NAME: Obasi Kosisochukwu Cindy

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COURSE: Histochemistry

1. If Nissl stain is used to demonstrate RNA/DNA in the neurons of CNS, what staining technique is used for identifying the same in peripheral neurons?

The staining technique used for identifying DNA and RNA in peripheral neurons is Fluorochrome Acridine Orange.

Acridine orange 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 clinical specimen with acridine orange at low pH (Acridine orange Acid Stain)

- Requirements: Acridine orange, Glacial Acetic acid, Distilled water
- Preparation of reagent: 50 mg acridine orange is dissolved in 10 ml of distilled water to prepare a stock solution and stored in the refrigerator.1 ml of Acridine orange stock solution and 0.5 ml of glacial acetic acid is added to 50 ml of distilled water to prepare a working solution.
- **Staining procedure:**

1. Prepare a smear in a clean grease free slide and allow it to air dry.
2. The slide is then fixed with methanol and dried again.

3. It is then put in trough with acridine orange staining working solution (i.e 0.01 per cent).
4. After 2 minutes of staining, the slides are washed gently with water and dried and then examined in a fluorescent microscope.

Observance: Bacteria stain orange against a green to yellow background of human cells and debris.

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.

Fuelgen Technique can also be used

The Feulgen technique selectively stains DNA, and under controlled conditions, can be used for the photometric determination of DNA content. The reaction consits of two steps. Fixed material is treated for 8-10 min with 1N HCl in a water bath or oven at 60°C. Afterwards, the material is immediately transferred into Schiff's reagent at room temperature (for at least 30 min or until the tissue stains deep purple). The material is then squashed in acetocarmine or aceto-orcein. It is recommended the material be analyzed the same day, however, it can be kept at 4°C for a several days if necessary. Acid hydrolysis removes purin bases(adenine and guanine) from the DNA, thereby unmasking free aldehyde groups. The aldehyde groups then react with Schiff's reagent, which results in the purple staining. RNA is not hydrolyzed by the HCl treatment and, thus, the reaction is DNA-specific. The sugar part that remains reacts as an aldehyde ultimately forming a visible color. **Schiff's reagent**

Schiff's reagent is prepared by pouring 200 mL of boiling distilled water over 1-g basic fuchsin. Shake thoroughly, cool to 50°C, filter, and add 30 mL 1N HCl to the filtrate. Cool to room temperature and add 1 g potassium metabisulfite ($K_2S_2O_5$). Allow the solution to stand overnight in the dark or until a light straw or faint pink color develops. If not completely decolorized, add 0.5 g charcoal powder, shake, filter through a coarse filter, and refrigerate in a tightly-stoppered bottle in the dark.

<u>METHOD:</u>

1. Fix the tissue with 10% formalin.
2. Paraffin sections cut at 5 microns

STAINING PROCEDURE :

1. Prepare Hydrochloric Acid working solution; combine and mix well.

    Hydrochloric Acid, 20% aqueous 16ml

Distilled water. 24ml

    Preheat and maintain Hydrochloric Acid working solution at 60. Prior to use .

2. Deparaffinizesections thoroughly in three changes of xylene, 3 minutes each. Hydrate through two changes each 100% and 95% alcohols, 10 dips each. Wash well with distilled water.
3. Hydrolyze sections in hydrochloric acid working solution at 60 for 10 minutes .
4. Place slides directly in shift reagent, McManus for 4t minutes.
5. Wash in running tap water for 5 minutes, rinse in distilled water.
6. Counterstain in light Green SF Yellowish stain 0.2%, Aqueous for 1 minute.
7. Dehydrate in two changes each of 95% and 100% ethyl alcohol. Clear in three changes of xylene, 10 dips each.
8. Coverslip with compatible mounting medium.

RESULT :

DNA= Red-purple

Nuclei= Red-purple

Background= Green

PRECAUTION:

1. Do not allow sections to dry out at any point during staining procedure.
2. Drain staining rack/slides after each step to prevent solution carry over
3. Bouinfixed tissue is unsatisfactory for use with FUELGENreaction

2. If Luxol Fast Blue Stain is also used to detect demyelination in the PNS?

Explain the procedure involved in the demonstration of demyelination in the PNS.

Luxol Fast blue stain is not used to detect demyelination in the PNS.

The term demyelination in the peripheral nervous system describes a pathologic process of destruction of myelin-supporting cells in the peripheral nervous system that is the Schwann cells and / or the myelin lamellae with relative preservation of axons.

On the basis of histochemistry studies, it has been known that normal myelin sheath contains acidic polysaccharide and this compound is liberated during demyelination. The disintegration of myelin sheaths of degenerating peripheral nerves occurs in two stages. The early stage is characterized by a physical disruption of myelin sheaths without chemical alteration. The late stage, beginning about 1 week after atonal section, consists of a chemical degradation of myelin lipids. In degenerating peripheral nerves these changes develop within Schwann cells, indicating that these cells are capable not only of forming but also of digesting myelin sheaths. In the central nervous system the breakdown of myelin sheath after atonal degeneration occurs at a much slower pace. This temporal difference suggests that oligodendrocytes, which form the myelin sheaths of central nerve fibers, do not behave like Schwann cells, but that myelin digestion must await the arrival of macrophages. Myelinated axons can be observed in the fresh state by phase-contest microscopy of teased nerves or of squash or touch preparations of central nervous tissue. Living myelinated fibers can be seen in thin tissue cultures derived from sensory or autonomic ganglia or from immature brain or spinal cord . The nervous tissue is fixed, sectioned and stained to provide permanent preparations. When a neuron dies, or if it's axon has been severed, the part that has been cut off from the neuronal cell body becomes beaded and then breaks into a string of fragments which are eventually taken up by phagocytosis cells. This process is known as wallerian degeneration

In the peripheral nervous system, the pathologist is usually looking for the absence of myelin at sites where it would normally be present or for abnormal myelin that has not yet been removed by phagocytosis. Fragmentation of axons and their myelin sheaths, followed by loss of the debris, occurs throughout a nerve distal to a site of transection. In demyelinating neuropathy, of which there are many types: autoimmune, toxic, etc. The Schwann cells fail individually, so that abnormalities appear in individual internodes, a change known as segmental demyelination. Pathological changes of degeneration and demyelination are well shown in teased fibers. Teased preparations of nerve biopsies require a more complicated procedure than the one given here, applied to glutaraldehyde-fixed specimens Teased preparations of nerve biopsies are not routinely made in all laboratories because the procedure is labor-intensive and the information needed for diagnosis can usually be obtained from semithin (1 m) sections of tissue embedded in epoxy resin and stained with alkaline toluidine blue. The method for demonstrating a degenerating myelin is :

SWANK AND DAVENPORT's MARCHI METHOD FOR DEGENERATING MYELIN: The technique incorporates improvements that minimize artifacts such as sporadic staining of normal

myelinated fibers. Specimens are fixed for 2-4 days in phosphate buffered formaldehyde (2-3 weeks for human or other large brain) and then cut into slices no more than 3mm thick.

Solution needed **:**

Staining solution.

This can be kept for a few months in a suitable bottle. It is used only once. It includes ;

- Potassium chlorate 15g
- Water 200ml
- Osmium tetroxide 0.5g
- Formalin (37-40%) 0.5ml
- Glacial acetic acid 2.5ml

Result **:**

Normal myelin is brownish-orange.

Degenerating myelin is black.