Utility:
(1) Assess degree of fibrosis. H&E stain is not reliable for demonstration of fibrosis especially in early stages. Pericellular fibrosis around the central vein in steatohepatitis can be difficult to identify on the H&E stain.
(2) Distinguish fibrosis from necrosis. In acute hepatitis, areas of confluent necrosis can be difficult to distinguish from fibrosis and can lead to an erroneous interpretation of advanced fibrosis. Areas of necrosis show pale staining compared to dense staining of fibrous septa.
(3) Raising the suspicion for amyloidosis. Amyloid deposits show pale homogenous staining compared to dark staining of collagen. In the absence of clinical suspicion, subtle amyloid deposits can be overlooked on the H&E stain. Trichrome stain can highlight the pale staining amyloid and definite identification can then be performed with Congo red stain.

Pitfalls:
(1) Overstaining: Normal sinusoids should not stain with trichrome stain. If high background staining is present, it can lead to erroneous interpretation of pericellular fibrosis in steatohepatitis. It is also not possible to distinguish necrosis from fibrosis in overstained sections.
(2) Understaining: This can lead to understaging and does not enable distinction of necrosis from fibrosis.

Guidelines for interpreting trichrome stain for staging of chronic hepatitis (Batts-Ludwig system)
(1) Understaging is likely in limited or fragmented biopsies.
(2) Tangentially cut or branching portal tracts: If the fibrous tissue “expansion” or “septa” contains an artery and/or duct, it is likely an extension of the portal tract rather than true fibrosis.
(3) Large portal tracts can be mistaken for portal fibrosis. More connective tissue is normal in this setting and the larger size of duct and artery are helpful. Nerves within fibrous zones also indicate a large portal tract. If ductular reaction is present at the edge of a large fibrous zone, it is more likely to be true fibrosis.
(4) Subcapsular biopsy can lead to overstaging as nodularity can be normally seen in this region and the portal tracts in this region have more connective tissue.
(5) Periportal ductular reaction or hepatocytes entrapped at the edge of the portal zone or surrounded by strands of fibrous tissue helps in identification of stage 1 fibrosis.
(6) Portal tract that are much larger than expected for the size of the interlobular bile duct and interlobular artery indicate stage 1 fibrosis.
(7) If periportal fibrous septa are thin, it qualifies for stage 2 even if there is focal portal-portal bridging as long as there are no nodular regenerative changes.
(8) Several thin bridges would qualify for architectural distortion and should be labeled as stage 3. If bridging fibrosis in some areas is accompanied by portal tracts lacking significant fibrosis in other areas, stage 3 (and not stage 4) is appropriate.
(9) Resolution of fibrosis (for example, with treatment) can lead to findings that are not diagnostic of advanced fibrosis or cirrhosis as clinically suspected.

Technical Aspects:
The technique uses three dyes to highlight nuclei, cytoplasm and collagen with three different colors. In the most commonly used technique (Masson trichrome), three dyes are used sequentially iron hematoxylin (nuclei: red to dark blue black), Beibrich scarlet (cytoplasm including muscle: red) and aniline
blue or aniline light green (collagen: light blue or green). The Gomori technique is a one-step method with simultaneous use of three dyes along with phosphotungstic acid and acetic acid: iron hematoxylin (nuclei), chromotrope 2R (cytoplasm) and aniline blue or aniline light green (light blue or green).

**ROUTINE USE**
Routinely used for assessment of fibrosis as this may be underestimated on H&E stain.

### Reticulin stain

**UTILITY**
1. Highlights the architecture of liver cell plates; helpful in demonstrating collapse of reticulin network in parenchymal necrosis. Collagen typically stains brown compared to black elastic fibers and this distinction can be made in a well performed stain.
2. Highlights the nodular architecture in nodular regenerative hyperplasia and demonstrates compression of cell plates at the periphery of the nodules.
3. Demonstrates loss or fragmentation of reticulin network in hepatocellular carcinoma.

**PITFALL**
Reticulin network can be disrupted in areas of steatosis and can be confused with reticulin loss seen in hepatocellular carcinoma.

**TECHNICAL ASPECTS**
The ability of reticulin fibers to reduce silver from silver nitrate (argyrophilia) is exploited by reticulin stains. The Gomori stain for reticulin fibers involves oxidation of the hexose carbohydrate chains in reticulin fibers to aldehydes. After addition of ammonium sulfate followed by silver solution, metallic silver is deposited on the reticulin fibers by a reduction reaction facilitated by the exposed aldehyde groups. Formaldehyde enables further reduction of the diamine silver (‘developing’). In the last step, gold chloride is added, and silver is replaced by metallic gold (‘toning’), yielding the black color.

**ROUTINE USE**
Often obtained routinely in liver biopsies. However, it is unlikely to yield useful information in most liver biopsies and can be restricted to situations outlined above where it is likely to be relevant.

### Iron stain

**UTILITY**
Distinguishes hemosiderin from other pigments in the liver (bile, lipofuchsin, copper).

**PITFALL**
Faint blush of ferritin seen in cytoplasm of hepatocytes should not be confused with granular deposits of hemosiderin. Since ferritin is an acute phase reactant, any chronic inflammatory disorder can show ferritin blush on iron stain.

**TECHNICAL ASPECTS**
Perls stain (based on Prussian blue reaction) is most commonly used and demonstrates only ferric form of iron. In the first step, ferric ions are released from binding proteins by dilute hydrochloric acid followed by potassium ferrocyanide. The resulting ferric ferrocyanide has a bright blue color (Prussian blue).

**INTERPRETATION**
A. **Patterns of iron overload**: The significance of iron depends upon the compartment in which it is present.
(a) Hepatocellular iron: When present predominantly or exclusively in periportal location, it suggests hereditary hemochromatosis. In advanced HH, deposition of iron in the bile duct epithelium and extracellular iron in the fibrous septa is a characteristic finding. These features are not specific and warrant confirmation of diagnosis based on correlation with serum iron indices and HFE mutation testing. In most instances, randomly distributed hepatocellular iron is of secondary origin, especially if associated with Kupffer cell iron.

(b) Kupffer cell iron: In most instances, this represents iron accumulation secondary to hemolysis or another inflammatory disease (steatohepatitis, hepatitis C, rheumatoid arthritis etc.). This may be accompanied by varying degree of hepatocellular iron, usually mild to moderate and randomly distributed. In some instances like hepatitis C cirrhosis and alcoholic liver disease, there can be marked accumulation of hepatocellular and Kupffer cell iron in the absence of hereditary hemochromatosis.

<table>
<thead>
<tr>
<th>Types of hereditary hemochromatosis</th>
<th>Genetics</th>
<th>Liver biopsy</th>
<th>Clinical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 (HFE HH)</td>
<td>Autosomal recessive C282Y homozygous, C282Y and H63D double heterozygous</td>
<td>Iron overload in hepatocytes, most prominent in periportal region</td>
<td>3rd or 4th decade Liver, pancreas, heart, skin, joints can be involved</td>
</tr>
<tr>
<td>Type 2 (Juvenile HH)</td>
<td>Autosomal recessive Mutations involving hemojuvelin (2A) or hepcidin (2B)</td>
<td>Iron overload in hepatocytes</td>
<td>1st three decades More severe disease compared to HFE HH</td>
</tr>
<tr>
<td>Type 3</td>
<td>Autosomal recessive Transferrin receptor type 2 mutation</td>
<td>Iron overload in hepatocytes</td>
<td>Similar to HFE HH. Severity intermediated between HFE HH and juvenile HH</td>
</tr>
<tr>
<td>Type 4</td>
<td>Autosomal dominant Ferroportin mutation</td>
<td>First subtype: Iron overload in hepatocytes Second subtype: Iron overload in Kupffer cells</td>
<td>4th or 5th decade Severity varies with type of mutation</td>
</tr>
</tbody>
</table>

B. Grading of hepatic iron: Scheuer system is one of many systems used for semi quantitatively grading liver iron (Table). I personally find it difficult to use this grading system and prefer a simple subjective 0-4 scale system of minimal, mild, moderate and severe siderosis, with separate assessment of hepatocytes and Kupffer cells.

Quantitative iron can be determined from fresh or paraffin embedded liver tissue. The level is <400 µg/g of liver tissue in normal states (3). Hepatic iron index was considered a more accurate indicator of iron overload as it takes both age and liver iron into account, with values >1.9 being characteristic of HH. However, this is not specific for HH, and quantitative iron testing has become uncommon with the availability of HFE mutation testing.

Scheuer system of grading liver iron

<table>
<thead>
<tr>
<th>Grade 0</th>
<th>Granules absent or barely discernible on 400x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>Granules barely discernible on 250x, easily seen at 250x</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Discrete granules seen at 100x</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Discrete granules seen at 25x</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Masses visible at 10x or naked eye</td>
</tr>
</tbody>
</table>

Routine use

The amount of iron is often underestimated on H&E stained slides and hence the routine use of iron has been advocated. The presence of hepatic siderosis has been associated with disease progression in chronic liver diseases like hepatitis C. However, the significance of mild hepatic siderosis is unclear and use of iron stains in specific situations rather than on a routine basis is acceptable practice.
Copper stain

Rhodanine, rubeanic acid and orcein stains can be used to demonstrate copper. Orcein stain highlights copper binding proteins and hence is an indirect stain for copper. Orcein stain also stains elastic fibers and ground glass hepatocytes in chronic hepatitis B.

UTILITY
(1) Distinguishes copper from other pigments in the liver (bile, lipofuchsin, iron).
(2) Since copper is excreted in the bile, it accumulates in periportal hepatocytes in cholestatic conditions. Demonstration of copper in periportal hepatocytes helps in the distinction of biliary from hepatitic diseases.
(3) Wilson disease is associated with elevated hepatic copper, which may be demonstrated on copper stain.

PITFALLS
(1) Copper accumulation can occur in a nonspecific fashion in any liver disease with advanced fibrosis and cirrhosis. In this setting, it has limited utility in the distinction of biliary and hepatitic diseases.
(2) Periportal hepatocellular copper in cholestatic diseases can be focal and careful evaluation is necessary.
(3) Absence of periportal copper does not exclude biliary disease.
(4) Copper stain is unreliable for the diagnosis of Wilson’s disease. The positive staining on copper stain can be patchy and even negative. Quantitative copper analysis from the block is a more reliable assay for hepatic copper.

TECHNICAL ASPECTS
The stain is based on the reaction of 5 p-dimethylaminobenzylidine rhodanine with copper-binding protein.

Routine use
Not necessary.

PAS with diastase (PAS-D)

UTILITY
(1) Demonstrate cytoplasmic globules of alpha-1-antitrypsin deficiency.
(2) Highlight basement membrane of bile ducts and can be helpful to evaluate bile duct damage and ductopenia.
(3) Highlight macrophages in acute and resolving hepatitis.
(4) Identify abnormal glycogen inclusions in combination with PAS stain.

PITFALLS
(1) Inadequate digestion can lead to staining of residual glycogen in the hepatocytes. This can be misinterpreted as cytoplasmic globules of alpha-1-antitrypsin deficiency. Unlike the large globules seen in alpha-1-antitrypsin deficiency, glycogen particles in an incompletely digested stain are smaller, randomly distributed and have a granular rather than globular appearance.
(2) Presence of PAS-D positive cytoplasmic globules does not establish the diagnosis of alpha-1-antitrypsin deficiency. Heterozygous disease can also show cytoplasmic globules, but these tend to be smaller and less numerous compared to homozygous disease. Protease inhibitor phenotype testing by isoelectric focusing is necessary to establish the diagnosis. Globules can be seen in acute hepatitic conditions due to transient defects in secretion. Cytoplasmic globules with similar appearance can be seen in other settings like fibrinogen storage diseases.
(3) Use of PAS stain without diastase digestion can occasionally be used to highlight glycogen in the hepatocytes. In most other instances, it is of limited use in liver biopsy evaluation.

TECHNICAL ASPECTS
PAS stain involves oxidation of hydroxyl groups in carbohydrate chains into aldehydes by periodic acid. The aldehyde groups react with Schiff reagent (basic fuschin and sulfurous acid) to produce a bright pink color. Common substances highlighted by the PAS stain are glycogen, mucin and basement membrane. Pre-treatment with diastase leads to digestion of glycogen and/or other carbohydrate moieties and hence absence of PAS staining. The enzyme diastase contains alpha and beta amylase. Alpha-amylase is preferred over diastase in the staining reaction, and hence the procedure is also referred to as PAS with amylase.

Routine use
The cytoplasmic globules of alpha-1-antitrypsin deficiency can be easily missed on H&E stain. Hence routine use of PAS-D has been advocated. It offers an added advantage of evaluation of bile ducts by highlighting the basement membrane. However, its routine use is not standard practice.

Elastic stain

UTILITY
Distinguish confluent or bridging necrosis from fibrosis. Elastic fibers are not seen in areas of necrosis while fibrous septa are richly endowed with elastic fibers. The connective tissue in normal portal tracts also contains elastic fibers and serves as the internal control.

PITFALL
The adequacy of staining must be checked by ensuring that elastic fibers are present in portal tracts and vessel walls. In my experience, orcein stain (which highlights copper associated proteins as well as elastic fibers) yields better results in the liver compared to elastic stains such as Verhoeff’s stain.

ROUTINE USE
Not necessary.

Other stains

Oil Red O: Used in frozen sections to highlight fat and has been used for the diagnosis of Reye syndrome and assessment of fat in donor biopsies. Oil Red O dissolved in alcohol is used for staining. It has higher solubility in fat than in alcohol, and moves from the staining solution to the tissue fat. Since tissue fat is removed by the alcohols during paraffin block processing, this stain can be performed only on frozen sections. It is seldom used in clinical practice.

Victoria blue: Stains copper associated protein, hepatitis B surface antigen, and elastic fibers.

Sirius red: Most often used in research applications to enhance the natural birefringence of collagen. Collagen, reticulin fibers and basement membranes are stained red. Under polarized light, collagen is selectively visualized as bright yellow to orange fibers.

Bile stain (Fouchet): Demonstrate bile and distinguish it from other liver pigments. Seldom used in diagnostic pathology.

Masson Fontana: Can highlight lipofuchsin as well as cytoplasmic pigment in Dubin Johnson syndrome.
<table>
<thead>
<tr>
<th>Stain</th>
<th>Should the stain be routinely obtained on liver biopsies?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichrome</td>
<td>Yes</td>
</tr>
<tr>
<td>Reticulin</td>
<td>No</td>
</tr>
<tr>
<td>Iron</td>
<td>Yes</td>
</tr>
<tr>
<td>PAS-D</td>
<td>No</td>
</tr>
<tr>
<td>Copper</td>
<td>No</td>
</tr>
<tr>
<td>Elastic</td>
<td>No</td>
</tr>
<tr>
<td>Bile</td>
<td>No</td>
</tr>
</tbody>
</table>

* There is wide variability in the choice of routinely obtained special stains. These recommendations are based on the author's personal opinion.