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**Question 1:**

Acridine Orange is the stain used for identifying DNA and RNA in Peripheral neurons. It is a dye that intercalates or binds with the nucleic acid (either DNA or RNA) present in organisms and fluoresce to emit various colors that help in differentiation of cellular organelles. This binding is the result the electrostatic interactions of acridine molecule between the nucleic acid base pairs. Acridine Orange (AO), due to its metachromatic properties, is commonly used in fluorescence microscopy and flow cytometry analysis of cellular physiology and cell cycle status, including the fluorescent microscopic examination of microorganisms.

**Principle** : Acridine orange is a cell-permeable, nucleic acid selective dye that emits green fluorescence when bound to dsDNA(at 520 ) and red fluorescence when bound to ssDNA or RNA(at 650 nm).Since it is a cationic dye, it also  enter acidic compartments such as lysosomes  which in  low pH conditions, will emit orange light.

**Staining Procedure:**

* For staining cells for analysis by flow cytometry.

**Requirements:** 0.1M Citric Acid (dissolve 1.921g per 100ml distilled water) ,0.2M Dibasic Sodium Phosphate  (dissolve 2.839g per 100ml distilled water) ,Triton X-100 (Baker),0.5M EDTA, Sodium chloride(NaCl), Acridine Orange (Powder) and Sucrose.

**Preparation of reagents:**

Stock Buffer I :20mM Citrate-Phosphate, pH 3.0, 0.1mM EDTA, 0.2M Sucrose, 0.1% Triton X-100
(To 125ml distilled water add 40µl 0.5M EDTA, 26.48ml 0.1M Citric Acid, 6.85ml 0.2M Dibasic Sodium Phosphate, 13.69g Sucrose, 0.2ml Triton X-100 .**QS**to 200ml and 0.2µ filter. Store at 4oC)
Stock Buffer II :10mM Citrate-Phosphate, pH 3.8, 0.1M NaCl

(To 150 ml distilled water add 9.92ml 0.1M Citric Acid, 5.46ml 0.2M Dibasic Sodium Phosphate, 1.7g NaCl. QS to 200ml and 0.2m filter. Store at 4oC)

**Staining Procedure :**

1. Make a 2mg/ml solution of Acridine orange in distilled water and dilute to 1:100 in Buffer II
2. Aliquot cells: 105- 106 in 100µl PBS or media .
3. Add Buffer I (0.5ml) at room temp, agitate to suspend .
4. Add Buffer II + AO (0.5ml) at room temp, agitate to suspend.
5. Run on flow cytometer. Excitation 488 nm; dot plot of green fluorescence at 530nm versus red fluorescence >600 nm).

**Observance:**

Green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA.

**Applications:**

* For analyzing mitochondria and lysosomal content by flow cytometry.
* For visual detection of nucleic acids on agarose and polyacrylamide gels.
* For enumerating the microbial load in a sample since acridine orange binds with the nucleic acid of both living and dead bacteria.
* For  identifying engulfed apoptotic cells, because it will fluoresce upon engulfment.
* For differential staining  of human cells and prokaryotic cell with a fluorescence microscope. Human cells are stained black to faint green in which Bright orange organisms are easily detected .

**Question 2:**

Luxol Blue Stain **cannot** be used to detect demyelination in Peripheral Nervous System. Rather, it is used to detect demyelination in the Central Nervous system.

**Procedure involved in demonstrating demyelination in Peripheral Nervous System:**

**Adams’s OTAN Method for Normal and Degenerating Myelin:**

The abbreviation is for osmium tetroxide and alpha-naphthylamine. The technique is applied to cryostat sections, ideally after calcium-formaldehyde fixation.

**Solutions Needed**

A. Osmium Tetroxide-Potassium Chlorate. This is made up as required and used only once.

Osmium tetroxide, 2% stock solution in water: 5 mL Potassium chlorate (KClO3), 1% stock solution in water: 30 mL Water: 5 mL

B. Saturated α-Naphthylamine Solution. Dissolve a few crystal of α-naphthylamine in 40 mL of water at 40°C. Filter. This solution is used at 37°C

**Procedure**

1. Treat the sections with osmium tetroxide-potassium chlorate (Solution A) overnight at room temperature, in a tightly closed glass container.

2. Wash the sections for 10 min in water (3 changes with occasional agitation).

3. Treat the sections with saturated α-naphthylamine solution (B) for 20 min at 37°C.

4. Wash the sections for 5 min in water (3 changes with occasional agitation).

5. Apply coverslips, using an aqueous mounting medium.

**Result:**

Normal myelin is brownish–orange. Degenerating myelin (late products only) is black. Fat, if present in the tissue, is also blackened.