Special Stains in Interpretation of Liver Biopsies

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Special histochemical stains are routinely used for the interpretation of liver biopsies. Most laboratories have standard protocols for liver biopsies so that special stains are automatically performed for these specimens without having to be specially ordered by the pathologist. The panel of special stains varies from laboratory to laboratory, depending on tumor versus non-tumor cases, or transplant versus non-transplant liver biopsy specimens.

Commonly Used Special Stains
Most anatomic pathology laboratories perform a trichrome stain along with two or three H&E stained sections. The remaining panel of special stains varies depending on the laboratory and consists of a variable combination of reticulin silver stain, Perl's iron stain and Periodic Acid-Schiff (PAS) stain with and without enzyme digestion.

Special Stains for Special Circumstances
Some stains are ordered for special purposes when indicated by the clinical situation. For instance, the Ziehl-Neelsen stain is ordered for mycobacteria, and Grocott's silver methanamine stain is used when granulomas are seen or infection is suspected. The Congo Red stain is requested when amyloid is suspected to be present. Rhodanine stain, Victoria blue or orcein stain is ordered to detect copper deposition when there is clinical suspicion of Wilson's disease (see Glossary). The latter two may also be used for the detection of hepatitis B surface antigen; but most pathologists prefer to use immunohistochemistry to detect hepatitis B surface antigen. The Oil Red O can only be performed on frozen sections and therefore is not widely applied in routine practice. In clinical practice, the Oil Red O stain is mainly ordered on frozen sections of liver biopsy specimens to assess the amount of fat in donors for liver transplantation. This stain is also popular with researchers when they are looking for fat in the liver. The Sirius red and aniline blue stains are used for selective staining of collagen when quantitative measurement of fibrosis by morphometry is required. The Hall's stain for bile pigment seems to be obsolete today and is hardly used in clinical practice or research applications.

Trichrome Stain
Principles of Staining: As the name implies, the trichrome stain uses three dyes to impart three different colors, on collagen, cytoplasm and nuclei respectively. The three most common trichrome stains are Masson's trichrome, Gomori's one-step trichrome and the Van Gieson stain. Of these, the first 2 are used in the majority of laboratories. The Masson's trichrome stain consists of sequential staining with iron hematoxylin which stains nuclei black; Biebrich scarlet which stain cytoplasm red and aniline blue or aniline light green which stain collagen blue or green respectively.

The Gomori's One-Step method employs the same principles except that all dyes are present in a single solution together with phosphotungstic acid and glacial acetic acid. The red color in this Gomori's one-step method is imparted by chromotrope 2R.
Figure 1. Trichrome stained liver showing fibrous tissue. The fibrous tissue is stained blue while the cytoplasm of hepatocytes are stained red. The nuclei can be seen as dark red to black structures within cells; Collagen is the fibrous tissue are stained Blue (with aniline blue) or very light green (by aniline light green).

Figure 2. Trichrome stain showing cirrhotic liver. As it is evident, the normal architecture of the liver is destroyed in this disease and the liver shows nodules surrounded by fibrous bands.
**Utility of Trichrome Stain:** The trichrome stain is performed on medical liver biopsies to assess the degree of fibrosis in the liver. A large number of liver diseases such as hepatitis B and C viral infections, fatty liver disease, alcoholic liver disease and chronic biliary diseases show the formation of fibrous tissue including bridging fibrous septa leading to the end-stage process called cirrhosis (Fig. 2). The aim of the treatment in these disease processes is to halt the progression of fibrosis. Therefore, every liver biopsy report that comes from a pathologists office contains a statement about the degree of liver fibrosis, also known as the stage of disease. The degree of liver fibrosis provides hepatologists the necessary information regarding the advancement of the disease thereby helping them to make the required therapeutic decisions. Comparison of the degree of fibrosis in pre- and post treatment biopsies indicate if the treatment has been effective or to what degree has it been effective. Comparison of fibrosis is important in clinical trials to assess the success of different medications. The assessment of fibrosis is mostly carried out with a trichrome stain making this staining procedure a preferred method on medical liver biopsies.

In summary, a trichrome stain is used to assess fibrosis, which gives important information about stage and progression of disease. The stain is used to make treatment decisions; utilized to assess the effect of therapy, medications in clinical trials and is needed for all liver biopsy specimens.

**Reticulin Stain**

**Principles of Staining:** Reticulin stains are silver stains based on the argyrophilic properties of reticulin fibers. The 2 most common reticulin stains are the Gomori's stain and Gordon & Sweet's stain. The first step in the staining procedure consists of oxidation of the hexose sugars in reticulin fibers to yield aldehydes. The second step is called "sensitization" in which a metallic compound such as ammonium sulfate is deposited around the reticulin fibers, followed by silver impregnation in which an ammonical or diamine silver solution is reduced by the exposed aldehyde groups to metallic silver. Further reduction of the diamine silver is achieved by transferring the sections to formaldehyde; this step is called “developing”. The last step consists of “toning” by gold chloride in which the silver is replaced by metallic gold and the color of the reticulin fibers changes from brown to black.

**Utility of Reticulin Stain:** Reticulin fibers are thin fibers composed of collagen III which form a delicate stromal network in many organs. The reticulin network is particularly rich in the liver and can be seen along hepatic trabecula. Since reticulin provides the stromal support for the parenchyma, the reticulin stain provides important information about the architecture of the liver. When hepatocytes are damaged and undergo necrosis, the reticulin fibers surrounding them collapse in the empty space left behind. Areas of reticulin crowding thus indicate focal hepatocyte loss (Fig. 4). Large areas of cell necrosis appear as reticulin collapse. On the other hand, when hepatocytes regenerate, the reticulin fibers show thickening of the hepatic cell plates which appear as two or three- cell thick plates instead of the usual 1-cell thick plates (Fig. 5). Fibrous tissue composed of collagen type I appear brown on a reticulin stain and thus can be distinguished from reticulin fibers (Fig. 3).

In summary, a reticulin stain is useful for demonstrating liver architecture; hepatocyte necrosis and hepatocyte regeneration.

**Iron Stain**

**Principles of Staining:** The Perl's stain is the most commonly used method for staining of iron. It depends on the Prussian blue reaction. The tissue is first treated with dilute hydrochloric acid to release ferric ions from binding proteins. The freed ions then react with potassium ferrocyanide to produce ferric ferrocyanide which is an insoluble bright blue compound called Prussian blue.

**Utility of Iron Stain:** The liver is one of the main organs for storage of excess iron. Iron may be stored in cells as a soluble compound called ferritin or an insoluble form called hemosiderin. Ferritin cannot be seen on an H&E stain while hemosiderin appears as coarse golden-brown refractile granules (Fig. 7). On the Perl's stain, ferritin appears as a faint bluish blush while hemosiderin appears as coarse blue granules. Hemosiderin may accumulate in hepatocytes or Kupffer cells. Accumulation in Kupffer cells usually occurs in secondary hemosiderosis in which there is an underlying cause for iron accumulation such as hemolysis and transfusions (Fig. 8). Deposition in hepatocytes occur
Figure 3. Dark black staining of the reticulin fibers (arrowheads) with reticulin stain. Collagen fibers are stained brown (arrows), nuclei red and the background in grey, or light pink if overtoned. The other white circular structures near the reticulin fibers are fat globules (asterix*).

Figure 4. Reticulin stain shows an area of collapse of reticulin fibers (between arrows) corresponding to an area of cell loss.
in primary hemochromatosis which is due to a genetic mutation in a key gene involved in iron metabolism (Fig. 6). The iron stain helps to define the pattern of iron deposition and provides a clue to the possible underlying causes of excess iron. Iron deposition also occurs in a wide variety of other diseases such as hepatitis C and fatty liver disease. In the former, iron deposition has been thought to affect response to therapy. The iron stain also gives an estimate of the degree of the iron deposition and various grading methods exist to grade the extent of deposition in the liver.

In summary, the iron stain is useful for demonstrating excess iron deposition in the liver providing information about the degree of iron deposition and clue to the underlying causes leading to the iron deposition.

PAS Stain With and Without Enzyme Digestion

Principles of Staining: The PAS stain is based on exposing aldehyde groups in sugars by oxidation with periodic acid. The exposed aldehyde groups react with the chromophores in Schiff’s reagent (produced by treating basic fuschin with sulfurous acid) to produce a bright pink color. The PAS stain demonstrates glycogen, neutral mucosubstances and basement membranes. Treatment with alpha-amylase digests glycogen and the PAS staining performed following enzyme digestion shows no staining. Enzyme digestion is also called diastase digestion. The word diastase comes from malt diastase which contains both alpha and beta amylase. However, alpha-amylase derived from various sources is preferred to malt diastase.

Utility of PAS Stain: The normal liver contains a large amount of glycogen. This, therefore leads to the staining of hepatocytes intensely pink with a PAS stain (Fig 9). This staining is abolished following treatment with alpha-amylase as this enzyme selectively digests glycogen. The major utility of the PAS stain with enzyme digestion is for demonstration of alpha-1 antitrypsin globules within hepatocytes (Fig. 10). Alpha-1 antitrypsin is a major serum anti-protease which protects the body from the destructive actions of endogenous enzymes. Some individuals have genetic mutations that lead to an abnormal alpha-1 antitrypsin which accumulates in hepatocytes and can be demonstrated by the PAS stain. PAS stain with enzyme digestion also highlights storage cells in Gaucher’s disease and Niemann-Pick disease. Finally, the stain is useful for the demonstration of basement membranes around bile ducts which may be destroyed or thickened in biliary diseases.

In summary, the PAS stain is useful for demonstrating alpha-1 antitrypsin globules in hepatocytes; storage cells in Gaucher’s and Niemann-Pick disease, and abnormalities of the bile duct basement membrane in biliary diseases.

Oil Red O Stain

Principles of Staining: The Oil Red O stain is based on the greater solubility of the dye in neutral fats than in the solvent in which it is dissolved. Oil Red O dissolved in an alcohol is used for staining. In tissues containing fat, the Oil Red O moves from the staining solution to the tissue fat because of its greater solubility in the latter than in alcohol. Oil Red O staining can only be performed on frozen sections since tissue fat is removed by the alcohols and clearing agents used for paraffin processing. The sections are counterstained with hematoxylin and mounted in an aqueous medium or a synthetic medium that will not dissolve the tissue fat.

Utility of Oil Red O Stain: Oil Red O is used to demonstrate neutral fats in liver tissue. It is used to assess the presence and extent of neutral fat in 2 main situations. First, it used on frozen sections of liver donors for transplantation and second in experimental studies which require the evaluation of the presence and extent of fat in liver tissue.

Rhodanine Stain

The rhodamine stain is used to demonstrate excess copper in hepatocytes in Wilson’s disease, a disease in which a genetic mutation leads to an excessive storage of copper within the body. The stain is also used to demonstrate copper deposition in chronic biliary diseases. The stain is based on the reaction of 5 p-dimethylaminobenzylidine rhodanine with copper-binding protein associated with deposited copper.

Orcein Stain

Orcein is a natural dye obtained from lichens which are found to stain copper-associated protein, elastic fibers and hepatitis B surface antigen, all of which stain dark brown.

The orcein stain is used for diagnosis of hepatitis B infection as well as copper accumulation in Wilson’s disease or in chronic biliary diseases.
Figure 5. Reticulin stain shows hepatic plates which are more than 1-cell thick indicating regeneration of hepatocytes.

Figure 6. Perl's iron stain shows accumulation of dark blue granules of hemosiderin within hepatocytes. This pattern of iron deposition occurs in genetic hemochromatosis. The coarse blue granules are hemosiderin, and the bluish blush is Ferritin.
Figure 7. Hemosiderin appears on an H&E stain as coarse, dark-brown, refractile granules.

Figure 8. Perl's iron stain showing hemosiderin accumulation in Kupffer cells. This pattern is seen in secondary hemosiderosis.
Figure 9. PAS stain shows intense staining of hepatocytes (arrowheads) and basement membranes of bile ducts (arrow). Glycogen, neutral polysaccharides and basement membranes are stained bright pink.

Figure 10. A PAS stain with enzyme digestion shows globules of alpha-1 antitrypsin (arrows). Neutral polysaccharides and basement membranes are stained bright pink and glycogen is seen as colorless areas.
Figure 11. Oil Red O stain shows orange-red droplets of fat of varying sizes. Neutral fats are stained Orange-red and the nuclei are seen in blue.

Figure 12. Rhodanine stain shows bright orange to brown granules within hepatocytes. Copper is stained bright orange to brown and nuclei in blue.
Figure 13. Methylene blue staining of a cirrhotic liver. Collagen appears dark blue with methylene blue.
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<th>Stain</th>
<th>Staining characteristics</th>
<th>Utility</th>
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<td>Masson’s Trichrome</td>
<td>Collagen: blue</td>
<td>To assess the degree of fibrosis</td>
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<td></td>
<td>Hepatocytes: red</td>
<td>To make treatment decisions</td>
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<td></td>
<td>Nuclei: dark red to black</td>
<td>To assess the effect of therapy</td>
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<td>To assess medications in clinical trials</td>
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<tr>
<td>Gordon and Sweet’s silver</td>
<td>Reticulin fibers: black</td>
<td>Demonstrates the liver architecture</td>
</tr>
<tr>
<td></td>
<td>Collagen fibers: brown</td>
<td>Demonstrates hepatocyte loss or necrosis</td>
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<td></td>
<td>Background: grey to light pink (depending on toning)</td>
<td>Demonstrates proliferation of hepatocytes</td>
</tr>
<tr>
<td>Perls iron stain</td>
<td>Hemosiderin: Coarse dark blue granules</td>
<td>Demonstrates presence of hemosiderin deposition</td>
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<td></td>
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<td>Demonstrates extent of hemosiderin deposition</td>
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<td>Demonstrates the site of hemosiderin deposition (primary vs secondary)</td>
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<td>PAS stain</td>
<td>Glycogen, neutral polysaccharides, basement membranes: bright pink</td>
<td>Demonstrates the presence of storage cells in Gaucher’s disease and Niemann-Pick disease</td>
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<td>Demonstrates bile duct basement membrane damage in biliary diseases</td>
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<td>Demonstrates the presence of alpha-1 antitrypsin globules</td>
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<td>Rhodanine</td>
<td>Copper-associated protein: bright orange to red</td>
<td>Demonstrates copper deposition in Wilson’s disease and chronic biliary diseases</td>
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<tr>
<td>Orcein</td>
<td>Copper associated protein, elastic fibers, hepatitis B surface antigen: dark brown</td>
<td>Demonstrates the presence of hepatitis B infection</td>
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Summary of Special Stains in Diagnostic Liver Pathology.
Victoria Blue
The Victoria Blue stain demonstrates hepatitis B surface antigen, copper-binding protein and elastic fibers, all of which stain blue.

Sirius Red Stain
Sirius Red F3B (Direct Red 80) is an extremely hydrophilic dye which stains collagen I fibers, reticulin fibers, basement membranes, and some mucins. The stain is most often used in research applications to enhance the natural birefringence of collagen. When observed by brightfield microscopy, collagen, reticulin fibers, basement membranes and some mucins appear red, while the counterstained nuclei appear grey-brown to black. However, under polarized light, collagen is selectively visualized as bright yellow to orange fibers.

Methylene Blue Stain
The methylene blue stain is used to selectively stain collagen in cirrhotic liver for digitized morphometry applications (Fig. 13).

Summary
In summary, special stains are very important in the interpretation of liver biopsies. The trichrome stain imparts valuable information about the stage of chronic liver diseases and effects of treatment. Reticulin stain is used for assessment of hepatic microarchitecture. The PAS stain with diastase highlights storage of abnormal metabolites and alpha-1 antitrypsin. The Perl’s and rhodanine stains allow detection of intracellular deposits of iron and copper respectively.

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<tr>
<td>Sirius Red</td>
<td>Collagen, reticulin fibers, basement membranes, some mucins: red</td>
<td>Enhances the natural birefringence of collagen under polarized light</td>
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<tr>
<td>Aniline Blue</td>
<td>Collagen: blue</td>
<td>Exclusively stains collagen for digitized morphometric analysis</td>
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<td>Oil Red O</td>
<td>Neutral fats: orange-red</td>
<td>Demonstrates the presence of fat in frozen sections of liver biopsies from donor livers for transplantation</td>
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<td>Demonstrates the presence of fat in liver in experimental studies</td>
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Summary of Special Stains in Diagnostic Liver Pathology.

Bibliography

Glossary
- Gaucher’s disease: Gaucher’s disease is caused by an inherited deficiency of an enzyme that is involved in lipid metabolism. As a result, an abnormal biochemical metabolite accumulates in various tissues such as the spleen, liver, bone marrow and kidneys.
- Genetic hemochromatosis: Genetic hemochromatosis is a genetic disorder that causes the body to absorb excess iron from the diet. The excess iron accumulates in the liver, pancreas, heart, and joints. Excess iron is toxic to these tissues.
- Niemann-Pick disease: Niemann-Pick disease is caused by an inherited deficiency of an enzyme involved in lipid metabolism. As a result, an abnormal biochemical metabolite accumulates in various tissues such as the brain, liver and spleen.
- Wilson’s disease: Wilson’s disease is a genetic disorder that prevents the body from getting rid of extra copper. In Wilson disease, copper builds up in the liver, parts of the brain and eyes. Excess copper is toxic to these tissues.