2014). Research on zebrafish ROS provides insights into oxidative stress-related diseases and therapeutic strategies, highlighting their relevance in biomedical research.

   Embryos or larvae can be collected in a 1.5 ml tube in 1X E3 media. Freshly make 10 mM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) stock solution by solubilizing the salt in dimethyl sulfoxide (DMSO). Incubate the embryo or larvae in 10 µM H_2DCFDA solution for 30 min. After the incubation rinse the sample three times for 5 min. Samples should be kept afterwards for 1.5 h at 28˚C, safely away from light exposure by keeping the samples in 1X E3 media. H_2DCFDA is quite light-sensitive. So, precautions should be taken even during imaging. After the end of incubation, embryos or larvae can be anesthetized by using Tricaine solution (working concentration: 164 mg/l) (Collymore et al., 2014). The embryos/larvae can finally be mounted using 1% low-melting agarose. The slides can be imaged under a fluorescence or confocal microscope. Cautions should be taken not to expose the sample for too long under microscope. The fluorescence intensity can be measured by ImageJ and plotted in MS Excel or GraphPad PRISM.

8. **RNA purification:** RNA purification in zebrafish involves isolating high-quality RNA from zebrafish tissues or embryos for downstream applications like gene expression analysis, reverse transcription, and real-time PCR.

1. Homogenize the embryo/larvae in 200 µl Trizol reagent or phenol-chloroform mixture by repeated crushing and spinning down (pulse, 8000 rpm) in a 1.5 ml tube until the solution becomes clear (translucent). Crushing should be done while keeping the vials on ice.
2. After the solution has become translucent, spin down the debris by centrifuging at 12,000 g for 2 min at 4°C.
3. Collect the supernatant in a fresh tube.
4. Allow the homogenate collected, to stand at room temperature, for 10 min. This step is to ensure complete dissociation of nucleo-protein complexes.
5. Add 200 µl Chloroform to the supernatant and mix vigorously for 15 sec.
6. Incubate at room temperature for 15 min.
7. Centrifuge at 12,000 g for 15 min at 4°C, to separate the aqueous layer from the organic layer.
8. Carefully collect the aqueous layer, in a fresh tube**.** Conserve the organic layer and interphase, for subsequent isolation of DNA/Protein, if desired, at -80°C.
9. Repeat steps 'd-g' to get rid of any genomic DNA contamination that might have been collected with the aqueous layer by mistake during the first round of collection.
10. Add 200 µl isopropanol to the final collected aqueous layer and mix by inverting, for 10-15s.
11. Incubate the samples at -20°C for a minimum of 1 h.
12. Centrifuge at 12,000 g for 10 min at 4°C, to pellet down the precipitate. If precipitate is not seen or very less of it is seen, try incubating for more than 10 min at RT. If the same condition persists, incubate at -20°C for at least 1 h.
13. Carefully decant the supernatant, by keeping eyes on the pellet and watching it fervently, to avoid losing it by mistake.
14. After decanting the supernatant add about 500 µl (or so) of 75% ethanol, prepared in DEPC-treated water.
15. Mix by inverting the tubes for 15 sec and then tap gently for about 10 times (DO NOT VORTEX)**,** and then centrifuge at 12,000 g for 15 min at 4°C, to wash the pellet.
16. Air dry the pellet while keeping the mouth of the tube open and keeping it on ice itself.
17. Resuspend the pellet in 15 µl DEPC treated water and proceed for
    (i) Nanodrop evaluation.
    (ii) Preparation of RNA sample for denaturing agarose gel electrophoresis.
    (iii) 1 µg of RNA can further be considered to convert into cDNA.

Consider the following protocol for cDNA conversion:

a. Prepare the following mixture: -

   RNA Template: Equivalent of 1 µg
   Oligo dT primer: 1 µl
   Autoclaved double distilled water: Adjustable up to 20 µl

b. Keep at 70°C for 5 min followed by a quick centrifugation and subsequently on ice.

c. Further add to the mix:

   (i) 5X reaction buffer: 4 µl

(ii) 10 mM dNTPs: 2 µl

d. Spin down and incubate at 25°C for 10 min
e. Mix 1 µl Thermo Fischer RevertAid enzyme solution.
f. Incubate at 42°C for 1 h.
g. Incubate at 70°C for 10 min.
h. Store in -20°C till further use.

A normal PCR should be run to check for the possible contamination with genomic DNA (gDNA). Primers should be designed to span one intron between two exons. This way, the PCR will be able to detect the presence of gDNA.

9. Real-time PCR: Real-time PCR (qPCR) is crucial in zebrafish research due to its sensitivity, specificity, and quantitative gene expression analysis. It allows precise measurement of gene expression, essential for understanding gene function, regulation, and genetic modifications. qPCR is vital for validating high-throughput sequencing results, assessing environmental toxin effects, and exploring molecular mechanisms. qPCR help in determining real-time monitoring of gene expression during development and experimental treatments. Overall, qPCR is a powerful tool for advancing zebrafish research and understanding vertebrate biology and disease models.

Prepare the qPCR reaction mix, typically including SYBR Green or TaqMan Master Mix, forward and reverse primers, cDNA template, and nuclease-free water.

Example reaction setup for a 10 µl total volume:

a. 5X SYBR Green Master Mix
b. 0.5 µl forward primer (10 µM) and 0.5 µl reverse primer (10 µM)
c. 1 µl cDNA template (5 ng)
d. 6 µl nuclease-free water

Real-Time PCR Cycling Conditions:

a. Initial denaturation: 95˚C for 2 min
b. 40 cycles of: Denaturation for 95˚C for 15 sec, annealing for 60˚C for 30 sec, extension for 72˚C for 30 sec

Analyze the qPCR data to determine the relative expression levels of the target gene.

Normalize the target gene expression using primers for any housekeeping gene as an internal control.

Calculate the relative expression using the $^{\Delta\Delta}C_t$ (cycle threshold) method.

QUESTIONS FOR FURTHER STUDIES

1. Why are zebrafish considered a bridge between invertebrate and mammalian models in biomedical research? Discuss specific advantages and limitations compared to both *Drosophila* and mouse models.
2. How does the optical transparency of zebrafish embryos enhance the study of dynamic cellular processes such as apoptosis, morphogenesis, and organogenesis? What other techniques could complement this advantage?

3. Suppose zebrafish embryos exposed to a certain pollutant show delayed hatching and pericardial edema. How would you design a follow-up experiment to identify whether the pollutant affects cardiac development or enzymatic hatching pathways?
4. Among twitching ability, heart rate, and escape response, which assay would you consider most sensitive for early detection of neurotoxicity? Justify your reasoning and suggest how statistical reliability can be improved.
5. If phalloidin staining reveals disrupted actin filament organization in treated embryos, what cellular processes might be affected? How could you determine whether this disruption is reversible?
6. Discuss the ethical advantages of using zebrafish embryos for toxicological testing compared to mammalian models. How does early developmental testing align with the 3Rs (Replacement, Reduction, Refinement) in research ethics?
7. Based on your understanding of zebrafish as a model, propose one novel research question or experiment using zebrafish to study a human disease (e.g., cancer, neurodegeneration, or metabolic disorder). Explain the cellular or molecular rationale behind your choice.

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