MEASUREMENT OF THE SIZE OF CELLS AND SUBCELLULAR COMPONENTS IN LIGHT MICROSCOPE

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.

MATERIALS REQUIRED

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

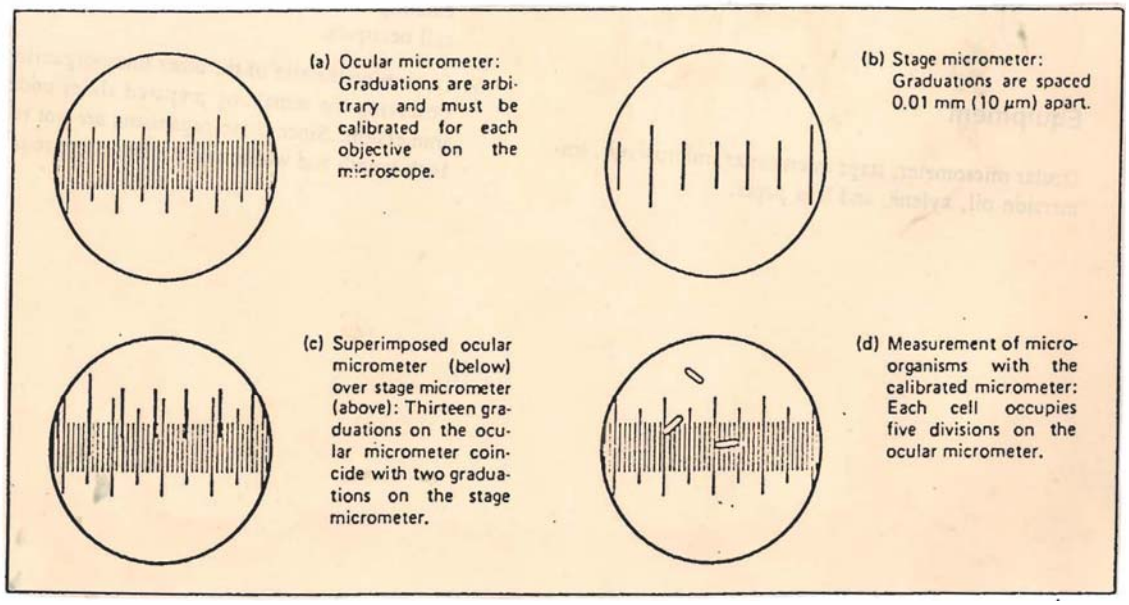
PROCEDURE

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.
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 (10µm) apart.
3. Superimpose the two scales and record the number of ocular division 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 (2X10µm=20µm) of stage micrometer

   Then 1 ocular division = $\frac{20\mu m}{13 \text{ divisions}} = 1.54\mu m$
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. 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 1.54µm = 7.7µm
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.



(a) Ocular micrometer: Graduations are arbitrary and must be calibrated for each objective on the microscope.

(b) Stage micrometer: Graduations are spaced 0.01 mm (10 μm) apart.

(c) Superimposed ocular micrometer (below) over stage micrometer (above): Thirteen graduations on the ocular micrometer coincide with two graduations on the stage micrometer.

(d) Measurement of microorganisms with the calibrated micrometer: Each cell occupies five divisions on the ocular micrometer.

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OBSERVATION OF HUMAN CHEEK EPITHELIAL CELLS

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.

MATERIALS REQUIRED

Ethanol soaked tooth pick, slide, methylene blue stain

PROCEDURE

6. 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.
7. 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.
8. 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.
9. After 5 min of staining, rinse cells once with distilled water so 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. The live deeper layer of cheek cells have slightly larger nuclei.

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STAINING OF MITOCHONDRIA IN HUMAN CHEEK EPITHELIAL CELLS

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.

MATERIALS REQUIRED

Ethanol soaked tooth pick, slide, cover glass, 0.01% Janus green B stain 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 Janus green stain and leave for 5-10 min for staining.
4. After 5 min of staining, rinse cells once with distilled water so 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. 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.

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DIFFERENTIAL STAINING FOR DNA AND RNA IN HUMAN CHEEK EPITHELIAL CELLS

INTRODUCTION

In eukaryotes the genomic DNA is compartmentalized in the nucleus. The genes get transcribed in the nucleus and majority of the transcrips are transported to the cytoplasm for translation or for various other functions. Thus nucleus is rich in DNA and a good amount of RNA, while the cytoplasm is rich in RNA.

Methyl green stain specifically binds with DNA, while Pyronine Y binds can bind with DNA as well as RNA. However, in presence of methyl green stain it binds with RNA alone and thus the DNA rich regions (nucleus) and regions having RNA (nucleolus and cytoplasm) can be differentially stained by using a mixture of methyl green and pyronine Y stain.

MATERIALS REQUIRED

Ethanol soaked tooth pick, slide, cover glass,

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 Methyl green-Pyronine Y stain and leave for 5-10 min for staining.
4. After 5 min of staining, rinse cells once with distilled water and mount the cells in a drop of distilled water with a cover glass and observe under the bight field students' microscope.

OBSERVATION

The nucleus stains dirty green (a mixture of green and pink stain as it contain DNA as well as RNA), while the cytoplasm appears pink.

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STUDY OF DIFFERENT STAGES OF MITOSIS IN ONION ROOT TIP CELLS

Onion root tip has meristematic tissue just behind the root cap, hence this serves as a good material for studying various stages of mitosis. The roots can be easily grown if an onion 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 two days time 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.

MATERIALS REQUIRED

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

PROCEDURE

Fixation of root tips:

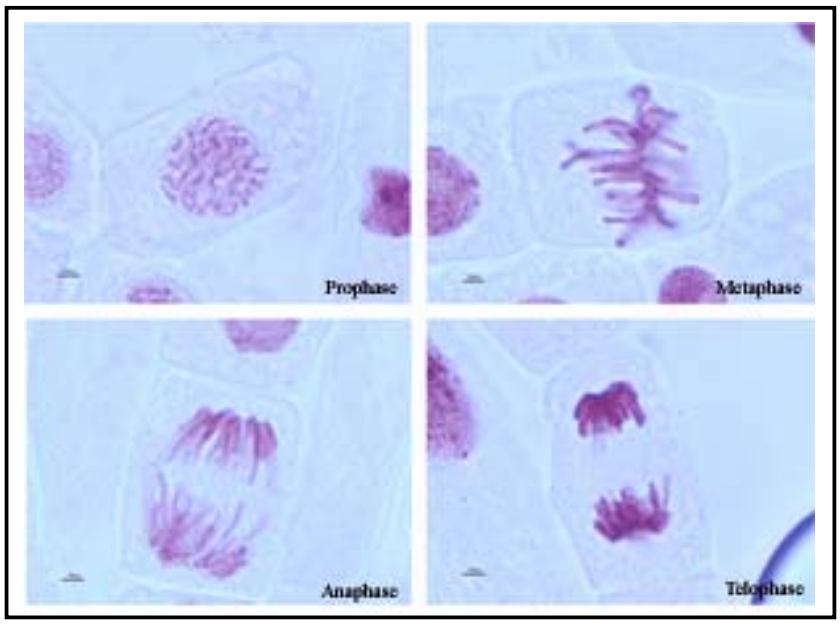
1. Fix the freshly cut ~1 cm long root tips in acetomethanol fixative for overnight in a specimen tube.
2. Remove fixative and add 90% ethanol, leave for 2hr.
3. Decant 90% ethanol and add 70% ethanol. The root tips can be stored in 70% ethanol for a long period of time if the tube is tightly closed. 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 was 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 can be observed as shown in the figures below:



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

Colchicine is very well known as mitotic poison which inhibits tubulin polymerization causing a block on spindle fibre 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.

MATERIALS REQUIRED

Onion root tips, 0.05% colchicine in water, 1N HCl, 1:3 acetomethanol fixative, 70% and 90% ethanol, 2% acetocarmine stain (2gms of carmine mixed with 100ml of 45% acetic acid and boiled using a reflux conderser for 1 hr to dissolve carmine), 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 one 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 colchicine. Let it be exposed to colchicine for 4 hrs 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..
5. Seal the edges of the cover glass with molten was 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 can be observed in control samples while no anaphases will be observed and chromosomes will be randomly dispersed in the cells in the colchicine treated samples.

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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.

Temporary squash preparation

MATERIALS REQUIRED

Male grasshopper, insect saline (0.67% NaCl), 1:3 acetomethanol fixative, 70% and 90% ethanol, 2% acetocarmine stain (2gms of carmine mixed with 100ml of 45% acetic acid and boiled using a reflux conderser for 1 hr 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 acetomethanol fixative, close the tube and leave for overnight.
3. Remove fixative and add 90% ethanol, leave for 2hr.
4. 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. Stain the fixed testis in acetocarmine 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 was 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.

MATERIALS REQUIRED

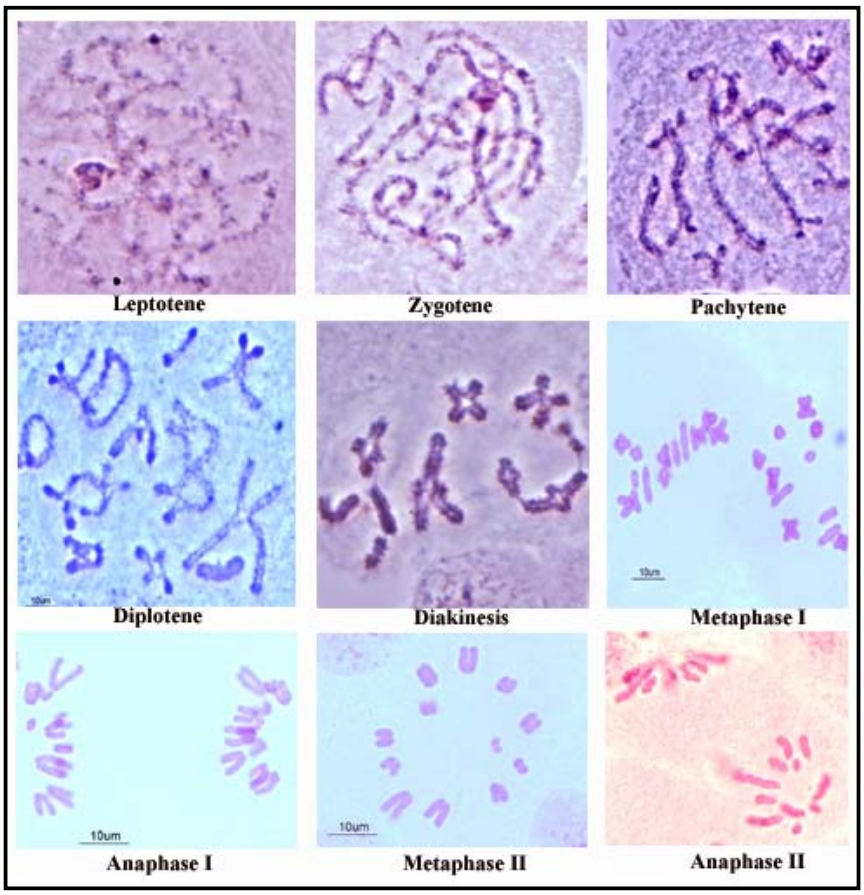
Male grasshopper, insect normal saline (0.67% NaCl), fixative (1:3 acetic acid-methanol), 60% acetic acid, centrifuge tubes, centrifuge, slides, cover glasses, Giemsa stain.

PROCEDURE

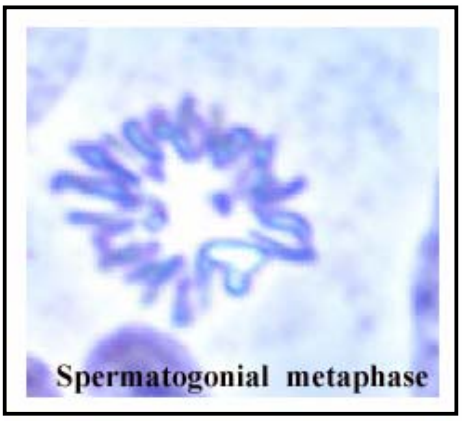
1. Dissect out testes from male grasshopper.
2. Keeping the testes in normal saline, remove the yellow fat.
3. Fix the testis in fixative in a centrifuge tube for 30 min.
4. Remove fixative and add about 0.5 ml of 60% acetic acid, leave for 2-3 min till the testis appears nearly dissolved.
5. Add 5-6 ml of fixative to the tube without removing the acetic acid.
6. Centrifuge at 1,200 rpm for 5 min.
7. Gradually remove the supernatant and add a few drops (~0.2 ml) of fresh fixative and make a suspension.
8. Drop a few drops of cell suspension on a slide and flame dry.
9. Stain the slides with Giemsa stain, rinse in water, dry and observeunder a microscope.
10. The slide can be mounted with DPX before observing in oil immersion lens.

OBSERVATIONS

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



A mitotic metaphase from spermatogonium is observed as shown below :



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ASEPTIC TECHNIQUE AND GOOD CELL CULTURE PRACTICE

AIM

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

- Chloros / Presept solution (2.5g/l)
- 1% formaldehyde based disinfectant e.g. Virkon,Tegador
- 70% ethanol in water

EQUIPMENT

- 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 with 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. Culture discard in chloros (10,000) ppm must be kept in the cabinet for a minimum of two hours (preferably overnight) prior to discarding down the sink with copious amounts of water.

STERILIZATION TECHNIQUES

AUTOCLAVE

AIM

Autoclaving is used for:

- Sterilisation of solutions, media and equipment required for growing microorganisms, culturing cells and tissues *in vitro*. (Solutions which are not heat sensitive are only autoclaved).
- Sterilisation of used cultures and waste materials prior to disposal.

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

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

Air removal is achieved by:

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

Downward Displacement Steriliser

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 should discharge stop. (This can be done manually or automatically).

High Vacuum Autoclaves

High Vacuum Autoclaves are not suitable for the sterilisation 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 sterilisers should be fitted with appropriate air filters to prevent the release of infectious aerosols into surrounding areas.

Sterilisation Times

The sterilisation 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 sterilisation 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 STERILISATION.

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). 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 cookers, which are convenient and economic autoclaving equipment for the small laboratory.

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 Degrees Celcius. 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 a 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.

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 minutes 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 a crack. Beware of rush of steam.
- After the slow exhaust cycle, open autoclave door and allow liquids to cool for 20 minutes 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 minutes 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 hours. 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 (i.e. labeling tape), or other flamable 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 (i.e. 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 0.22µm pore size filter that is appropriate to the solvent used. 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 the filters for.

Coconut water is sometimes filter sterilized instead of autoclaved. To filter sterilize coconut water, filter the water using a 0.45µm pore size filter, before using a 0.22µm 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 filteration with syringe-tip filter

Materials

Sterile:

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

Nonsterile:

- Solution for sterilization

Procedure:

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 Sterilisation

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 (eg. 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 sterilisation efficacy.

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

Check each lot by confirming survival for 5 minutes 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 centre of large liquid loads.
- They are less accurate in determining holding times than temperature measurement.
- They cannot be used for sterilisation 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.

Reference

Biological Safety Principles and Practices edited by Diane Fleming & Debra Hunt, ASM Press, Washington D.C., page 393-395, 2000.

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SHORT TERM CULTURE OF WHOLE BLOOD AND PREPARATION OF METAPHASE CHROMOSOMES

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 (10ml, 5ml. 1ml), culture vials (universal containers), conical flask 100ml, Syringes (5ml, 1ml), 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, 0.2µg/ml colcemid, 1N HCl, 90% alcohol

It is ideal to check sterility of different solutions at least 24h prior to setting up a culture by keeping each one of them at 37ºC 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,

<u>Giemsa stain</u>

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

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

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

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

PROCEDURE

Setting the culture

1. About 5ml 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 (10ml serum to 100ml medium - serum can be added separately to the culture). Since commercial media have phenol red as indicator, color 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 (5ml in sterile distilled water). This can be stored under sterile conditions for 1-2 months at 4^oC
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	5ml
Fetal bovine serum	1ml (if not already added)
Blood	0.3ml
PHA	0.1ml

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 37^0C for 48 to 72 hours
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 hours prior to harvesting the culture for chromosome preparation, colcemid (working conc. 0.02μg/ml) is added.

Chromosome Preparation

1. Transfer the culture to a centrifuge tube and centrifuge at 1000 to 1200rpm for 5min
2. Decant the supernatant and make a fresh suspension of cells in prewarmed 0.56% KCl (hypotonic). Initially add slowly and agitate the sediment. Once the cells come in suspension, make up the volume to 8-10ml. Keep in incubator (37^oC) for 18-20min
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 ~8-10ml. Keep for 15min
4. Recentrifuge (1000-1200 rpm) for 5min, decant the supernatant and resuspend the pellet in fresh fixative (~ 8-10ml). Keep for 10min.
5. Recentrifuge (as in #4) and discard the supernatant. Add only about 0.2-0.4ml 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 24x60mm coverglass

PRECAUTIONS

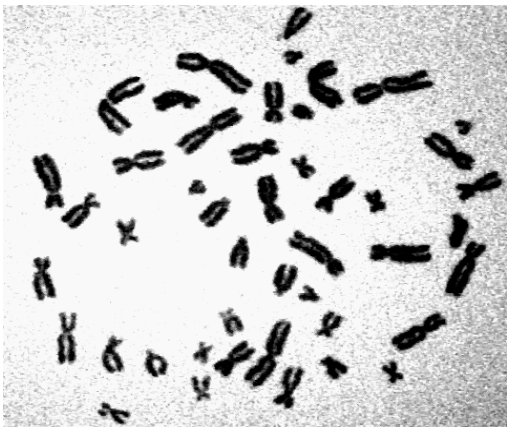
Success of tissue culture work depends largely on the personal habits of the user. Tissue culture medium is rich not only for the cells that have been provided but also to any other kind of cells, like bacteria and fungi, that can find access to it. Therefore, standard of hygiene and sterilization has 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 glassware are kept in concentrated nitric acid over-night and then washed in running tap water for 3h. They are rinsed first in steamed distilled water and then in two changes of double distilled water. They are dried in incubator at ~60^0C.

Dried glasswares are sterilized either dry or wet. For the former they are kept in an oven at 160^oC to 200^oC for 2h. Wet sterilization is done in an autoclave in which the sterilization is done by the pressure created by steam. The contents are autoclaved for 20-30min at 15-20lbs/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 0.22μm 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.



A metaphase spread from human lymphocyte culture

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CHROMOSOME PREPARATION FROM CELL LINE

MATERIALS REQUIRED

Centrifuge tubes (15ml graduated), Pasteur pipettes, agitator, colchicine, incubator (37-38^{o}C), clinical centrifuge

Solutions

Hypotonic Solution: 0.56% KCl (should be kept pre-warmed to 37^{o}C before use)

Fixative

Glacial acetic acid	25ml
Ethanol or Methanol	75ml

Giemsa stain (see page 62)

PROCEDURE

1. Treat 48-72 h old culture with colchicine (0.5µg/ml) for 2-3h at 37^{o}C
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 1000rpm 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 37^{o}C.
4. Recentrifuge (1000-1200 rpm) for 5min, decant the supernatant and resuspend the pellet in fresh fixative (~ 8-10ml). Keep for 20min.
5. Recentrifuge (as in #4) and discard the supernatant. Add only about 0.2-0.4ml of fixative. Agitate to resuspend the cells.
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 24x60mm coverglass

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PREPARATION OF METAPHASE CHROMOSOMES FROM BONE MARROW

INTRODUCTION

The marrow from long bones, like the limb bones, provides the most handy material for analysis of karyotype of small animals. 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 they 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.

MATERIALS REQUIRED

Rat or mouse, dissection set, centrifuge tubes (15ml graduated), Pasteur pipettes, agitator, brown tape, colchicine, syringe (5ml) and needle (#22, #18), incubator (37-38^{o}C), clinical centrifuge

Solutions

Hypotonic Solution: 0.56% KCl (should be kept pre-warmed to 37^{o}C before use)

Fixative

Glacial acetic acid	25ml
Ethanol or Methanol	75ml

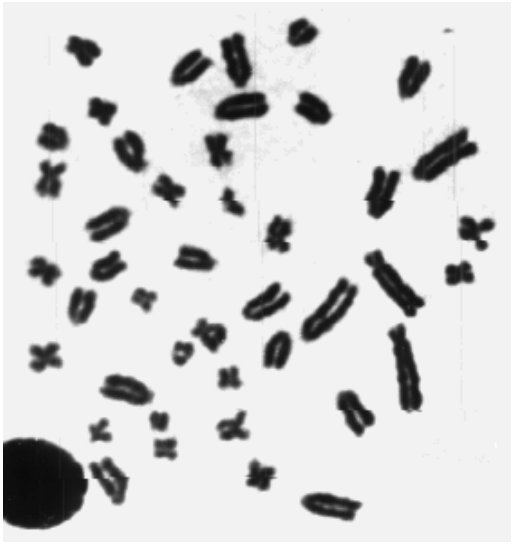
Giemsa stain (see page 62)

PROCEDURE

1. Inject colchicine (0.2mg/kg body weight) to the animal 2-3h prior to sacrifice
2. Sacrifice by cervical dislocation and dissect out femur bone (any long bone will do)
3. Cut both ends of the bone. Take about 0.5ml of prewarmed hypotonic solution in a 5ml 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 10ml and keep in incubator (37^{o}C)
5. Centrifuge the cell suspension (1,000 rpm) for 5min 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 0.5ml) and agitating the tube to make a suspension. Make up the volume to 10ml with the fixative. Keep for 15min
7. Repeat # 6
8. Repeat # 6 but instead of making up larger volume, suspend cells in a small volume of fixative (0.3 - 0.5ml) and using a Pasteur pipette place 2-3 drops on a clean slide and gently flame it

9. After proper drying, stain the slide with Giemsa for 3-4min 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 coverglass

10. 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.



A metaphase spread from bone marrow of Rat

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G- and C- BANDING OF METAPHASE CHROMOSOMES

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-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 (30mg/ml in 0.9% NaCl, stored at $-20^{o}C$), 0.9% NaCl (stored at $4^{o}C$), phosphate buffer (stored at $4^{o}C$), Giemsa stain, distilled water

Phosphate buffer

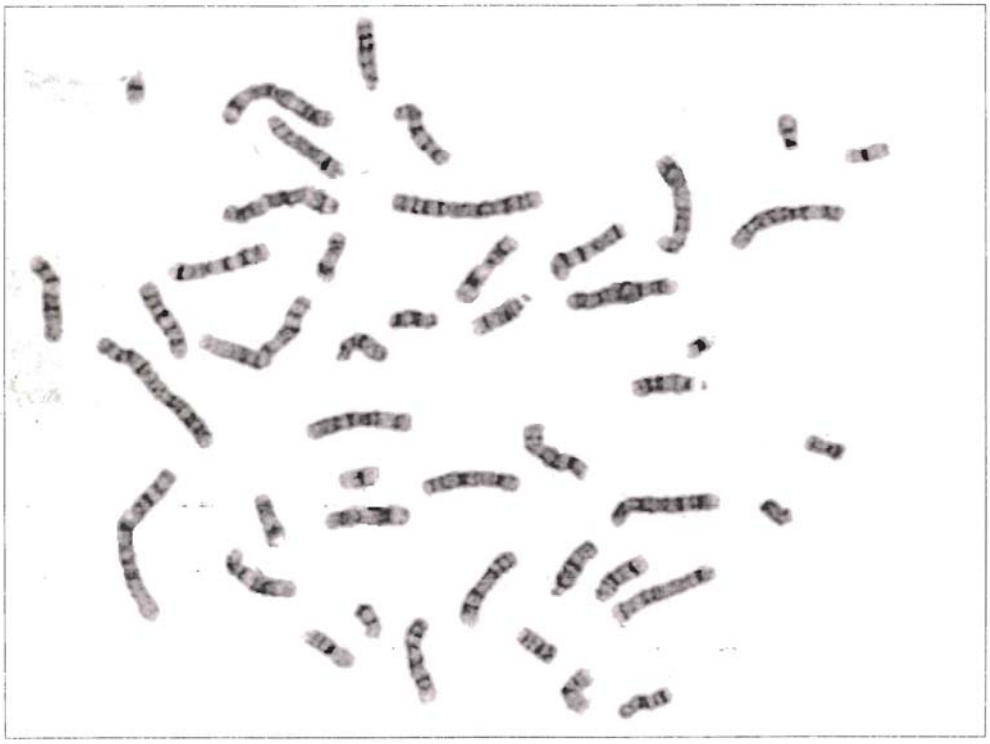
Equal parts of solution 1 and solution 2
Solution 1 : 9.073g KH_2PO_4 in 1000 ml dist water
Solution 2 : 11.87g $Na_2HPO_4.2H_2O$ in 1000 ml dist water

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 1M NaOH required).
2. Add 50ml 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 seconds (take 3-4 slides, but process only one slide at a time and go to the next only when results of the first one are clear)
5. Rinse the slide in phosphate buffer immediately after trypsin exposure.
6. Stain the slide for 3-5min in Giemsa
6. 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

7. Dry the slide, mount in DPX and observe



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B. C-BANDING OF METAPHASE CHROMOSOMES

MATERIALS REQUIRED

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

Solutions

5% Barium Hydroxide

Barium Hydroxide	5g
Dist. water	100ml

Boil 100ml of dist. water in a conical flask and add 5g $Ba(OH)_2$ 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 50ºC in a waterbath

0.2N HCl

Conc. HCl	1ml
Dist.. water	54ml

2xSSC (pH 7.2)

Giemsa stain

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 waterbath maintained at 60ºC for 1-2h
5. Rinse in dist. water, air dry and stain with Giemsa for 15-20min (monitor the staining after different time intervals). After appropriate staining, air dry and mount with DPX.

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

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.

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 37oC for 10 min.

2. Treat the cells with dexamethasone (10 µM) for 0, 1, 2 and 3 hours to induce apoptosis.

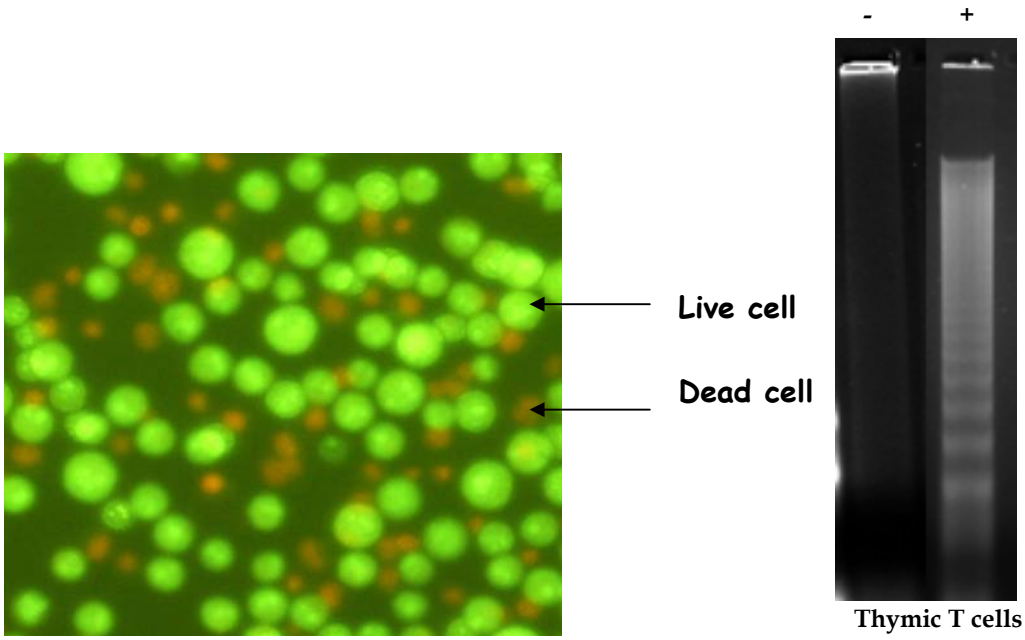
3. Wash the cells in PBS and resuspend in PBS and stain them with 1:1 mixture of acridine orange and propidium iodide (10 µg/ml each, in PBS) for 5 to 10 min without any fixation (fixation disrupts membrane and even the live cell take up Propidium iodide and stain orange).

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.



Photmicrograph showing counter staining of live (green) and apoptotic cells(red) with Acridine orange/propidium iodide staining of T- cells exposed to Dexamethasone.

Agarose gel electrophoresis showing apoptosis in T cells exposed to Dexamethasone (+) for 2hrs (thymic T cells).

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CELL VIABILITY ASSAY BY TRYPAN BLUE EXCLUSION

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 (e.g. trypan blue) that is visible when it leaks into cells that have damaged plasma membranes.

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.

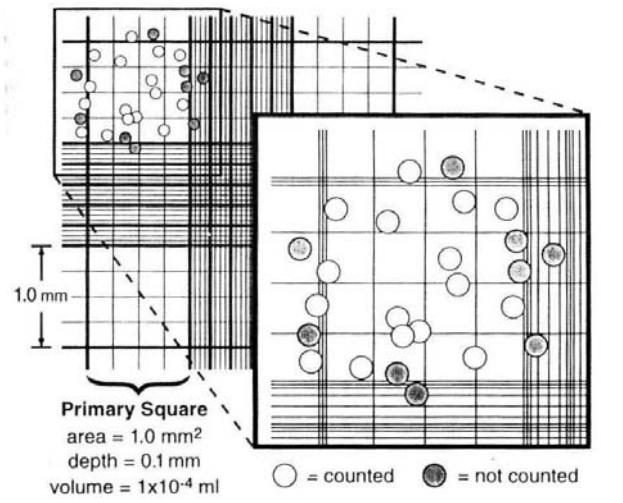
2. Add equal volume of 0.4% trypan blue prepared in PBS to the cell suspension.

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 showing blue color).

4. One can also use this method for counting viable cells in a suspension using a hemocytometer

OBSERVATION

Live cells will not be stained while dead cells will be brightly stained.



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MTT ASSAY FOR CELL VIABILITY

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.

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 (10µM; or any other agent which causes cell death) to the four tubes and incubate them for 0, 1, 2 and 3 hours, respectively in water bath at 37^0C to induce apoptosis. The untreated tube will have live cells.

3. Take 200µl of suspension from each tubes in fresh microfuge tubes. Add 20µl of MTT (5 mg/ml in PBS) in each and incubate at 37^oC for 2 hr.

4. Centrifuge the incubation mixtures at 5000rpm for 5 min in a microfuge, remove the supernatant and resuspend the pellets in 100µl 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 540nm to measure the intensity of colour developed.

OBSERVATION

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

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ASSAYING PHAGOCYTOSIS IN MOUSE MACROPHAGES

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 hours. Then they migrate into the tissue, where they become macrophages and move within the tissues. In the presence of an inflammation site, 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 peritoneum cavity of the mice by injecting chilled PBS and then making them adhere again on glass slide for studying phagocytosis.

MATERIALS REQUIRED

Mice, Dissecting tray & tools, petridish, 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 gms of crystal violet mixed with 2ml of ethanol to dissolve and finally made 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 pertidish and pour 200-500µl 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 hr.

4. In the mean while prepare yeast cell suspension. Heat kill the yeast cells by boiling Baker's yeast (3mg/ml) in PBS in a test tube in a boiling bath for 15 min., wash 2-3 times with PBS. Keep this 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 hr 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.

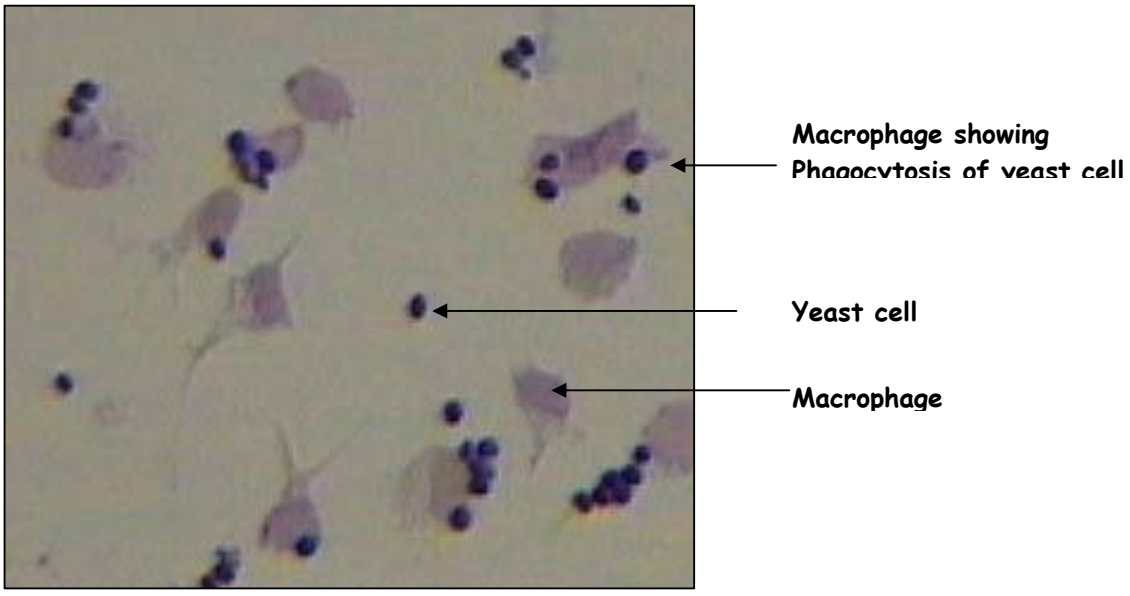
10. Stain the cells with crystal violet for 5 min or with Giemsa for 20 min and observe under the microscope.

OBSERVATION

Look for macrophages (Flattened cell showing several processes radiating out). Several of them, you would find, are seen at different stage of engulfing dead yeast cells. Score the macrophage showing phagocytosis and calculate the % phagocytosis and phagocytic index :

$$\% \text{ Phagocytosis} = \frac{\text{No. of macrophage showing phagocytosis}}{\text{Total no. of macrophage}} \times 100$$

$$\text{Phagocytosis index (PI)} = \frac{\text{Total no. of yeast phagocytosed}}{\text{Total no. of macrophage showing Phagocytosis}} \times 100$$



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SQUASH PREPARATION OF POLYTENE CHROMOSOMES FROM *DROSOPHILA* LARVAE

INTRODUCTION

Polytene chromosomes are well known for their use in a variety of genetical, cytogenetical 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 many cells in this tissue. 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 8 or 9 rounds of replication. All the resulting chromatids (2^8 or 2^9 chromatid fibrils) 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 pericentromeric heterochromatic regions of different chromosomes remain in close association with each other and together they form the chromocentre. The euchromatic arms of different chromosomes appear to radiate from this common chromocentre (in some dipterans, like *Chironomus*, chromocentre formation does not occur in polytene cells). A major part of the DNA in chromocentre 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 in other somatic cells of *Drosophila* and many other diptera, the homologous chromosomes in polytene cells also remain tightly synapsed. As a result, 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.

MATERIALS REQUIRED

Healthy late 3rd instar larvae, blotting paper, droppers, dissecting needles and fine forceps, cavity slides, clean glass slides, coverglasses, 37°C incubator, stereobinocular microscope, cloth for cleaning slides, diamond marker, marker-pen, slide box, razor-blade, dipping jars, slide tray, microscope for examination of preparations.

Solutions

Poels' Salt Solution (pH 6.8)

NaCl	86mg
KCl	3l3mg
$CaCl_2.2H_2O$	ll6mg
$NaH_2PO_4.2H_2O$	88mg
$KHCO_3$	l8mg
$MgSO_4.7H_2O$	5l3mg
Dist. H_2O	l00ml

Adjust pH to 7.0 with 1M NaOH and filter.

50% acetic acid

Aceto-Methanol 1:3 - freshly prepared

Aceto-Orcein (2%)

Dissolve 2g Orcein in 50% Acetic acid by boiling for 30min under a reflux condenser; filter when cool. It is strongly desirable to filter the stain every time before use

Aceto-Carmine (2%)

Dissolve 2g Orcein in 50% Acetic acid by boiling for 2 hours under a reflux condenser; filter when cool. It is strongly desirable to filter the stain every time before use

Lacto-Aceto-Orcein (2%)

Dissolve 2g Orcein in a solution containing 51ml Glacial Acetic Acid, 34ml distilled water and 15ml of 85% Lactic Acid by boiling for 2 hours on a very low flame in a flask fitted with a reflux condenser. Filter the stain when cooled to room temperature. It is strongly desirable to filter the stain every time before use

DPX mountant

Ethanol grades 70%, 90%, Absolute alcohol

Cleaning of slides and coverglasses

To obtain good squash preparations, it is absolutely essential that the slides and coverglasses 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 coverglasses in 90% ethanol in suitable containers and wipe them dry, immediately before use, with a clean soft silken cloth. These are stored covered till used.

PROCEDURE

Method I (Temporary preparations)

1. Take late 3rd instar larvae (about 5 day old if grown at 24^{o}C) 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 etc with water and transfer to a 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 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 drops (drop-by-drop) of freshly prepared fixative. Wipe out excess fixative with a piece of filter paper (the total duration of fixation should not exceed 1min since longer fixation makes chromosomes brittle and difficult to spread). Add a few drops of aceto-orcein stain and leave the slide covered with a watch glass for a few minutes (a better staining is obtained by a mix of Aceto-Orcein and Aceto-Carmine stains: add 2 drops of Aceto-Carmine and l drop of Aceto-Orcein and keep covered).

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

5. For squashing, put the slide with its coverglass between folds of a clean filter paper and lightly tap the coverglass 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 coverglass on the slide may be desirable but too strong a tapping would break chromosomes in pieces). Hold the coverglass in position with fingers of one hand placed over the filter paper such that they press on two diagonal corners of coverglass. Apply firm pressure of thumb of the other hand on the coverglass. This act of squashing spreads the polytene chromosome arms of a nucleus and makes them flat in one plane. Any lateral movement of coverglass 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 coverglass with 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; temporary preparations will last longer if the squash preparation is made in Lacto-Aceto-Orcein instead of 50% acetic acid and if the sealed slides are stored in refrigerator at 4^{o}C).

Method II (Permanent preparations)

1-5. same as in Method I. (in certain cases staining with aceto-orcein is not done: the glands are directly transferred to 50% acetic acid after fixation; for certain situations, it may also be desirable that squashes are made on slides which are gelatin-subbed using coverglasses which are siliconized: gelatin subbing makes chromosomes better stick to slide while siliconization prevents chromosomes from sticking to coverglasses).

6. Transfer the slides with coverglass to a slide box which has been pre-chilled and immediately store the slide box at -70^{o}C. Keep at -70^{o}C for a few hours or over-night.

7. After the desired period of freezing, quickly flip off the coverglass using a sharp razor-blade and immediately transfer the slide to a 1:1 solution of 50% acetic acid and 50% ethanol (slides are taken out one by one from the freezing chamber to ensure their frozen state). Pass the slides through 70%, 90%, absolute alcohol and air-dry.

   Alternatively, after chilling at -70^{o}C for 15-20min, briefly dip the slides in liquid nitrogen followed by quick flipping off of the coverglass with a blade. Immediately transfer the slide to a 1:1 solution of 50% acetic acid and 50% ethanol (slides are taken out one by one from the freezing chamber to ensure their frozen state). Pass the slides through 70%, 90%, absolute alcohol and air-dry.

8. Observe the slides under phase-contrast microscope (bright-field microscope can also be used if the light is dimmed and substage diaphragm is closed).

9. Slides can be stained with Giemsa and mounted with DPX (some times, most of the spread chromosomes may stick to the coverglass and when the coverglass is flipped off, little material is seen on the slide; in such cases, the coverglass may be mounted with D.P.X. or other mounting medium on a clean slide in such a way that the chromosomes remain on upper exposed surface of the coverglass; after the mounting medium has dried, these can be processed further like other slides).

OBSERVATIONS

A good squash preparation reveals many polytene nuclei with well spread polytene chromosome arms connected to a common chromocentre. The chromocentre 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 chromocentre. 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. 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.

PRECAUTIONS

Getting good “squash preparations” is an art and requires some practice and care. Following are some of the commonly encountered problems.

Excessive tapping may lead to breaking of individual chromosome arm into pieces; likewise, excessive pressure during squashing may cause over-stretching of some chromosome regions. Tapping and the thumb pressure have to be "optimal". In a well-spread preparation, all chromosome regions remain in focus at one plane.

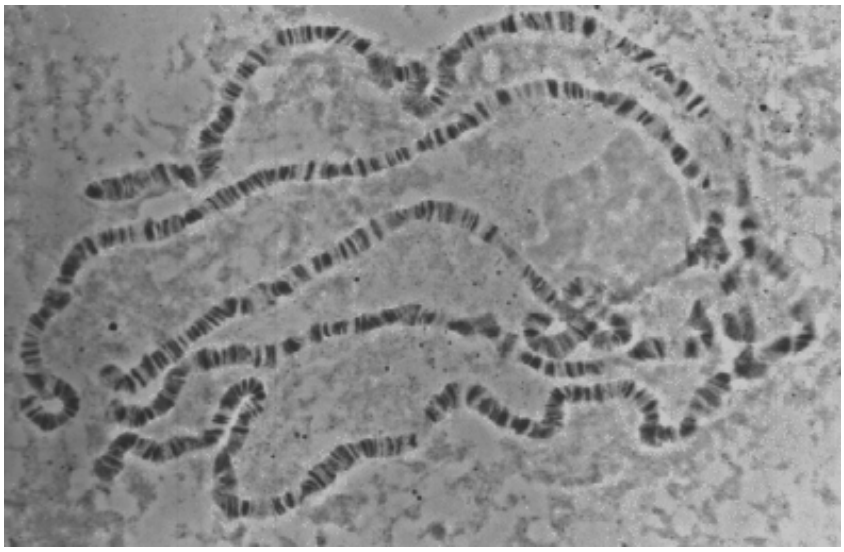
Any lateral movement of the coverglass relative to the slide during squashing results in chromosomes being “rolled” and fragmented: such chromosomes appear as small “rolls” of homogeneously stained material..

Imbalanced salt concentrations in the Ringers' or the saline solution in which the glands are dissected or incubated may cause poor morphology of the chromosomes (the bands do not appear "crisp") or the nuclei may not open up at all (most of the polytene nuclei remain rounded up with their nuclear membrane remaining intact).

Salivary glands from larvae that have not reached the late third instar stage or that are weak due to over-crowding or growth at higher temperature, do not provide well spread, thick and distinctly banded chromosomes due to their reduced levels of polyteny.

Any trace of grease on slides or coverglasses hampers good spreading of chromosomes. The slides and coverglasses must be kept in 95% alcohol for some time and should be wiped dry just before use with fresh clean and soft cloth - examination of the slide and coverglasses under reflected light (from a lamp) will reveal if traces of oiliness or fibers etc are present on their surface.

Any pieces of tracheae or fibers (e.g., from filter-paper) or stain particles or any cuticular structures (e.g., mouth parts) of larvae left on the slide will not permit good squashing. All these must be carefully removed before applying the coverglass.



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 chromocentre (phase-contrast optics, x 550 approx.)

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IN-SITU HYBRIDIZATION ON NUCLEAR DNA OR CHROMOSOMES

INTRODUCTION

Nucleic acid hybridization provides a means of evaluating homology between single stranded DNA and/or RNA molecules (also see SOUTHERN HYBRIDIZATION on p. 60). When one of the hybridization partner 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.

MATERIALS REQUIRED

1. Incubators set at 37º, at 42º and at 60ºC
2. Acid-cleaned glass slides and coverglasses (optional: coverglasses may be siliconized)
3. Slide racks, slide tray, couplin jars, magnetic stirrer,
4. micropipettes, pipette tips, plastic box, forceps

SOLUTIONS REQUIRED

1. 20x SSC (3M sodium chloride, 0.3M sodium citrate)
2. 0.1% gelatin solution (freshly prepared)
3. 3M sodium acetate (pH 5.2 with the help of glacial acetic acid)
4. RNase 10mg/ml (Stock solution)
5. 70%, 90% and absolute alcohol
6. 1M Tris pH 7.5, 1M Tris pH 8.0, 1M Tris pH 9.5
7. 5M sodium chloride
8. 0.5M EDTA
9. TE (10mM Tris, pH 8.0; 1mM EDTA
10. Digoxigenin-dUTP labeled DNA probe
11. Salmon sperm DNA 10mg/ml
12. Hybridization mix
13. Color detection buffers

    Buffer I (100mM Tris, pH 7.5; 150mM NaCl)
    Buffer II (0.5% w/v Blocking reagent in Buffer I)
    Buffer III (100mM Tris, pH 9.5; 100mM NaCl, 50mM $MgCl_2$)
    Buffer IV (TE)
    Color Developing Solution (to be prepared fresh just before use)

Nitroblue Tetrazolium (NBT, 75mg/ml in Dimethyl Formamide)	4.5µl
5-Bromo-4-Chloro-3-Indolyl Phosphate (50mg/ml in Dimethyl Formamide)	3.5µl
Buffer III to make	1ml

14. Safranin stain

Safranin	100mg
Dist. Water	100ml

    (Dissolve Safranin powder in water at room temperature: the prepared stain is stable for long time and can be used repeatedly)
15. Entellan mountant (E.Merck), Immersion oil

Coverglass siliconization

Put fresh coverglasses in a Petri dish containing absolute alcohol and wipe them dry with a clean silken cloth. Dip alcohol cleaned coverglasses in 1% siliconizing fluid. Wash in running water for 1-2h. Rinse in distilled water and dry in oven

PROCEDURE

Labeling of probe DNA with digoxigenin dUTP by random priming method

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

1. Take the required amount of DNA (linear) in an eppendorf tube and denature by heating in boiling water for 10min. Quickly chill the tube on ice.
2. Add the following in sequence to the same eppendorf tube

a. Hexanucleotide mix	2µl
b. dNTP labeled mix	2µl
c. Distilled water to make	19µl
d. Klenow enzyme (3-5 units)	1µl
Final volume	20µl

3. Incubate at 37^{o}C for 1h
4. Stop reaction by adding 0.8ml of 0.5M EDTA (Final concentration 20mM)
5. Precipitate labeled DNA by adding the following:
   2.0µl of salmon sperm DNA (10mg/ml)
   2.5µl of 4M Lithium Chloride
   75µl of pre chilled (-20^{o}C) ethanol
   Leave at -70^{o}C for 2hrs
6. Centrifuge at 12,000rpm for 30min 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) 10pg, 1pg and 0.1pg of the prepared probe. Let it dry at room temperature (~30min) following which cross-link the probe DNA with the membrane either by 3-4min exposure to UV on a transilluminator or by heating the filter at 70^{o}C for 2h.
2. Wash the filter briefly in Buffer I
3. Incubate in Buffer II for 30min 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
5. Incubate in Anti-DIG Antibody-Enzyme conjugate (1µl in 4ml of Buffer I) for 30min at room temp

6. Wash twice at 15min interval in Buffer I
7. Briefly rinse in Buffer III
8. Put the blot in a small polythene bag and working in dim light add color developing solution (4.5µl of NBT and 3.5µl of BCIP in 1ml of Buffer III) and seal the bag
9. Incubate the blot within the sealed bag in dark (e.g., 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, 0.1pg of probe gives a detectable signal within 30min). 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 5sec in a freshly prepared 0.1% solution of Gelatin (100mg Gelatin dissolved in 100ml dist. water at 70^{o}C for 1h). 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, see Lakhotia, S.C., Sharma, A., Mutsuddi, M. and Tapadia, M.G. 1993. Gelatin as a blocking agent in southern blot and chromosomal *in situ* hybridizations. TRENDS IN GENET. 9: 261)
2. Arrange the slides in a moist chamber containing filter papers soaked in 2x SSC. Place 100µl of RNase (100µg/ml in 2X SSC) over the preparation on each slide and cover with 22mm^2 coverglasses (no air bubbles should be trapped) to remove RNA from preparations. Incubate the slides at room temperature for 2 hr
3. Remove the coverglasses gently by dipping slides into a beaker containing 2x SSC. Coverglasses will fall in solution
4. Wash slides in 2X SSC (3 times 5min each), in 70% ethanol (2 times 10min each) and in 95% ethanol for 5min. 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 0.07N NaOH for exactly 3min to denature chromosomal DNA
6. Wash slides in 3 changes of 70% ethanol (10min each) and 2 changes of 95% ethanol (5min each). Air dry. Slides are now ready for hybridization

HYBRIDIZATION MIXTURE

Formamide	500µl
20X SSC	250µl
DIG labeled probe (10-20ng/slide)	as required
H_20 to make TOTAL volume to	1000µl

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

Hybridization

1. Denature labeled probe DNA by placing the tube in boiling waterbath for 10min. Add the desired amount of denatured probe to the hybridization mix.

2. Add 20µl of hybridization mixture containing 10-20ng of labeled probe. Place a coverglass over the hybridization mixture and seal the edges with DPX. No air bubbles should be trapped
3. Incubate slides at 37^{o}C in a closed moist chamber. Allow hybridization to proceed for 12-14hrs (overnight)

Washing

1. Peel off DPX sealing with the help of forceps. Remove coverglasses by dipping slides in 2X SSC
2. Wash slides in 1x SSC, (3 times 15min each) at 60^{o}C

Colour detection

1. Rinse slides for 1min in buffer I (100mM Tris pH7.5, 150mM NaCl)
2. Place them in buffer II (0.5% W/V Blocking reagent in Buffer I) and leave for 30min
3. Wash again in buffer I for 1min
4. Incubate in anti-Digoxigenin antibody alkaline phosphatase conjugate (diluted 1: 5000 in buffer I) for 30min
5. Wash in buffer I (2 times 20min each)
6. Rinse in buffer III (Tris pH 9.5 100mM, NaCl 100mM, $MgCl_2$ 50mM)
7. Prepare fresh color reaction reagent. Put 20-30µl of color reaction reagent on the slide, cover with a coverglass, seal with DPX and leave the slide in a dark chamber at room temperature for 1-12h, depending upon the time required for optimal signal development.
8. Stop reaction in Buffer IV (10mM Tris pH 8.0, 1mM EDTA) 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 5-6min (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 coverglass for 5-6min. Following the staining, quickly rinse slides in two changes of 70% alcohol 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 (E.Merck).

    Alternatively, mount the slides temporarily using the stop buffer or the immersion oil; after examination the coverglasses 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 (e.g., DPX) causes fading of color.

OBSERVATIONS

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

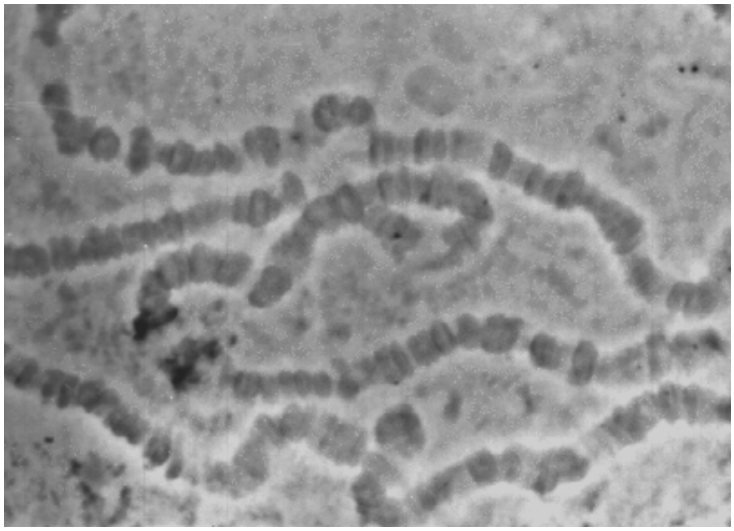
PRECAUTIONS

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 coverglasses at the various steps since any trapped bubble would not permit the reaction in the local region and thereby prevent the hybridization signal.



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)

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IN-SITU HYBRIDIZATION TO STUDY PATTERN OF EXPRESSION OF A GENE

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 oligonucleotide (DNA or RNA) probe, location of the complementary nucleotide sequences in cells can be determined. *In situ* hybridization studies on chromosomes provide an approach to genetic mapping of the sequence of interest. Likewise, 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 37^{o}, at 42^{o} and at 60^{o}C
2. Acid-cleaned glass slides and coverglasses
3. Micropipettes, pipette tips, moist chambers, forceps

SOLUTIONS REQUIRED

1. 20X SSC (3M sodium chloride, 0.3M sodium citrate)
2. 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
3. 4% paraformaldehyde in PBS
4. PBT (PBS + 0.1% Tween20)
5. ProteinaseK(10µg/ml) in PBT
6. Glycine (2mg/ml) in PBT
7. Hybridization buffer A : 50% deionized formamide + 5X SSC (pH- 5.00)
8. Hybridization buffer B :
   50% formamide
   5X SSC (pH- 5.00)
   10µg/ml yeast tRNA
   100µg/ml sheared salmon sperm DNA
   50µg/ml Heparin
   0.1% Tween20
   Dissolved in DEPC treated water. Adjust the pH of SSC with citric acid, filter and autoclave. SS DNA should be boiled for 10 min, chilled for 5 min before adding to the hybridization mix
9. TBS:

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

10. Staining Buffer:

NaCl	100mM
Tris-HCl (pH 7.5)	100mM
$MgCl_2$	50mM

Tween20 0.1%
Levamisol 1mM (optional)

11. Coloring Solution:
    NBT – 4.5µl (50mg/ml in 70% DMFO),
    BCIP – 3.5µl (50mg/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 X-100 in PBS at room temperature (RT) for 20 min. (In tissues like salivary glands, one can go for 0.5% NP-40 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 (PBS + 0.1% Tween 20) 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(10µg/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 (2mg/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 1-12 hrs at 42^0C.
15. Hybridize with riboprobe in Hybridization buffer B for 24 hrs at the desired temperature mentioned above.

<u>Washing</u>

16. Wash in Hybridization buffer A at least for 1 hr (15 min each for 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 4^0C overnight)

Colour detection

19. Incubate the tissues in Anti DIG antibody (1:2000 in PBT) for 2-3 hrs at room temperature in a shaker. The antibody should be preabsorbed in lesser dilution (1:200) on fixed larval tissues (steps 2-6 without active DEPC treatment) overnight at 4^0C.
20. Wash in PBT 5 times for 5 min each. (You can store the tissues at 4^0C)
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. Wash the tissues in freshly made staining buffer for 15-20 min.
23. Add freshly made colouring 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 PBS or PBT.
25. Incubate the tissues in 50%-80% glycerol in PBS for 2-4 hrs before mounting in the same solution.

OBSERVATIONS

Hybridization of the probe results in appearance of purplish-blue colour deposit at the site of hybridization.

PRECAUTIONS

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

It is always better to make fresh glycine.

Colourless amorphous precipitate forms if the colouring solution is contaminated with PBT.

If little blue crystals are seen on tissues, they should be washed thoroughly in PBT.

If too much background appears, reduce the probe concentration, increase the Hybridization temperature, increase the proteinase concentration or treatment time and even vary the pH of the Hybridization buffer.

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

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IMMUNOSTAINING

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.

MATERIALS REQUIRED

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

2. Dissect tissues from crawling third instar larvae in Poels' salt solution (pH-7.0) or 1X PBS (pH-7.4).
3. Transfer tissues to cavity slide in IX PBS.
4. Fix in freshly prepared PBS-PFA for 20 mins at room temperature.
5. Wash the tissues in PBST for 10 min (x3)
6. Incubate the tissues in blocking solution at room temperature for 2 hrs.

7. Add primary antibody at a dilution of 1:10 in blocking solution and incubate at 4^0C over night.
8. Take off the supernatant. This can be saved for second use.
9. Rinse once with PBST and then wash 2 X 10 min each in PBST.
10. Add AlexaFluor-488 conjugated secondary antibody (or a desired secondary antibody) diluted in blocking solution and incubate for 2 hrs at room temperature with gentle shaking.
11. Take off the supernatant. Wash as in step 8 and counter stain in DAPI for 10 min.
12. Rinse with PBS and mount in mounting medium seal and store at –20C till further observation.
13. Observe under a fluorescence microscope at the desired excitation wave lenth of light..

OBSERVATIONS

Green fluorescence will be observed at those specific sites where the antigen-primary antibody complex is present when viewed at 494nm 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.

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IDENTIFICATION AND LOCALIZATION OF NOR ON CHROMOSOMES

INTRODUCTION

In eukaryotes the genomic DNA is compartmentalized in the nucleus. The genes get transcribed in the nucleus and majority of the transcrips are transported to the cytoplasm for translation or for various other functions. Thus nucleus is rich in DNA and a good amount of RNA, while the cytoplasm is rich in RNA.

Methyl green stain specifically binds with DNA, while Pyronine Y binds can bind with DNA as well as RNA. However, in presence of methyl green stain it binds with RNA alone and thus the DNA rich regions (nucleus) and regions having RNA (nucleolus and cytoplasm) can be differentially stained by using a mixture of methyl green and pyronine Y stain.

MATERIALS REQUIRED

Chromosome spreads, 5% silver nitrate, 3% formalin, ammonical silver nitrate (dissolve 4gm $AgNO_3$ in 5ml conc ammonium hydroxide, add slowly 7.5ml dist water. No precipitate should appear. Store at 4^oC in dark bottle).

PROCEDURE

1. Place 3 drops of 5% AgNO3 on the chromosome spread, gently place a cover glass on the drop and incubate the slide in an incubator at 60oC until AgNO3 is crystalline.
2. Rinse slide in dist 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 dist water. The cover glass will fall off.
3. Air dry the slide and observe NOR on specific chromosomes under high magnification of microscope.

OBSERVATION

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

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FEULGEN STAINING FOR DNA

INTRODUCTION

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

Schiff's reagent, 1N HCl, sodium metabisulphite, freshly prepared bleaching solution (5 ml of 10% sodium metabisulphite + 5 ml of 1N HCl + 90 ml of distilled water), fast green FCF (0.5% in 90% ethanol), tissue fixed in Carnoy fixative.

PROCEDURE

The slides containing the section was processed serially through following steps :

1. Xylene 2 chnages of 5 min each
2. Absolute ethanol 5 min
3. 90%, 70%, 50%, 30% ethanol 5 min each
4. Distilled water 5 min
5. 1N HCl (at room temperature) 2 min
6. 1N HCl (at 60oC in water bath) 6 min
7. 1N HCl (at room temperature) quick rinsing
8. Distilled water rinse once
9. Schiff's reagent 2h in dark
10. Bleaching solution 1 min
11. Distilled water rinse once
12. 30%, 50%, 70%, 90% ethanol 5 min each
13. Fast green (FCF) 1 or 2 quick dips
14. Absolute ethanol 10 min
15. Xylene 10 min
16. Air dry, mount in DPX and observe under a microscope.

Step numbers 5, 6, 7, 9 and 10 are critical and should be done carefully.

OBSERVATION

Each cell will have clear cytoplasm and pink staining in the nucleus. Pink staining will be directly proportional to the amount of DNA present per unit area and the intensity can be quantitated in a cytophotometer. With proper control the DNA quantity per cell nucleus can be presented.

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TO STUDY PINOCYTOSIS AND DYE EXCLUSION TEST IN CELLS.

INTRODUCTION

Membranes normally do not allow larger molecules (such as dyes) to enter a cell through simple diffusion. If a dye is presented to an amoeba or paramecium or macrophage, however, the dye will be incorporated as part of what is referred to as receptor mediated endocytosis. In more general terms, the cell will drink or eat the dye, i.e. pinocytosis (cell drinking) or phagocytosis (cell eating). The dye will enter into internal vacuoles, known as vacuoles.

With some dyes, the cell will actively transport the dye back out of the cell. It requires energy for this dye (trypan blue, a vital dye) exclusion from metabolism. Energy will only produced in living cells, and the phenomenon can be monitored in what is known as the dye exclusion test. The dye exclusion text becomes a way to monitor cell viability.

It implies that we can monitor the health of a cell by timing the movement of dye, and we can observe alterations in membrane function by alterations in the process of dye movement. A healthy, functioning cell will not stain and will have a minimum number of dyed vacuoles. Its membrane will remain intact and there will be minimum interruption of its ligand-mediated endocytic processes. A damaged cell, however, will undergo physiological and morphological changes as the membrane receptor sites become irreversibly bound to dye. As it loses its ability to excrete the dye, it will become stained, round up and ultimately die.

MATERIALS

- Paramecium or Amoeba or macrophage cultures.
- 0.001 M Alcian blue (Mol. Wt.- 1289.9).
- 0.01 M Sodium azide.
- Slides, cover slips.
- Microscope.
- Hemocytometer with cover slips, micro tips, pipettes etc.
- Trypan blue 0.4%.

PROCEDURE FOR PINOCYTOSIS

1. Form a small ring of petroleum jelly onto a slide.
2. Add a small drop of *paramecium or Amoeba or macrophage* suspension, and add 10 µl of Alcian blue to the slide. Note the time. Immediately place a cover slip onto the slide and gently seal the jelly ring.
3. Observe the cell type with 10X magnification.
4. Record the time for each of the following events:
    a. Surface staining.
    b. Rounding up of the cell.
    c. Formation of rosette (crinkling of cell)
    d. Channeling (flattened pseudopodia and investigation of membranes).
    e. Pinosome formation (small vesicles of dye pinched off from invaginated membranes).

5. Prepare another slide with a jelly rins; add 10 μl of Sodium azide along with the cell. Allow the cell type to remain in the azide solution for about 5 minutes and then add the Alcian blue and a cover slip. Repeat steps 3 and 4.
6. Compare the tome for each event of step 4 for the control and for those cell stained with the azide.

PROCEDURE FOR DYE EXCLUSION TEST:

1. Prepare a cell suspension, either directly from a cell culture or from a concentrated or diluted suspension (depending on the cell density) and combine 50 μl of cells with 50 μl of trypan blue suspension (0.1%). Mix thoroughly.
2. With the cover slip in place, transfer a small amount of trypan blue-cell suspension to both 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.
3. Starting with 1 chamber of the hemocytometer, count all the cells in the 1 mm center square and four 1 mm corner squares. Keep a separate count of viable and non-viable cells.
4. If there are too many or too few cells to count, repeat the procedure either concentrating or diluting the original suspension as appropriate.
5. Include cells on top and left touching middle line. Do not count cells touching middle line at bottom and right. Count 4 corner squares and middle square in both chambers and calculate the average.
6. Each large square of the hemocytometer, with cover slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to approximately 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 cells} x 100.

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CELL MIGRATION ASSAY

(Modified Boyden Chamber assay)

INTRODUCTION

Cell migration is the movement of cell from one place to another, generally happens for the purpose of embryonic development, wound healing, angiogenesis, tumor growth and metastasis. It is one of the important cellular activities for normal physiological and pathological purpose of the body.

PRINCIPLE

Cells can show its migratory behavior towards a chemo-attractant. If cells can be kept in chamber which is separated by porous membrane and chemo-attractant like serum/growth factor is placed to the other chamber, cell can move through the porous membrane to reach towards the attractant. Migration analysis can be achieved by the counting of the cells that reach to the second chamber in the given time

MATERIALS

a) cell that has properties of migration like endothelial cell (human umbilical cord vascular endothelial cells- HUVEC), tumor cells { the protocol is based on HUVEC cell}

b) 12 well /24 well cell culture plate { can be used Falcon cell culture plate or any Indian make cell culture plate}

c) 12 well /24 well cell culture inserts with membrane of 8.0µ pores { can be used Falcon cell culture insert}

d) Serum containing and Serum free medium {like MCDB 131}

e) Collagen type – I

f) DAPI {any other stain like methylene blue for light microscopic study can be used in place of DAPI}

g) Phosphate buffer

h) Methanol

PROCEDURE

1. Coat a thin film collagen type I to the upper surface of the membrane of the cell culture insert for half an hour
2. Add 500 µl / 750 µl of serum containing medium to the cell culture plate.{ chemo-attractant}
3. Add the appropriate number of cells {1x 105 for 24 well cell culture plate}to the cell culture insert with 250µl serum free medium.
4. Place the cell containing insert to the cell culture plate and allowed to migrate the cell for 18 – 20 hours in a CO2 incubator {Normal incubator may be used}. {cell from the upper surface of the insert will migrate to the lower surface}
5. Take out the insert, decant the culture medium from the insert and remove cells of the upper surface of the membrane by cotton swab.
6. Fixed the cells of the lower surface of the membrane with 100% methanol for 5 minutes

7. Wash and rinsed the cells with PBS for 5 minutes.

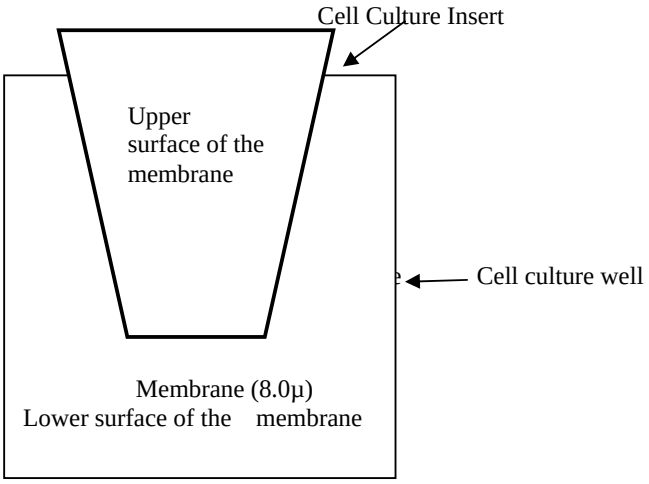
8. Cells can be stained with methylene blue or other stain like Hematoxylin , and then membrane can be cut apart from the insert and mount to the slide with mounting medium like DPX for light microscopic study, otherwise alternative method can be followed for fluorescence microscopy as mentioned below

8a. Cut the membrane from insert

9. Mount the membrane to the glass slide with vetashiled containing DAPI to stain the cell nucleus.

10. Study and count the cell under Light Microscope or Fluorescence Microscope

11. The number of cells as counted is the assessment of migratory cells



Assembly for the modified Boyden chamber cell migration assay.

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TO MEASURE LONG-TERM CYTOTOXICITY OR DELAYED GROWTH ARREST OF CELLS USING THE COLONY FORMATION ASSAY

INTRODUCTION

Some drugs/toxins may cause a slowing of growth. Others may cause reversible groth inhibition. How can we measure these actions of drugs? The common short term cytotoxicity assays (e.g. MTT or LDH release) cannot detect these long term effects of drug. So, we need the **Colony Formation assay (CFA)**

The **Colony Formation assay (CFA)** measures clonogenic potential, i.e. proliferative ability of single cells to form a clone, which then results in a colony. This assay measures colony formation and survival, and is routinely used as a sensitive model for testing and predicting cytotoxicity of anticancer drugs (Yalkinoglu 2000). Colony formation assay was necessary for assessment of long-term cytotoxicity of drugs which cause reversible growth inhibition (Saotome et al 1989). In a fluorescence based assay used to distinguished viable from non viable cells, microscopic detection of one viable pre-stained cell per 10^5 unstained cells, was as accurate and sensitive as the clonogenic assay for measuring cytotoxicity (Duerst et al 1985).

PROCEDURE

Plating of cells

CHO cells are plated in 6 well tissue culture plates at a density of 100-200 cells/ well containing 2.5ml growth medium/well. The growth medium was DMEM supplemented with 10% Fetal Bovine Serum, penicillin (200U/ml), streptomycin (200µg/ml), and L-Glutamine (2mM).
Note: One should start with both 100cells/well and 200 cells/well and determine which density gives the best colony formation.

Preparation of Herbal extract

Authentic stocks of Powders are solubilized in triple distilled water (10mg/ml) by limited autoclaving (5 pounds pressure for 7 min -we used a pressure cooker with a pressure gauge). This extraction method yielded extracts with reproducible sterility and biological activity. Herbal stocks (10mg/ml) were diluted with growth media to reach a final concentration of 25, 50, or 100 microG/mL. Then, this growth media with the diluted herb is filter sterilized (13mm, 0.45 micron Cellulose nitrate membrane) before addition to cells. We used *W. somnifera* root powder, and *E. officinalis* or *(Phyllanthus emblica)* fruit powder.
Note: The herbal powders must be fresh (1-14 days old) to get best results. Store them in a cool (not cold) room with dessicant. Aged stocks of these powders from Ayurvedic shops or other sources don't work.

Treatment with test drug

After allowing cells to attach for a 24hour period, designated wells were treated with/without sterile aqueous herbal extract. Plates were incubated at 37deg in a 5% CO2 incubator WITHOUT MEDIA CHANGE, for 8-12 days. This is essential to prevent disturbance of colonies. The growth media added on first day will be sufficient. Note that herbal drug is present throughout the 8-12 days.
Note: If herbal drug is prepared correctly-it should not alter the pH of growth medium.

Observing cultures
In controls (no drug treatment) colonies containing 5-10 cells should start to form at Day 4-5. Observe colonies again on Day 7 or 8 to see if colonies are increasing in number and whether each colony is increasing in diameter. If there is a visually significant difference in the size and number of colonies in controls versus drug treated wells, one can stop the experiment and stain colonies on Day 8. Otherwise one can check on Day 10 and Day 12. It is best to stain between Day 8-10, as the media will start getting acidic (remember-no media change in the experiment)

Preparation of Fixative and Stain
Prepare the fixative ahead of time and store at -20^{o}C. The stain can also be prepared ahead of time and stored at room temp, but it should be filtered twice through pre-wetted Whatman #1 papers just before use. Use glass distilled water for all steps.
Note: The stain can be saved, re-filtered and reused at least 2-3 times.

Visualization of Colonies by staining
Gently remove the media from each well. Gently wash each well twice with PBS (2mL/wash). Now Colonies are fixed in cold ethanol:acetic acid:3:1, for 30 minutes at room temperature. Then, remove fixative gently, and stain with crystal violet stain (0.25% aqueous-not in PBS) for 8-12 hours (overnight is ideal). The stain is then removed. Each well can now be washed gently with water 2-3 times. One can keep a wide beaker of tap water and gently immerse the 6 well plate few times. Once the purple unbound dye moves out—you can stop. Place the plates at an angle on a clean table to dry. The correct labelled lid should be near each plate—otherwise you may not know what was added to each well. One should see 80-100 well separated Colonies in each control well. Mark the location of each colony and count it as you mark it (use red pen to mark the bottom of each colony, so if one is interrupted, one knows where to continue the count).

$$\text{Calculations: } \frac{\text{Number of colonies in drug treated wells}}{\text{Number of colonies in control wells}} \times 100$$

OBSERVATION

Extracts of *W. somnifera* root powder, but not *E. officinalis* fruit powder; cause reproducible inhibition of colony formation of CHO cells.
Note: SiHA cervical cancer cell line can be used-although we got more reliable results with CHO cells in terms of effects of these 2 herbal drugs.

Questions
1. Does your test drug dose dependently inhibit (or stimulate) colony formation? How reproducible is the data?
2. Can you make a simple modification in the experimental conditions—so that you measure effect of drug on cell attachment-not on colony formation?
3. Test the same drug for short term cytotoxicity on CHO cells grown in 96 well plates. For the 96 well experiment—the protocol is given (Sumantran et al 2007).
3a. Does the drug have short term and long term cytotoxicity-or only one type of cytotoxicity for CHO cells?
3b. If you got both types of cytotoxicity? what was the dose range for short versus long term cytotoxicity?
3c. Which drug would be a better growth inhibitor/anticancer drug—one which gives both types of cytotoxicity? Or one which gives only one type of cytotoxicity?

3d. If the number of colonies remains constant even as you increase the drug concentration, we can conclude that the drug may be cytostatic (causes growth arrest).

Test drug

We have used aqueous extracts of Indian herbal drugs. However, one can use other agents known to inhibit CHO cells. Students should be encouraged to search the literature, identify a drug, and design the experiment. If possible—they should do this experiment also.

Important tips

If you use an anticancer drug or commercial growth inhibitor—make sure you dissolve it in appropriate solvent. Ensure that this solvent itself is not toxic to CHO cells! In fact, one should first only do experiments on CHO cells treated with/without solvent to establish whether the solvent is cytotoxic or not. Minor toxicity of solvent (10-15%) maybe accepted. One should account for toxicity of solvent in CHO (or SiHA cells) in final calculations on drug toxicity.

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HAEMATOXYLIN SQUASH TECHNIQUE FOR PLANT AND ANIMAL CHROMOSOMAL PREPARATIONS

PLANT MATERIAL

Root meristematic tissues are fixed in a mixture of ethyl alcohol and glacial acetic acid (3:1) for 24 hours.
The fixed roots are brought down to water,after changes in ethyl alcohol and 70% ethyl alcohol.
They are hydrolysed in 1N HCl at 60° C for 10 - 12 minutes.
Roots are then washed in distilled water.
They are 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.
They are then stained with 0.5% solution of haematoxylin for 20 -25 minutes.
The stained roots are washed thoroughly in water.
Each root tip is softened with 45% acetic acid and teased into framents 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.

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

ANIMAL MATERIAL

Chromosomal preparations from grasshopper testes could also be obtained employing haematoxylin procedure. The method, in brief, is the following:

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 hours and then stored in 70% methyl alcohol.
The tissue is hydrolysed in 1N HCl at 60^{o} C for 6 min.
After thorough washing in distilled water,the tissue is moranted with 4% iron alum (Ferric Ammonium sulphate) for about 25 min. It is washed repeatedly in distilled water and stained with 0.5 % solution of haematoxylin for 30 minutes.
The tissue is teased into small fragments in a drop or two of 45% acetic acid on
a slide and squashed under a coverslip.
The preparation is sealed with paraffin to have a temporary preparation.

It has been observed that a large number of Departments of Botany and Zoology make use of the haematoxylin technique for chromosomal preparations from plant using meristematic cells and from animal material employing grasshopper testes.
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. The teachers and students need to be aware of the publications which had reported these procedures in Current Science.

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ISOLATION OF DNA FROM MOUSE/RAT LIVER CELLS

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.

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) etc.

Normal saline- (0.9% NaCl)- weigh out 0.9 g NaCl and dissolve in 100 ml distilled H_20.

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 bit for proper dissolution. Make-up the volume to 50 ml with distilled H_20, 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 H_20. 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^oC before use.

PROCEDURE

1. Sacrifice a mouse/rat by cervical-dislocation, and quickly remove the liver. (Alternatively, **freshly excised** liver of any animal (chicken prefrred) 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 1 g, use 19 ml of water or saline).

   DNA content in rat liver is approx. 2 mg/g wet weight of tissue. Hence a 5% homogenate gives a concentration of approx. 100 ug/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 ug/ml. Very low DNA concentration (less than 10 ug/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 the 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, the 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 inter phase 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 hour.

OBSERVATION

White fibrous DNA can be seen as precipitate which can be spooled on a glass rod.

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RAPID ISOLATION OF PLASMID DNA

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 host, they are widely used as vectors in recombinant DNA technology. A desired gene can be cloned into 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 site 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. The small amount of resulting plasmid DNA can be used to screen for the presence of desired clones in a population by restriction enzyme digestion pattern or for further transformation.

MATERIALS REQUIRED:

1. Transformed *E.coli* colonies containing the desired plasmid
2. Autoclaved bacterial culture tubes with 5ml LB medium
3. Desired antibiotic solution
4. Shaker incubator set at 37ºC; Waterbath with boiling water
5. Eppendorf tubes, eppendorf tube stand, microfuge, micropipettes and tips
6. Lysis buffer

1M Tris-HCl (pH 8.0)	100µl
0.5M EDTA	2µl
Sucrose	15mg
Lysozyme	2mg
Pancreatic RNase	0.2mg
Bovine Serum Albumin	0.1mg
Distilled Water	to 1ml

7. LB Medium

Bacto tryptone	10gm
Bacto yeast	5gm
Sodium chloride	10gm
Distilled water	1000ml

Adjust pH to 7.5 and autoclave for 20min at 15lb/inch^2 pressure

PROCEDURE

1. Inoculate 5ml of LB medium containing the appropriate antibiotic with a single bacterial colony. Incubate overnight at 37^{o}C with vigorous shaking (at ~ 150 rpm)
2. Pour 1.5ml of overnight culture into an eppendorf tube. Centrifuge in a microfuge (~10000 rpm) for 5min 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 30µl of Lysis buffer and gently tap the tube to allow cells to be uniformly suspended and incubate at room temperature (~25^{o}C) for 5min
6. Place the tube in a boiling water bath exactly for 1min and immediately chill on ice for at least 1min
7. Spin in microfuge (1000 to 12000 rpm) for 15min at room temperature
8. Carefully collect the supernatant and directly use for restriction digestion etc.

(Approximate yield of plasmid DNA is about 2µg/1.5ml of bacterial culture)

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AGAROSE GEL ELECTROPHORESIS FOR DNA

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 (254nm or 310nm or 354nm) using a Transluminator.

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

MATERIALS REQUIRED

A. Solutions

1. Tris-Borate-EDTA (TBE) stock solution (5X)

Tris base	54.0gm
Boric acid	27.5gm
0.5M EDTA (pH 8.0)	20.0ml
Distilled water to make	1000ml

2. Working Buffer: 1X or 0.5X TBE

3. Loading Buffer (lOx)
   0.25% Bromophenol blue
   O.25% Xylene cyanol
   25% Ficoll (Type 400) in dist. water
   Store at room temp.

B. Preparation of Agarose gel

For analyzing restriction digested genomic DNA, usually a 0.8% gel is made in 0.5X or 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 0.5-0.1mm 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 320mg of agarose in 40ml of 0.5xTBE)
3. Boil till the agarose dissolves (total of about 10min heating on a heater)
4. Cool solution to 50^{o}C and add ethidium bromide to a final concentration of 0.5μg/ml. (A stock solution of 10mg/ml ethidium bromide can be made and stored in a colored 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 to the table or the gel mold while polymerization is in progress)

6. After the gel is completely set (30-45min 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 or 0.5x TBE) to let the gel just submerge
8. Prepare DNA samples for electrophoresis as follows

DNA (at least 150-200ng) + distilled water	18µl
10x loading Buffer	2µl

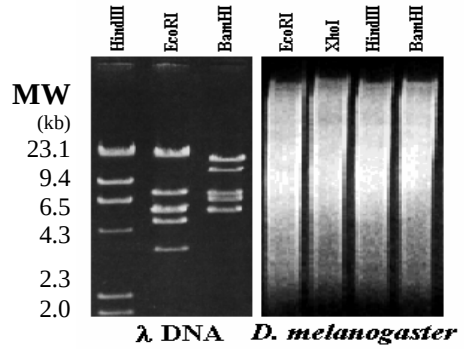
(N.B. Volume of water can be adjusted according to the volume of DNA sample)
Vortex the samples and centrifuge briefly before loading in the wells. Switch on the power supply at 50-150 volts depending upon the gel size (5 volts/cm being the optimal). After the run is over, examine the gel in ultra violet light using a transluminator (**UV light is harmful to eyes: use protective glasses or the perspex shield**).

OBSERVATIONS

Lambda DNA digested with Hind III restriction enzyme is usually run as molecular weight 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 enzyme digested plasmid or phage DNA gives sharp band/s of expected molecular weight size/s. However, similarly digested genomic DNA shows a smear.



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 viewd on UV transluminator

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SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS FOR PROTEINS

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-do-decyl-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 ration being 29:1 in all cases):

% Acrylamide Concentration	Size (in kd) in Linear Range of Separation
15%	12 to 43kd
10%	16 to 68kd
7.5	36 to 94kd
5.0	57 to 212kd

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 etc. 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.

MATERIALS REQUIRED

1. Acrylamide Stock Solution (acrylamide: bis-acrylamide:: 29: 1)
   - Acrylamide 29gm
   - N,N'-methylene-bis-acrylamide 1gm
   - Deionised Water 100ml
   - 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 10gm
   - Deionized Water to make 100ml
   - Store at room temperature
3. Tris Buffers (1M Stock) - pH 8.8 and 6.8
   - Tris base 12.1gm
   - Dist. Water 100ml
   - Adjust to pH 8.8 or to 6.8 with 1N-HCl
4. Ammonium persulfate (APS) stock solution
   - Ammonium persulfate 10gm

Dist. Water to make 100ml

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 15.1gm
   - Glycine 94.0gm
   - Dist. Water to make 100ml
   - (let the contents dissolve fully before adding SDS solution and water)
   - 10% SDS 50ml
   - Dist. Water to make 1000ml
   - **pH should be 8.3**
7. 3M Sodium Acetate (pH 5.2)
   - Sodium acetate 40.81gm
   - Dist. Water 80ml
   - dissolve and adjust pH to 5.2 with Glacial Acetic Acid and make the volume to 100ml
8. 10mM Sodium Acetate (pH 5.2)
   - 3M Sodium Acetate (pH 5.2) 3.3µl
   - Dist. Water to make 1ml
9. 1M Dithiothreitol (DTT)
   - DTT 150mg
   - 10mM Sodium Acetate (pH 5.2) 1ml
10. 100mM Phenyl methyl sulfonyl fluoride (PMSF)
   - PMSF 17.4mg
   - Abs. Ethanol 1ml
11. Sample buffer
   - 1M Tris (pH 6.8) 50µl
   - 1M DTT 100µl
   - 10% SDS 200µl
   - 1% Bromophenol Blue 100µl
   - Glycerol 100µl
   - 100mM PMSF 20µl
   - Dist. Water to make 1ml
12. Coommassie Brilliant Blue (CBB) Staining Solution
   - CBB R 250 2gm
   - Methanol 45ml
   - Glacial Acetic Acid 10ml
   - Distilled Water to make 100ml
   - Store the stain in tightly stoppered bottle. The staining solution may be reused several times.
13. Destaining solution
   - Glacial Acetic Acid 20ml
   - Methanol 10ml
   - Dist. Water to make 100ml
14. Gel casting glass plates, spacers and combs (for 15cm x 15cm x 0.6mm gel)

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 1.5ml Eppendorf tube, close the tube tightly and immediately keep the tube in a boiling waterbath for 5-10min. Make sure that the tube cap does not open in between. After

10min, briefly spin the tube and load the sample in the gel or store the sample at -70^{o}C till use.

B. CASTING OF VERTICAL SLAB GEL

i. SEPARATING OR 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

Dist. Water	3.46ml
1M Tris (pH 8.8)	7.50ml
Acrylamide Stock solution	8.36ml
50% Glycerol	0.40ml
10% SDS Stock solution	0.20ml

Mix, filter and degas for 5min under vacuum (presence of dissolved oxygen inhibits polymerization of Acrylamide)

Add the following polymerizing catalysts

10% Ammonium persulfate	100µl
TEMED	10µl

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 45-60min 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.

ii. STACKING OR THE UPPER GEL

1. Prepare the following stacking gel (5% Acrylamide) solution

Dist. Water	3.46ml
1M Tris (pH 6.8)	0.50ml
Acrylamide stock solution	0.66ml
10% SDS	40µl

Mix and degas under vacuum for 5min and add the TEMED and APS as follows

10% APS	40µl
TEMED	4µl

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 45-60min 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.)

C. 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 5mm x 0.6mm well, 20µl of sample containing about 40µg protein is adequate).
4. Apply current (20mA constant for a 15cm x 15cm x0.6mm slab gel) till the dye enters the separating gel. At that time increase the current to 25mA (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. FIXING AND STAINING OF THE GEL

1. Remove the gel from the supporting glass plate and carefully transfer it to a suitable size tray containing 5 volumes of the CBB staining solution. Leave overnight in the stain at room temperature.
2. Next morning, remove the stain and store for reuse. Add the gel de-staining solution. Replace the de-staining solution 3 times at 10-15min intervals
3. After the final removal of destaining solution, give two changes in 15% Acetic acid at 10min intervals and finally leave the gel in 10% acetic acid when the protein bands appear bright purple stained against an almost clear background.
4. The gel may be stored in 10% acetic acid or may be dried before storage. If the proteins were labeled with radioactive precursors, the gel may be processed for autoradiography or fluorography as desired.

PRECAUTIONS

1. The gel assembly must remain free of any vibrations during the period of polymerization since vibrations cause unevenness in the polymerized matrix which results in distortions in protein bands. The table/platform on which the gel assembly is kept should be vibration free. If not, the whole assembly may be placed on a flat wooden board kept on a tray full of sand.
2. Gel must not get heated during run: this causes the typical "smiling" effect. Excessive heating may be prevented by regulating the current flow and by keeping the gel in cold room or preferably by circulating cold water through the gel tank.

3. The wells must be thoroughly cleaned: any pieces of polymerized gel or other debris will result in distorted bands.
4. All the samples should be quickly loaded in respective wells to prevent diffusion of sample. While loading the samples, care must be taken to avoid any bubbles in the sample.

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