B.Sc. ZOOLOGY LAB MANUAL

2nd Semester

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Cell viability study by Trypan blue exclusion method

The Trypan blue (TB) dye exclusion test is one of the most common methods for measuring cell viability. It is used to determine the number of viable cells present in a cell suspension. TB ($C_{34}H_{28}N_6O_{14}S_4$) is a toluidine-derived, approximately 960 Daltons molecule. Azidine Blue, Benzamine Blue, Chlorazol Blue and Diamine blue are synonyms for TB. A cell suspension is simply mixed with the dye and then visually examined to determine whether cells take up or exclude the dye.

Principle

The cell membrane of live cells is impermeable to TB and therefore it only enters cells which have compromised membrane. After TB enters into the cell, it binds to intracellular proteins and the dead, apoptotic and necrotic cells appear blue under bright field microscope whereas the colour of living cells remains unchanged. Hence, it allows a direct identification and enumeration of live (unstained) and dead (blue) cells in a given population.

Materials required

i. PBS or serum-free complete medium.
ii. Commercially available 0.4% trypan blue solution (pH 7.2-7.3; stored in dark bottle; over time TB may form dye aggregates and crystals, it is therefore recommended that dye is filtered using a 0.2 μ filter before use).
iii. 70% ethanol.
iv. Single cell suspension of any origin.

Protocol

- Centrifuge the cell suspension being tested for 5 min at 100 × g and discard supernatant.
- Resuspend the cell pellets in 1 mL PBS or serum-free complete medium. *Note: Serum proteins stain with trypan blue and can produce misleading results. Determinations must be made in serum-free solution.*
- 0.4% trypan blue and cell suspension are mixed. Allow mixture to incubate 3 min at room temperature.
  Note: Trypan blue should be sterile filtered before using in order to get rid of particles in the solution that would disturb the counting process.

Mixing can be performed in a well of a microtiter plate or a small plastic tube using 10-20 µL cell suspension and trypan blue.

- Apply a drop of the trypan blue/cell mixture to a hemacytometer. Place the hemacytometer on the stage of a binocular microscope and focus on the cells. *Note: Cells should be counted within 3-5 min after mixing with trypan blue, as longer incubation periods will lead to cell death and reduced viability counts.*
- Count the unstained (viable) and stained (nonviable) cells separately in the hemacytometer. To obtain the total number of viable cells per mL of aliquot, multiply the total number of viable cells by the dilution factor for trypan blue. To obtain the total number of cells per mL of aliquot, add up the total number of viable and nonviable cells.
  Note: The aliquot should contain a convenient number of cells to count in a hemacytometer when suspended in 1 mL PBS (e.g., 5 x 10^5 cells/mL).
- Calculate the percentage of viable cells as follows:

$$\text{Viable cells (\%)} = \frac{\text{total number of viable cells}}{\text{total number of cells}} \times 100$$

STUDY OF DIFFERENT STAGES OF MEIOSIS IN GRASSHOPPER TESTIS CELLS

Grasshopper testis is an ideal material for studying various stages of meiosis. 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.

MATERIALS REQUIRED

i. Male grasshopper,

ii. Insect saline (0.67% NaCl),

iii. 1:3 aceto-ethanol fixative, 70% and 90% ethanol,

iv. 2% acetoorcein stain

v. 45% acetic acid,

vi. Slide,

Cover glass,

vii. 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-ethanol fixative, close the tube and leave for 20 minutes.

3. Remove fixative and add 90% ethanol, leave for 2hr.

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 aceto-orceine for 30 min.

2. Take a drop of 45% acetic acid on slide, place a few 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.

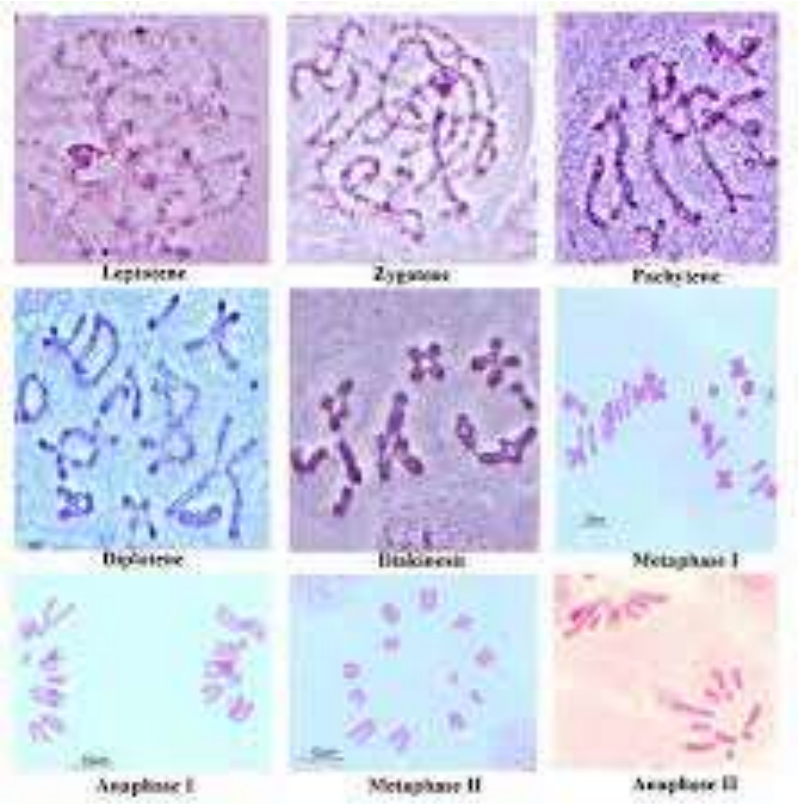
3. Place a cover glass on the tubules and squash using a rubber-end pencil under the folds of a blotting paper.

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.

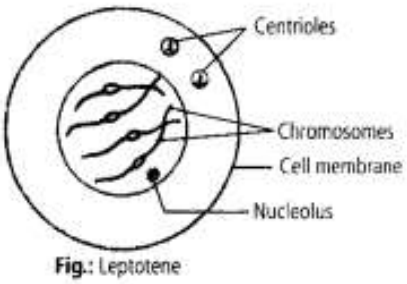
5.The slide is ready for observation under a microscope.

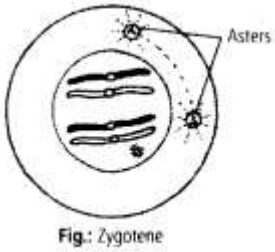
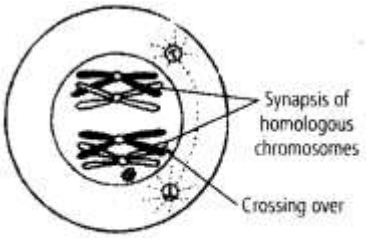
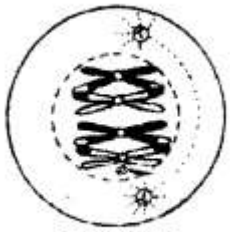
OBSERVATIONS

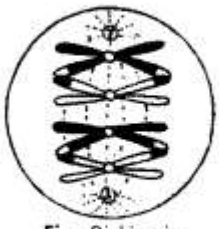
Different phases of mitosis can be observed as shown in the figures below:

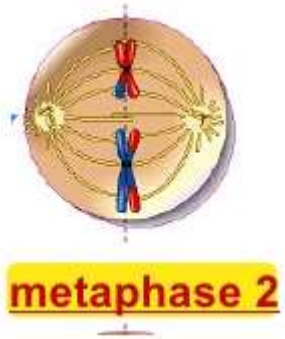
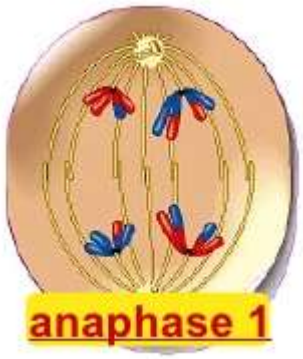


MEIOSIS PHASE

 **Fig.:** Leptotene	LEPTOTENE: • The reticulum of meiotic nucleus is opened out. • Chromosome is more distinct. • Chromosome appears as long slender and threads. • Chromosome numbering is not possible. • Chromosome looks beaded like.

 Fig.: Zygotene	ZYGOTENE: • Pair chromosome intimately associated. • Forms bivalent. • Chromosome are much shorted and thicker.
 Fig.: Pachytene	PACHYTENE: • Chromosome are thicker than the earlier stage. • Chromosome are associated in pair through out their length. • Presence of nuclear membrane. • Formation of chaiasma.
 Fig.: Diplotene	DIPLOTENE: • Nuclear membrane present. • Separation of paired chromosome noted. • Bivalents with four chomatids visible. • Formation of chaisma also seen. • Single unpaired x chromosome is short and thicker.

 Fig.: Diakinesis	DIAKINESIS: • Chromosome shorter and thicker. • Loops few in number. • Terminalisation in the way of completion. • Some of the bivalent completely terminalized showing end to end attachment with the figure like circle. • Terminalized and incompletely terminalized chromosome are scattered throughout the cytoplasm. • Single rod shaped X-chromosome is present.
 	PROPHASE II: • Chromosome become visible due to dehydration of cell. • Each arm of chromosome passes two chromosome. • Nuclear membrane and gradually disappear. • Centreols move to the opposite site.
 	METAPHASE I: • Each chromosome pairs a position on the equatorial plates. • Polar view gives an arrangement just as a line of the circle.

	• Almost all chromosome are terminalized. • Chromosome are exceptionally short and thicker.
	METAPHASE II: • Nuclear membrane have broken down again. • Short rod shaped chromosome are arranged in the equatorial plates in pair parallely. • Absence of chiasma. • Polar view shows as circular arrangement at the periphery.
	ANAPHASE I: • Chromosome forms a dyad. • Movement of chromosomes towards the pole by separation of chromatids. • Each pole contain a set of chromosome. • Sex chromosome at one pole.
	ANAPHASE II: • Chromosomes move towards pole by separation of chromatids. • Chromosome rod shaped. • Chromosome are shorter and thicker.

	• All each side of the equatorial plate equal numbers of chromosome are present.
	TELOPHASE I: • Chromosome reaches at pole. • Chromosome appears elongated and thin. • Cytokinesis may or may not occur. • Nuclear membrane appears around each group of chromosome(not visible).
	TELOPHASE II: • Presence of shorter cluster of chromosome at each pole. • Cytokinesis is visible. • Nucleolus and nuclear membrane are appear.

Preparation of temporary stained squash of onion root tip to study various stages of mitosis

Introduction:

An ordinate division of Chromosomes routed through consecutive changes on resulting in increase of cell number will equal chromosomal contain, is termed as mitosis. The process includes the replication of chromosomeand distribution of two sets of chromosome into two daughter nuclei.

Mitosis is arbitrarily divided into 4 stages:

1. Prophase
2. Metaphase
3. Anaphase
4. Telophase

Materials Required:

i. Onion bulb (*Alliumcepa*)

ii. Aceto-orcein (2%)

iii. Acitic acid (45%)

iv. HCl

v. Coverslip

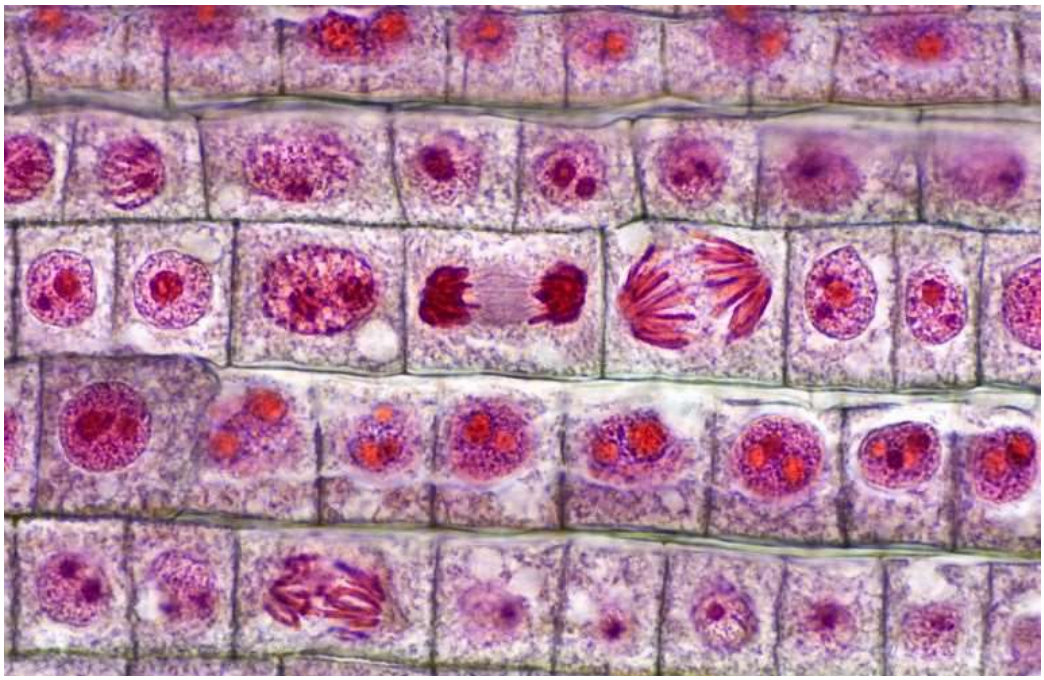
vi. Compound Microscope

Methods:

1. Roots are grown from bulb of onion in aerated distilled water at room temperature. Vigorously growing 2-3 days old healthy roots of 1 cm in length can be used.
2. Fresh young roots of onion about 2-3 cm long are cut and washed in running tap water to remove dirt.
3. About 5 mm of the tip portion of the root is cut out.
4. The root tips are fixed in aceto- alcohol i.e. acetic acid: absolute methanol ethanol (1:1 v/v) in specimen tube for 1 hour.
5. The tips are transferred into mixture of 2% aceto-orcein and normal HCl (9%:1 v/v).
6. Gently heated for a few seconds without boiling.
7. The root tips are kept in mixture for 15 minutes to half an hour.
8. The root tips are transferred into a clean grease free slide.
9. About 2 mm of the exact portion of the root tips is cut out.

10. About a drop of n1% aceto-orcein solution is added over the root tips.and coved with a coverslip.
11. Excess stain is blotted away
12. The squashing (in 45% acetic acid) is done by applying uniform vertical pressure on the cover slip with a thumb or by tapping the contents under coverslip with the aid of the flat end of abrush.
13. The squashed preparations are observed under compound microscope. If preparations are desired nature. The coverslip is covered with paraffin wax temporary.

Observation



INTERPHASE:

- Nuclear membrane is present.
- Coiled chromosome are dense.
- Nucleolus can be found.

PROPHASEE:

- Nuclear membrane almost disappear.
- Chromosome cell is not vivid.
- Chromatids are not distinct.
- Nucleolus disappear

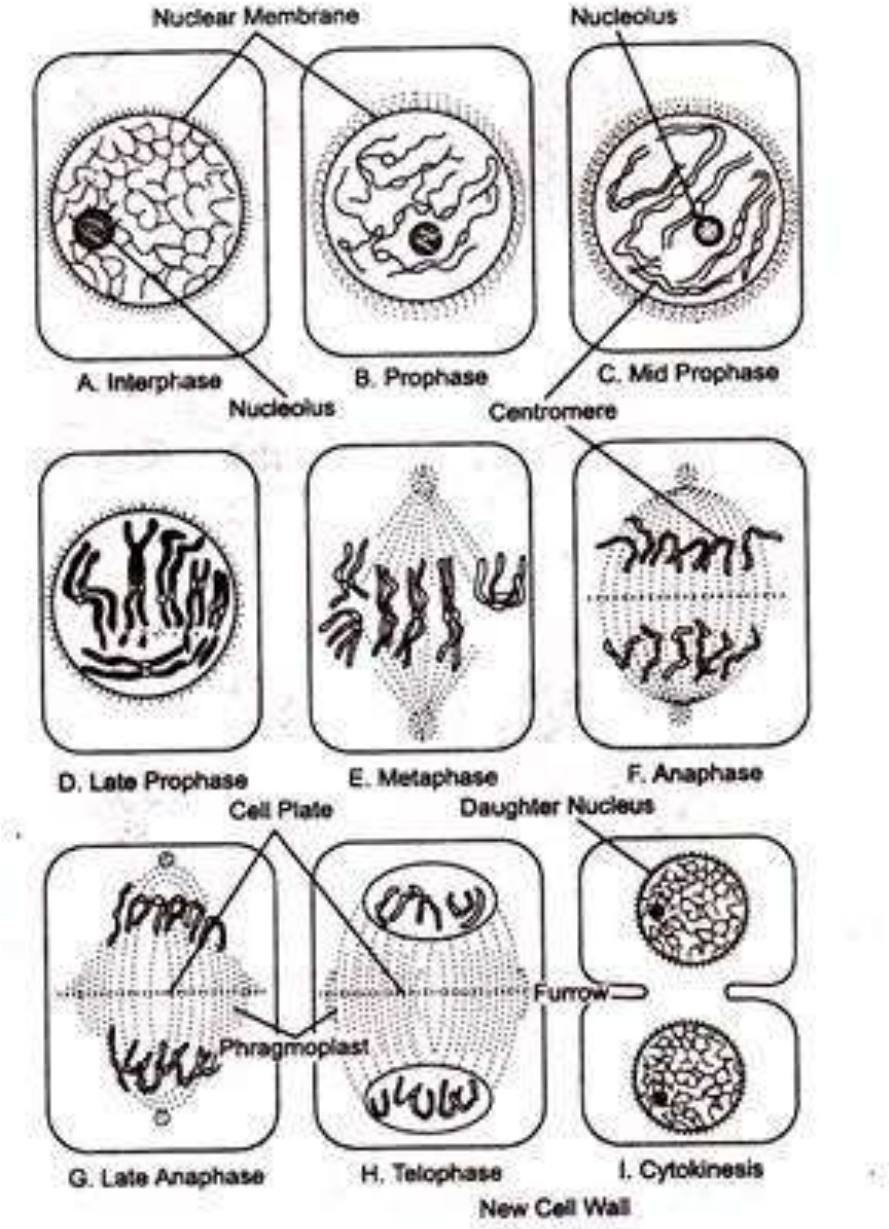


Fig. 11.4 A Various stages of mitosis in plant cells.

METAPHASE:

- Spindle fibre has been formed.
- Chromosomes are attached with the spindle by their centromeres.
- Chromatids are extended on either side.
- Axis that is equatorial in position.

ANAPHASE:

- Chromosome centromere divides and daughter chromosome arte formed.
- Spindle fibre extended more.
- Daughter chromosome moves to the pole of the spindle.

TELOPHASE:

- Spindle disappears.
- Daughter chromosome are assembled to the opposite pole.
- Nuclear membrane formation again start.

CYTOKINESIS:

- Chromosome become indistinct.
- Cell plate is formed.
- Nuclear membrane reappears.

Preparation of Permanent Slide to Show the Presence of Barr body in human female blood cells/ cheek cells

Introduction:

Barr body or sex chromatin refers to a condensed mass of chromatin representing an inactive X chromosome seen iin the nuclei of somatic cells of female mammals. In 1949, Murry Barr and E. G Bertram found in the nerve cells as well as in most of the body cells of a female cat a small dark body. They recognised this later on as sex chromatin. This sex chromatin is also called the Barr body after its discoverer. Later on, it was also found in the cells of other mammals but was further noticed that such structures are absent in the male counterpart and only found in females. In humans this body is easily detected in the female's buccal mucosal cells and fibroblast but not in males. This is a highly condensed structure with a diameter ofabout 1µm and lines against the nuclear enveloped. It is Feulgen positive and hence it has DNA in it. Later it has been proved that this body is an inactive X chromosome.

Materials required:

i. Spatula
ii. Glass slides
iii. Coverslips
iv. Compound Microscope

Reagents required:

i. Giemsa Stain
ii. Ethanol and Methanol
iii. Hcl
iv. Xylene
v. DPX

Methods:

i. At first glass slides are cleaned with 95% ethanol and marked on one side with glass marker. After that slides is covered with a thin film of Mayer's glycerol albumin.

ii. After that wash the mouth with tap water and scraped firmly glass slide against the fingers held outside the cheek.
iii. Buccal smears are immediately dipped into the fixative kept in coplin jar.
iv. Ethanol, ethanol+ ether or methanol may be used as fixatives.
v. In case of ethanol it should kept for 15-30 minutes however in case of methanol it should kept for overnight.
vi. After fixation slides are taken out and are air dried.
vii. Air-dried slides are then transferred into the coplin jar containing 6N HCL for …. minutes at 20-22ºC.
viii. After acid hydrolysis slides are taken out and kept under tap water for 10 mnutes.
ix. Finally the slides are air dried and ready for staining.
x. Slides are immersed in phosphate buffered (ph 6.4) 4% giemsa stain for 10 minutes,
xi. After that slides are transferred to distilled water for differentiation.
xii. The slides are then taken out and air-dried.
xiii. The air dried slides are cleared in xylene and mounted in DPX.

Observation:

The slide is observed under oil immersion lense of the compound microscope to find out the deeply stained small body i.e Barr body attached to the nuclear membrane.