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MOLECULAR BIOLOGY PRACTICALS.
Practical 3. Methyl green - pyronin method for DNA and RNA detection in cells.

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Theoretical background. Methyl green-pyronin (MGP) staining is a classical technique utilizing two basic dyes for the detection and differentiation of DNA and RNA. This cytochemical procedure allows to study nucleic acids without their extraction from the cells [24].

Methyl green (MG) or CI 42585 is a stain with positive charge (Fig. 1, A). Friedrich Miescher, a Swiss physician and biologist discovered the DNA in 1869 and was first who used MG for staining of this macromolecule preparations [2]. Currently, the fluorescent DNA staining of nuclei by MG is used for far-red visualization of live cell nuclei and in malignant tumor prognosis [11, 19, 20]. MG excited by 244 or 388 nm light in aqueous solution with pH7, emits fluorescent light at 488 or 633 nm, respectively. In DNA bound state at neutral pH in water, MG becomes fluorescent in the far red region with an Ex_{max} = 633 nm and an Em_{max} = 677 nm also [19]. It is flat molecule (Fig.1, A) able to insert into DNA major groove. Its interaction with the DNA is proved to be electrostatic [10]. MG has affinity to A-T reach regions of the major groove [20] and is specific to negatively charged phosphate radicals in the double strand DNA (dsDNA). It stains the dsDNA blue-green.

MG is an effective and cheap dye for DNA staining in polyacrylamide or agarose gels with detection limits for faint bands as well as relative densitometric quantitation equivalent to another fluorescent dye, the ethidium bromide [17].

MG is used in *fluorometric assays*, *flow cytometry* (FC), fluorescent embryo labeling [19, 20] and as an exclusion *viability stain* for cells.

MG may be used as a pH indicator. Its solution is a suitable counterstain for chloroacetate esterase, nonspecific esterase, alkaline phosphatase, peroxidase, naphthol AS acetate esterase, and acid phosphatase. A typical working concentration of MG as a counter stain is 0.1- 0.5% [14]. It is used in screening of nuclease producers in microbiological studies [16].

MG powders often contain crystal violet. Its presence in MG solution makes interferes with staining of DNA. Thus, crystal violet must be removed by extraction several times with chloroform until no traces of violet stain could be seen in extracts.

Pyronin Y or CI 45005 (syn. = pyronin G, pyronin J, pyronin) (P) is a cationic dye also (Fig. 1, B). However, it has no specific affinity to dsDNA and its attachment to the negatively charged RNA results in pink-red color. P intercalates RNA. In combination with Hoechst 33342 pyronin Y (PY) can be used in method of differential staining of RNA at the DNA presence [4].

The P dye accumulates in the mitochondria of living cells. At low concentrations the *cytostatic* effect is presented as cell arrest in the G1 phase, whereas at high concentrations the *cytotoxic* effect is manifested as cell arrest in the G2 and S phase of the cell cycle [3]. In combination with Alcian blue P is used in studies of lungs diseases [15].

P is a fluorescent compound with an excitation peak at 547 nm and an emission peak at 566 nm [9]. P stains the cytoplasm and most nucleoli, as RNA containing materials, in pink-red. MG stains DNA blue-green [13]. Thus, combined use of P with MG in the MGP staining allows DNA and RNA differentiation [12, 24].

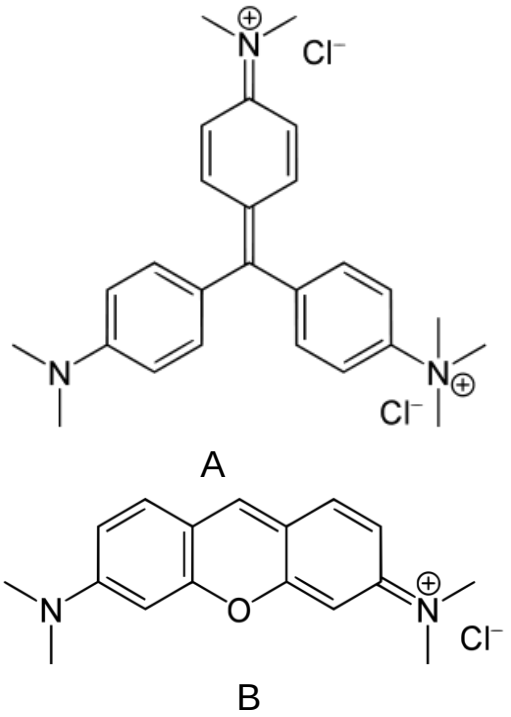

Fig. 1. Skeletal formulae of MG (A) and P (B). *By Yikrazuul,* 2008 [6, 7].

MGP staining distinguishes proliferating from differentiated non-proliferating cell nuclei after acid denaturation of DNA [22]. The MGP method is useful in identifying the distribution of Nissl substance in bodies of neurons cell [23].

MGP method utilizes formalin fixed tissue or alcohol-ether fixed smears [18, 19]. The technique is widely used in diagnostic cytology of human and animal diseases [5]. An example of MGP stained preparation is presented in Fig. 2. *Theileria annulata*, a tickborne intracellular protozoan parasite of cattle, present in Southern Europe, North Africa, the Near and Middle East, Central Asia, India, China. It causes both: mortality and reduced production in animals [8].

Experiment 1. Demonstration of DNA and RNA nucleic acids in tissues

Materials and equipment. Laboratory gloves, racks, glass slides and cover slips, tubes, flasks, pipettes and fluorescence microscope. Stains MG (CI 42585) and P Y (CI 45005) (Sigma-Aldrich).

Fixative: 10% buffered neutral formalin is used as fixative [18] . Other fixative which may be used are Carnoy's, formol saline and Zenker's. Any well fixed paraffin embedded tissue can be used. Paraffin tissue sections cut at 5-6 μm [1,10], xylene or xylene substitute for deparaffinization, fresh reagent alcohol.

0.2M acetic acid - Na acetate, pH 4.2. Prepare 0.2 M sodium acetate (Na acetate anhydrous, 2.05 gm + distilled water to total of 125 mL), 0.2M acetic acid (acetic acid, glacial, 1.5 mL + distilled water, 123.5 mL), than mix 0.2M acetic acid, 35 mL + 0.2M sodium acetate 12.5 mL. Adjust, if necessary, pH to pH 4.2 with 0.2M acetic acid or 0.2M Na acetate solutions. Store at 4^{o}C.

0.5% MG Solution (0.2M acetate buffer, pH 4.2, 5 ml + MG, 0.025 gm)

MGP solution (0.5% MG solution, 5 ml + P, CI 45005, 0.0025 gm)

The P content in commercial preparations falls between 50% and 90%. For good staining, it is necessary for some samples of P to increase its amount up to 0.005 gm.

Sigma-Aldrich, Inc. sells the ready to use MGP solution (Cat. No. HT7016-500) made of certified MG, 0.012% and P (certified), 0.01% methanol, 0.75%, in deionized water. MGP solution should be stored at room temperature (18–26°C).

Procedure.

1. Put laboratory gloves on clean hands.
2. Deparaffinize tissues using xylene or xylene substitute and hydrate through alcohols to distilled water.
3. Rinse slide thoroughly in distilled water.
4. Flood sections with MGP solution of room temperature. Let stand for 2 to 7 minutes.
5. Note: Increasing stain time intensifies P staining (red color); decreasing time intensifies MG staining (blue-green to green color) [12].
6. Dip quickly 1-2 times in distilled water of room temperature.
7. Blot with blotting paper.
8. Dehydrate slide through 3 changes of fresh reagent alcohol.
9. Place in acetone for 10 seconds.

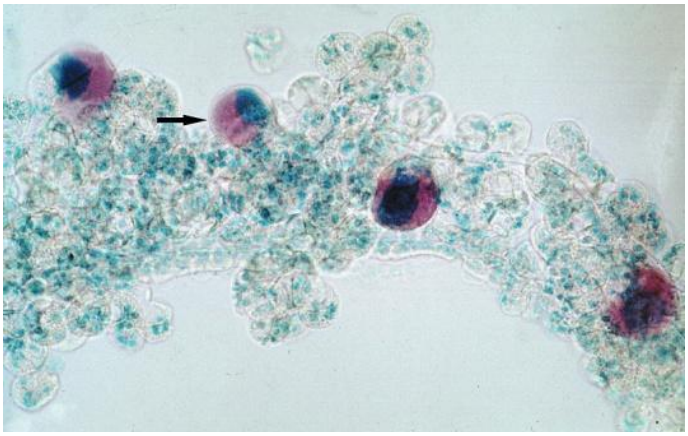

Fig.2. *Hyalomma anatolicum anatolicum* salivary gland with sporoblast stage (arrow) of *T. annulata*. *By A.R. Walker* 2012 [8].

10. Dip 3 times in equal parts acetone /xylene.
11. Clear in fresh xylene (not in a xylene substitute), 3 changes.
12. Mount with permanent mounting medium.
13. Observe the stained tissue slides under fluorescence microscope.

Staining Results: The cells nuclei are blue to blue-green due to DNA presence. The nucleoli and cytoplasm are pale pink to red due to RNA staining by P.

Experiment 2. Detection of DNA in protozoan cells and avian blood cells

In contrast to the discoid red blood cells of human and other mammals, the erythrocytes of birds are ellipsoid and contain the nuclei [21]. Thus, they are good for DNA staining in the cell nuclei.

Materials and equipment. Laboratory gloves, racks, glass slides and cover slips, tubes, flasks, pipettes and fluorescence microscope.

Protozoa culture, chiken blood, formo-saline, acetone, distilled water, glycerine, heparin 10 000 IU, MGP solution as above, acetic acid, xylene, laboratory gloves, tubes, racks, glass slides and cover slips, flasks, pipettes with sterile noses, syringes, and fluorescence microscope.

Solution of heparinized saline: Add sodium heparin to physiological saline (0.9%) to make a final concentration of 200 IU/ml.

Solution of formo-saline: Dissolve 10 mL formalin (40% solution of formaldehyde) in 90 mL of normal (0.9%) saline.

Ready to use MGP solution (Cat. No. HT7016-500) made of certified MG, 0.012% and P (certified), 0.01% methanol, 0.75%, in deionized water (Sigma-Aldrich, Inc.).

Procedure.

Protozoan Culture Preparation Collect soft plant remnants from the ground into dark jar half-filled with pond water. Incubate the jar without cover at room temperature for development of protozoa culture.

1. Put laboratory gloves on clean hands.
2. Place a drop of protozoa culture onto washed dry glass slide.
3. Add a drop of acetic acid (for 2 minutes) to fix the microbes.
4. Rinse the slide with distilled water.
5. Apply a drop of MGP stain for 15 minutes.
6. Again wash with distilled water and then dehydrate it with acetone.
7. Finally, wash it with xylene.
8. Mount the slide with glycerine.
9. Observe the green blue color of DNA in nuclei under the fluorescence microscope.

Preparation avian blood smear

1. Take 1 mL of chicken blood in a syringe containing 1 mL of heparinized saline. Blood can be taken from jugular vein, wing or brachial vein and medial metatarsal vein.
2. Place a drop of chicken blood on slide and make a thin smear as shown in Fig. 3.
3. Dry the smear in sterile air flow.
4. Fix the blood cells onto surface of glass slide by addition of few drops of formal saline (for 7 minutes).
5. Cover smear with several drops of MGP stain for 15 minutes.
6. Dip quickly 1-2 times in distilled water of room temperature.
7. Blot with blotting paper.
8. Dehydrate preparation in acetone.
9. Wash glass slide smear with xylene.
10. Place 15 µL drop of glycerine onto smear and cover slide with cover slip.
11. Observe smears under fluorescence microscope.

Staining Results: The nuclei in the chicken RBC are green-blue due to stained DNA and cytoplasm is pink colored due to RNA presence.

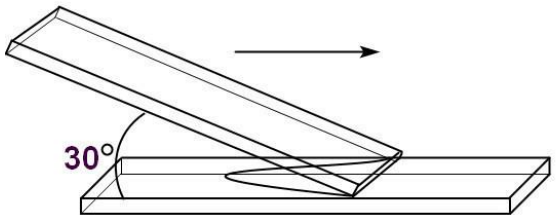

Fig. 3. Preparation of blood smear.
By D.B. Pylypiv.

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