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 = 20µm
   ---------- = 1.54µm
   13 divisions

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.
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 bright 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 phosphorylation. 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 transcripts 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 bright 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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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, solubilizes the membranes, and denatures the proteins, thus helps in the release and deproteinization of DNA. The phenol, an aromatic alcohol, selectively solubilizes 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 H2O.

SDS (10%) solution- Weigh out 5 g sodium dodecyl sulfate (SDS) and dissolve in 40 ml distilled H2O. The solution may be warmed a little bit for proper dissolution. Make-up the volume to 50 ml with distilled H2O, 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 H2O. 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° C before use.

PROCEDURE
1. Sacrifice a mouse/rat by cervical-dislocation, and quickly remove the liver. (Alternatively, freshly excised liver of any animal (chicken preferred) can be taken from a slaughter house.

2. Transfer the liver to ice-cold normal saline, and wash to remove the adhering blood. Blot the liver dry with the help of a blotting paper.

3. Weigh the liver and keep in a watch-glass in an ice-box.

4. Mince the liver and make a 5% homogenate (W/V) in ice-cold water or normal saline (For example if the weight of the liver is 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 1/9th 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 1/5th 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°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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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 in such a way that the onion disk touches water or else making the onion to sit on wet sand in such a way that the bulb is buried partially in sand. In two days 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 condenser 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°C is even better.

Staining and making squash preparation:

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

Different phases of mitosis 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 condenser 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 wax or with nail polish immediately to prevent drying of acetic acid film and entry of air bubbles.

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

OBSERVATIONS

Different phases of mitosis 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 condenser 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 4ºC 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 wax or with nail polish immediately to prevent drying of acetic acid film and entry of air bubbles.

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

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 observe under 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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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 37°C for one night and examining if any of them becomes turbid the next day. Sterile solutions remain clear.

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

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

Incubator, centrifuge, clean-sterile work bench,

Giemsa stain

<table>
<thead>
<tr>
<th>Stock soln -</th>
<th>Giemsa Powder</th>
<th>380mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol</td>
<td>25ml</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>25ml</td>
</tr>
</tbody>
</table>

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

Working soln -

<table>
<thead>
<tr>
<th>Stock soln</th>
<th>2.5ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>1.5ml</td>
</tr>
<tr>
<td>Giemsa water</td>
<td>50.0ml</td>
</tr>
</tbody>
</table>

Giemsa water -

| 0.2M Na2HPO4 | 80ml |
| Distilled water | 800ml |

Adjust pH to 6.8 with 0.1M Citric acid (roughly 24ml) and then make up the volume to 1 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 CO2 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°C
5. Before using pipettes, container, culture vial etc, flame them gently but use only after cooling
6. For each culture, add the following in the order given:
   - TC medium 5ml
   - Fetal bovine serum 1ml (if not already added)
   - Blood 0.3ml
   - PHA 0.1ml
7. In order to buffer the pH, blow CO2 from a CO2 cylinder or bubble exhaled air orally through a cotton-plugged-pipette
8. Culture is kept in an incubator at 37°C for 48 to 72 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°C) 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 overnight 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 ~600C.

Dried glasswares are sterilized either dry or wet. For the former they are kept in an oven at 1600C to 2000C 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/inch² 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 HNO3 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°C), clinical centrifuge

Solutions
- Hypotonic Solution: 0.56% KCl (should be kept pre-warmed to 37°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°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°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°C), clinical centrifuge

Solutions

- Hypotonic Solution: 0.56% KCl (should be kept pre-warmed to 37°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°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 distinctively 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°C), 0.9% NaCl (stored at 4°C), phosphate buffer (stored at 4°C), Giemsa stain, distilled water

Phosphate buffer

Equal parts of solution 1 and solution 2

Solution 1 : 9.073g KH₂PO₄ in 1000 ml dist water
Solution 2 : 11.87g Na₂HPO₄.2H₂O 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
7. Rinse in water, and monitor under the microscope. If stain is less, put again in Giemsa; if treatment is under, put again in trypsin for a few seconds and repeat these steps until

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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 50°C (for Ba(OH)₂ solution) and at 60°C (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 50°C 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 60°C 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 37°C 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.
Photomicrograph 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°C 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°C 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°C, Water bath at 37°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°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 3\(^{rd}\) instar larvae, blotting paper, droppers, dissecting needles and fine forceps, cavity slides, clean glass slides, coverglasses, 37\(^{\circ}\)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)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>86mg</td>
</tr>
<tr>
<td>KCl</td>
<td>313mg</td>
</tr>
<tr>
<td>CaCl(_2).2H(_2)O</td>
<td>116mg</td>
</tr>
<tr>
<td>NaH(_2)PO(_4).2H(_2)O</td>
<td>88mg</td>
</tr>
<tr>
<td>KHCO(_3)</td>
<td>18mg</td>
</tr>
<tr>
<td>MgSO(_4).7H(_2)O</td>
<td>513mg</td>
</tr>
<tr>
<td>Dist. H(_2)O</td>
<td>100ml</td>
</tr>
</tbody>
</table>

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°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 1 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°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°C. Keep at -70°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°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 37°, at 42° and at 60°C
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 MgCl2)
   **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°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°C) ethanol

   Leave at -70°C for 2hrs

6. Centrifuge at 12,000rpm for 30min at 4°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°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°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°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 22mm² 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

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>500μl</td>
</tr>
<tr>
<td>20X SSC</td>
<td>250μl</td>
</tr>
<tr>
<td>DIG labeled probe (10-20ng/slide)</td>
<td>as required</td>
</tr>
<tr>
<td>H2O to make TOTAL volume to</td>
<td>1000μl</td>
</tr>
</tbody>
</table>

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 22mm² 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°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°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, MgCl₂ 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 37o, at 42o and at 60oC
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 Na₂HPO₄, 18.6 mM NaH₂PO₄, pH 7.4)
   Prepare 1X PBS by diluting 1:10 with dH₂O
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
    MgCl₂ 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.
14. Wash in Hybridization buffer B for 10 min at RT.

Prehybridization and Hybridization

14. Prehybridize in Hybridization buffer B for 1-12 hrs at 42°C.
15. Hybridize with riboprobe in Hybridization buffer B for 24 hrs at the desired temperature mentioned above.

Washing

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°C 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°C.

20. Wash in PBT 5 times for 5 min each. (You can store the tissues at 4°C)

21. Incubate the tissues in TBS for 15-20 min, as the phosphate molecules in the PBS can decrease the phosphatase activity so as to result in weak signal, in higher pH (9-9.5) PBS tends to precipitate also to give crystal like sediments which one can see the moment you add staining buffer.

22. 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, polysaccharides 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 permeabilized 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 CaCl2.2H2O, 88mg NaH2PO4, 18mg KHCO3, 513mg MgSO4.7H2O, 100ml distilled water, pH6.8]

10X PBS - [175.0 mM NaCl, 84.1 mM Na2HPO4, 18.6 mM NaH2PO4, pH 7.4]
Prepare 1X PBS by diluting 1:10 with dH2O.

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

PBS-PFA - [1X PBS, 4% paraformaldehyde]
Dissolve at 60°C 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°C overnight.

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 –20°C till further observation.

13. Observe under a fluorescence microscope at the desired excitation wave length of light.

OBSERVATIONS

Green fluorescence will be observed at those specific sites where the antigen-primary antibody complex is present when viewed at 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 transcripts 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 AgNO3 in 5ml conc ammonium hydroxide, add slowly 7.5ml dist water. No precipitate should appear. Store at 4°C 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 60°C 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 changes 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 rinse; 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 underfill 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 mm³ or 10⁻⁴ cm³. Since 1 cm³ 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 10⁴;

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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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 growth 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 100 cells/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°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).

Calculations:  Number of colonies in drug treated wells  x 100
Number of colonies in control wells

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 fragments in a drop or two of 45% acetic acid on a slide and squashed under a coverslip. The coverslip is then sealed with paraffin wax to obtain a temporary preparation.

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°C for 6 min.
After thorough washing in distilled water, the tissue is mordanted 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.

References:


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