S. S. College, Jehanabad

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Topic: Histochemical localization of glycoproteins

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Teacher: Praveen Deepak

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HISTOCHEMICAL LOCALIZATION OF GLYCOPROTEINS

Glycoproteins or mucopeptide are branched molecules containing sialic acid rich oligosaccharide chains covalently attached to amino acid side chains. The presence of sialic acid at the free end of the glycoproteins makes it a negatively charged compound. Because of the –OH groups of sugars, glycoproteins are more hydrophilic than simple proteins. This means glycoproteins are more attracted to water than ordinary proteins. The hydrophilic nature of the molecule also leads to the characteristic folding of the protein's tertiary structure.

The carbohydrate moiety is a short molecule in a glycopeptide molecule which is often branched and may consists of following:

- Simple sugars (e.g., glucose, galactose, mannose, xylose)
- Amino sugars (sugars that have an amino group, such as N-acetylglucosamine or N-acetylgalactosamine)
- Acidic sugars (sugars that have a carboxyl group, such as sialic acid or N-acetylneuraminic acid)

Most of the glycoproteins compose the integral membrane protein, where they have an essential role in cell-cell interaction. These glycoproteins are formed in the subcellular organs through the process known as glycosylation of protein. There are two types glycosylation occurring in the subcellular organs in the cytoplasm namely O-linked glycoproteins and N-linked glycoproteins.

O-linked glycoproteins: O-linked glycoproteins are ones in which the carbohydrate bonds to the oxygen atom (O) of the hydroxyl group (-OH) of the R group of either the amino acid threonine or serine. O-linked carbohydrates may also bond to hydroxylysine or hydroxyproline. The process is termed O-glycosylation. O-linked glycoproteins are bound to sugar within the Golgi complex.

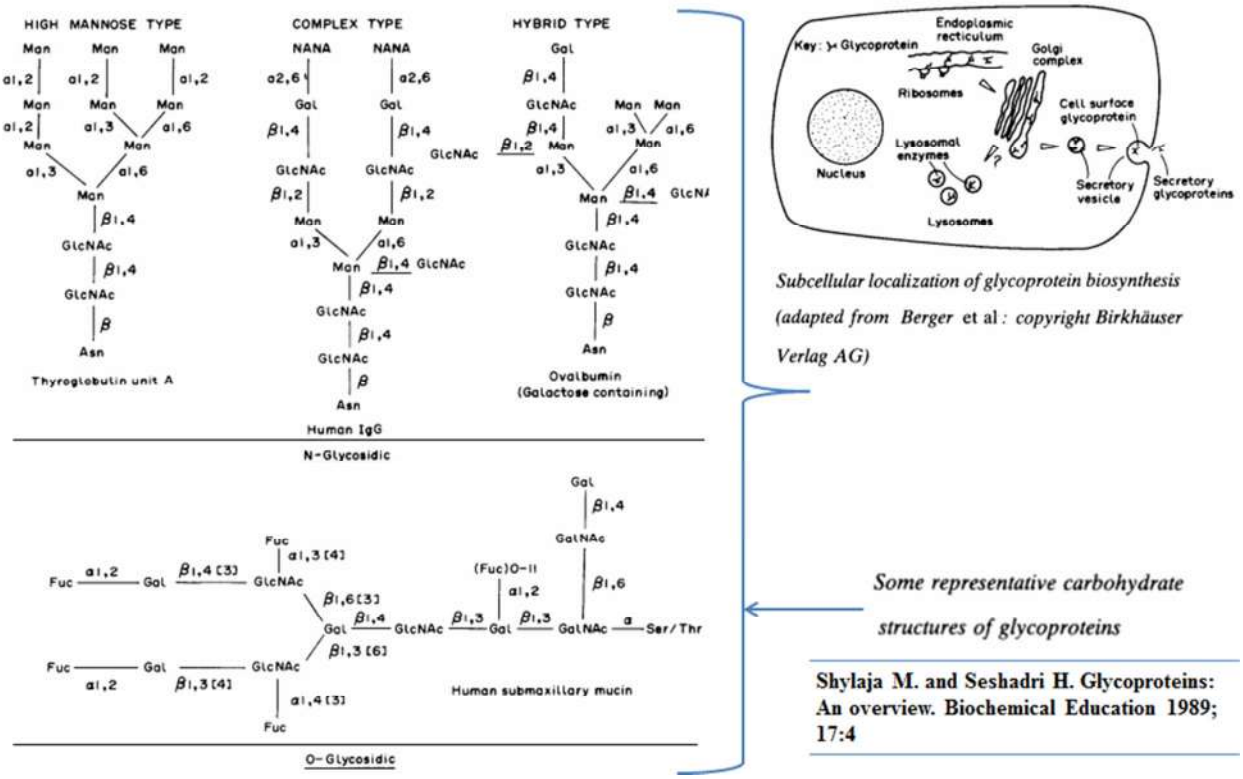
N-linked glycoproteins: N-linked glycoproteins have a carbohydrate bonded to the nitrogen (N) of the amino group ($-NH_2$) of the R group of the amino acid asparagine. The R group is usually the amide side chain of asparagine. The bonding process is called N-glycosylation. N-linked glycoproteins gain their sugar from the endoplasmic reticulum membrane and then are transported to the Golgi complex for modification.

While O-linked and N-linked glycoproteins are the most common forms, other connections are also possible:

- P-glycosylation occurs when the sugar attaches to the phosphorus of phosphoserine.
- C-glycosylation is when the sugar attaches to the carbon atom of an amino acid. An example is when the sugar mannose bonds to the carbon in tryptophan.
- Glypiation is when a glycophosphatidylinositol (GPI) glycolipid attaches to the carbon terminus of a polypeptide.

Methods of histochemical localization or demonstration: The methods of localization of glycoproteins are the same that are applied for the histochemical analysis of glycosaminoglycans

(GAGs), for example, PAS (Periodic acid Schiff) reaction, alcian blue, and Cuprolinic blue staining method.



PAS reaction

PAS reaction has been already discussed in previous class of histochemical localization of glycogen. The periodic-Schiff (PAS) reaction, the term was introduced by McManus, is one of the most widely used histochemical method to visualize glycogen and glycoprotein in a tissue specimen. In this reaction, periodic acid oxidizes the carbon to carbon bond forming aldehydes which react to the fuchsin-sulfurous acid to form the magenta colour. In brief, PAS reaction is following:

Materials required: Tissue specimen, Periodic acid solution (1 gm periodic acid in 100 ml distilled water), Schiff's reagent (add 1 gm basic fuchsin in 100 ml boiling distilled water then add 2 gm Sodium metabisulfite and 2 ml HCl), and hematoxylin.

Procedure:

1. Deparaffinize the section and wash it in distilled water.
2. Treat the section with periodic acid for 5 minutes.
3. Rinse the section well with distilled water.
4. Put the section in Schiff's reagent for 10-15 minutes.
5. Wash the section well under distilled water to remove the extra chemicals.
6. Counterstain the section with hematoxylin for 15 seconds.

7. Wash the section again with distilled water.
8. Rinse the section with alcohol.
9. Clean the slide with xylene and mount it.

Hyaluronic acid is also a nonsulfated glycosaminoglycan distributed widely throughout the connective tissue, cannot be stained with PAS. Complex substances such as chondroitin and keratan sulfate also give a negative PAS test.

Alcian blue

Alcian blue is a copper phthalcyanin dye and contains positively charged groups capable of salt linkage with certain polyanions. These polyanions consist of the sulfate and carboxyl radicals of the acid mucins and the phosphate radicals of the nucleic acids that do not react. Consequently, only the acid mucins are stained. By varying the pH of the solution of Alcian blue more information can be gained concerning the types of acid mucin present. Therefore, because of its basic nature, it mainly stains acidic mucosubstances that are carboxylated and sulfated by forming a salt bridge with them.

Technique

- Sections of 4 or 5µM are cut in a microtome and gently put on a slide.
- Tissue section is then spread with the help of a water bath top.
- Spread section is then processed to make ready for the staining

Equipment

- Slides
- 22mm square coverslips
- Ceramic staining rack
- Columbia staining dish
- Columbia staining jar
- Forceps
- Latex globes

Reagents

- 3% acetic acid solution
- 1.0 gm Alcian blue
- 0.1 gm nuclear fast red or Kernechetrot
- 5.0 gm aluminum sulfate
- Thymol
- 200 ml distilled water
- 100% ethyl alcohol
- Xylene

Solution

1. Alcian blue solution

- Prepared by adding 1 gm alcian blue in 100 ml of 3% acetic acid solution – pH maintained to pH2.5.

2. Nuclear fast red solution

- Prepared by dissolving 5.0 mg aluminum sulfate in 100 ml distilled water, adding 0.1 gm nuclear fast red and then heating to boil. This solution is then filtered and a grain of thymol is added as a preservative.

3. Alcohol 50 %

- Reagent alcohol ~50 ml
- Deionized water ~50 ml

4. Alcohol 70 %

- Reagent alcohol ~70 ml
- Deionized water ~30 ml

5. Alcohol 80 %

- Reagent alcohol ~80 ml
- Deionized water ~20 ml

6. Alcohol 95 %

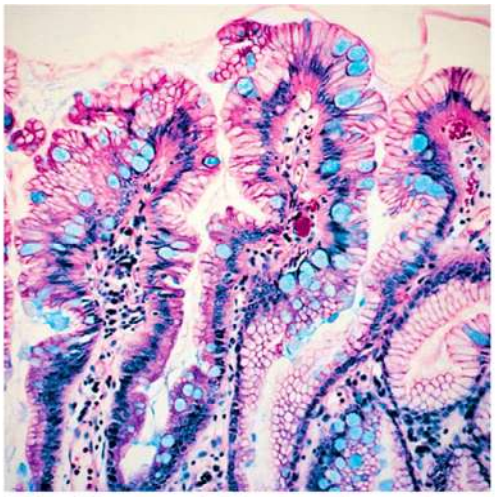
- Reagent alcohol ~95 ml
- Deionized water ~ 5 ml

Procedure

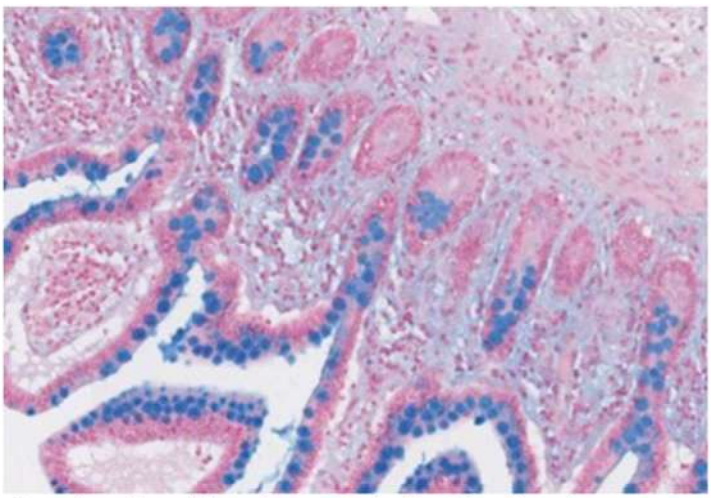
1. Deparaffinize the tissue and wash it under running distilled water.
2. Stain the section of tissue in the alcian blue solution for 30 minutes.
3. Rinse the section in distilled water to remove the extra stain.
4. Counterstain the section with nuclear fast red for 5 minutes.
5. Wash the section again in distilled water for 1 minute.
6. Dehydrate the section, clear and mount it for observation.

Results

Nuclei stain in pink to red, mucosubstances in blue color and cytoplasm is stained in pale pink color.



Alcian Blue control slides contain a section of positive staining tissue from small intestine.



A section of small intestine stained with alcian blue demonstrates both sulfated and carboxylated mucopolysccharide at pH2.5.

Iron diamine method

O-sulfate esters are stained brown-black with HID generated "in-situ" by the oxidation of a mixture of hydrochlorides of N, N-dimethyl meta- and para-phenylene diamines with ferric chloride.

This method either singly used in histological study or used alcian blue at pH2.5 to stain sialic acid and carboxyl groups of glycoproteins, which stains it in aquamarine blue colour.

Technique

- Sections of 4 or 5µM are cut in a microtome and gently put on a slide.
- Tissue section is then spread with the help of a water bath top.
- Spread section is then processed to make ready for the staining

Equipment

- Slides
- 22mm square coverslips
- Ceramic staining rack
- Columbia staining dish
- Columbia staining jar
- Forceps
- Latex globes

Reagents

- N, N-dimethyl-*m*-Phenylenediamine
- N, N-dimethyl-*p*-Phenylenediamine

- Disodium phosphate (Na_2HPO_4)
- HCl
- Anhydrous Ferric chloride (FeCl3)
- Alcian blue (optional)
- Distilled water
- 100% ethyl alcohol
- Xylene

Solution

1. Diamine solution

- Dissolve 30 mg N, N-dimethyl-rn-phenylenediamine (HC1) 2 ml and 5 mg N, N-dimethyl-p-phenylenediamine (HC1) in 50 ml distilled water and then adjust the pH to 3.4-4.0 with 0.2 M Na2HPO4 (0.15-0.65 ml).

2. Alcian blue solution (optional)

- Prepared by adding 1 gm alcian blue in 100 ml of 3% acetic acid solution – pH maintained to pH2.5.

3. 1 N HCL solution

- Prepared by addin 36.46 gm of HCL in 1 litre of water (Molar mass of HCL = 36.46 g/mole)

4. Ferric chloride solution

- Prepared by dissolving 8.11g anhydrous FeCl3 in some water and then dilute to 500 mL either in a volumetric flask or a graduated cylinder.

5. Alcohol 50 %

- Reagent alcohol ~50 ml
- Deionized water ~50 ml

6. Alcohol 70 %

- Reagent alcohol ~70 ml
- Deionized water ~30 ml

7. Alcohol 80 %

- Reagent alcohol ~80 ml
- Deionized water ~20 ml

8. Alcohol 95 %

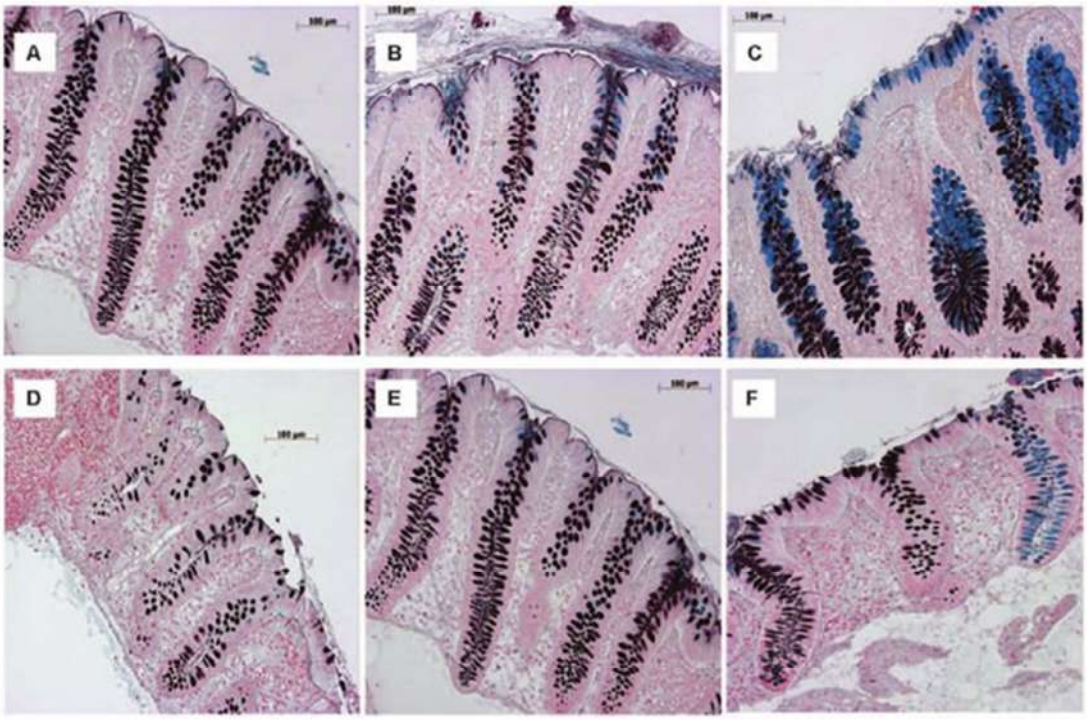
- Reagent alcohol ~95 ml
- Deionized water ~ 5 ml

Procedure

1. Deparaffinize the slide and hydrate it in graded alcohol.
2. Hydrolyze the section in 1 N HCl for about 10 minutes (to remove interfering stains).
3. Wash the slides in running water for 5 minutes.
4. Stain the section in the diamine solution for 20-48 hours. The diamine solution should be freshly prepared.
5. Wash the slides in running water for 5 minutes (optional for alcian blue staining step).
6. Stain in Alcian blue solution (pH2.5) for 10-20 minutes.
7. Rinse the section with 95 % alcohol and then with absolute alcohol.
8. Dehydrate the section in xylene and mount it in mounting medium.

Result

Mucosubstances (acid sulfmucus) is observed in black-brown color. If Alcian blue step is crried out, then caroboxylated acid mucus is stained in blue, and further, if nucleolar counterstain such as nuclear fast red is used then nuclei is appeared as red.



High iron diamine and alcian blue (pH 2.5) stained sections of human colon. Sulfomucin is s tained black/brown and sialomucin is stained blue. Top 3 panels demonstrate typical spatial variation among 3 colonic regions within one individual: A) Right colon B) Left colon C) Rec tum. Bottom 3 panels demonstrate typical interin. Croix et. a. PLoS ONE 2011; 6(9):e24447

Precaution

- Prepare high iron diamine solution prior to use, and throw it off after use. The solution can be used once or twice if well stored in a refrigerator. However, the background and specificity may be influenced and changed.
- Place smear in working solution at room temperature between 20~25℃. At lower room temperature extend staining time.
- Diamine salt is toxic. Avoid contact with skin.

Hale's colloidal iron method

At very low pH, carboxyl and sulfate-containing substances absorb the colloidal ferric ions. Prussian blue staining reaction then stains the absorbed ferric substance in blue.

Technique

- Sections of 4 or 5μM are cut in a microtome and gently put on a slide.
- Tissue section is then spread with the help of a water bath top.
- Spread section is then processed to make ready for the staining

Equipment

- Slides
- 22mm square coverslips
- Ceramic staining rack
- Columbia staining dish
- Columbia staining jar
- Forceps
- Latex globes

Reagents

- 12% acetic acid solution
- Glacial acetic acid
- 2% aqueous potassium ferrocyanide
- Stock colloidal iron suspension
- 2% ferrocyanide
- 2% HCl
- Distilled water
- 100% ethyl alcohol
- Xylene

Solution

1. Perl's solution

- Prepared by mixing 2% ferrocyanide and 2% HCl in equal volumes.

2. Working colloidal iron suspension

- Prepared by adding 10 ml stock colloidal iron in 18 ml distilled water and mixed it with 12 ml glacial acetic acid.

3. Alcohol 50 %

- Reagent alcohol ~50 ml
- Deionized water ~50 ml

4. Alcohol 70 %

- Reagent alcohol ~70 ml
- Deionized water ~30 ml

5. Alcohol 80 %

- Reagent alcohol ~80 ml
- Deionized water ~20 ml

6. Alcohol 95 %

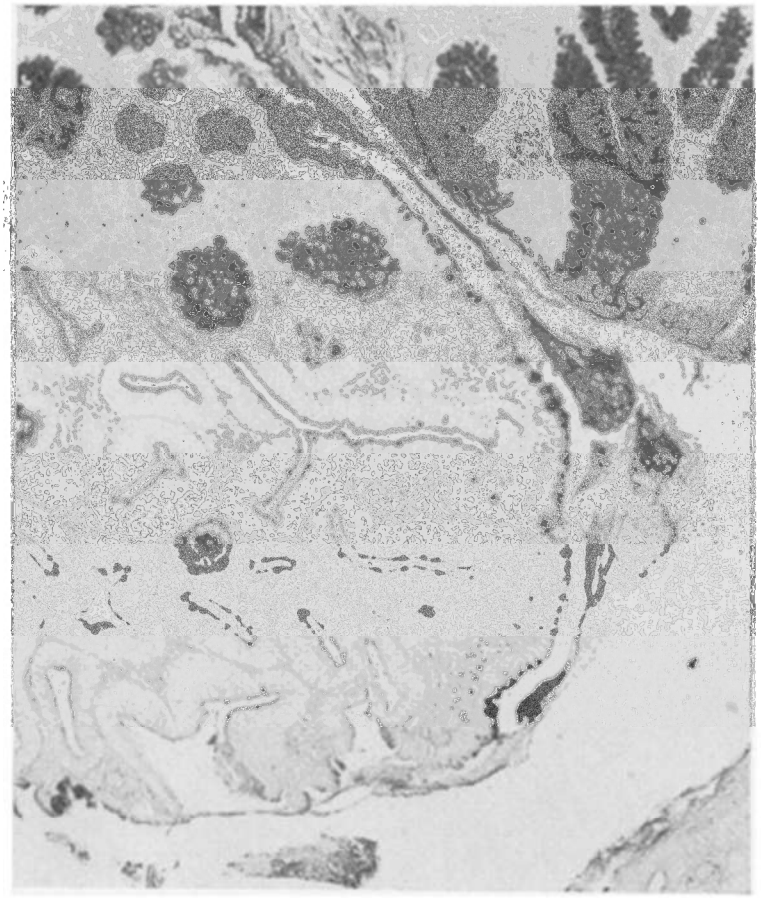
- Reagent alcohol ~95 ml
- Deionized water ~ 5 ml

Procedure

1. Deparaffinize the section and rinse it with distilled water.
2. Again, rinse the slides well in the 12% acetic acid.
3. Put the section in a working colloidal solution for 15-20 minutes.
4. Rinse the section three times with a 12 % acetic acid solution.
5. Put the section in Perl's solution for 20 minutes.
6. Wash the section with distilled water to remove the extra solution.
7. Counterstain the section with nuclear-fast-red for 1 minute.
8. Dehydrate the section, and mount it in DPX.

Results

Acid mucopolysccharides and sialomucins are observed as deep blue in colour. If nuclear counter stain as nuclear fast red is used, then nuclei stained as pink to red. Importantly, tissue sections should be rinsed well in distilled water, dehydrated with 95% and absolute alcohols, cleared and cover-slipped.



Adenomatous polyp of rectum showing an area of malignant transform-ation with practically no mucous secretion.
Colloidal iron method. × 64.

Gut, 1969, 10, 577-586

Metachromatic staining of glycoprotein

There are certain basic dyes belonging to aniline group that will differentiate particular tissue components by giving them a different color to that of original dye. The phenomenon is known as metachromasia. It takes place when certain negatively charged groups on the tissue react with cationic dyes. On polymerization the original colour of the dye changes to another colour (e.g. mast cell stain pink with toluidine blue). Thionin and toluidine blue dyes are commonly used for quick staining of frozen selection using their metachromatic property to stain nucleus and cytoplasm differently. Metachromasia is enhanced when intermolecular distances are reduced.

Factors which enhance metachromasia are

- Increasing concentration of dye.
- Decreasing temperature.
- pH
- Water a polar solvent, contributes to the efficiency of van der Waal's forces by which the molecules are held together.

In tissues, where there is a high concentration of anions e.g. in sulphated mucopolysaccharides, the cationic dye molecules may be held in such close proximity to one another that van der

Waal's forces can exert their influence and cause the dye to polymerize. Consequently the colour changes from blue to red.

- Tissue components often demonstrated by metachromatic stains:
- Amyloid material, Mast cell granules

Various stains are used to demonstrate amyloid or other mucopeptide in the tissue specimen, one of the stain crystal violet stain for detection of mucopeptide is given below;

Crystal violet stain for amyloid

Amyloid (a glycoprotein) exhibits metachromasia in tissue sections when stained with crystal violet and other cationic dyes.

Technique

- Sections of 4 or 5µM are cut in a microtome and gently put on a slide.
- Tissue section is then spread with the help of a water bath top.
- Spread section is then processed to make ready for the staining

Equipment

- Slides
- 22mm square coverslips
- Ceramic staining rack
- Columbia staining dish
- Columbia staining jar
- Forceps
- Latex globes

Reagents

- Crystal violet
- 100% ethyl alcohol
- Distilled water
- Aqueous mounting medium
- Nail polish for sealing

Solution

1. Crystal violet stock solution

- Prepared by adding 14 gm of crystal violet in 100 ml 95% ethyl alcohol.

2. Crystal violet working solution

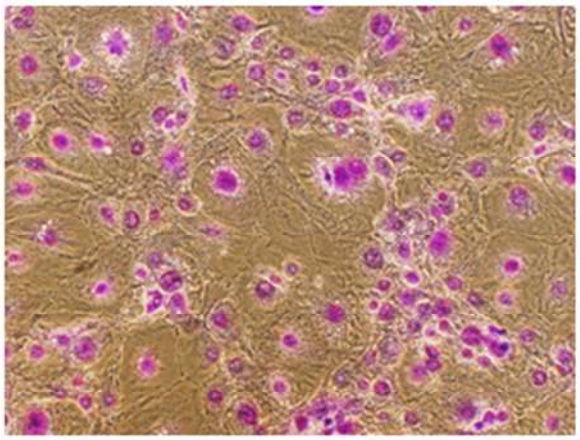
- Stock solution 10ml

Procedure

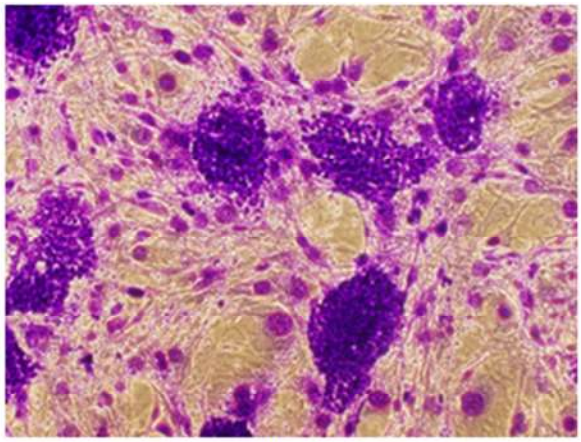
1. Deparaffinize and bring the sections to water.
2. Put working crystal violet solution for 1 to 2 minutes and check under microscope.
3. Rinse in tap water.
4. Mount in water or in water soluble media.
5. Put on the coverslip seal the edges with nail polish (Do not let it dry.)

Result

Amyloid is seen purple violet in colour, while other tissues can be observed with blue.



Feeder cells stained with crystal violet and imaged with a 40X objective.



Embyonic Stem cell colonies growing over feeder cells. Stained with crystal violet and imaged with a 40X objective.

<u>Congo-Red stain for amyloid</u>

Diazo dye attaches itself to amyloid fibrils. The union is affected by H bonds between the OH groups of amyloid and amino side groups of the dye. Congo red dye forms non-polar hydrogen bonds with amyloid. The green birefringence of congo red stained amyloid by polarized light is considered diagnostic of amyloid.

Technique

- Sections of 4 or 5µM are cut in a microtome and gently put on a slide.
- Tissue section is then spread with the help of a water bath top.
- Spread section is then processed to make ready for the staining

Equipment

- Slides

- 22mm square coverslips
- Ceramic staining rack
- Columbia staining dish
- Columbia staining jar
- Forceps
- Latex globes

Reagents

- Congo red
- Lithium carbonate
- 100% ethyl alcohol
- 1% acid alcohol
- Distilled water
- Xylene
- DPX

Solution

1. Congo red solution

- Prepared by dissolving 1.0gm Congo red in 100 ml distilled water.

2. Lithium carbonate saturated solution

- Prepared by dissolving 1.3gm Lithium carbonate in 100 ml distilled water.

3. Alcohol 50 %

- Reagent alcohol ~50 ml
- Deionized water ~50 ml

4. Alcohol 70 %

- Reagent alcohol ~70 ml
- Deionized water ~30 ml

5. Alcohol 80 %

- Reagent alcohol ~80 ml
- Deionized water ~20 ml

6. Alcohol 95 %

- Reagent alcohol ~95 ml

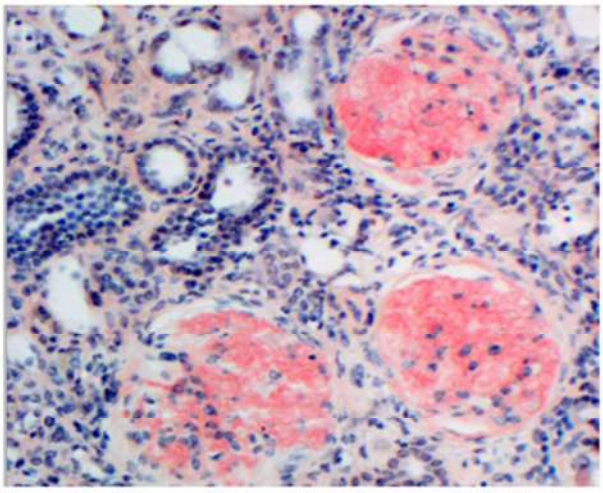
- Deionized water ~ 5 ml

Procedure

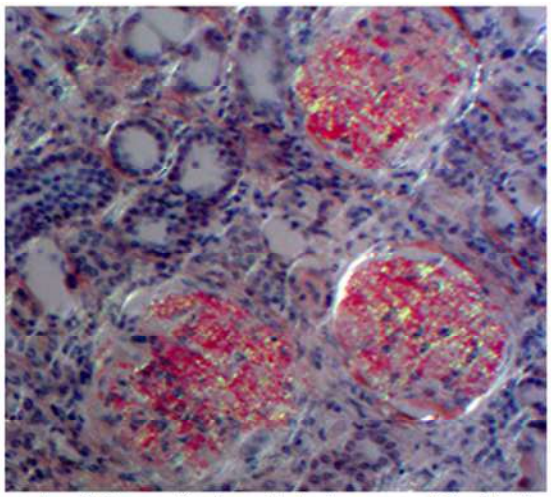
1. Pour Congo red solution for 20 minutes.
2. Pour off the solution and cover the slide with lithium carbonate for 1.5 minutes to differentiate.
3. Wash with water.
4. Counter-stain with hematoxyline for 5 minutes (Optional).
5. Differentiate with 1% acid alcohol.
6. Wash in running tap water.
7. Dehydrate, clear in xylene and mount in DPX.

Result

Amyloid in a specimen is stained bright red in colour which gives apple green birefringence in polarized light. Nuclei are, on the other, hand stained with blue, if hematoxyline is used, and other structures are seen unstained to yellow.



Example of Congo Red positivity in congophilic fibrillary GN ×100. Alexamnder et. al., AJKD© National Kidney Foundation.



Example of Congo Red positivity in congophilic fibrillary GN ×100. Alexamnder et. al., AJKD© National Kidney Foundation.

Precautions

1. Sections must be cut at 8 to 10 microns for birefringence.
2. Solution must be filtered through glass wool, not paper filters for birefringence to occur.
3. Tissue fixed in solutions other than formalin may display false positive birefringence

Reference

1. Shylaja M. & Seshadri H. Glycoproteins: An overview. Biochemical Education 1989; 17:4.

2. Sheehan D. & Hrapchak B. Theory and practice of histotechnology. 2nd Ed, Battelle Press, Ohio, 1980; 164-166.
3. Theonard J.C. & Scherp W. H. Histochemical demonstration of acid mucopolysccharides in human gingival epithelial intercellular spaces. Archives Oral Biol. 1962; 7(2): 125-136.
4. Anjali Singh. Histochemical techniques to demonstrate carbohydrates – polysaccharides. https://conductscience.com/histochemical-techniques-to-demonstrate-carbohydrates-polysaccharides/.
5. Baso Histology Series: High iron diamine – alcian blue stain, http://www.baso.com.cn/en/view.asp?id=77#:~:text=Principle%3A,Alcian%20Blue%20at%20pH%202.5.
6. Anjali Singh. Histochemical techniques to demonstrate carbohydrates – polysaccharides. https://conductscience.com/histochemical-techniques-to-demonstrate-carbohydrates-polysaccharides/.
7. Metachromatic staining. https://nios.ac.in/media/documents/dmlt/HC/Lesson-12.pdf.