

# Histotechnology

## A Self Instructional Text

4th Edition

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| 26 | <b>Hallmarks of good fixation</b>        |
| 26 | <b>Troubleshooting fixation problems</b> |
| 26 | Autolysis                                |
| 26 | Incomplete fixation                      |
| 29 | <b>Learning activities</b>               |
| 29 | <b>References</b>                        |

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| 32 | <b>Dehydration</b>                                   |
| 32 | Alcohols                                             |
| 33 | Ethyl alcohol (ethanol)                              |
| 33 | Methyl alcohol (methanol)                            |
| 33 | Isopropyl alcohol (isopropanol)                      |
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| 33 | Acetone                                              |
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| 34 | <b>Clearing</b>                                      |
| 34 | Xylene                                               |
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| 35 | Benzene                                              |
| 35 | Chloroform                                           |
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| 39 | Microwave oven processing                            |
| 39 | Water soluble waxes                                  |
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| 41 | Agar & gelatin                                       |
| 41 | <b>Troubleshooting processing</b>                    |
| 41 | Precipitate in the processor chamber & in the tubing |
| 41 | Overdehydration                                      |
| 41 | Poor processing                                      |
| 42 | Sponge artifact                                      |
| 42 | Tissue accidentally desiccated                       |
| 42 | Embedding & specimen orientation                     |
| 45 | <b>Troubleshooting embedding</b>                     |

|    |                                                       |
|----|-------------------------------------------------------|
| 45 | Soft mushy tissue                                     |
| 45 | Incorrect orientation                                 |
| 46 | Tissue carryover                                      |
| 46 | Tissue not embedded at the same level                 |
| 46 | Pieces of tissue missing from the block               |
| 46 | <b>Special techniques in processing</b>               |
| 46 | Decalcification                                       |
| 47 | Acid methods                                          |
| 48 | Chelating agents                                      |
| 48 | End point of decalcification                          |
| 49 | Undecalcified bone                                    |
| 49 | Troubleshooting decalcification                       |
| 50 | Frozen sections                                       |
| 51 | Frozen sectioning formalin fixed tissue               |
| 51 | Troubleshooting processing tissue for frozen sections |
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| 52 | <b>References</b>                                     |

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| 54 | Light microscope                                                               |
| 55 | Polarizing microscope                                                          |
| 56 | Phase-contrast microscope                                                      |
| 56 | Darkfield microscope                                                           |
| 56 | Fluorescence microscope                                                        |
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| 58 | Sliding microtome                                                              |
| 58 | Clinical freezing microtome                                                    |
| 58 | Microtome blades                                                               |
| 60 | Troubleshooting microtomy                                                      |
| 60 | Crooked ribbons                                                                |
| 61 | Block face unevenly sectioned                                                  |
| 61 | Holes in the section                                                           |
| 62 | Failure of ribbon to form                                                      |
| 62 | Lifting of the section from the blade as the block is raised                   |
| 62 | Washboarding or undulations in the section                                     |
| 64 | Chatter, or microscopic vibration, in the section                              |
| 64 | Skipped or varied thickness of sections (thick & thin sections)                |
| 64 | Compressed, wrinkled, or jammed sections                                       |
| 64 | Lengthwise scratches or splits in the ribbon                                   |
| 65 | Fragmented or torn sections                                                    |
| 66 | Sections flying and sticking to nearby objects or other parts of the microtome |

|    |                                             |
|----|---------------------------------------------|
| 66 | <b>Cryostat</b>                             |
| 67 | Troubleshooting cryotomy                    |
| 67 | Poorly adjusted antiroll plate              |
| 67 | Incomplete sections                         |
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| 71 | Automatic coverslipper                      |
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| 77 | Slide & cassette printers                   |
| 78 | Circulating water bath                      |
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| 78 | pH meters                                   |
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| 79 | Solvent recycler                            |
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| 80 | Equipment malfunction                       |
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| 91 | Fire & explosive hazards            |
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#### 106 The nucleus

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106 Nucleolus

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#### 107 The cytoplasm

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108 Lysosomes

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109 Nuclear staining

109 Cytoplasmic staining

### 110 The dyes

#### 111 Factors affecting dye binding

#### 111 Differentiation

#### 112 The nuclear dyes

113 Harris hematoxylin

114 Mayer hematoxylin

114 Ehrlich hematoxylin

114 Gill hematoxylin 1

115 Scott solution

115 Weigert hematoxylin

#### 115 Hematoxylin substitutes

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116 Gallein iron hematoxylin

#### 116 Plasma stains

116 Eosin solution

117 Eosin-phloxine B solution

#### 117 H&E staining

117 Manual progressive staining method

118 Manual regressive staining method

#### 118 Automated staining

119 Note on results of H&E staining

119 Hints to help achieve good H&E staining

120 Restoring tissue basophilia

#### 121 Troubleshooting the H&E stain

121 Incomplete deparaffinization

121 Nuclear staining is not crisp

122 Pale nuclear staining

122 Dark nuclear staining

122 Red or red-brown nuclei

122 Pale cytoplasmic staining

123 Dark cytoplasmic staining

124 Eosin not properly differentiated

124 Blue-black precipitate on top of sections

124 Water & slides turn milky when slides are placed in water following alcohol during deparaffinization

124 Slides are hazy or milky in last xylene before applying cover glass

#### 124 Uneven H&E staining

125 Dark basophilic staining of nuclei & cytoplasm, especially around tissue edges

125 Poor contrast between nucleus & cytoplasm

#### 126 Nucleic acid stains

126 Feulgen reaction

127 Methyl green-pyronin Y

#### 129 Polychromatic stains

130 May-Grunwald Giemsa stain

### 131 Mounting stained sections

#### 131 Resinous media

#### 131 Aqueous mounting media

#### 132 Coverslips

#### 132 Troubleshooting mounted stained sections

132 Water bubbles noted in mounted sections

132 All areas of section cannot be brought into focus

134 Cornflaking artifact seen on mounted sections

134 Mounted stained sections are not as crisp as usual when viewed microscopically

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138 Group III: glycoproteins (mucins, mucoid, mucoprotein, mucosubstances)

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#### 139 Special staining techniques

139 PAS reaction

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141 PAS reaction with diastase digestion

143 Best carmine

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147 Alcian blue, pH 2.5

149 Alcian blue with hyaluronidase

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| 151 | Müller-Mowry colloidal iron      |
| 154 | <b>Amyloid</b>                   |
| 154 | Alkaline Congo red method        |
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| 158 | <b>Learning activities</b>       |
| 158 | <b>References</b>                |

## Connective & Muscle Tissue

### Chapter 08

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| 165 | Gomori 1-step trichrome stain                                               |
| 166 | van Gieson picric acid-acid fuchsin stain                                   |
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| 180 | PTAH without mercuric solutions                                             |
| 180 | <b>Staining technique for basement membranes</b>                            |
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| 183 | <b>Staining techniques for lipid</b>                                        |
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| 203 | Astrocytes: Cajal stain                                                                                       |
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|-----|--------------------------------------|
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| 292 | <b>References</b>                    |

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| 302 | Naphthol AS-D chloroacetate esterase technique                |
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## Chapter 14 **Cytopreparatory Techniques**

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| 326 | <b>OG-6</b>                                                   |
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| 327 | Papanicolaou (Pap) stain                                      |
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## Chapter 15 **Laboratory Informatics**

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## Preface |

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After each edition was done, I have vowed that it would be my last one; however, something usually comes up that entices me to work on another one. Laboratory information systems and the ability to track specimens from accessioning to sign-out have led to this edition. The current texts for the histotechnology laboratory don't touch upon informatics, so a chapter on laboratory informatics has been added to this edition. Since the certification exams no longer include electron microscopic techniques, we have deleted this chapter. Fixatives for electron microscopy are discussed in the fixation chapter. Other chapters are updated, many new images have been added, as well as a glossary.

Our thanks go to Donna Willis, HT(SCP)HTL, Anatomic Pathology Manager, Baylor University Medical Center, Dallas, Texas for her help in obtaining the many of the new images; to Maureen Doran, HTL(ASCP), Chair of the Health and Safety Committee of the National Society for Histology for reviewing the safety chapter and keeping me from making incorrect statements, while adding new information; Lori Schmitt, BS, HT(ASCP)QIHC, Anatomic Pathology Manager at the Children's Hospital of Pittsburgh for information on the interference of eosin with FISH, and its replacement with methylene blue; and to Amy Watts, HT(ASCP), Field Application Specialist, Roche Tissue Diagnostics for reviewing the chapter on Laboratory Informatics.

While this edition, again, is written with histotechnology students foremost in mind, I also hope that practicing technicians, technologists, residents, and pathologists will find it useful. Finally, I give my thanks to all of the students who have shared their appreciation for the help that they have received from the previous 3 editions.

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# Connective & Muscle Tissue

## Chapter 8

## Objectives

On completing this chapter, the student should be able to do the following:

1. Identify the 3 connective tissue fibers and briefly describe each
2. Identify the 3 types of muscle fibers and briefly describe each
3. Identify 5 connective tissue cells and briefly describe each
4. Classify the following techniques as to the fiber(s) or cell demonstrated:
  - a. Masson trichrome
  - b. Gomori 1-step trichrome
  - c. van Gieson
  - d. Verhoeff
  - e. Gomori aldehyde fuchsin
  - f. orcein
  - g. Russell modification of the Movat pentachrome stain
  - h. Gomori silver impregnation
  - i. Gordon & Sweets silver impregnation
  - j. methenamine-silver
  - k. Mallory phosphotungstic acid-hematoxylin
  - l. oil red O
  - m. Sudan black B
  - n. osmium tetroxide
  - o. toluidine blue
  - p. methyl green-pyronin
5. Outline each of the above staining procedures, considering the following characteristics:
  - a. most desirable fixative
  - b. if another fixative has been used, what can be done
  - c. primary reagents or dyes and their purpose
  - d. results of the stain
  - e. appropriate control material
  - f. sources of error and appropriate correction
  - g. mode of action
  - h. special requirements (eg, chemically clean glassware)
  - i. microscope used
6. Identify reasons for using each of the techniques listed in objective 4
7. Describe the tissue preparatory techniques(s) used for the following stains:
  - a. oil red O
  - b. osmium tetroxide
  - c. Sudan black B
8. Identify a unique feature of mast cells that aids in their demonstration
9. Identify the cytoplasmic substance stained by pyronin
10. Define impregnation
11. Identify the name of a modification of the periodic acid-methenamine silver technique for demonstrating basement membranes that uses a hematoxylin and eosin counterstain

## Connective tissue

Connective tissue is one of the 4 basic tissue types, and it functions to provide structural and metabolic support for the other tissues and organs in the body. It consists of 3 different components: fibers, cells, and amorphous ground substance. These components vary in amount within the different connective tissue types, which are (1) connective tissue proper, (2) cartilage, and (3) bone. Blood also is sometimes classified as a connective tissue.

Most commonly, when we use special stains to demonstrate connective tissue elements in the histopathology laboratory, we are interested in the fibers or cells of connective tissue proper. The ground substance of connective tissue proper is a mucopolysaccharide and is demonstrated with carbohydrate staining techniques. The fibers of connective tissue proper are described below:

1. Collagen fibers provide strength; the more collagen present, the stronger the tissue is. A dense regular arrangement of collagen fibers is found in tendons, organ capsules, and the dermis. At least 27 different types of collagen have been identified [Young 2006], with type 1 being the most common in humans. The differences in the different types are unnecessary for our understanding of the affinity of collagen for certain stains. Type 1 collagen is found in fibrous supporting tissue, the dermis of skin, tendons, ligaments, and bone. It is very eosinophilic, readily visible with light microscopy birefringent upon polarization, and reveals a characteristic pattern of cross-striations with the electron microscope. When necessary, collagen may be differentiated from smooth muscle using the Masson and Gomori trichrome techniques, and the van Gieson stain. The procedures are given later in the chapter.
2. Elastic fibers are present in most fibrous connective tissue, but are most abundant in tissue requiring flexibility, because the elastic fibers allow tissues to stretch. The size and arrangement vary among different tissues, from fenestrated sheets or lamellae in the aorta to scattered fibers in loose connective tissue. These fibers usually cannot be seen on hematoxylin-eosin (H&E) stained sections but require special stains such as the Verhoeff iron hematoxylin, Weigert resorcin fuchsin, orcein, or Gomori aldehyde fuchsin stains for demonstration.
3. Reticulin fibers have been identified as a type of collagen (type III). They form a delicate supporting network for many highly cellular organs such as endocrine glands, lymph nodes, and liver. These fibers are not apparent in ordinary H&E stained sections but may be demonstrated with an argyrophilic reaction, because they have the ability to adsorb silver from solution. The silver may then be reduced chemically to its visible metallic form. Reticular fibers form delicate networks and are much smaller than most collagen fibers.

The cells found in connective tissue proper, whether fixed, or free and transient, are:

1. Fibroblasts, the most common cell in connective tissue, produce the connective tissue fibers (extracellular, nonliving elements). Frequently, only flattened fibroblast nuclei can be distinguished among bundles of collagen. These cells are not commonly of interest in tissues; rather, it is the product of fibroblasts that we demonstrate with special techniques.
2. Mesenchymal cells, which may be indistinguishable from fibroblasts. These are primitive, relatively undifferentiated cells that may develop into various differentiated cell types if the need arises for replacement.
3. Adipose or fat cells, which synthesize and store lipid and are common in most loose connective tissue. In some areas of the body, this is the predominant cell type and the tissue is known as adipose tissue. The nucleus in an adipose cell becomes very flattened as the lipid accumulation of the cell grows. These are very long lived cells, and even though the fat store may be depleted, the cell remains, awaiting a new accumulation. Fat, or simple lipid, is not preserved in paraffin sections, but can be demonstrated in frozen sections with special techniques. Lipids are discussed separately in most special staining manuals, but because adipose tissue is a type of connective tissue, I have elected to present the techniques for simple lipids in the connective tissue section.
4. Mast cells contain abundant secretory granules that, with special stains, frequently obscure the nucleus. Mast cell granules contain histamine and heparin, and exhibit metachromasia when stained with toluidine blue, a reaction that primarily is the result of the heparin content of the granules. Mast cells are most prominent along small blood vessels, and they closely resemble the basophilic leukocyte found in blood. Both mast cells and basophils can degranulate to increase vascular permeability; however, there are structural differences in these 2 cells, which suggests that mast cells are not merely basophils resident in the tissues [Young 2006].
5. Macrophages are “big eaters” or scavenger cells that are found not only in connective tissue proper but in various other tissues such as liver, and myeloid and lymphatic tissues. Monocytes (blood leukocytes) are the precursor of macrophages, and are also known as histiocytes. These cells play an important role in immune mechanisms, processing antigenic material for presentation to the lymphocytes. We are not asked to demonstrate this cell in routine histopathology.
6. Plasma cells, which are derived from B lymphocytes, produce immunoglobulins. Before immunoenzyme techniques were used for the demonstration of immunoglobulins, the methyl green-pyronin stain was frequently used to help identify immunoblastic sarcomas, a type of B cell lymphoma.

7. Blood cells of all types may be found in tissue, but these will not be discussed in this chapter.

### Basement membrane

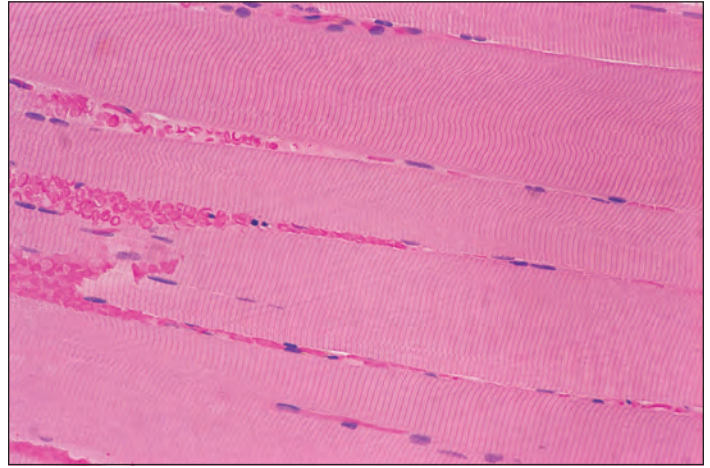
The basement membrane, frequently referred to as the *basal lamina*, is found beneath epithelium and separates the epithelium from the underlying connective tissue. The basement membrane consists of type IV collagen, the glycoproteins laminin, fibronectin, and entactin, and a proteoglycan rich in heparin sulfate. A similar structure surrounds muscle cells, Schwann cells, and other cells of mesenchymal origin. Basement membranes are illustrated by techniques that demonstrate the carbohydrate component. This is due to the glycoproteins present in the membrane, and to the fact that the collagen present in the basement membrane contains much more sugar in some of its side chains than is normally present in ordinary collagen.

The primary function of the basement membrane is to provide physical support for epithelium; it also provides for cell attachment and for ultrafiltration. In the kidney, the basement membrane of capillary endothelium acts as a sieve by holding back molecules on the basis of size, shape, and electrostatic charge. The glomerular basement membrane stained with periodic acid-Schiff (PAS) is demonstrated in Chapter 7 [i7.2](#) and stained with a silver technique in this chapter.

### Muscle

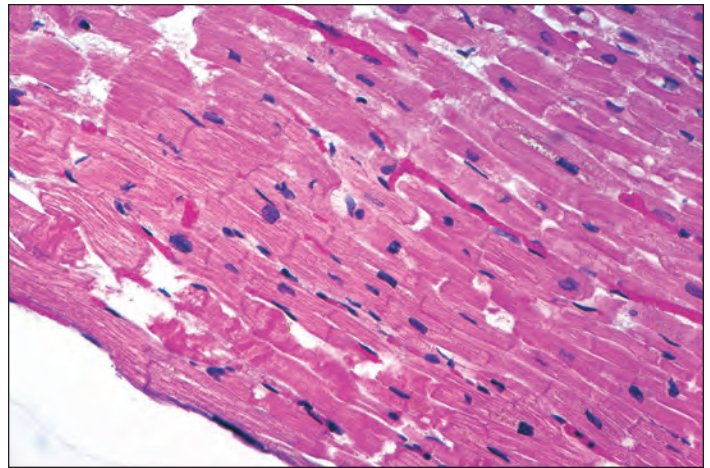
Muscle is also 1 of the 4 basic types of tissue and, based on the differences in structure and in function, is classified as follows:

1. Skeletal muscle. This type of muscle also may be classified as (1) striated because of the characteristic dark (A) and light (I) bands seen microscopically, and (2) voluntary because contraction can be brought about at will. Because cardiac muscle is also striated, skeletal muscle should be referred to as skeletal muscle, not striated muscle. Individual skeletal muscle fibers, or cells, may be quite long and contain multiple peripherally located nuclei [i8.1](#). Actin and myosin are the major contractile proteins of skeletal muscle, and the arrangement of these 2 proteins imparts the striated appearance seen in longitudinal sections. Traditionally, the phosphotungstic acid-hematoxylin (PTAH) method of Mallory was used to demonstrate these cross-striations but today monoclonal antibodies are used with immunohistochemical methods to demonstrate pathologic skeletal muscle components.
2. Cardiac muscle. This is also a striated but involuntary type of muscle. It is similar to skeletal muscle, except that the cells branch and anastomose, and each cell usually has only 1 centrally located nucleus. Cardiac muscle cells anastomose with specialized intercellular junctions called intercalated discs. Cardiac muscle can be seen in [i8.2](#).



**i8.1**

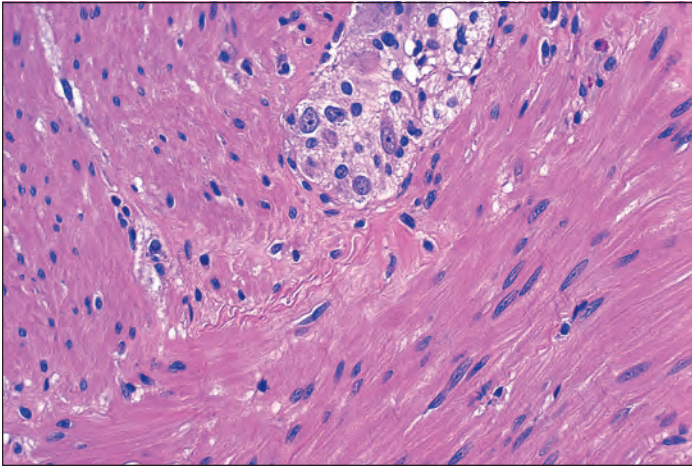
Skeletal muscle in longitudinal section. The muscle was isometrically fixed so that the dark (A) and light (I) bands, which give skeletal muscle its characteristic striated appearance, are demonstrated with the H&E stain. Note that each muscle cell or fiber contains multiple peripheral nuclei.



**i8.2**

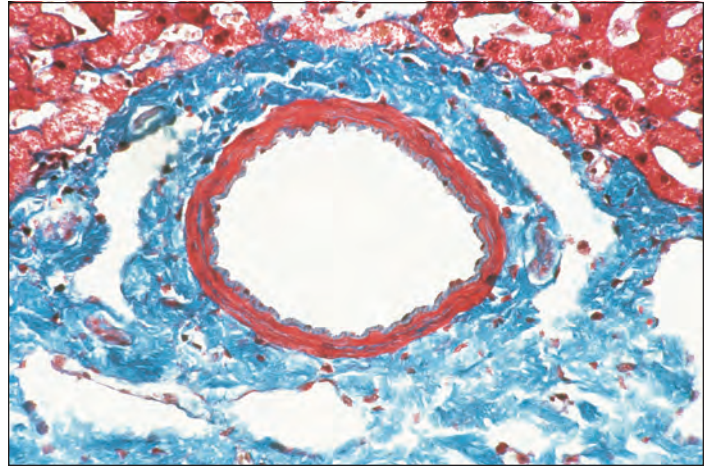
Cardiac muscle in longitudinal section. Note that the fibers branch, and each contains 1 centrally located nucleus. Although cardiac muscle is striated, the striations are not as obvious as in skeletal muscle [i8.1](#). A dark line, identified as an intercalated disk, can be seen crossing the fibers occasionally.





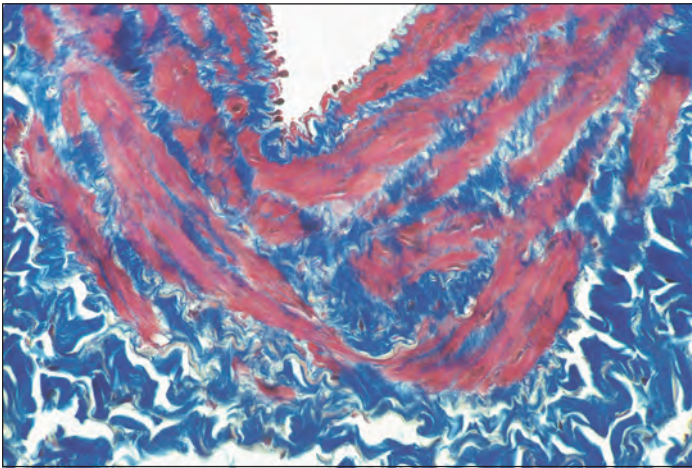
i8.3

Smooth muscle in cross (top left) and longitudinal (bottom right) section, characteristic of the external muscle layers of the gastrointestinal tract. The single nuclei are elongated and centrally placed in the longitudinally sectioned fibers, and round and centrally placed in the cross-sectioned fibers. The arrow is located in a ganglion or a collection of nerve cells occurring outside the central nervous system.



i8.5

Masson trichrome stained section of liver shows the portal vein containing a few layers of smooth muscle in a portion of the portal triad. A large vein or a muscular artery in a section is best for judging the quality of staining. Red staining of epithelial cells but not of smooth muscle cells indicates a problem with the procedure or reagents.



i8.4

Masson trichrome stained section of a large vein. The lumen can be seen at the top. Smooth muscle cells are bright red and collagen fibers are blue.

- Smooth muscle. This is a nonstriated, involuntary type of muscle that is commonly arranged in layers. The muscle fibers are long and tapered and contain a single centrally located nucleus [i8.3](#). Actin and myosin are also present in smooth muscle, but they are present in a different ratio than in striated muscle, and these proteins also are organized very differently. Smooth muscle regulates luminal size in hollow organs and tubular structures. [i8.4](#), [i8.5](#) show smooth muscle fibers in the wall of a blood vessel stained red.

## Staining techniques for connective tissue fibers

### Masson trichrome stain [Luna 1968, Sheehan 1980]

#### Purpose

Trichrome stains are frequently used to differentiate collagen from smooth muscle in tumors and to identify increases in collagenous tissue in diseases such as cirrhosis of the liver.

#### Principle

Trichrome procedures are so named because 3 dyes, which may or may not include the nuclear stain, are used. The mechanism of the stain is not totally understood and may be related in part to the size of different dye molecules. Sections are first stained with an acid dye such as Biebrich scarlet; all acidophilic tissue elements such as cytoplasm, muscle, and collagen will bind the acid dyes. The sections are then treated with phosphotungstic and/or phosphomolybdic acid. Because cytoplasm is much less permeable than collagen, phosphotungstic and phosphomolybdic acids cause Biebrich scarlet to diffuse out of the collagen but not out of the cytoplasm of cells. Phosphotungstic and phosphomolybdic acid have numerous acidic groups that most likely act as a link between the decolorized collagen and aniline blue dye. Also the pH of the phosphotungstic/phosphomolybdic acid solution probably increases selective collagen staining and aids in the diffusion or removal of Biebrich scarlet.

#### Fixative

Bouin solution is preferred, but 10% neutral buffered formalin may be used.

#### Equipment

56°C to 58°C oven, Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes.

**Technique**

Cut paraffin sections at 4-5  $\mu\text{m}$ .

**Quality control**

Practically every tissue has an internal control, so no other control sections are needed; however, if a control is desired, uterus, small intestine, appendix, or Fallopian tube will provide good material

**Reagents****Bouin solution**

|                                         |       |
|-----------------------------------------|-------|
| Picric acid, saturated aqueous solution | 75 mL |
| Formaldehyde, 37%-40%                   | 25 mL |
| Glacial acetic acid                     | 5 mL  |

**Weigert iron hematoxylin solution****Solution A**

|              |          |
|--------------|----------|
| Hematoxylin  | 10 g     |
| Alcohol, 95% | 1,000 mL |

**Solution B**

|                                       |        |
|---------------------------------------|--------|
| Ferric chloride, 29% aqueous solution | 20 mL  |
| Distilled water                       | 475 mL |
| Glacial acetic acid                   | 5 mL   |

**Working solution**

Mix equal parts of solutions A and B

**Biebrich scarlet-acid fuchsin solution**

|                                       |        |
|---------------------------------------|--------|
| Biebrich scarlet, 1% aqueous solution | 360 mL |
| Acid fuchsin, 1% aqueous solution     | 40 mL  |
| Glacial acetic acid                   | 4 mL   |

**Phosphomolybdic/phosphotungstic acid solution**

|                      |          |
|----------------------|----------|
| Phosphomolybdic acid | 25 g     |
| Phosphotungstic acid | 25 g     |
| Distilled water      | 1,000 mL |

**Aniline blue solution**

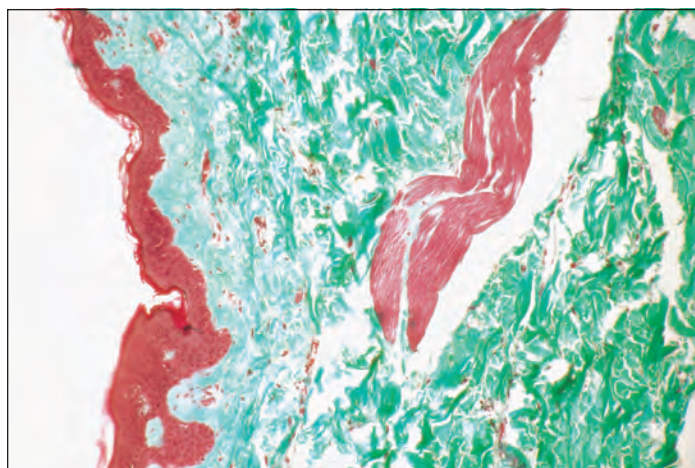
|                     |          |
|---------------------|----------|
| Aniline blue        | 25 g     |
| Glacial acetic acid | 20 mL    |
| Distilled water     | 1,000 mL |

**Acetic acid, 1% solution**

|                     |       |
|---------------------|-------|
| Glacial acetic acid | 1 mL  |
| Distilled water     | 99 mL |

**Conventional procedure**

1. Deparaffinize sections and hydrate to distilled water
2. Rinse well in distilled water
3. Mordant formalin fixed sections in Bouin solution for 1 hour at 56°C
4. Remove slides from oven, allow to cool, and wash in running water until the yellow color disappears
5. Rinse in distilled water
6. Stain sections in Weigert iron hematoxylin for 10 minutes
7. Wash in running water for 10 minutes
8. Rinse in distilled water

**i8.6**

A section of skin stained with Masson trichrome. Light green has been used as the counterstain instead of aniline blue. An erector pili muscle and the epithelium are stained red, and the collagen is stained green.

9. Stain sections in Biebrich scarlet-acid fuchsin solution for 2 minutes. If desired, the solution may be saved for 1 more run only
10. Rinse in distilled water
11. Place the slides in phosphomolybdic/phosphotungstic acid solution for 10-15 minutes; discard this solution
12. Stain sections in aniline blue solution for 5 minutes; if desired, the solution may be saved for 1 more run only
13. Rinse the slides in distilled water
14. Place the slides in 1% acetic acid solution for 3-5 minutes; discard this solution
15. Dehydrate with 95% and absolute alcohols, 2 changes each
16. Clear with 2 or 3 changes of xylene and mount with synthetic resin

**Results i8.4, i8.5**

|                                   |       |
|-----------------------------------|-------|
| Nuclei                            | Black |
| Cytoplasm, keratin, muscle fibers | Red   |
| Collagen and mucin                | Blue  |

**Technical notes**

1. If desired, collagen may be counterstained with light green instead of aniline blue **i8.6**. The following changes are made:

Step 11: Place the sections in a 5% aqueous solution of phosphotungstic acid

Step 12: Stain 5 minutes in 2% light green



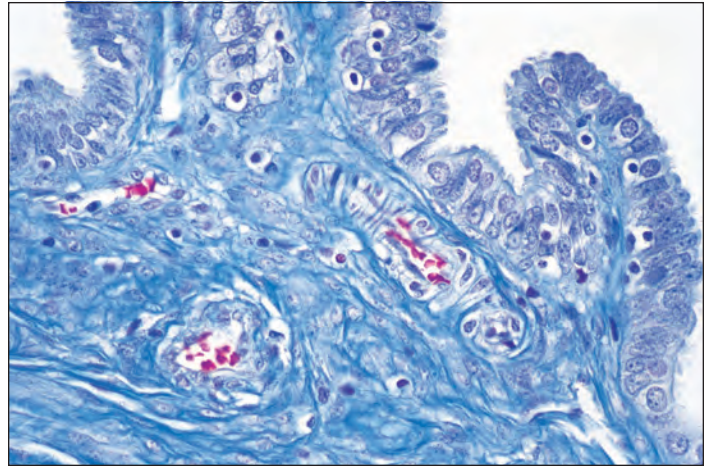
|                           |       |
|---------------------------|-------|
| Light green, SF yellowish | 2 g   |
| Distilled water           | 99 mL |
| Glacial acetic acid       | 1 mL  |

Light green is a better counterstain when collagen is predominant, however, when only small amounts are to be demonstrated, the aniline blue is the better counterstain.

- Decreased red staining usually indicates that the staining solution has aged or been overused and should be discarded **i8.7**. If blue staining of connective tissue appears faded, the section has probably been overdifferentiated in the acetic acid solution. Pathologically altered collagen, such as that seen in burns, may lose its affinity for aniline blue and bind the acid dye instead [Vacca 1985].
- Sections fixed in 10% neutral buffered formalin will stain poorly and unevenly. Cross-linking fixatives such as formaldehyde and glutaraldehyde mask reactive chemical groups that would otherwise bind the acid dyes used in trichrome techniques. Formalin fixed tissues will stain brilliantly when mordanted in either Bouin or a mercuric chloride solution; however, mercuric fixatives should not be used because of the toxicity. Zinc formalin has been shown to give satisfactory results [Della Speranza 2009].
- An iron hematoxylin solution is used for nuclear staining in the trichrome procedures because iron hematoxylin is more resistant than aluminum hematoxylin to decolorization in the subsequently used acidic dye solution.
- Although most texts state that Weigert iron hematoxylin should be prepared fresh, I find that it is good for several days.
- Picric acid containing less than 10% water is very explosive; therefore, it is important that solutions not be spilled in the oven and then allowed to evaporate. For this reason, the staining jar containing picric acid (Bouin solution) should be placed inside another container while in the oven.

#### **Microwave procedure** [Crowder 1991]

- Deparaffinize the sections and hydrate to distilled water.
- If the sections were not originally fixed in Bouin solution, they should be mordanted by placing in a vented plastic Coplin jar of Bouin solution. Microwave at 70% power for 45 seconds. If originally fixed in Bouin solution, skip to step 4.
- Rinse sections in running water until colorless.
- Place slides in Gill hematoxylin III in a vented plastic Coplin jar. Microwave on high for 15 seconds.
- Rinse slides in tap water.
- Blue the nuclei with an alkaline blueing solution.
- Rinse well with tap water.
- Rinse slides in distilled water.



**i8.7**

A section of fallopian tube stained with Masson trichrome. Note that neither the smooth muscle surrounding the blood vessels nor the epithelium is stained red, but the red blood cells do show red staining. Red blood cells should never be used to judge the quality of the stain. This is a bad stain and should be repeated with new reagents.

- Place sections in Biebrich scarlet-acid fuchsin solution in a vented plastic Coplin jar. Microwave at 70% power for 30 seconds.
- Rinse sections in distilled water.
- Place sections in phosphomolybdic/phosphotungstic acid solution in a vented plastic Coplin jar. Microwave at full power for 15 seconds. *Do not rinse.*
- Place in aniline blue solution in a vented Coplin jar. Microwave at 70% power for 30 seconds.
- Rinse slides in distilled water.
- Place slides in 1% acetic acid for 2-3 minutes.
- Dehydrate sections with 95% and absolute alcohol, 2 changes each.
- Clear with xylene and mount with synthetic resin.

#### **Results i8.4, i8.5**

|                                   |           |
|-----------------------------------|-----------|
| Nuclei                            | Dark blue |
| Cytoplasm, keratin, muscle fibers | Red       |
| Collagen and mucin                | Blue      |

#### **Technical notes**

- All reagents should be discarded after use.
- Because picric acid is explosive when allowed to dry, the Coplin jar should be placed in a partially closed plastic bag to trap any boil-over or spill of this reagent.



**Gomori 1-step trichrome stain** [Gomori 1950b, Sheehan & Hrapchak 1980]**Purpose**

To identify an increase in collagenous connective tissue fibers or to differentiate between collagen and smooth muscle fibers.

**Principle**

In the 1-step trichrome procedure, a plasma stain (chromotrope 2R) and a connective tissue fiber stain (fast green FCF, light green, or aniline blue) are combined in a solution of phosphotungstic acid to which glacial acetic acid has been added. Phosphotungstic acid favors the red staining of muscle and cytoplasm. The tungstate ion is specifically taken up by collagen, and the connective tissue fiber stain is subsequently bound to this complex.

**Fixative**

Any well fixed tissue may be used. Bouin solution is used as a mordant to intensify the color reactions.

**Equipment**

56°C-58°C oven, Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes.

**Technique**

Cut paraffin sections at 4-5  $\mu$ m.

**Quality control**

Practically every tissue has an internal control, so no other control sections are needed; however, if a control is desired, uterus, small intestine, appendix, or Fallopian tube will provide good material.

**Reagents****Bouin solution**

|                                         |       |
|-----------------------------------------|-------|
| Picric acid, saturated aqueous solution | 75 mL |
| Formaldehyde, 37%-40%                   | 25 mL |
| Glacial acetic acid                     | 5 mL  |
| Weigert iron hematoxylin solution       |       |

**Solution A**

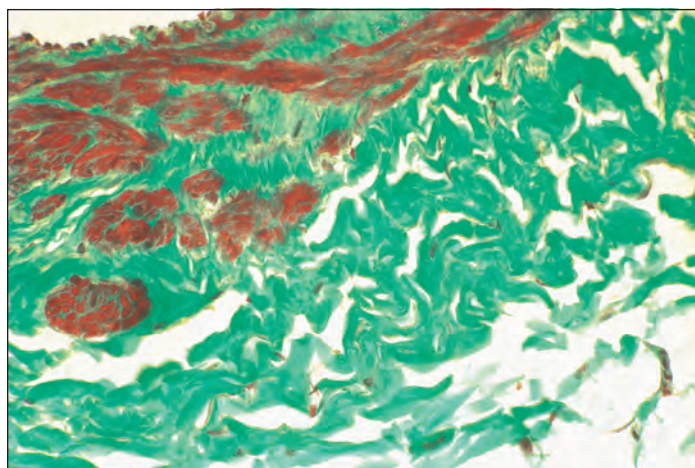
|              |          |
|--------------|----------|
| Hematoxylin  | 10 g     |
| Alcohol, 95% | 1,000 mL |

**Solution B**

|                                       |        |
|---------------------------------------|--------|
| Ferric chloride, 29% aqueous solution | 20 mL  |
| Distilled water                       | 475 mL |
| Glacial acetic acid                   | 5 mL   |

**Working solution**

Mix equal parts of solutions A and B.

**i8.8**

Gomori 1-step trichrome stained section of a large vein. Light green was used instead of aniline blue in preparing the solution. Smooth muscle cells are stained bright red and collagen fibers are stained green.

**Gomori trichrome stain**

|                                              |        |
|----------------------------------------------|--------|
| Chromotrope 2R                               | 0.6 g  |
| Fast green FCF, light green, or aniline blue | 0.3 g  |
| Phosphotungstic acid                         | 0.8 g  |
| Glacial acetic acid                          | 1 mL   |
| Distilled water                              | 100 mL |

Store this solution in the refrigerator.

**Acetic acid, 0.5% solution**

|                     |         |
|---------------------|---------|
| Glacial acetic acid | 0.5 mL  |
| Distilled water     | 99.5 mL |

**Procedure**

1. Deparaffinize sections and hydrate to distilled water
2. Rinse well in distilled water
3. Mordant sections in Bouin solution for 1 hour at 56°C
4. Remove slides from the oven, allow to cool, and wash in running water until the yellow color disappears
5. Rinse in distilled water
6. Stain sections in Weigert iron hematoxylin for 10 minutes
7. Wash in running water for 10 minutes
8. Stain sections for 15-20 minutes in Gomori trichrome stain
9. Differentiate for 2 minutes in 0.5% acetic acid
10. Dehydrate, clear, and mount with synthetic resin

**Results i8.8**

|                                   |               |
|-----------------------------------|---------------|
| Nuclei                            | Black         |
| Cytoplasm, keratin, muscle fibers | Red           |
| Collagen and mucin                | Green or blue |

**Technical notes**

1. Sweat [1968] states that coloration of fine connective tissue fibers is affected by the dye solution pH, with maximum binding occurring around pH 1.3. The pH of Gomori trichrome is ~2.5, which decreases affinity for anions by ~50%, so these investigators suggest that by replacing the acetic acid with hydrochloric acid, a pH of ~1.3 can be obtained. The intensity of coloration of the fine connective tissue fibers can be varied by altering the pH.
2. Churukian [1993] finds that zinc formalin allows good trichrome staining without mordanting in Bouin solution.

**van Gieson picric acid-acid fuchsin stain** [Mallory 1942, Sheehan 1980]**Purpose**

Although the van Gieson technique may be considered a primary connective tissue stain, it is rarely used as such; however, it serves as an excellent counterstain for other methods such as the Verhoeff elastic technique, referred to in many institutions as the Verhoeff-van Gieson (VVG) stain. Other institutions refer to it as the elastic-van Gieson (EVG) stain.

**Principle**

In a strongly acidic solution, collagen is selectively stained by acid fuchsin, an acid aniline dye. Picric acid provides the acidic pH necessary and also acts as a stain for muscle and cytoplasm. The low pH is very important, as selective staining of collagen will not occur at higher pH levels. The addition of 0.25 mL of hydrochloric acid to 100 mL of van Gieson solution will sharpen the differentiation between collagen and muscle [Lillie 1976]. Saturated picric acid solutions are important in the preparation of the stain, and again for the selective staining of collagen. If the picric acid solution is not saturated, collagen may stain pale pink to pale orange, and collagen, cytoplasm, and muscle may all stain the same color.

**Fixative**

Any well fixed tissue may be used.

**Equipment**

Mechanical stirrer, Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes.

**Technique**

Cut paraffin sections at 4–5  $\mu\text{m}$ .

**Quality control**

Practically every tissue has an internal control, so no other control sections are needed; however, if a control is desired, uterus, small intestine, appendix, or Fallopian tube will provide good material.

**Reagents****Weigert iron hematoxylin solution****Solution A**

|              |          |
|--------------|----------|
| Hematoxylin  | 10 g     |
| Alcohol, 95% | 1,000 mL |

**Solution B**

|                                       |        |
|---------------------------------------|--------|
| Ferric chloride, 29% aqueous solution | 20 mL  |
| Distilled water                       | 475 mL |
| Glacial acetic acid                   | 5 mL   |

**Working solution**

Mix equal parts of solutions A and B

**Acid fuchsin, 1% solution**

|                 |        |
|-----------------|--------|
| Acid fuchsin    | 1 g    |
| Distilled water | 100 mL |

**Picric acid, saturated solution**

|                 |          |
|-----------------|----------|
| Picric acid     | 13 g     |
| Distilled water | 1,000 mL |

Stir the solution on the mechanical stirrer for several hours. Some picric acid should remain undissolved in the bottom of the flask. The solubility of picric acid is 1.23 g/100 mL water at 20°C. The amount used may have to be adjusted, depending on whether water has been added to the stock powder to ensure that the water content does not drop below at least 10%.

**van Gieson solution**

|                                 |       |
|---------------------------------|-------|
| Acid fuchsin, 1% solution       | 5 mL  |
| Picric acid, saturated solution | 95 mL |

**Procedure**

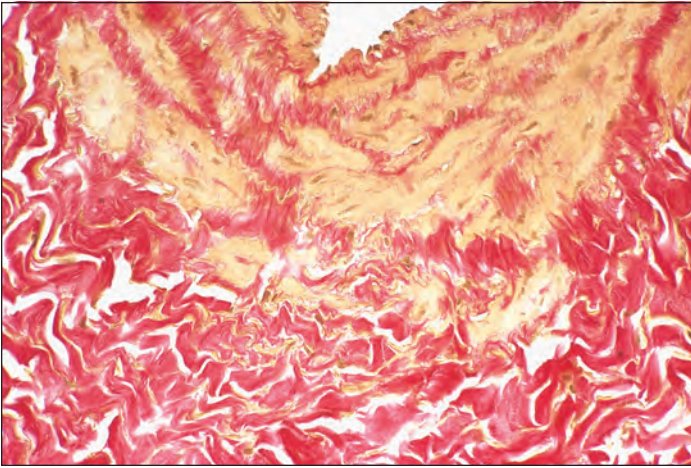
1. Deparaffinize sections and hydrate to distilled water
2. Stain sections with Weigert iron hematoxylin for 10–20 minutes; sections should be overstained, as they will be slightly decolorized by the picric acid
3. Wash in running tap water for 10 minutes
4. Stain sections in van Gieson stain for 5 minutes; discard solution
5. Place slides in 95% alcohol
6. Dehydrate as usual, clear with xylene, and mount with synthetic resin

**Results i8.9**

|                      |               |
|----------------------|---------------|
| Nuclei               | Black         |
| Collagen             | Brilliant red |
| Muscle and cytoplasm | Yellow        |

**Technical notes**

1. An iron hematoxylin solution is used for nuclear staining in the trichrome procedures because iron hematoxylin is more resistant than aluminum hematoxylin to decolorization in subsequent acidic dye solutions.



i8.9

van Gieson stained section of a large vein. Smooth muscle cells are stained yellow and collagen fibers are stained red.

- Although many texts state that Weigert iron hematoxylin should be prepared fresh, I find that the solution is good for several days.
- If a sharp color differentiation is not obtained between collagen and muscle, check the preparation of the saturated picric acid solution, as the acidic pH provided by this solution is very important. Also the addition of 0.25 mL of hydrochloric acid to 100 mL van Gieson solution may sharpen the color differentiation.

### Verhoeff elastic stain [Mallory 1942, Sheehan 1980]

#### Purpose

Elastic fiber techniques are used to demonstrate pathologic changes in elastic fibers. These include atrophy of the elastic tissue; thinning or loss that may result from arteriosclerotic changes; and reduplication, breaks, or splitting that may result from other vascular diseases. The techniques also may be used to demonstrate normal elastic tissue, as in the identification of veins and arteries, and to determine whether or not the blood vessels have been invaded by tumor.

#### Principle

The tissue is overstained with a soluble lake of hematoxylin-ferric chloride-iodine. Both ferric chloride and iodine serve as mordants, but they also have an oxidizing function that assists in converting hematoxylin to hematein. The mechanism of dye binding is probably by the formation of hydrogen bonds, but the exact chemical groups reacting with the hematoxylin have not been identified. Because this method requires that the sections be overstained and then differentiated, it is a regressive method. Differentiation is accomplished by using excess mordant, or ferric chloride, to break the tissue-mordant-dye complex. The

dye will be attracted to the larger amount of mordant in the differentiating solution and will be removed from the tissue. The elastic tissue has the strongest affinity for the iron-hematoxylin complex and will retain the dye longer than the other tissue elements. This allows other elements to be decolorized and the elastic fibers to remain stained. Sodium thiosulfate is used to remove excess iodine. Although van Gieson solution is the most commonly used counterstain, other counterstains may be used.

#### Fixative

Any well fixed tissue may be used, but neutral buffered formalin or Zenker solution is preferred.

#### Equipment

Mechanical stirrer, Coplin jars, Erlenmeyer flasks, graduated cylinders.

#### Technique

Cut paraffin sections at 4-5  $\mu$ m.

#### Quality control

Most laboratories use a section of aorta embedded on edge, but I prefer a cross-section of a muscular artery.

#### Reagents

##### Lugol iodine

|                  |          |
|------------------|----------|
| Iodine           | 10 g     |
| Potassium iodide | 20 g     |
| Distilled water  | 1,000 mL |

Put the iodine and potassium iodide in a flask with 200 mL of the water. Stir on a mechanical stirrer until the iodine dissolves and then add the remaining water.

##### Ferric chloride, 10% solution

|                            |        |
|----------------------------|--------|
| Ferric chloride            | 50 g   |
| Distilled water            | 500 mL |
| Store in the refrigerator. |        |

##### Ferric chloride, 2% solution

|                               |       |
|-------------------------------|-------|
| Ferric chloride, 10% solution | 10 mL |
| Distilled water               | 40 mL |

##### Alcoholic hematoxylin, 5% solution

|              |        |
|--------------|--------|
| Hematoxylin  | 5 g    |
| Alcohol, 95% | 100 mL |

##### Verhoeff elastic stain

Prepare fresh each time and mix in order:

|                               |       |
|-------------------------------|-------|
| Alcoholic hematoxylin, 5%     | 30 mL |
| Ferric chloride, 10% solution | 12 mL |
| Lugol iodine                  | 12 mL |

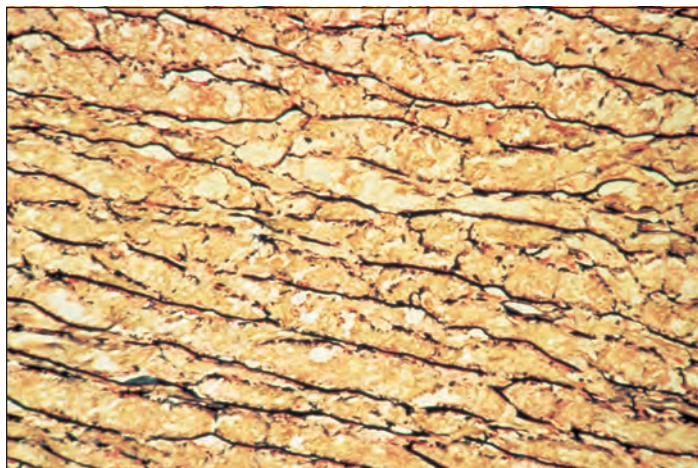
##### van Gieson solution

(See under van Gieson picric acid-acid fuchsin stain)

##### Sodium thiosulfate, 5% solution

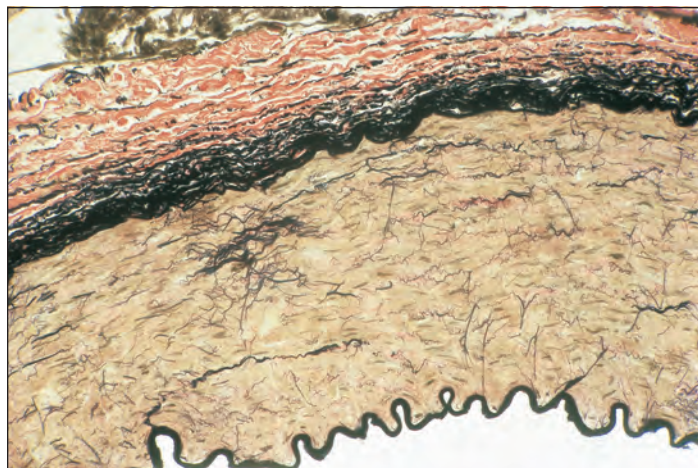
|                    |          |
|--------------------|----------|
| Sodium thiosulfate | 50 g     |
| Distilled water    | 1,000 mL |





i8.10

A Verhoeff-van Gieson stained section from the wall of the aorta, an elastic artery. The tunica media (middle layer) is composed of concentrically arranged perforated laminae of elastic tissue stained black. The spaces between the elastic laminae are occupied by smooth muscle cells (yellow)



i8.11

Part of a cross-section of a muscular artery stained with the Verhoeff-van Gieson stain. Both the internal and external elastic laminae are very prominent, and very fine elastic fibers (black) can be seen in the yellow muscular tunica media. The collagen fibers of the tunica adventitia are stained red.

### Procedure

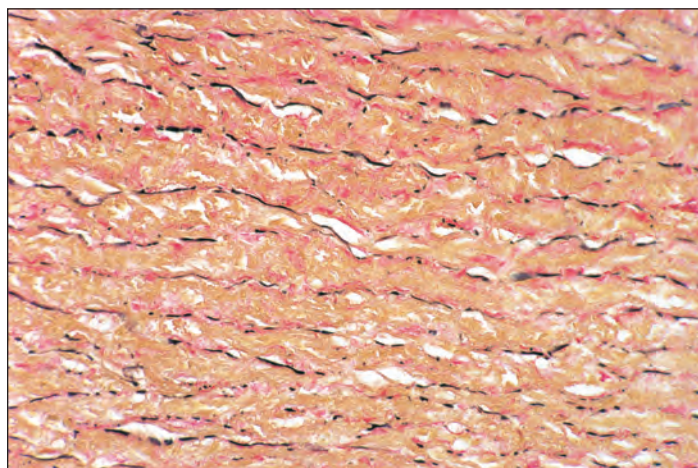
1. Deparaffinize sections and hydrate to distilled water
2. Place sections in Verhoeff elastic tissue stain for 1 hour
3. Wash in 2 changes of distilled water
4. Differentiate sections microscopically in 2% ferric chloride until the elastic fibers are distinct and the background is colorless to light gray; if the sections are differentiated too far, restain
5. Rinse sections in distilled water
6. Place section in sodium thiosulfate for 1 minute
7. Wash in running tap water for 5 minutes
8. Counterstain sections in van Gieson stain for 1 minute
9. Differentiate in 95% alcohol
10. Dehydrate in absolute alcohol, clear in xylene, and mount with synthetic resin

### Results i8.10, i8.11

|                       |                     |
|-----------------------|---------------------|
| Elastic fibers        | Blue-black to black |
| Nuclei                | Blue to black       |
| Collagen              | Red                 |
| Other tissue elements | Yellow              |

### Technical notes

1. It is easy to overdifferentiate this stain **i8.12**. If the background is completely colorless, so that a clear yellow counterstain is obtained, the section may be overdifferentiated. It is probably better to err on the side of underdifferentiation.
2. Overdifferentiated sections may be restained at any step, provided they have not been treated with alcohol.

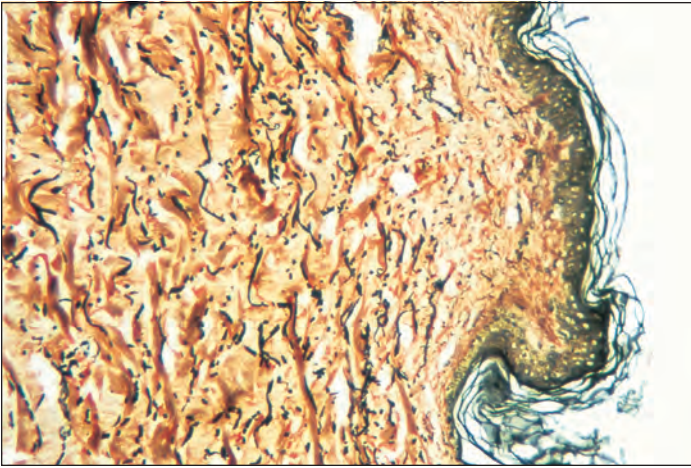


i8.12

A duplicate of **i8.10** stained with the Verhoeff-van Gieson stain, but overdifferentiated. Note how much less elastic tissue is demonstrated. The lack of the slightly grayed background, along with very clear, bright yellow muscle fiber staining, is frequently indicative of overdifferentiation. It is imperative that each section be differentiated microscopically.

3. Do not prolong staining with van Gieson solution, because picric acid also will differentiate the stain further.
4. It is not necessary to remove mercury deposits before staining, because they will be removed by the staining solution; however, because of the toxicity, mercuric fixatives should not be used.
5. The preparation of van Gieson solution is critical for proper differentiation of muscle and collagen. If the picric acid is not saturated, collagen will not stain red, and cytoplasm, muscle, and collagen may all stain the same color **i8.13**.





i8.13

A section of skin stained with the Verhoeff-van Gieson stain. The collagen should be stained bright red in a correctly done stain, but it is yellow-orange in this section, indicating that the picric acid used to prepare the van Gieson stain was not saturated. This stain should be repeated with correctly prepared van Gieson stain.

- To prepare Verhoeff elastic staining solution, the reagents must be added in the order given, with mixing after each addition, or poor staining may result.
- The staining jar that contained Verhoeff solution may be cleaned easily by transferring the 2% ferric chloride to the jar for a few minutes before discarding the solution.
- For optimum results, slides must be individually differentiated, because the time of differentiation is somewhat dependent on the amount of elastic tissue present. Do not depend on the control for timing the differentiation of all sections.
- Because proper differentiation is sometimes difficult, it is helpful to use duplicate sections differentiated to a slightly different end point.

#### Aldehyde fuchsin elastic stain [Gomori 1950a, Sheehan & Hrapchak 1980]

##### Purpose

Refer to the Verhoeff elastic stain.

##### Principle

Hydrochloric acid and paraldehyde are added to an alcoholic solution of basic fuchsin to form aldehyde fuchsin. Schiff bases are formed by the aldehyde and the fuchsin, but the affinity of elastic fibers for this solution is not understood. A number of other tissue elements will also stain with aldehyde fuchsin. These elements include pancreatic  $\beta$  cell granules and sulfated mucosubstances. Staining is intensified by prior oxidation. With variations in the technique, aldehyde fuchsin also has been used to stain highly sulfated mucosubstances, pancreatic  $\beta$  cells, and hepatitis b antigen.

##### Fixative

10% neutral buffered formalin is preferred; chromate fixatives should be avoided. Formalin and Bouin fixed tissues will show a colorless background and mercury fixed tissue show a pale lilac background [Sheehan & Hrapchak 1980].

##### Equipment

Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes, and Whatman No. 2 filter paper.

##### Technique

Cut paraffin sections at 4-5  $\mu$ m.

##### Quality control

Use a section of aorta embedded on edge or a cross section of a muscular artery. Skin also provides a good control.

##### Reagents

###### Aldehyde fuchsin solution

|                                          |        |
|------------------------------------------|--------|
| Pararosaniline (basic fuchsin, CI 42500) | 1 g    |
| Ethyl alcohol, 70%                       | 200 mL |
| Hydrochloric acid, concentrated          | 2 mL   |
| Paraldehyde (must be fresh)              | 2 mL   |

Mix well and let stand at room temperature for 2-3 days or until the stain is deep purple. Store in the refrigerator.

###### Light green stock solution

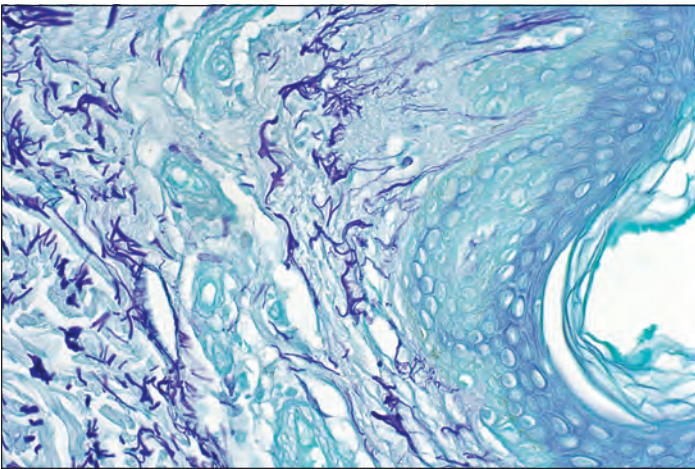
|                          |        |
|--------------------------|--------|
| Light green SF yellowish | 0.2 g  |
| Distilled water          | 100 mL |
| Glacial acetic acid      | 0.2 mL |
| Mix well.                |        |

###### Light green working solution

|                            |       |
|----------------------------|-------|
| Light green stock solution | 10 mL |
| Distilled water            | 50 mL |

##### Procedure

- Deparaffinize sections and hydrate to 70% alcohol
- Stain sections in aldehyde fuchsin solution for 10-40 minutes; with good solutions, 10 minutes is usually sufficient for staining
- Rinse off the excess stain with 70% alcohol
- Wash the sections in water and check microscopically for staining of elastic fibers. If a deeper stain is desired, rinse sections briefly in 70% alcohol and return to the aldehyde fuchsin. If further differentiation is needed, return sections to the 70% alcohol. Differentiation is stopped by rinsing the sections with distilled water. The stain may be filtered and reused.
- Rinse the sections with distilled water
- Counterstain sections with the light green working solution for 1-2 minutes; discard the solution
- Dehydrate in 2 changes each of 95% and absolute alcohols, clear in xylene, and mount with synthetic resin



**i8.14**  
A section of skin stained with the Gomori aldehyde fuchsin procedure. Elastic tissue is stained a dark purple. The keratinized stratified squamous epithelium, or the epidermis, and the dense irregular connective tissue of the dermis are all stained green.

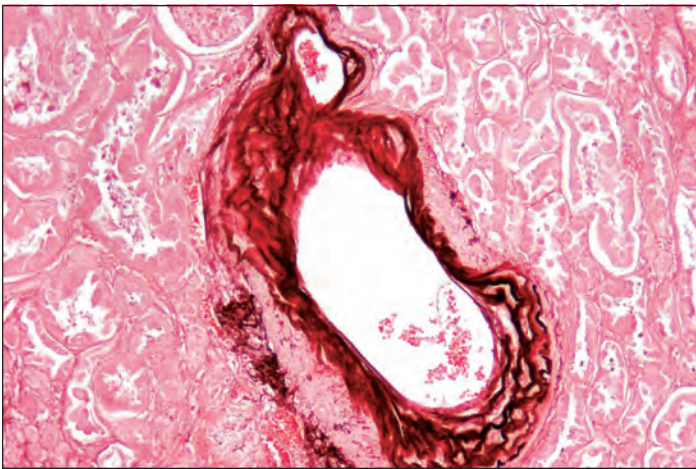
Results i8.14

|                       |                     |
|-----------------------|---------------------|
| Elastic fibers        | Deep blue to purple |
| Other tissue elements | Green               |

Technical notes

1. The paraldehyde used for preparation of the aldehyde fuchsin reagent should be fresh. Do not use reagent that was opened previously.
2. Old solutions of aldehyde fuchsin may not stain well, and the staining time may need to be prolonged.
3. Do not use rosaniline (CI 42510) for the preparation of this reagent, it is not satisfactory [Mowry 1979].
4. The shelf life of aldehyde fuchsin may be prolonged by refrigerating a small amount and freezing aliquots of the remainder.
5. Acetaldehyde is cheaper and may be obtained without a DEA number. The solution can be prepared as follows [Wenk 1995 ]:

| Alcoholic basic fuchsin, 0.5% solution                                         |        |
|--------------------------------------------------------------------------------|--------|
| Basic fuchsin (CI 42500)                                                       | 2.5 g  |
| Ethyl alcohol, 70%                                                             | 500 mL |
| Stir until dissolved and store at room temperature. Stable for several months. |        |
| Aldehyde fuchsin solution                                                      |        |
| Alcoholic basic fuchsin, 0.5% solution                                         | 50 mL  |
| Acetaldehyde                                                                   | 2.5 mL |
| Hydrochloric acid, concentrated                                                | 1.0 mL |



**i8.15**  
Orcein stained sections of a small artery demonstrating the elastic fibers.

Stir until dissolved. Cover tightly and allow to stand overnight at room temperature. Filter and store at 4°C. Allow to warm to room temperature and shake before using. The solution may be reused until staining is weak. It is stable for ~3 weeks.

Notes on other elastic stains

In the United States, the Verhoeff-van Gieson stain is probably the most widely used for the demonstration of elastic fibers. The second most widely used is the aldehyde fuchsin stain. Other stains for elastic tissue that are not used often in the United States, and not given in this text, are orcein and resorcin fuchsin. Orcein is 1 of the oldest methods for elastic fibers; however, it gives a less intense color than the Verhoeff. Orcein is used in an acidified alcoholic solution, and elastic fibers are stained brown with this stain i8.15. This procedure may be found in Kiernan [2008]. Resorcin fuchsin uses an acidified alcoholic solution of resorcin fuchsin and usually the counterstain is van Gieson; the results look very much like the Verhoeff-van Gieson. This procedure may be found in Vacca [1985].

1. In Europe, the Miller technique for elastic tissue is widely used. This is 1 of the modifications of the original Weigert resorcin fuchsin method. Miller elastic stain is composed of a mixture of Victoria blue 4R, new fuchsin, and crystal violet dissolved in water, to which the following is added in order: resorcin, dextrin, and ferric chloride. Finally hydrochloric acid is added. This procedure was described by Luna [1992].

## Russell modification of the Movat pentachrome stain [Luna 1992]

### Purpose

The demonstration of mucin, fibrin, elastic fibers, muscle, and collagen.

### Principle

Acidic mucosubstances are stained by alcian blue. The alkaline alcohol solution that follows converts the alcian blue to monastral fast blue, which is insoluble. Complete conversion is necessary because alcian blue will be decolorized during the remainder of the procedure. Iron hematoxylin is used to stain the elastic fibers, which are then differentiated with ferric chloride (see the Verhoeff method). Sodium thiosulfate removes any residual iodine. Crocein scarlet and acid fuchsin are acid dyes that stain muscle, cell cytoplasm, collagen, and ground substance. Phosphotungstic acid differentiation removes the stain from the collagen and ground substance. The acetic acid removes the phosphotungstic acid, and collagen is then counterstained with alcoholic safran.

### Fixative

10% neutral buffered formalin is preferred.

### Equipment

Coplin jars, Erlenmeyer flasks, graduated cylinders, pipettes.

### Technique

Cut paraffin sections at 4-5  $\mu$ m.

### Quality control

Use a section of lung, skin, or colon.

### Reagents

| Alcian blue, 1% solution                                                                                 |        |
|----------------------------------------------------------------------------------------------------------|--------|
| Alcian blue-GS                                                                                           | 1 g    |
| Distilled water                                                                                          | 100 mL |
| Glacial acetic acid                                                                                      | 1 mL   |
| Mix well and store at room temperature.                                                                  |        |
| Alkaline alcohol solution                                                                                |        |
| Ammonium hydroxide                                                                                       | 10 mL  |
| Alcohol, 95%                                                                                             | 90 mL  |
| Prepare fresh.                                                                                           |        |
| Iodine-iodide solution                                                                                   |        |
| Iodine                                                                                                   | 2 g    |
| Potassium iodide                                                                                         | 4 g    |
| Distilled water                                                                                          | 100 mL |
| Add the iodine and potassium iodide to ~25 mL of water and mix until dissolved. Add the remaining water. |        |

### Absolute alcoholic hematoxylin, 10% solution

|                                                                 |        |
|-----------------------------------------------------------------|--------|
| Hematoxylin                                                     | 10 g   |
| Absolute alcohol                                                | 100 mL |
| Mix until dissolved. Cap tightly and store at room temperature. |        |

### Ferric chloride, 10% solution

|                                                    |        |
|----------------------------------------------------|--------|
| Ferric chloride                                    | 10 g   |
| Distilled water                                    | 100 mL |
| Mix until dissolved and store at room temperature. |        |

### Working hematoxylin solution

|                                     |       |
|-------------------------------------|-------|
| Absolute alcoholic hematoxylin, 10% | 25 mL |
| Absolute alcohol                    | 25 mL |
| Ferric chloride, 10% aqueous        | 25 mL |
| Iodine-iodide solution              | 25 mL |
| Prepare just before use.            |       |

### Ferric chloride, 2% (for differentiation)

|                               |       |
|-------------------------------|-------|
| Ferric chloride, 10% solution | 10 mL |
| Distilled water               | 40 mL |
| Prepare just before use.      |       |

### Sodium thiosulfate, 5% solution

|                                                    |        |
|----------------------------------------------------|--------|
| Sodium thiosulfate                                 | 5 g    |
| Distilled water                                    | 100 mL |
| Mix until dissolved and store at room temperature. |        |

### Crocein scarlet-acid fuchsin solution

#### Solution A (stock)

|                                                    |         |
|----------------------------------------------------|---------|
| Crocein scarlet                                    | 1 g     |
| Distilled water                                    | 99.5 mL |
| Glacial acetic acid                                | 0.5 mL  |
| Mix until dissolved and store at room temperature. |         |

#### Solution B (stock)

|                                                     |         |
|-----------------------------------------------------|---------|
| Acid fuchsin                                        | 0.1 g   |
| Distilled water                                     | 99.5 mL |
| Glacial acetic acid                                 | 0.5 mL  |
| Mix until dissolved, and store at room temperature. |         |

#### Working solution

|                          |       |
|--------------------------|-------|
| Solution A               | 40 mL |
| Solution B               | 10 mL |
| Prepare just before use. |       |

### Phosphotungstic acid, 5% solution

|                                                    |        |
|----------------------------------------------------|--------|
| Phosphotungstic acid                               | 5 g    |
| Distilled water                                    | 100 mL |
| Mix until dissolved and store at room temperature. |        |

### Alcoholic safran solution

|                                                                                                                                                               |        |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| Safran du Gatinais                                                                                                                                            | 6 g    |
| Absolute alcohol                                                                                                                                              | 100 mL |
| Place in an air-tight container, and mix well. Keep at 56°C-58°C for 48 hours, and keep tightly closed in a brown bottle to prevent hydration or evaporation. |        |



**Procedure**

1. Deparaffinize and hydrate to distilled water
2. Stain in alcian blue for 20 minutes
3. Wash in running tap water for 5 minutes
4. Place slides in alkaline alcohol for 30 minutes
5. Wash in running tap water for 10 minutes
6. Rinse in distilled water
7. Stain in working hematoxylin solution for 15 minutes
8. Rinse in several changes of distilled water
9. Differentiate in 2% aqueous ferric chloride until the elastic fibers contrast sharply with the background
10. Rinse in distilled water
11. Place slides in sodium thiosulfate for 1 minute
12. Wash in running tap water for 5 minutes; rinse in distilled water
13. Stain in Crocein scarlet-acid fuchsin solution for 1 minute
14. Rinse in several changes of distilled water
15. Rinse in 0.5% acetic acid solution for 30 seconds
16. Place slides in 5% phosphotungstic acid solution, 2 changes of 5 minutes each
17. Rinse in 0.5% acetic acid solution
18. Rinse in 3 changes of absolute alcohol
19. Stain in alcoholic safran solution for 15 minutes
20. Rinse in 3 changes of absolute alcohol
21. Clear in 2 or 3 changes of xylene and mount with synthetic resin

**Results i8.16, i8.17**

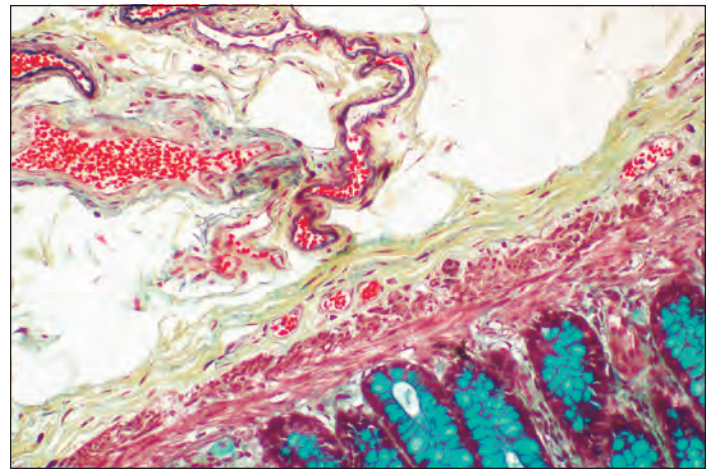
|                            |             |
|----------------------------|-------------|
| Nuclei and elastic fibers  | Black       |
| Collagen                   | Yellow      |
| Ground substance and mucin | Blue        |
| Fibrinoid, fibrin          | Intense red |
| Muscle                     | Red         |

**Technical notes**

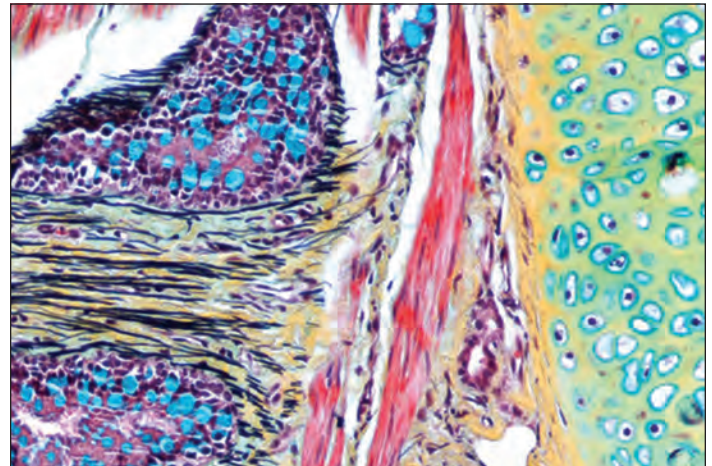
1. Allison [2014] prepares the stock Crocein scarlet (solution A) as follows:

|                     |         |
|---------------------|---------|
| Crocein scarlet MOO | 0.1 g   |
| Distilled water     | 99.5 mL |
| Glacial acetic acid | 0.5 mL  |

She recommends staining with the Crocein scarlet MOO-acid fuchsin working solution for 90 seconds to 3 minutes depending on desired redness **i8.17**.

**i8.16**

A section of colon stained with the Movat pentachrome procedure. Note the blue stained mucin in the goblet cells, red stained smooth muscle, yellow stained collagen, and black elastic fibers.

**i8.17**

A Movat pentachrome stained section, showing blue stained mucin, red smooth muscle, yellow collagen, and black elastic fibers. Cartilage is seen on the right [image courtesy of Akemi Allison].

2. Allison [2014] suggests pouring the used safran solution back into the stock bottle (will last ~6 months). Store the safran solution in a 60°C-65°C oven and also stain at this temperature. Use very pure Safran du Gatinais and keep tightly capped at all times. The heat helps give proper staining for collagen and reticular fibers.
3. The differentiation of the elastic fibers is usually complete in 2-3 minutes.
4. The complete removal of alkaline alcohol with running water is very important. Failure to remove all of the alkaline alcohol will inhibit the subsequent staining steps.
5. This stain also may be used to demonstrate *Cryptococcus neoformans*, staining the organism a bright blue.



**t8.1 Variations in the first 4 steps of 6 of the most common methods for reticulin**

| Reagent               | Snook                                                                                                          | Gordon & Sweet                                                                                                 | Gomori                                                                                                         | Laidlaw                                                                                                      | Nasher & Shanklin                                                                                        | Wilder                                                                                                         |
|-----------------------|----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|
| Oxidizer              | Potassium permanganate                                                                                         | Potassium permanganate                                                                                         | Potassium permanganate                                                                                         | Potassium permanganate                                                                                       | Potassium permanganate plus sulfuric acid                                                                | Phosphomolybdic acid                                                                                           |
| Sensitizer            | Uranyl nitrate                                                                                                 | Ferric ammonium sulfate                                                                                        | Ferric ammonium sulfate                                                                                        | None                                                                                                         | Silver nitrate                                                                                           | Uranyl nitrate                                                                                                 |
| Impregnating solution | Ammoniacal silver (silver nitrate plus sodium hydroxide, precipitate almost dissolved with ammonium hydroxide) | Ammoniacal silver (silver nitrate plus sodium hydroxide, precipitate almost dissolved with ammonium hydroxide) | Ammoniacal silver (silver nitrate plus sodium hydroxide, precipitate almost dissolved with ammonium hydroxide) | Lithium silver (silver nitrate plus lithium carbonate, precipitate almost dissolved with ammonium hydroxide) | Ammoniacal silver (ammonium hydroxide plus silver nitrate until slight turbidity; pyridine added at end) | Ammoniacal silver (silver nitrate plus sodium hydroxide, precipitate almost dissolved with ammonium hydroxide) |
| Reducing solution     | Formaldehyde                                                                                                   | Formaldehyde                                                                                                   | Formaldehyde                                                                                                   | Formaldehyde                                                                                                 | Formaldehyde plus absolute alcohol                                                                       | Formaldehyde plus uranyl nitrate                                                                               |

*The remaining steps (gold chloride, sodium thiosulfate, and counterstain) may vary only in reagent concentration or counterstain choice [table courtesy of NSH/CAP HQIP Final Critique 2004A]*

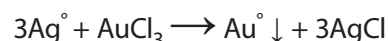
**Silver techniques for reticular fibers**

Many variations of silver techniques can be used for the demonstration of reticular fibers; the principles, however, are the same for most of the techniques, and closely resemble those of the PAS technique. The major steps are:

1. Oxidation of the adjacent glycol groups of the hexose sugars in reticulin to aldehydes. Reagents vary with the technique used, but the most frequently used oxidizers are phosphomolybdic acid, potassium permanganate, and periodic acid.
2. Sensitization, which usually is a metallic impregnation step. Impregnation is the deposition of metallic salts on or around, but not in, the tissue element to be demonstrated. The exact chemical reaction of sensitizers is not known, but according to Sheehan & Hrapchak [1980], the metallic salt used in this step probably forms a metal-organic compound with the reticulin. The sensitizing metal is then replaced by silver. Commonly used sensitizers are uranyl nitrate, ferric ammonium sulfate, and dilute solutions of silver nitrate.
3. Silver impregnation involves treating tissue with an ammoniacal or diamine silver complex,  $[\text{Ag}(\text{NH}_3)_2]^+$ . Kiernan [2008] states that 4 atoms of silver will be deposited at the site of each reactive sugar residue in the reticulin, and that the aldehyde groups present will reduce the diamine silver to metallic silver; however, this is not enough silver to provide adequate visibility. Further deposition occurs when incompletely washed sections are transferred to formaldehyde.
4. Reduction uses formaldehyde in all methods. Residual silver diamine ions are reduced to metallic silver by the formaldehyde. Kiernan [2008] states that metallic silver catalyzes the reaction, so the metal precipitates due to formaldehyde reduction mainly at the original sites of the sugar molecules of the reticulin. This further

deposition of silver by the formaldehyde reduction gives a highly visible precipitate. The reduction step in silver impregnation techniques is sometimes termed *developing*.

5. *Toning* is the term used when bound metallic silver is treated with gold chloride, and the color of the impregnated component is changed from brown to black. The metallic silver is replaced by metallic gold in the following reaction:



A more stable compound is formed, and section contrast and clarity are improved. The yellow color is removed from the background by this step; however, toning can be overdone and a violet to red background instead of the desired gray one will result.

6. Unreduced silver is removed by treating the sections with sodium thiosulfate (hypo). This step will prevent any nonspecifically bound silver remaining in the section from being reduced by later exposure to light.
7. A counterstain may or may not be used, depending on the type of tissue stained and personal preferences of the pathologists.

Only 2 representative, reliable methods for reticular fibers will be presented. Silver stains are notoriously capricious, and the method that consistently gives the best results in your laboratory should be used. If 1 of these methods does not work well in your hands, refer to the literature for other techniques. In my experience, the results of the 2 methods presented are consistently better than those obtained with either the Wilder or the Snook technique. It is very important that the amount of reticulin to be demonstrated in the control section be known and that it be checked carefully each time to determine how completely the reticulin is stained. A summary of the most common reticulin methods and the variations in the first 4 steps is given in **t8.1**.

**Gomori stain for reticular fibers** [Luna 1968]**Purpose**

The demonstration of reticular fibers in tissue sections can be important in the differential diagnosis of certain types of tumors. A change from the normal reticular fiber pattern, as seen in some liver diseases, is also an important diagnostic finding.

**Principle**

The hexose sugars of reticulin are demonstrated by oxidation to aldehydes. Potassium permanganate is the oxidizing agent in this procedure and the excess is removed by potassium metabisulfite. Ferric ammonium sulfate acts as the sensitizer and is subsequently replaced by silver from the diamine silver solution. Following impregnation, formalin is used to reduce the silver to its visible metallic form. Toning with gold chloride and removal of unreacted silver with sodium thiosulfate follow. The final step is to counterstain if desired.

**Fixative**

10% neutral buffered formalin is preferred.

**Equipment**

Nonmetallic forceps, chemically clean glassware (Coplin jars, graduated cylinders, Erlenmeyer flasks, and pipettes), filter paper.

**Technique**

Cut paraffin sections at 4-5  $\mu$ m.

**Quality control**

Liver is a very good control tissue.

**Reagents****Silver nitrate, 10% solution**

|                 |        |
|-----------------|--------|
| Silver nitrate  | 10 g   |
| Distilled water | 100 mL |

**Potassium hydroxide, 10% solution**

|                     |        |
|---------------------|--------|
| Potassium hydroxide | 10 g   |
| Distilled water     | 100 mL |

**Ammoniacal silver solution**

Combine 20 mL of 10% silver nitrate solution and 4-5 mL of 10% aqueous solution of potassium hydroxide. Add concentrated ammonium hydroxide, drop by drop, while shaking the container continuously, until the precipitate is completely dissolved. Cautiously add 10% silver nitrate solution, drop by drop, until 1 drop causes the solution to become permanently cloudy. Only a faint cloudiness is desirable. Measure the resulting solution, dilute with an equal amount of distilled water, and filter into a chemically clean Coplin jar.

**Potassium permanganate, 0.5% solution**

|                        |        |
|------------------------|--------|
| Potassium permanganate | 2.5 g  |
| Distilled water        | 500 mL |

**Potassium metabisulfite, 2% solution**

|                         |        |
|-------------------------|--------|
| Potassium metabisulfite | 10 g   |
| Distilled water         | 500 mL |

**Ferric ammonium sulfate, 2% solution**

|                         |        |
|-------------------------|--------|
| Ferric ammonium sulfate | 10 g   |
| Distilled water         | 500 mL |

**Formalin solution**

|                       |       |
|-----------------------|-------|
| Formaldehyde, 37%-40% | 10 mL |
| Distilled water       | 40 mL |

**Gold chloride, 0.2% solution**

|                                   |       |
|-----------------------------------|-------|
| Stock gold chloride solution (1%) | 10 mL |
| Distilled water                   | 40 mL |

**Sodium thiosulfate, 2% solution**

|                    |        |
|--------------------|--------|
| Sodium thiosulfate | 10 g   |
| Distilled water    | 500 mL |

**Nuclear fast red (Kernechtrot) solution**

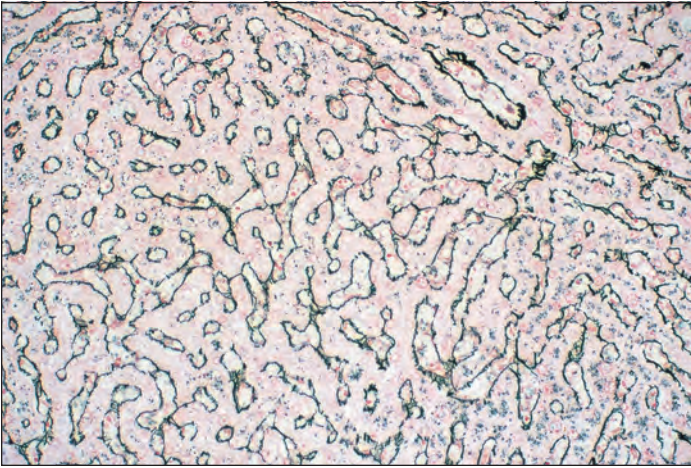
|                                |        |
|--------------------------------|--------|
| Nuclear fast red (Kernechtrot) | 0.5 g  |
| Aluminum sulfate               | 25 g   |
| Distilled water                | 500 mL |

Dissolve the aluminum sulfate in the distilled water and then dissolve the nuclear fast red in this solution using heat. Cool, filter, and add a few grains of thymol as a preservative.

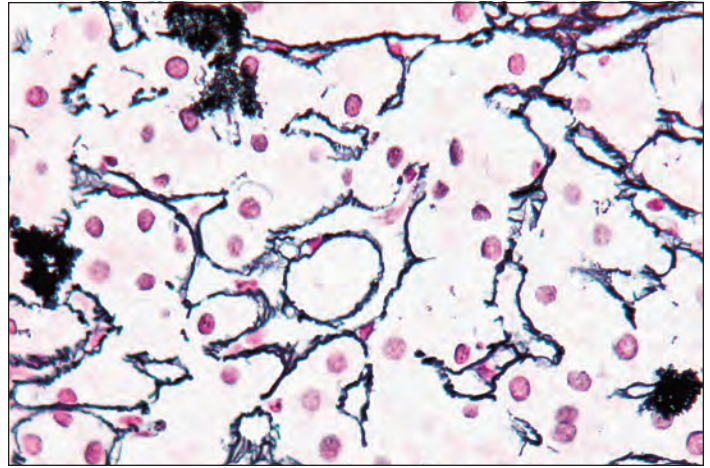
**Procedure**

1. Deparaffinize sections and hydrate to distilled water
2. Oxidize sections in 0.5% potassium permanganate solution for 1 minute
3. Rinse in tap water for 2 minutes
4. Differentiate in 2% potassium metabisulfite for 1 minute
5. Wash in tap water for 2 minutes
6. Sensitize sections in 2% ferric ammonium sulfate for 1 minute
7. Wash slides in tap water for 2 minutes followed by 2 changes of distilled water for 30 second each
8. Impregnate sections with the ammoniacal silver solution for 1 minute
9. Rinse in distilled water for 20 seconds
10. Reduce for 3 minutes in the 20% formalin solution
11. Wash in tap water for 3 minutes
12. Tone in 0.2% gold chloride solution for 10 minutes
13. Rinse in distilled water
14. Place sections in 2% potassium metabisulfite for 1 minute
15. Place sections in 2% sodium thiosulfate for 1 minute
16. Wash in tap water for 2 minutes
17. Counterstain if desired with nuclear fast red for 5 minutes

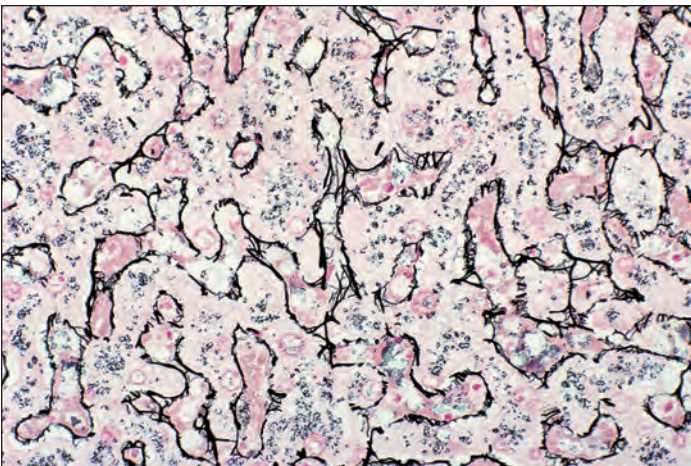


**i8.18**

A section of liver stained with the Gomori reticulin stain and lightly counterstained with nuclear-fast red. As in this photograph, the pattern should be readily assessed with the 10× objective.

**i8.20**

Marked precipitate is present in this reticulin stained liver section.

**i8.19**

The same section as shown in **i8.18** viewed with the 40× objective. The reticulin fibers stand out sharply in **i8.18**, **i8.19**. Some cellular organelles have stained black with the silver, and rare suggestions of silver precipitate can be seen in the sinusoids.

### Technical notes

1. It is important that a hint of turbidity remain in the silver solution. An excess of ammonia decreases the sensitivity and results in incomplete impregnation of reticular fibers.
2. The rinse between the diamine silver solution and the formaldehyde is critical for good reticulin demonstration. If the wash is prolonged, the staining of the reticulin will be reduced, and if it is insufficient, there will be excessive background staining.
3. The glassware must be chemically cleaned with commercial cleaning agents or bleach. The older method of using a mixture of sulfuric acid and potassium dichromate is not recommended because of the hazards involved in the preparation and use of this solution. Contaminated glassware, metallic instruments, salts from tap water, unfiltered silver solution, incorrect pH, and impregnation solution temperature incorrect [Brown 2009] are all possible causes of nonspecific silver staining and precipitation **i8.20**.
4. Metal forceps should not be used in any silver staining solutions. If chemicals are contaminated by metal forceps or metal measuring instruments, silver nitrate will be nonselectively precipitated onto the tissue [Swisher 1997]. Many types of plastic or coated (eg, Teflon) forceps are available commercially and should be used in this method. Metallic forceps can be coated with paraffin, but this is less than satisfactory because heated solutions or paraffin solvents must be avoided.
5. Some texts refer to the ferric ammonium sulfate as iron alum. Alum is a term used for salts that are double or bisulfates, such as potassium aluminum sulfate, ammonium aluminum sulfate, chromium potassium sulfate, and ferric ammonium sulfate.

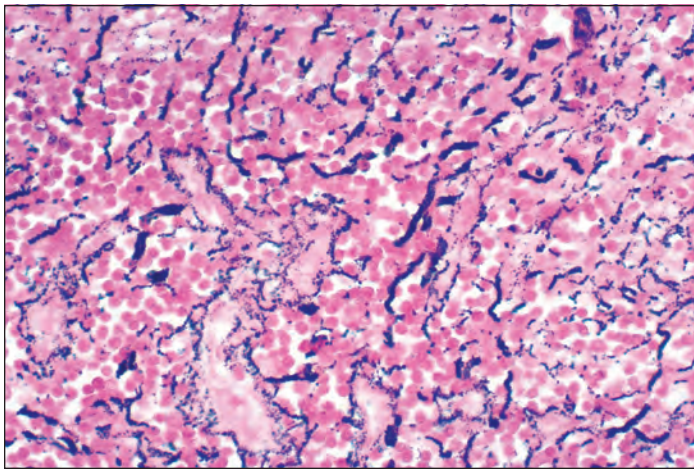
18. Wash well in tap water
19. Dehydrate in 95% and absolute alcohols
20. Clear in xylene and mount with synthetic resin

### Results **i8.18**, **i8.19**

|           |       |
|-----------|-------|
| Reticulin | Black |
| Collagen  | Taupe |

Other tissue elements reflect the counterstain used.





i8.21

A section of liver stained with the Snook reticulin stain [Sheehan 1980] shows granular, rather than linear, staining of the reticulin. This may be because of the use of old reagents, especially old uranium nitrate. This stain should be repeated.

6. If nuclear fast red is used as a counterstain, wash the slides well with water after staining. If the slides are not washed well, or are transferred from the counterstain directly to alcohol, they will develop a cloudiness that can be removed only by backing the slides up to water.
  7. Many times the pattern of reticular fiber staining is very important, as in liver biopsy specimens, and should be seen easily with the scanning lens of the microscope. The easy visualization of the reticulin pattern with the 10× objective is a guide to the quality of the stain. The counterstain, however, may obscure this easy visualization and therefore should not be used, or should be extremely light, when visualization of the pattern is important.
  8. If silver staining of the nuclei is a problem, this may be helped by using acetified potassium permanganate:
- |                              |         |
|------------------------------|---------|
| Sulfuric acid, 3%            | 2.5 mL  |
| Potassium permanganate, 0.5% | 47.5 mL |
9. When the ammonium hydroxide has lost strength because of the loss of ammonia from the solution, a fresh bottle should be opened. The decrease in strength is commonly noticed because more than the usual amount of ammonium hydroxide is required, and the reticulin may stain gray-black rather than a sharp black.
  10. Churukian [1993] used the ammoniacal silver solution for several days, storing it in the refrigerator, and bringing it to room temperature before use. He feels that the reticulin stains better after the ammoniacal silver has aged for a few days.
  11. The deposition of silver should be in a linear pattern. Stains exhibiting a granular deposition, as seen in i8.21, should be repeated.

12. Diamine silver is a very alkaline solution, with a pH in the range of 11-12, and sections will sometimes wash off the slides. It may be helpful to use special coated slides or charged slides for picking up the sections. If this is not sufficient, sections may be treated with celloidin before staining.
13. Ammoniacal silver solutions may form explosive compounds, so extreme care must be exercised in the preparation, use, and storage of these solutions. Storage of these solutions in the refrigerator inhibits the formation of explosive compounds; exposure to direct sunlight should be avoided.

### Gordon & Sweets stain for reticular fibers [Gordon 1936]

#### Purpose

The demonstration of reticular fibers in tissue sections can be important in the differential diagnosis of certain types of tumors. A change from the normal reticular fiber pattern, as seen in liver diseases such as cirrhosis, hepatocellular fibrosis, and/or necrotic liver disease, is also an important diagnostic finding.

#### Principle

The tissue is first oxidized by potassium permanganate to enhance subsequent staining of reticular fibers, and excess permanganate is removed by oxalic acid. Ferric ammonium sulfate acts as the sensitizer and is subsequently replaced by silver from the diamine silver solution. After impregnation, formalin is used to reduce the silver to its visible metallic form. Before toning with gold chloride, unreacted silver is removed with sodium thiosulfate. The final step is to counterstain if desired.

#### Fixative

10% neutral buffered formalin is preferred.

#### Equipment

Nonmetallic forceps, chemically clean glassware (Coplin jars, graduated cylinders, Erlenmeyer flasks, and pipettes), filter paper.

#### Technique

Cut paraffin sections at 4-5 μm.

#### Quality control

Liver is a very good control tissue.

#### Reagents

##### Silver nitrate, 10% solution

|                 |        |
|-----------------|--------|
| Silver nitrate  | 10 g   |
| Distilled water | 100 mL |

##### Sodium hydroxide, 3% solution

|                  |        |
|------------------|--------|
| Sodium hydroxide | 3 g    |
| Distilled water  | 100 mL |

**Ammoniacal silver solution**

Place 5 mL of 10% silver nitrate solution in an Erlenmeyer flask and add concentrated ammonium hydroxide, drop by drop, while shaking the container continuously, until the precipitate that forms is completely dissolved. Do not add any excess ammonium hydroxide. Add 5 mL of 3% sodium hydroxide solution and cautiously redissolve the precipitate with concentrated ammonium hydroxide until only a faint cloudiness remains. If this step is carried too far and no cloudiness remains, add 10% silver nitrate solution, drop by drop, until 1 drop causes the solution to become permanently cloudy. Only a faint cloudiness is desirable. Dilute the resulting solution to 50 mL with distilled water, and filter into a chemically clean Coplin jar.

**Potassium permanganate, 1% solution**

|                        |        |
|------------------------|--------|
| Potassium permanganate | 1 g    |
| Distilled water        | 100 mL |

**Oxalic acid, 1% solution**

|                 |        |
|-----------------|--------|
| Oxalic acid     | 1 g    |
| Distilled water | 100 mL |

**Ferric ammonium sulfate, 2.5% solution**

|                         |        |
|-------------------------|--------|
| Ferric ammonium sulfate | 2.5 g  |
| Distilled water         | 100 mL |

**Formalin, 10% solution**

|                       |       |
|-----------------------|-------|
| Formaldehyde, 37%-40% | 10 mL |
| Distilled water       | 90 mL |

**Gold chloride, 0.2% solution**

|                                   |       |
|-----------------------------------|-------|
| Stock gold chloride solution (1%) | 10 mL |
| Distilled water                   | 40 mL |

**Sodium thiosulfate, 5% solution**

|                    |        |
|--------------------|--------|
| Sodium thiosulfate | 5 g    |
| Distilled water    | 100 mL |

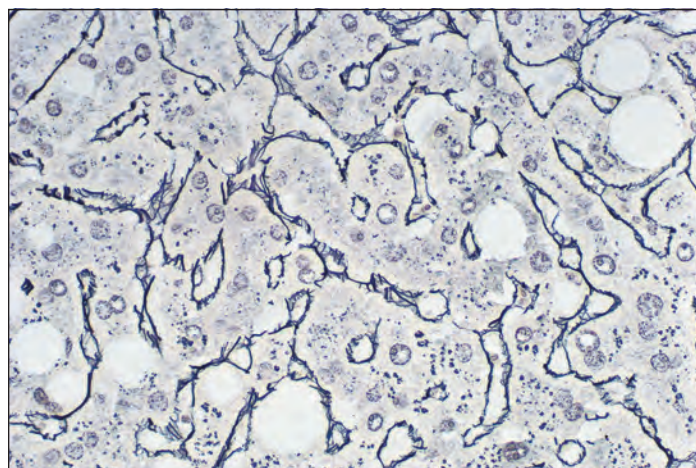
**Nuclear fast red (Kernechtrot) solution**

|                                |        |
|--------------------------------|--------|
| Nuclear fast red (Kernechtrot) | 0.5 g  |
| Aluminum sulfate               | 25 g   |
| Distilled water                | 500 mL |

Dissolve the aluminum sulfate in the distilled water and then dissolve the nuclear fast red in this solution using heat. Cool, filter, and add a few grains of thymol as a preservative.

**Procedure**

1. Deparaffinize sections and hydrate to distilled water
2. Oxidize sections in 1% potassium permanganate solution for 5 minutes
3. Rinse in tap water for 2 minutes
4. Bleach in 1% oxalic acid for 2 minutes or until sections are colorless
5. Wash in tap water for 2 minutes
6. Sensitize sections in 2.5% ferric ammonium sulfate for at least 15 minutes
7. Wash in several changes of distilled water

**i8.22**

A section of liver stained with the Gordon and Sweets technique. No counterstain has been applied, the reticular network lining the sinusoids is well demonstrated, and the reticulin pattern can be evaluated easily with lower magnification.

8. Impregnate sections with the silver solution for 2 minutes
9. Rinse well with distilled water
10. Reduce sections for 2 minutes in the 10% formalin solution
11. Wash in tap water for 3 minutes
12. Tone in 0.2% gold chloride solution for 10 minutes
13. Wash in tap water for 2 minutes
14. Place slides in 5% sodium thiosulfate for 1 minute
15. Rinse in distilled water
16. Counterstain, if desired, with nuclear fast red for 5 minutes
17. Wash well in distilled water
18. Dehydrate in 2 changes each of 95% and absolute alcohols, clear in xylene, and mount with synthetic resin

**Results i8.22**

Reticulin

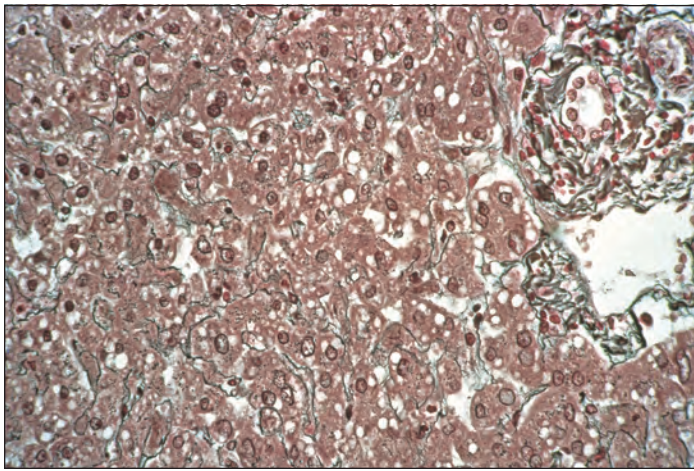
Black

Other tissue elements depend on counterstain used.

**Technical notes**

1. See the technical notes under the Gomori technique for reticular fibers.
2. The Gordon and Sweets method gives much less background and nuclear staining than most of the more common methods.





i8.23

This is an example of a poor reticulin stain. The reticulin pattern is totally obscured by the strong counterstaining. The stain is unacceptable and should be repeated.

- Silver stains are capricious, and when poor stains are obtained [i8.20](#), [i8.21](#), [i8.23](#), the stain should be repeated. All fresh reagents should be used, and particular attention should be paid to the preparation of the diamine silver solution.
- The 2 methods presented do not use uranyl nitrate, which has been banned in some laboratories because of its toxicity.

## Staining techniques for muscle

### Mallory PTAH technique for cross-striations & fibrin

[Mallory 1942, Carson 1984, Sheehan 1980]

#### Purpose

The demonstration of muscle cross-striations and fibrin. Cross-striations are a diagnostic feature of rhabdomyosarcomas or tumors arising from striated muscle. Nemaline rods, present in some skeletal muscle diseases, may also be demonstrated by the method. PTAH has also been used for the demonstration of glial fibers and myelin. This method is rarely used today because it has been replaced by immunohistochemical techniques.

#### Principle

The amount of phosphotungstic acid in the staining solution is far greater than the amount of hematein (20:1), and it is believed that tungsten binds all available hematein to give a blue lake. This metal-hematein lake stains selected tissue components blue, while the phosphotungstic acid is thought to stain the red-brown components. This stain has been referred to as a polychrome stain because 1 solution gives 2 major colors. The components colored red-brown will lose this color with water or prolonged alcohol washes, and therefore dehydration of the section after staining must be rapid.

#### Fixative

Zenker solution is preferred, but 10% neutral buffered formalin may be used.

#### Equipment

Mechanical stirrer, Coplin jars, Erlenmeyer flasks, graduated cylinders.

#### Technique

Cut paraffin sections at 4–6  $\mu\text{m}$ .

#### Quality control

Use longitudinal sections of skeletal or cardiac muscle to demonstrate cross-striations, a section containing fibrin for the demonstration of fibrin.

#### Reagents

##### PTAH solution

|                      |          |
|----------------------|----------|
| Hematoxylin          | 1 g      |
| Phosphotungstic acid | 20 g     |
| Distilled water      | 1,000 mL |

Dissolve the solid ingredients in separate portions of water, dissolving the hematoxylin with the aid of heat. When cool, combine. No preservative is necessary. The solution that is allowed to ripen naturally (~4–6 months) is a better stain and lasts longer, but if time is not available for natural ripening, 0.2 g of potassium permanganate may be added to the solution. This chemically ripened stain may be used immediately, but the best results are not obtained until the solution has been ripened for at least 2 weeks.

##### Gram iodine

|                  |        |
|------------------|--------|
| Iodine           | 3 g    |
| Potassium iodide | 6 g    |
| Distilled water  | 900 mL |

Place the iodine, potassium iodide, and ~150 mL of the water in a flask and stir until dissolved. Add remaining water.

##### Sodium thiosulfate, 5% solution

|                    |        |
|--------------------|--------|
| Sodium thiosulfate | 5 g    |
| Distilled water    | 100 mL |

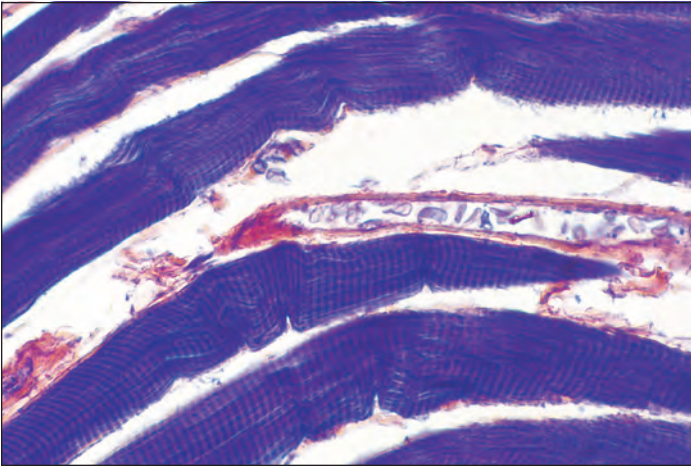
##### Potassium permanganate, 0.25% solution

|                        |        |
|------------------------|--------|
| Potassium permanganate | 0.25 g |
| Distilled water        | 100 mL |

##### Oxalic acid, 5% solution

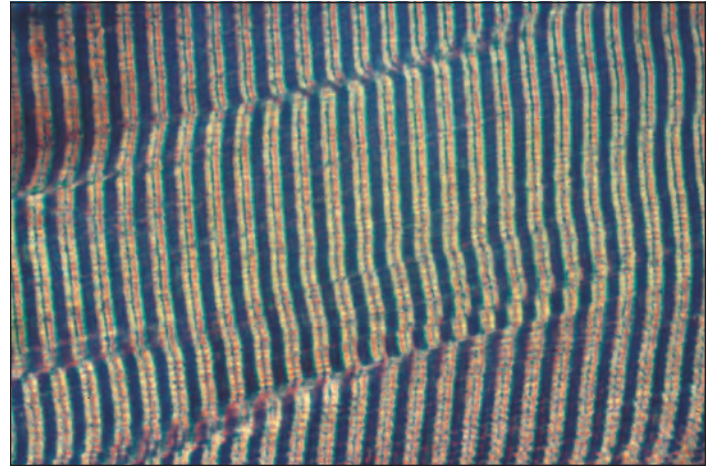
|                 |        |
|-----------------|--------|
| Oxalic acid     | 5 g    |
| Distilled water | 100 mL |





i8.24

A section of Zenker fixed skeletal muscle stained with the Mallory phosphotungstic acid-hematoxylin (PTAH) technique.



i8.25

A very high magnification of a section of Zenker fixed skeletal muscle stained with the Mallory PTAH technique. The A- and I-bands are well demonstrated, and the Z-lines can also be seen. The stain is rarely used today for the identification of cross-striation in tumors thought to be rhabdomyosarcomas; immunohistochemical staining is now preferred.

### Procedure

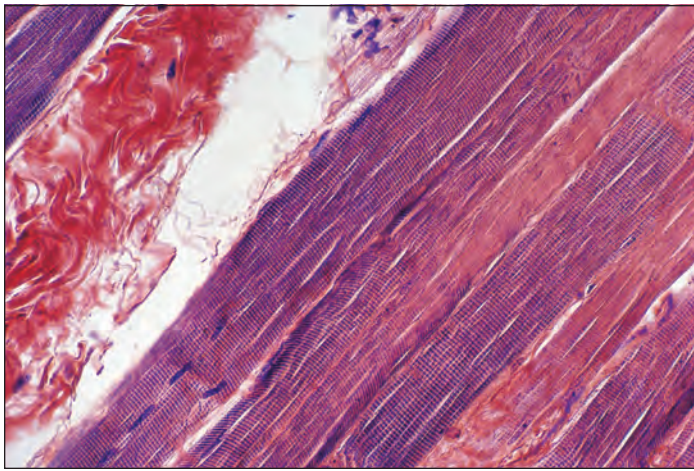
1. Deparaffinize sections and hydrate to distilled water
2. If sections are formalin fixed, mordant in Zenker fixative containing 5% acetic acid overnight at room temperature
3. Rinse in tap water
4. Place in Gram iodine for 15 minutes
5. Rinse in tap water
6. Place in 5% aqueous sodium thiosulfate for 3 minutes
7. Wash in tap water for 10 minutes
8. Place sections in 0.25% potassium permanganate for 5 minutes
9. Rinse in tap water
10. Place in 5% oxalic acid for 1 minute
11. Wash in running tap water for 10 minutes
12. Stain in PTAH solution overnight at room temperature
13. Dehydrate rapidly through 2 changes each of 95% and absolute alcohol, clear in xylene and mount with synthetic resin

### Technical notes

1. A chemically oxidized staining solution has a shorter shelf-life than a naturally ripened staining solution, because chemical oxidation may cause overoxidation. When this occurs, there is a failure to show the proper density of the blue tones. Solutions should be stored in amber glass bottles to retard overoxidation by light.
2. According to Meloan [1988], thorough washing of sections before staining is essential, because hydration of the tissue structures will greatly facilitate uptake of dye molecules. They also state that sodium thiosulfate interferes with binding of the PTAH, so sections should be washed very well after application of sodium thiosulfate.
3. Bouin solution will also work as a mordant. It should be used for 1 hour at 60°C, and then the sections should be washed in running water until the yellow color is gone. Skip the iodine step. This gives better results than mordanting in Zenker solution, or with the chromate method that follows [Wenk 2007]. Fixation in zinc formalin may also provide satisfactory PTAH staining, but I have not tried this variant.

### Results i8.24, i8.25

|                          |           |
|--------------------------|-----------|
| Cross-striations, fibrin | Blue      |
| Nuclei                   | Blue      |
| Collagen                 | Red-brown |



i8.26

A section of formalin fixed skeletal muscle stained with PTAH. The sections were mordanted overnight in Zenker fixative. This uneven staining appears to be characteristic of formalin fixed tissue. Note that the collagen is stained red in i8.24, i8.25, i8.26. This is an important differentiation in correctly performed PTAH staining.

4. Fortunately, the PTAH technique has been largely replaced by immunohistochemical methods; although fibrin will stain very well after formaldehyde fixation, we consistently get better results when staining for muscle cross-striations if the tissue is mordanted, or postfixed, in a mercuric solution. The stain is not as good when formalin fixed sections are mordanted as when the original fixative is mercuric; very uneven staining frequently occurs in tissue originally fixed in formalin i8.26. Stevens and Wilson [1996] described the following technique, which avoids mercuric solutions and uses acidic dichromate treatment instead.

### PTAH without mercuric solutions

#### Reagents

##### Acidic dichromate solution

|                                            |       |
|--------------------------------------------|-------|
| Hydrochloric acid, 10% in absolute alcohol | 12 mL |
| Potassium dichromate, 3% aqueous solution  | 36 mL |

##### Acidic potassium permanganate solution

|                                               |        |
|-----------------------------------------------|--------|
| Potassium permanganate, 0.5% aqueous solution | 50 mL  |
| Sulfuric acid, 3% solution                    | 2.5 mL |

##### Oxalic acid, 1% solution

|                 |        |
|-----------------|--------|
| Oxalic acid     | 1 g    |
| Distilled water | 100 mL |

#### Procedure

1. Deparaffinize sections and hydrate to distilled water
2. Place slides in acidic dichromate solution for 30 minutes
3. Wash in tap water
4. Place slides in acidic permanganate solution for 1 minute

5. Wash in tap water
6. Bleach sections in 1% oxalic acid solution
7. Rinse in tap water
8. Stain in PTAH solution overnight
9. Dehydrate rapidly through 95% and absolute alcohols, clear in xylene, and mount with synthetic resin

The results of this stain should be the same as those described for the technique using mercuric solutions for either primary fixation or postfixation mordanting.

#### Technical note

Potassium dichromate is a hazardous chemical that is toxic on inhalation and ingestion. It is corrosive to eyes, skin, and mucous membranes, and is also a carcinogen; however, with care, the solutions pose little risk when used under normal conditions. It cannot be put down the drain.

### Staining technique for basement membranes

#### Periodic acid-methenamine silver microwave procedure for basement membranes [Brinn 1983, Sheehan 1980]

#### Purpose

This procedure best delineates basement membranes, and is most often used in the histopathology laboratory for the detection of abnormalities or diseases manifested in the glomerular basement membrane.

#### Principle

The carbohydrate component of basement membranes is oxidized to aldehydes by periodic acid. The aldehydes formed by oxidation bind the silver ions from the methenamine silver complex and reduce the silver to its metallic form. Methenamine gives the solution the alkaline properties necessary for the proper reaction, and the sodium borate acts as a buffer. Toning is with gold chloride, and any unreduced silver is removed by sodium thiosulfate.

#### Fixative

10% neutral buffered formalin is preferred. Mercury-containing fixatives are not recommended.

#### Equipment

Microwave oven or 50°C-60°C water bath, vented plastic staining jars, graduated cylinders, Erlenmeyer flasks.

#### Technique

Paraffin sections cut at 2  $\mu$ m.

#### Quality control

Kidney has an internal control. No other control slide is necessary.



## Reagents

### Stock methenamine silver

|                                         |        |
|-----------------------------------------|--------|
| Methenamine, 3% aqueous (15 g/500 mL)   | 400 mL |
| Silver nitrate, 5% aqueous (5 g/100 mL) | 20 mL  |

Mix and keep solution refrigerated at 4°C.

### Borax (sodium borate), 5% solution

|                 |        |
|-----------------|--------|
| Sodium borate   | 5 g    |
| Distilled water | 100 mL |

### Working methenamine silver solution

|                                    |       |
|------------------------------------|-------|
| Stock methenamine silver           | 25 mL |
| Distilled water                    | 25 mL |
| Borax (sodium borate), 5% solution | 2 mL  |

Prepare just before use.

### Periodic acid, 1% solution

|                 |        |
|-----------------|--------|
| Periodic acid   | 1 g    |
| Distilled water | 100 mL |

### Sodium thiosulfate, 2% solution

|                    |        |
|--------------------|--------|
| Sodium thiosulfate | 10 g   |
| Distilled water    | 500 mL |

### Gold chloride, 0.02% solution

|                            |       |
|----------------------------|-------|
| Gold chloride, 1% solution | 1 mL  |
| Distilled water            | 49 mL |

### Stock light green, 0.2% solution

|                            |        |
|----------------------------|--------|
| Light green SF (yellowish) | 1 g    |
| Distilled water            | 500 mL |
| Glacial acetic acid        | 1 mL   |

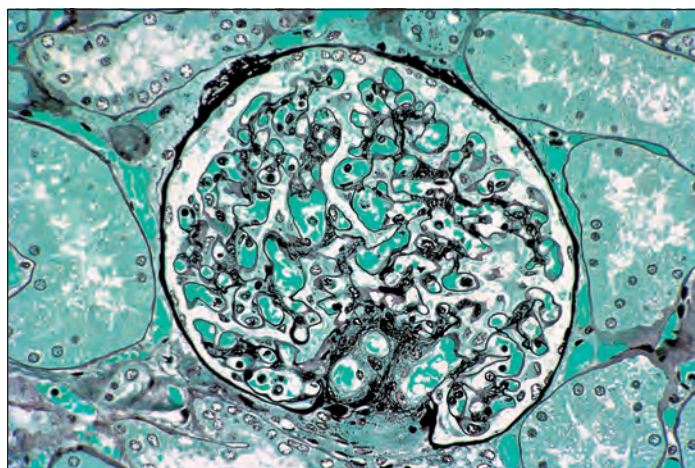
### Working light green solution

|                            |       |
|----------------------------|-------|
| Light green stock solution | 10 mL |
| Distilled water            | 50 mL |

## Procedure

This procedure is for 5 slides. If you do not have 5 slides, then include blank slides. Do not use >5 slides. This procedure should be followed exactly for optimum results.

1. Deparaffinize sections and hydrate to distilled water
2. Place sections in 1% periodic acid solution for 15 minutes at room temperature
3. Rinse in distilled water
4. Place slides (5) in a vented plastic Coplin jar containing 50 mL of methenamine working solution.\* Apply the vented cap and place in the microwave oven. Also place a vented plastic Coplin jar containing exactly 50 mL (measured) of distilled water in the oven. Microwave on full power for exactly 70 seconds (see technical note 2). Remove both jars from the oven, mix the staining solution with a plastic Pasteur pipette, and let stand on the counter. Check the slides frequently until the desired staining intensity is achieved. This will take ~15-20 minutes.
5. Rinse slides in the heated distilled water



**i8.27**

A kidney glomerulus stained with a periodic acid-methenamine silver technique. A good stain should look almost as if the basement membrane had been drawn with ink. Although the tubular basement membranes are well stained, they should not be used to judge the end point of the impregnation; use the glomerular basement membrane only.

6. Tone sections in 0.02% gold chloride\*
7. Rinse slides in distilled water
8. Treat sections with 2% sodium thiosulfate for 1 minute
9. Wash in tap water
10. Counterstain in the working light green solution for 1½ minutes
11. Dehydrate with 2 changes each of 95% and absolute alcohols
12. Clear with xylene and mount with synthetic resin

\*If for some reason, the microwave oven cannot be used, substitute the following solutions and staining times:

### Methenamine silver solution

|                                                                       |       |
|-----------------------------------------------------------------------|-------|
| Stock methenamine silver solution                                     | 50 mL |
| Borax, 5% solution                                                    | 5 mL  |
| Preheat the solution and stain slides at 56°C-60°C for 40-90 minutes. |       |

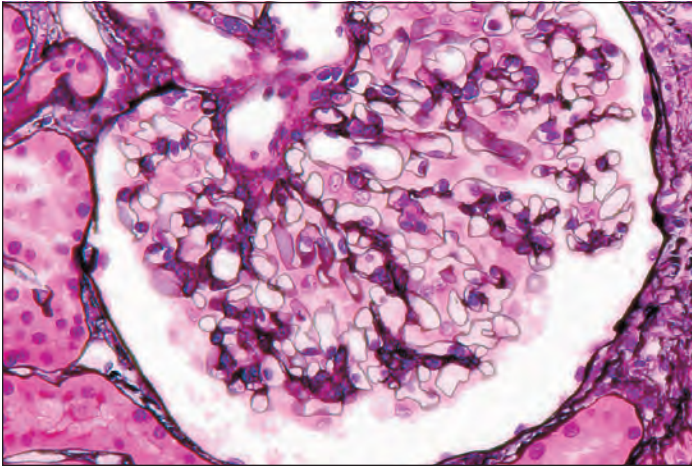
### Gold chloride, 0.2% solution

|                                  |       |
|----------------------------------|-------|
| Gold chloride, 1% solution       | 10 mL |
| Distilled water                  | 40 mL |
| Tone for 30 seconds to 1 minute. |       |

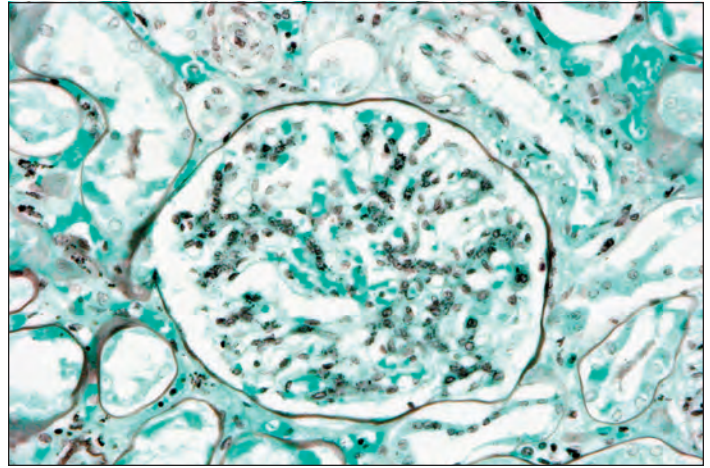
## Results i8.27

|                   |       |
|-------------------|-------|
| Basement membrane | Black |
| Background        | Green |

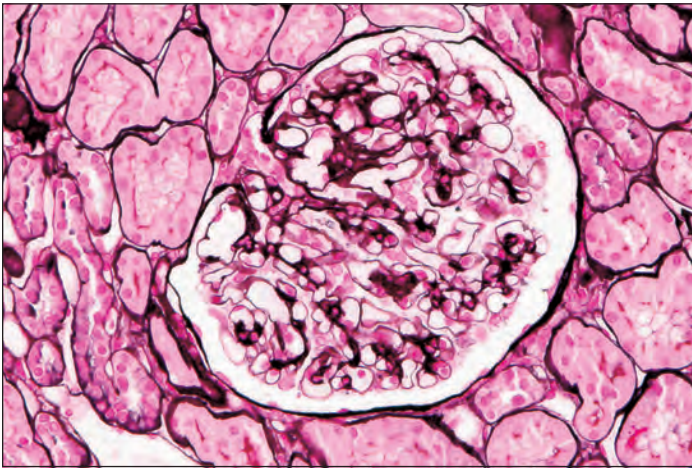


**i8.28**

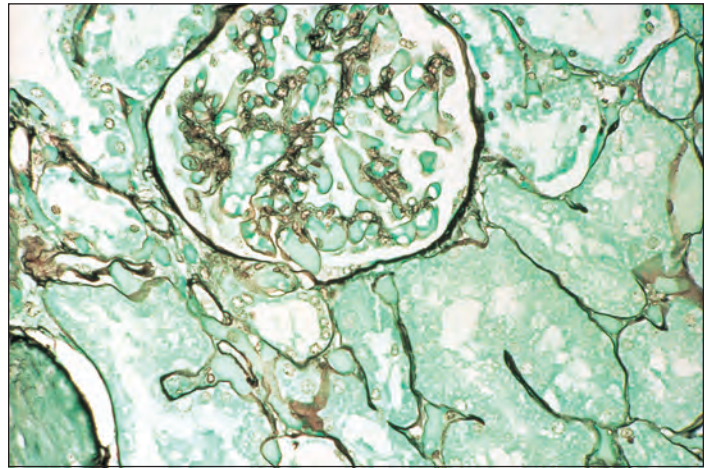
**i8.28** A kidney glomerulus stained with the Jones periodic acid-methenamine silver technique. Hematoxylin & eosin are the counterstains.

**i8.30**

Only the glomerular basement membrane should be used to determine the endpoint of staining. Although many tubular basement membranes are well stained in this section, no staining is seen in the glomerular basement membrane, and the stain is worthless.

**i8.29**

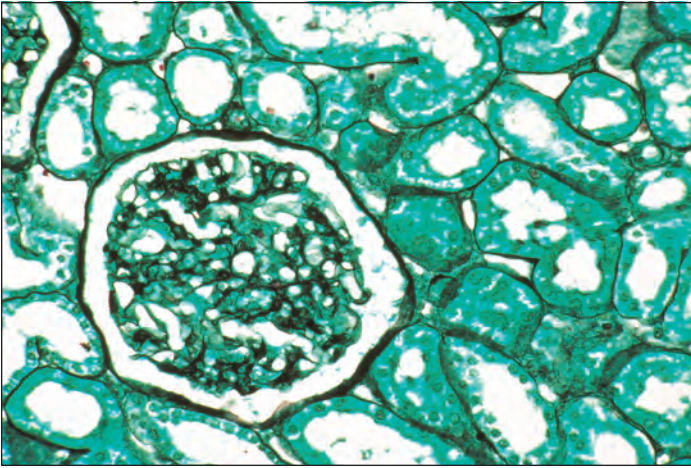
A section of kidney stained with the Jones periodic acid-methenamine silver technique and counterstained with nuclear fast red.

**i8.31**

This kidney glomerulus has been understained with a periodic acid-methenamine silver technique. Compare the staining here with that in **i8.27**. Note that the basement membrane exhibits continuous staining in **i8.27** and interrupted staining here.

### Technical notes

1. This nonmicrowave technique is a modification of the Jones method [1951], which heats the methenamine silver in a 60°C-70°C water bath for ~60-75 minutes with periodic checks on the depth of staining. When the basement membranes show a medium brown color, remove from the silver and rinse in 70°C distilled water. The other difference is in the counterstain used, usually hematoxylin and eosin **i8.28**. Nuclear fast red may also be used **i8.29**.
2. Sharper staining of the basement membrane and less background staining can be obtained with the use of the microwave oven for silver techniques.
3. The temperature is critical and should be just below boiling or ~95°C, immediately after removal from the microwave oven. Each oven should be calibrated for the time required to reach the correct temperature.
4. This is a very difficult stain to perform correctly. Although the tubular membranes will stain, the endpoint should be determined by the glomerular basement only **i8.30**. When well stained, the glomerular basement membrane should appear as a continuous black line. Stopping the silver impregnation too soon will result in uneven or interrupted staining **i8.31**. The application



i8.32

Too much counterstain has been applied to this section of kidney, and the periodic acid-methenamine silver staining is masked. The section is also too thick; sections should be 2  $\mu\text{m}$  thick for demonstration of the glomerular basement membrane.

of too much counterstain will mask the silver stain and decrease contrast **i8.32**.

- As with all silver stains, chemically cleaned glassware and plastic forceps should be used.
- The PAS technique is also used to stain basement membranes and can be found in Chapter 7, "Carbohydrates & Amyloid."

### Staining techniques for lipid

Because the lipid stains requested in the routine histopathology laboratory are for simple lipids, only a selection of techniques for simple fats will be presented.

#### Oil red O method for neutral fats [Pearse 1968]

##### Purpose

The demonstration of neutral lipids in frozen tissue sections. Fat occurring in an abnormal place such as fatty emboli that may develop after either a bone fracture or an injury that crushes a fatty body area may be demonstrated. The fat stain may verify that the emboli caused death. Degenerating material containing fat, such as cell membranes or myelin, may coalesce into fat droplets that are demonstrable with fat stains, and tumors arising from fat cells (liposarcomas) can be differentiated from other types of tumors.

##### Principle

Staining with oil soluble dyes is based on the greater solubility of the dye in the lipid substances than in the usual hydroalcoholic dye solvents. This is a physical method of staining and the dye used must (1) be more soluble in the tissue lipid than in the solvent in which it is dissolved, (2) not be water soluble, (3)

be strongly colored, and (4) act with tissue constituents only by solution. The solvent used is critical, with isopropanol removing a minimal amount of lipid and propylene glycol not extracting any lipid.

##### Fixative

10% neutral buffered formalin or calcium-formol. Because of the lipid-dissolving ability, no alcoholic fixative should be used.

##### Equipment

Cryostat, Coplin jars, Erlenmeyer flasks, graduated cylinders, filter paper.

##### Technique

Cut frozen sections at 10  $\mu\text{m}$ . Paraffin sections cannot be used because dehydrating and clearing agents dissolve the fat. If free-floating sections are not used, then sections of fixed tissue should be picked up on coated, charged, or subbed slides.

##### Quality control

Most tissue contains some fat, so normally a control is not used.

##### Reagents

###### Oil red O stock solution

|                  |        |
|------------------|--------|
| Oil red O        | 2.5 g  |
| Isopropanol, 98% | 500 mL |
| Mix well.        |        |

###### Oil red O working solution

|                           |       |
|---------------------------|-------|
| Oil red O, stock solution | 24 mL |
| Distilled water           | 16 mL |

Mix well and let stand for 10 minutes. Filter. The filtrate can be used for several hours.

##### Procedure

- Cut frozen sections, fix in 40% formaldehyde for 1 minute, and wash well in tap water; blot off excess water; if the tissue has been previously fixed, there is no need to refix
- Stain sections in oil red O for 10 minutes
- Wash sections in tap water
- Stain for 1 minute in Harris hematoxylin containing acetic acid (glacial acetic acid, 2 mL/hematoxylin, 48 mL)
- Wash section in tap water
- Blue in ammonia water
- Wash in tap water
- Mount sections with an aqueous mounting medium
- Seal the edges of the coverslip with fingernail polish



# Immunohistochemistry

## Chapter 12

### Objectives

On completing this chapter, the student should be able to do the following:

1. Define:
  - a. antigen
  - b. antibody
  - c. substrate
  - d. chromogen
  - e. fluorochrome
  - f. epitope
  - g. secondary antibody
  - h. multilink secondary antiserum
2. Differentiate between polyclonal antisera and monoclonal antibodies
3. Explain each of the following immunohistochemical techniques:
  - a. direct
  - b. indirect
  - c. peroxidase-antiperoxidase
  - d. avidin-biotin complex
  - e. polymeric detection system
4. List 2 fluorochromes
5. Identify 2 common chromogens used with immunoperoxidase techniques
6. Identify 2 chromogens used with immunoalkaline phosphatase techniques
7. Identify at least 3 enzyme systems used in enzyme immunohistochemistry
8. Describe the preferred method of specimen preparation for immunofluorescence
9. Identify problems that may be encountered with formaldehyde fixation of specimens for immunohistochemical staining
10. Identify fixatives other than formalin and describe any special issues with respect to immunohistochemistry
11. Describe 2 blocking reactions and identify the purpose of each
12. Identify the 5 heavy chains and 2 light chains that make up the different classes of immunoglobulins
13. Describe the use of negative tissue and reagent controls
14. List at least 6 problems that may occur with immunohistochemical staining and state a corrective action for each
15. State why preparing positive controls in the laboratory is preferred to purchasing them
16. List 2 methods of epitope retrieval
17. List at least 3 solutions that have been used for heat induced epitope retrieval
18. List at least 3 ways of heating in heat induced epitope retrieval methods (HIER)
19. List at least 3 solutions that have been used for enzyme induced epitope retrieval (EIER)
20. Identify alcohol soluble and nonalcohol soluble chromogens
21. List 4 chemicals used to intensify the 3,3'-diaminobenzidine (DAB) reaction
22. List common antibodies and the tumors they are used to identify



In the clinical laboratory, immunohistochemistry is a heavily used technique for assisting the pathologist in making a diagnosis. Immunohistochemistry is used to determine the origin of a tumor, prognosis, and treatment. It is important to understand the basics of immunology and the reagents and methods used, to achieve reproducible, high-quality patient care. Rigorous quality control (QC) is vital to ensure that results are accurate and consistent with expected staining patterns. The major role an immunohistochemist plays is troubleshooting for variables introduced by fixation, processing, and tissue types. The immunohistochemist should be familiar with both the controls used and the staining patterns of an antibody to troubleshoot unexpected results.

Techniques such as immunofluorescence and enzyme immunoassays have been available as diagnostic tools for over 50 years, but the development of immunodiagnostic methods, including immunohistochemistry, was much slower because of the unpredictable quality of polyclonal antibodies.

Since the immunohistochemistry revolution began in the 1980s, a number of dramatic advances have been made in immunology and immunodiagnostic methods; these advances have had a substantial impact on all phases of laboratory medicine. In most laboratories, the use of immunohistochemical stains has become as routine as the use of any other special stain, and in many instances, these stains have completely replaced the older histochemical or empirical methods.

The development of immunohistochemical staining standards and guidelines set by the College of American Pathologists (CAP) is related to predictive markers that are used for therapeutic patient care, and which qualify the patient for specific drug treatment. The laboratory's role is pivotal in ensuring that all specimens are processed and treated according to the recommended guidelines.

## General immunology

The immune system exerts its control through humoral (antibody) and cellular components. The overview of the immune system presented here will emphasize only the humoral or antibody system and how it relates to the discussion of immunohistochemical techniques.

### Antibody

An antibody is a host protein (immunoglobulin) produced in response to the presence of foreign molecules, organisms, or other agents in the body. Antibodies, commonly known as immunoglobulins, are proteins that are produced by B lymphocytes in response to antigenic stimulation. An immunoglobulin is a "Y"-shaped protein molecule that is composed of both heavy and light chains. Classes of antibodies differ in structure and function, with each immunoglobulin antigenically distinct. An immunoglobulin (Ig) is composed of 2 identical heavy chains ( $\gamma$ ,  $\alpha$ ,  $\mu$ ,  $\delta$ , or  $\epsilon$ ) that determine the Ig subclass (IgG, IgA, IgE,

IgD, and IgM). The light chain is either  $\kappa$  or  $\lambda$ . These proteins are expressed on the cell surface or membrane and secreted into blood and other fluids by plasma cells. The upper arms of the Y are the regions of the antibody that bind to the short arms of their specific antigen. This region of the antigen is known as an epitope, and is the site at which the antibody attaches to tissue. An antibody may target more than 1 antigen, but it is specific for only 1 epitope. Proteins are very good antigens because of their large size. The fact that antibodies (eg, immunoglobulins) are proteins makes them very potent antigens.

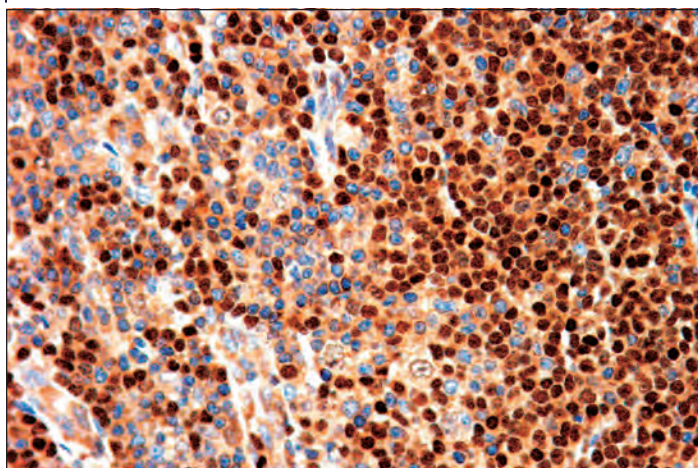
### Antigen

An antigen is a molecule made up of proteins, carbohydrates, or other polymers, and is capable of producing an immune response in animals or cell cultures for the production of antibodies. Antigens in tissues bind antibodies at different cell sites: the membrane, cytoplasm, nucleus, organelles, or a combination of sites. Many compounds are antigenic. Because of their large size, proteins are ideal for immunization and for binding, but polysaccharides, nucleic acids, and other polymers also can be antigenic. The most common antigens that induce antibody production by the body are bacteria and viruses.

### Polyclonal antisera

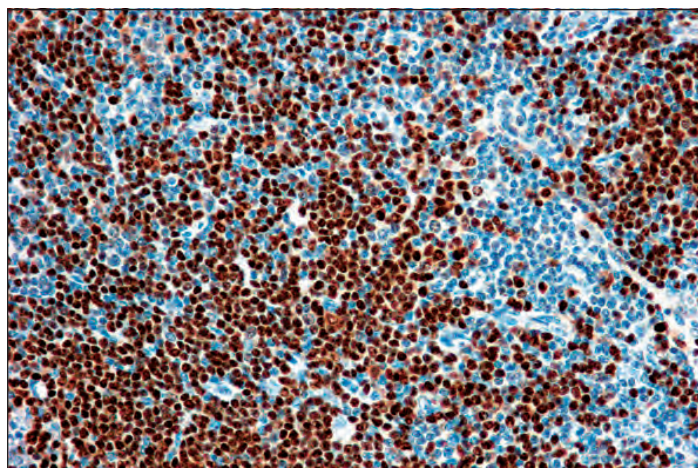
If blood was drawn from your arm and IgM antibody was isolated from it and injected into a rabbit, your IgM antibody would act as an antigen and stimulate the rabbit to make anti-human IgM antibody. Serum from the immunized rabbit could then be used as a polyclonal antihuman IgM antibody reagent. When lymphocytes are exposed to antigen, some of the lymphocytes proliferate, and each lymphocyte forms a cell line. All cells in a single clone, or cell line, produce an identical antibody, but various clones produce antibodies of different classes with specificities to different molecular sites (epitopes) on the antigen. The result of antigenic stimulation is the production of a mixture of antibodies from many clones of lymphocytes. This pool of antibodies is known as polyclonal antiserum.

Polyclonal antiserum is highly sensitive because it binds to multiple epitopes, but this also means that it is not as selective, resulting in some nonspecific staining (background). The titers used to stain tissue must be carefully selected to ensure no false positive staining. Examples of hosts that are used to produce polyclonal antiserum include rabbit, goat, horse, sheep, and human. Because of the variability of the immune response from 1 animal to another, polyclonal antibodies are difficult to standardize and can be used only in a limited number of immunoassays. Pooled antibodies made from many immunized animals of the same species are less likely to exhibit major batch-to-batch variations than pools made from only a few animals.



i12.1

A section stained with CyclinD1 mouse monoclonal antibody demonstrating poor signal-to-noise ratio and nonspecific background staining.



i12.2

A section stained with CyclinD1 rabbit monoclonal antibody demonstrating greater sensitivity and eliminating the nonspecific staining. Compare with i12.1.

## Monoclonal antibodies

The development of monoclonal antibody techniques revolutionized the whole science of immunology. Monoclonal antibodies are prepared by injecting mice with an antigen. B lymphocytes are fused with nonsecreting myeloma cells (non-secreting plasma cell tumor). This *in vitro* fusion yields hybrid cells (hybridoma) that retain the antibody secretion capability of the B cell and the immortality of the tumor cells. Hybridoma cells can be cloned, and a single clone is capable of producing antibody that is identical in molecular structure to the original. The antibody can be produced in unlimited quantities by tissue culture or by transplantation into the peritoneal cavities of mice. Although other animal species can be used, monoclonal antibodies for the histopathology laboratory are produced most commonly in mice. Monoclonal antibodies can be characterized, standardized, and produced in unlimited quantities. The advantages of monoclonal antibodies include high homogeneity, the absence of nonspecific antibodies, and no batch-to-batch or lot-to-lot variability. Mouse monoclonal antibodies are purer than polyclonal antibodies, and monoclonal antibodies display the most desirable attributes (high affinity and selectivity) of polyclonals without displaying most of the undesirable characteristics (lack of chemical homogeneity and lack of continuous supply).

## Rabbit monoclonal antibodies

Rabbit monoclonal antibody production was first reported by Spieker-Polet in 1995. Since that initial report, the production of rabbit monoclonal antibodies is making its way into the clinical laboratory. This technology follows the same principal of monoclonal hybridoma production, but uses “rabbit fusion partner cells,” which fuse with rabbit B cells. These antibodies

are proven to provide both sensitivity of a rabbit antibody and specificity of targeting a single epitope i12.1, i12.2.

## Tissue handling

### Frozen tissue fixation & processing

Before performing any immunologic procedure, the stability of the antigen must be considered. Because most antigens are soluble in aqueous solutions, the antigens must be fixed *in situ* when the technique permits.

Immunofluorescence, by today's standards, is a relatively insensitive method, and antigenic reactivity must be preserved to the maximal extent possible. Frozen sections of unfixed tissue constitute the classic preparation of tissue for immunofluorescence, because antigenic reactivity is minimally impaired and fluorescent antibody staining is strongest; however, in such sections, soluble antigens are lost [Sternberger 1979]. Formalin fixed paraffin sections are rarely used in immunofluorescence because of inconsistent results; it is suggested that, fixation and processing impair antigenic reactivity beyond the sensitivity of immunofluorescence [Sternberger 1979].

Dehydration, or drying, and fixation by chemical reagents are the 2 major categories of fixation. Dehydration unfolds and changes the solubility of the protein. Chemical fixation denatures and stabilizes proteins by coagulation, by forming additive compounds, or by a combination of the 2 actions. Acetone and alcohol are coagulating, nonadditive reagents; formaldehyde is a noncoagulating, additive fixative that cross-links proteins; and mercuric chloride is both a coagulating and additive fixative. The coagulant fixatives that do not form additive compounds permit good penetration of the antibody



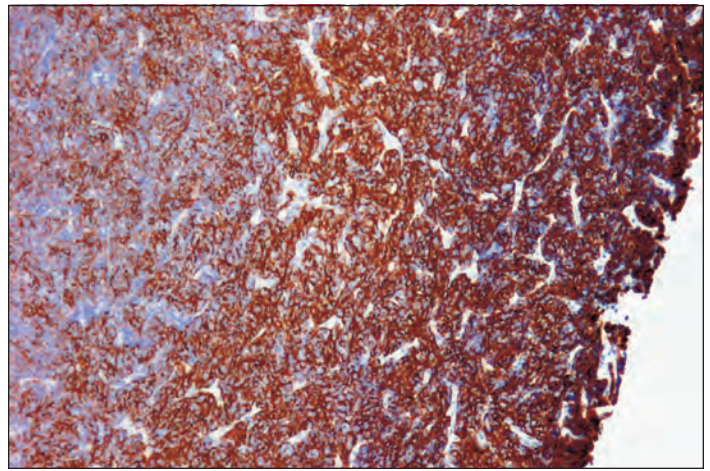
and do not block immunoreactive determinants. However, the treatment of cryostat sections with acetone, as currently practiced, does not achieve complete fixation. Extended immunohistochemical procedures may produce deleterious morphologic changes, including chromatolysis and loss of membranes [Farmilo 1989]. Allowing thin cryostat sections or cytospin preparations to dry for up to 48 hours before acetone fixation will improve morphologic preservation [Farmilo 1989]. It is my experience that frozen sections can be cut at 4–5  $\mu\text{m}$ , placed in cold 10% neutral buffered formalin for 5 minutes, then transferred to phosphate buffered saline (PBS; pH 7.4) and held for routine immunohistochemical staining. Muscle sections should be cut, air-dried for 1 hour, and immediately stained with antibodies targeted for dystrophin or other muscular dystrophy related proteins. Zenker and Bouin fixatives are also good for the preservation of some antigens, and alcohol based fixatives such as methacarn (methanol-Carnoy solution) or alcohol based proprietary reagents are reported to preserve antigens very well [Battifora 1986]. Elias [1985] states that there appears to be an inverse relationship with regard to preservation of antigenicity and morphology. Glutaraldehyde preserves morphology best, but it irreversibly blocks tissue antigenic determinants.

### Fixatives for paraffin processed tissue

Proper tissue handling, beginning with fixation and continuing through processing and microtomy, is pivotal to producing consistent results when performing immunohistochemical staining on paraffin sections. It is important for the immunohistochemist to understand the basics of how fixation, processing, temperature, and pH can affect the quality of a stain.

Regulations regarding predictive marker staining of tissue places responsibility on the clinical laboratory for documenting the time in fixative. For example, it is recommended that breast tissue from all invasive carcinomas be formalin fixed at room temperature for a minimum of 6 hours and a maximum of 48 hours [Wolff 2007] prior to HER2 testing. Breast tissue should be grossed and sectioned promptly to begin formalin fixation, and the time of fixation must be documented.

A fixative can positively or negatively influence the staining result with a given antibody. Not all antibodies are affected in the same way. Dr Timothy Drevyanko [1983] communicated that delayed fixation may have an effect on the staining results when diagnosing colorectal carcinoma with microsatellite testing. Specific concern related to false negative colon microsatellite marker results, when negative staining is interpreted as a positive result. It was suggested that there is a correlation between extended cold ischemic time and loss of antigen stability. The immunohistochemist should know which fixative was used before staining with an antibody. If the laboratory uses multiple fixatives, each antibody protocol should be validated with each type of fixative before staining patient tissue. If this is the case, it is desirable to have a multiple tissue control block containing tissue fixed with the different fixatives to ensure adequate



**i12.3**

A formalin fixed thymus stained with CK19 demonstrates an alcohol fixed center. This results from incomplete formalin fixation before beginning processing.

results. It is also likely that different antibody dilutions and staining times may be required based on the tissue fixative.

The procedures commonly used in diagnostic laboratories are based on tissue fixed in 10% neutral buffered formalin for an average of 6–48 hours. During fixation in neutral buffered formalin, hydrogen bonds are formed that must be reversed to have successful antibody binding to the antigen site. In rare cases, the epitope cannot be retrieved and frozen sections may be required. With the introduction of methods for unmasking or enhancing antigens, especially the microwave antigen retrieval methods, the time of exposure to formalin fixation is not emphasized as much as in the past. However, Taylor [1994] states that the first and perhaps most important thing that a laboratory can do is to standardize specimen fixation time. In most tissues, antigenicity can be restored by using 1 or a combination of several epitope retrieval methods. Using the mildest retrieval method with minimal damage to tissue morphology to achieve optimal staining is the goal. It is important to retain tissue morphology for diagnostic interpretation.

Herman [1988] demonstrated that zinc formalin preserved immunoreactivity remarkably well and that it can be used successfully on automated tissue processors. This report, and the search for better antigen retention and preservation, has led many laboratories to switch from 10% neutral buffered formalin to some formulation of a zinc formalin solution for routine processing. Zinc formalin is discussed in Chapter 1, “Fixation.”

Less common fixatives such as mercuric chloride (B5) and Bouins are still used. B5 is noted for producing excellent nuclear morphologic detail in hematopoietic tissues such as bone marrow or lymph nodes. Because of concerns about mercury as an environmental hazard, it is being replaced with substitutes-like acetic zinc formalin. Acetic zinc formalin is found to yield



comparable results to B5 in immunohistochemical staining [Bonds 2005].

Bouin fixative, composed of glacial acetic acid, formalin, and picric acid, is not ideal for immunohistochemical staining. The complete removal of picric acid is difficult yet vital for adequate staining. Crisp staining detail is lost with this fixative.

Because fixation varies markedly from laboratory to laboratory, vimentin provides an excellent way of determining when tissue has been overfixed. Vimentin staining is usually excellent in paraffin sections of tissue that has been optimally fixed in formalin but is progressively lost as the length of time in the fixative increases. When the vimentin stain is completely negative, the tissue is most likely overfixed and all antibody reactions should be interpreted with caution; if preservation of vimentin is uneven, then immunostains on parallel sections should be read in the area of most intense vimentin staining [Battifora 1991].

## Processing

Complete fixation before processing is necessary to achieve optimal results for stains performed at either a protein or molecular level. Thorough dehydration and infiltration are necessary for consistent staining in the tissue section. False negative or variable staining in the middle of a section in contrast to proper staining at the edges is a common artifact produced when the outer edge is formalin fixed and the center is alcohol fixed, the result of underfixation before processing **i12.3**.

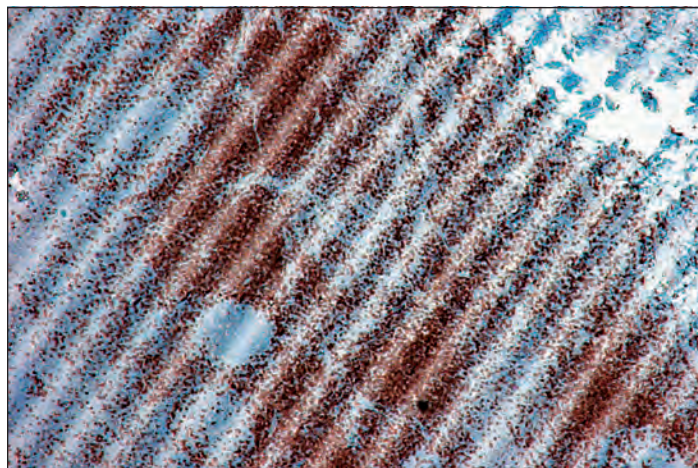
The paraffin that is used in both the processor and embedding center should have a low melting point. Paraffin containing dimethyl sulfoxide may require extended deparaffinization times to ensure complete removal. Incomplete removal of paraffin may obstruct antibody-binding sites, producing variation of staining intensity. Antigens are primarily proteins that can be altered by high temperatures. Tissue exposed to high temperatures during processing can accelerate the damage. Reprocessing tissue may alter the epitope sites, causing irreversible damage and leading to false negative results.

## Microtomy

The water bath should contain clean deionized water for immunohistochemical slide preparation. Albumin and other waterbath adhesives should be avoided because of potential non-specific staining over the slide and tissue. Gloves should be worn to avoid squamous cell contamination. Positively charged slides are recommended to ensure tissue adhesion during the epitope retrieval and rinsing steps.

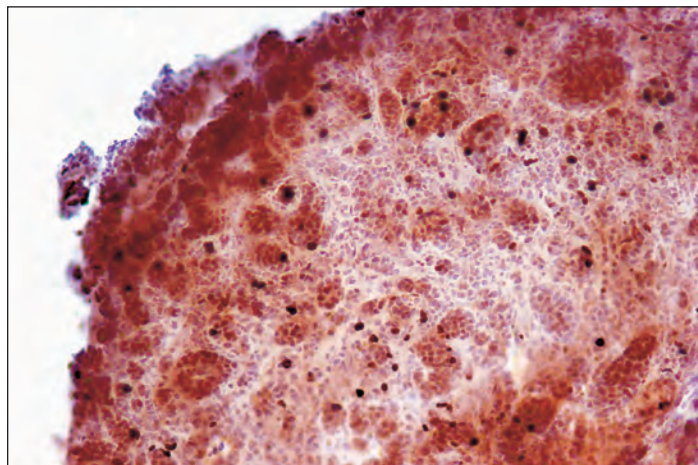
Staining quality is strongly affected by the quality of the tissue section. Sections should be free of wrinkles and folds. Good technique will decrease potential staining artifacts produced by reagents being trapped underneath the tissue or incomplete rinsing during staining **i12.4**, **i12.5**, **i12.6**.

Section thickness can affect staining intensity, therefore the patient tissue and control tissue should be cut at the same thickness; 3-4  $\mu\text{m}$  sections are recommended. Tissue sections



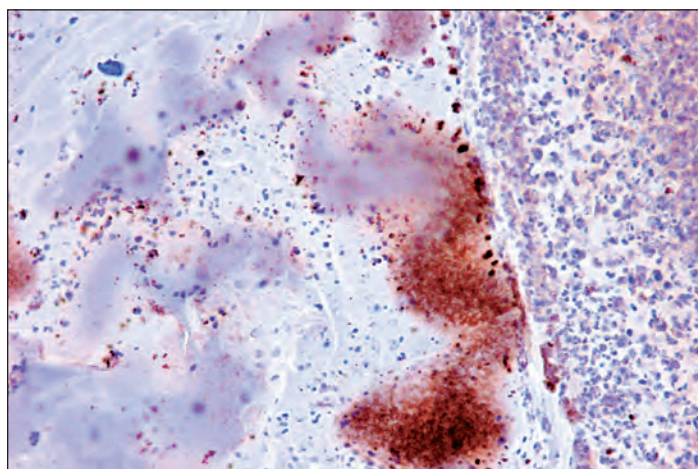
**i12.4**

Wavy section, poor tissue adherence from poor microtomy technique is demonstrated in this section.



**i12.5**

MIB-1 antibody, 3,3'-diaminobenzidine chromogen with nonspecific staining due to tissue lifting.



**i12.6**

3,3'-diaminobenzidine chromogen is trapped underneath the tissue in this section.



routinely should be picked up in the same area of the slide, typically the center, to ensure proper coverage of reagents. Avoid placing the tissue on the edges of the slide because this area is commonly missed by reagents or it dries out. Areas of incomplete coverage or drying may display false negative, or most likely nonspecific, staining.

To preserve precious tissue when the block is returned to the chuck for additional sections, the technician should avoid further trimming by carefully realigning the previously sectioned block with the blade.

Air drying overnight in front of a cool fan is optimal but is not realistic for a diagnostic laboratory. Once prepared, the tissue slide can be placed into a 55°C oven for 30 minutes. A convection oven that circulates the air can increase water evaporation and reduce drying time. Avoid high temperature ovens; many nuclear and cell surface markers are sensitive to dry heat in combination with high temperature, and false negative or weak staining may result.

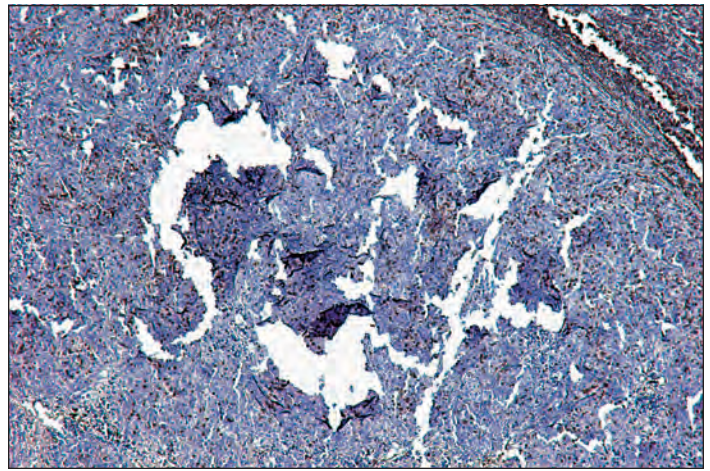
### Epitope enhancement or retrieval

There are 2 common categories of retrieval methods; the first is heat induced epitope retrieval (HIER) and the second is enzyme induced epitope retrieval (EIER). These methods are used when it is necessary to break down the hydrogen bonds that are formed during formalin fixation, because immunoreactivity can be compromised by some fixatives, especially aldehyde fixatives. Overfixation of tissue by formaldehyde can result in an antibody not having access to its epitope, thereby leading to a false negative result. The detectability of many antigens in formalin fixed tissue is greatly improved by epitope retrieval methods. Some of the advantages of epitope retrieval are [Lear 1995]:

- ability to further dilute antibodies
- exposure of epitope sites not previously detectable
- more intense reactions with decreased incubation times
- more uniform staining
- decreased background staining
- day-to-day consistency of stains
- improved standardization

### HIER

The commercial antibody specification sheet typically states the recommended method for antigen recovery. According to Bancroft and Gamble [2008], “major factors that govern the effectiveness of retrieval include the pH, volume of fluid, heating time and temperature.” Although there are many theories, it is not known exactly how and why the heating method works. The first method for antigen retrieval in formaldehyde fixed, paraffin embedded tissue sections was introduced by Shi [1991]; this revolutionized immunohistochemistry in the early 1990s. This method advocated immersing formalin fixed tissue sections in a metallic salt solution and heating in a microwave oven to



**i12.7**

The tissue has washed, or detached, in this section because of the antigen retrieval technique.

100°C. Many articles have appeared in the literature since then, which address various antigen retrieval solutions, pH, length of heating, and methods of heating.

Although high heat is the most important component of HIER, many studies have shown that the composition of the retrieval solution is also very important. The original retrieval solutions proposed by Shi [1991] (saturated lead thiocyanate and 1% zinc sulfate) have the disadvantage of potential toxicity, so numerous other less toxic solutions have been proposed. 2 of the most widely used are sodium citrate buffer (0.01M, pH 6.0) and ethylenediaminetetraacetic acid (EDTA; 1mM, pH 8.0). Other retrieval solutions that have been used are distilled water, glycine HCl (0.05M, pH 3.5), and 3M urea solution. Shi [1995] found that the pH value of the retrieval solution was more important than the composition, particularly for nuclear and cell surface antigens; they suggested that the critical influence of pH in antigen retrieval may provide an explanation of reported inconsistencies in HIER immunohistochemistry. They recommended that retrieval solutions of high pH be used for most of the antibodies in surgical pathology, and stated that tris-hydrochloric acid or sodium acetate buffer solutions at pH 8-9 may be suitable for most antigens; however, certain nuclear antigens (estrogen receptor [ER], retinoblastoma protein [Rb], and MIB-1, monoclonal Ki-67) show optimal staining at low pH. The disadvantage of a high pH is the extent of damage to the tissue sections after heating in a strongly alkaline solution. High pH can promote tissue detachment resulting in holes in the tissue or in extreme cases complete removal from the slide **i12.7**. The observations of Shi [1995] further support the concept that the antigen retrieval methods loosen or break the crosslinkages caused by formalin fixation. Many other methods of heating are

also available, including modified pressure cooker, laboratory microwave oven, vegetable steamer, or circulating waterbath.

A standard method should be established in each laboratory, but at least 2 methods should be available. A higher-volume laboratory will most likely require multiple retrieval solutions and methods, as the increased variety of antibodies will require different pH solutions and retrieval techniques.

Technology has allowed for automated immunohistochemical stainers to perform the steps of heating, deparaffinization, and antigen retrieval, reducing human error and ensuring that the temperature and exposure time are consistent each time a stain is performed, thus improving reproducibility.

### EIER

In the EIER method, a proteolytic enzyme is used to expose epitope sites. Proteolytic enzyme digestion is the older of the 2 epitope enhancement methods, but is used much less frequently since the advent of the heat induced epitope enhancement methods. As Elias [1990] stated, there is no universal proteolytic agent, so a number of enzymes have been used. Some of the solutions that have been used are 0.1% pronase in 0.5M tris buffer, pH 7.5; 0.6% ficin; 0.1% protease in PBS, pH 7.4; and 0.4% pepsin in 0.01N hydrochloric acid. Different proteolytic enzymes may be needed for epitope enhancement of various antigens. Digestion times for these proteolytic enzymes vary from 1-60 minutes or more. It is important to know the concentration of the enzyme solution being used.

Although proteolytic enzyme digestion usually reduces nonspecific staining, it may increase nonspecific staining if not used carefully. It may weaken specific staining and create false negative results, and also cause fragmentation or loss of tissue sections. The term “overdigestion” of the tissue is used to describe distorted tissue morphology and poor staining results. This can be caused when the incubation time is extended or concentration of the enzyme is high. Commonly the result is loss of cellular detail, in combination with nonspecific staining or no staining. Excessive digestion can promote loss of tissue from the slide during the staining process.

Measures should be taken to ensure that when EIER is performed manually, the digestion time is controlled for staining consistency. Temperature can influence the rate at which the digestion is achieved; the temperature should be the same each time an enzyme is used. A temperature of 37°C is commonly used for enzyme incubation. This step should be performed in a humidity chamber to avoid drying of the tissue.

### Combined HIER & EIER

The use of enzyme digestion alone for epitope retrieval can reduce the quality of tissue morphology because of a lengthy exposure time. Therefore it may be useful to combine both methods of epitope retrieval to minimize the extent of enzymatic pretreatment required. In general, HIER is performed first, followed by rinsing, and then enzyme digestion for a short time (from 1-5 minutes).

## Methods of visualization

### Immunofluorescence

Immunofluorescence, the oldest of pathology's immunohistochemical techniques, is still widely used. This technique makes it possible to visualize antigens in tissue sections or in live cell suspensions.

A fluorochrome is a dye that absorbs light and then emits its own light at a longer wavelength. This phenomenon of absorption and emission of light is called *fluorescence*. When the fluorochrome is attached or conjugated to an antibody, the sites of reaction between antigen and the labeled antibody can be visualized easily.

The most commonly used fluorochromes in immunofluorescence techniques are fluorescein isothiocyanate (FITC) and rhodamine. Both of these dyes absorb light that is not visible to the human eye (UV) and emit light that is visible using a fluorescence microscope. Virtually any antigen can be detected with immunofluorescence. The combination of sensitivity, specificity, and simplicity makes the method very useful. In most routine histopathology laboratories, frozen kidney and skin biopsy specimens are examined with immunofluorescence techniques, but the differentiation of tumors is performed using enzyme immunohistochemical techniques suitable for light microscopy.

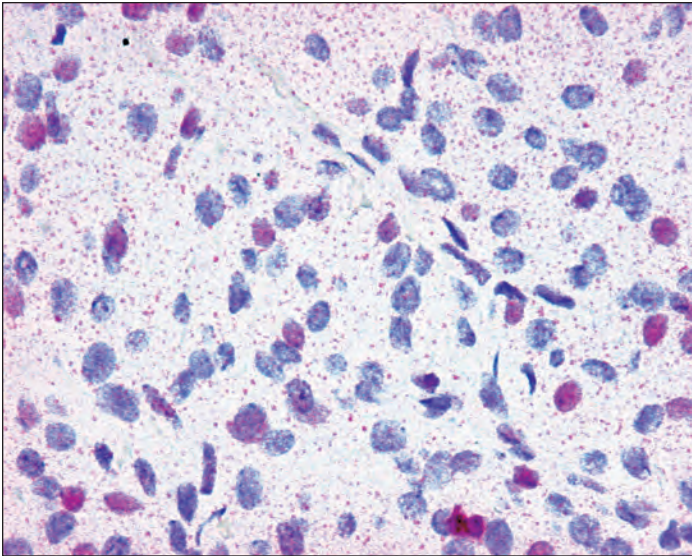
### Enzyme immunohistochemistry

The fundamental principle of enzyme labeled antibodies is similar to fluorescein labeled antibodies. The enzyme, in the presence of a substrate and a chromogen (may be the same or separate reagents), provides the indicator system to visualize the location of the antibody. Various enzymes such as alkaline phosphatase,  $\beta$ -galactosidase, glucose oxidase, and horseradish peroxidase (HRP) are used as markers.

Horseradish peroxidase and alkaline phosphatase are the enzymes most commonly chosen for antibody visualization. HRP, in the presence of hydrogen peroxide (substrate) and a chromogen such as 3,3'-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) will identify the sites of antibody binding by forming a colored compound. An HRP system routinely serves as a “work horse” in a clinical laboratory because it is consistent when producing large quantities of slides. AEC is soluble in organic solvents and requires an aqueous mounting medium, whereas DAB stained slides may be mounted with synthetic resins.

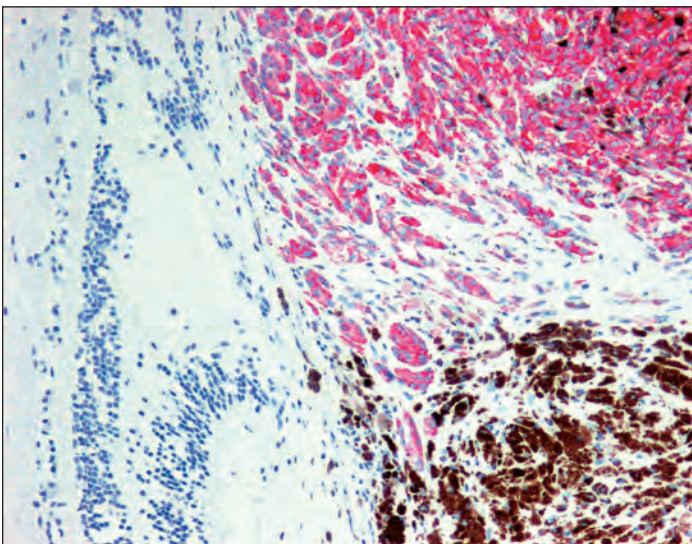
Alkaline phosphatase is demonstrated by the use of naphthol-AS-phosphate (substrate) and a chromogen such as fast red-violet LB, fast-red TR, or fast-blue BBN. Alkaline phosphatase is sensitive to heat and light which can lead to staining inconsistency. Semipermanent chromogens compatible with alkaline phosphatase allow the use of a permanent mounting media but require either no dehydration with air drying before coverslipping, or a quick dehydration to xylene. If not properly dehydrated, the alkaline phosphatase chromogen can break





i12.8

The semipermanent alkaline phosphatase red chromogen is breaking down in this section.

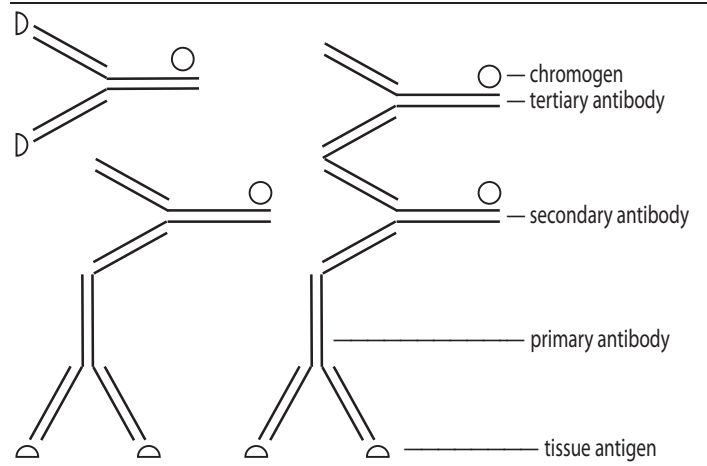


i12.9

A melanoma of the eye is seen in this section stained with HMB-45 using an alkaline phosphatase red chromogen to contrast with the melanin pigment.

down and precipitate across the tissue [i12.8](#). A red chromogen signal can assist in identifying antigen sites in those tissues containing melanin pigment which may obscure a brown signal [i12.9](#).

If hematoxylin is used as a counterstain, a formula such as Mayer hematoxylin is recommended because it does not contain alcohol. If Harris or another hematoxylin solution containing alcohol is used with AEC or the alkaline phosphatase chromogens, the reaction product will be dissolved and thus removed from the section. A false negative result will occur.



f12.1

A labeled antibody used in direct immunohistochemical techniques.

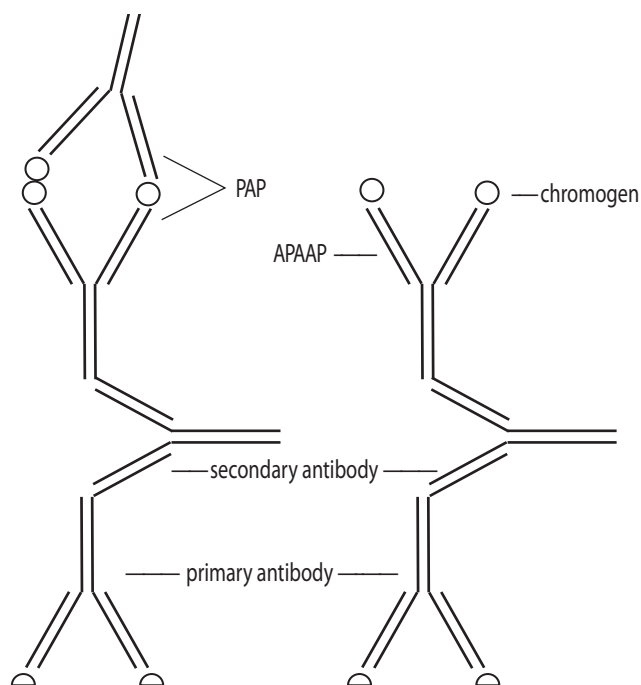
## Immunohistochemical staining methods

Various techniques are used to detect the presence of antigen in the patient's tissue or the presence of antibody in the patient's serum. References to "direct" and "indirect" methods are typically used in conjunction with immunofluorescence techniques, whereas the "unlabeled" or "soluble enzyme complex" and the "avidin-biotin-complex" (ABC) methods typically refer to visible light microscopic methods. The methods that will be described are:

- direct
- indirect
- unlabeled or soluble enzyme immune complex
- avidin-biotin
- polymeric and monomer

### Direct method

A labeled antibody of known specificity is used to identify antigens in the patient's tissue. An example is the detection of immune complex deposition in renal biopsy specimens using direct immunohistochemical techniques. In this method, the antibody may be labeled with FITC or other fluorescent dyes for fluorescence microscopy, or it may be labeled with an enzyme such as HRP, alkaline phosphate, or glucose oxidase for subsequent reaction with a chromogen and visualization with the light microscope [f12.1](#).

**f12.2**

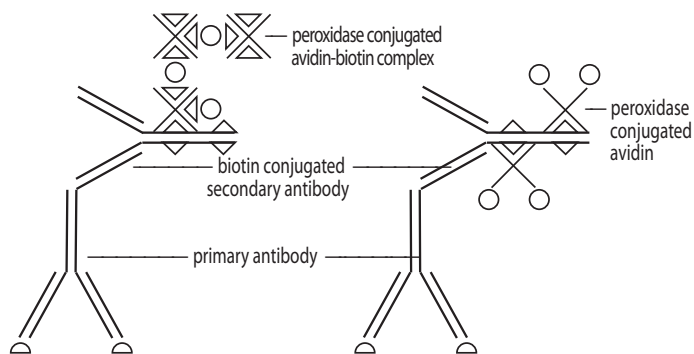
The peroxidase-antiperoxidase (PAP) immunohistochemical technique.

### Indirect method

In the indirect method, the patient's serum is added to tissue sections containing known antigens to test the patient for the presence of antibodies to those antigens. An example is the test for the presence of antinuclear antibodies. The patient's serum may also be added to a known bacterium to detect the presence of bacterial antibodies in the patient. A labeled antibody (anti-human Ig) must be used to detect the bound antibodies from the patient's serum. These indirect methods most frequently involve immunofluorescence microscopy.

### Unlabeled or soluble enzyme immune complex method

This is a 3-step method using primary antibody, linking or secondary antibody, and soluble enzyme-antienzyme complex. The primary and the enzyme-antienzyme complexes must be made in the same animal species for the secondary antibody to link them together. These techniques are based on the original peroxidase-antiperoxidase (PAP) technique of Sternberger [1979]. The most common techniques in this category involve the use of either a PAP or alkaline phosphatase-antialkaline phosphatase immune complex **f12.2**.

**f12.3**

The avidin-biotin-complex (ABC) immunohistochemical technique.

### Avidin-biotin methods

Avidin has a very high affinity for the vitamin biotin (>1 million times greater than the affinity of antibody for most antigens) and the binding is essentially irreversible. Avidin isolated from egg white was used first in the ABC methods, but streptavidin, isolated from the microorganism *Streptomyces avidinii*, is more commonly used today.

In the ABC method, the primary antibody is followed by a biotinylated secondary antibody (linking antibody). The third step in the ABC method is the application of a preformed avidin-biotin enzyme complex. This method has low background economy, and sensitivity reported to be up to 40× that of other immunoperoxidase methods [Hsu 1981]. The antibodies may be used at higher dilutions than in other techniques **f12.3**.

### Polymeric detection

The dextran polymer and monomer technology has quickly become a standard for clinical immunohistochemical staining. The polymer enzyme molecules such as HRP or alkaline phosphatase have been fused with the secondary antibody. Monomer technology uses a single strand, allowing for greater sensitivity and penetration at the antibody binding site. Turnaround times have been improved with the elimination of serum and avidin-biotin blocking steps necessary for the ABC method. In addition, this chemistry provides sensitivity without the risk of nonspecific staining when using the ABC method. Staining steps would be: antibody, polymer, and chromogen.

## Controls

### Positive controls

A positive control must be run with each antibody stain each time it is performed. This applies to both manual and automated methods. Although commercially prepared control slides can be used to check reliability of the reagents, these purchased slides should not be used as routine patient controls. The optimum control is one prepared in the laboratory under the exact same conditions as the diagnostic tissue, including type and timing of fixation and processing. Because fixation varies and affects the reactions so dramatically, commercially prepared control slides are not ideal to determine if a negative reaction on the diagnostic slide is a true negative or if it is the result of overfixation. The method described by Battifora [1986] of preparing sausage controls is excellent, though somewhat time consuming and difficult to execute. With careful planning the laboratory can make the most efficient use of available tissue to fulfill requirements of using controls containing both positive and negative tissue types. The author has found that a tissue microarray technique such as that available from Beecher Instruments (Sun Prairie, WI) is a more effective means for constructing multitissue control blocks (both positive and negative tissues) than previously reported manual methods. Technology can reduce the distortion of the tissue block and extend the use of a control tissue due to smaller precise core sizes [Bancroft 2008].

Best practice is to use a multitissue control that contains both positive and negative tissue for an antibody. This control specimen should be placed on the same slide as the patient's specimen to ensure that all of the steps, reagents, and temperature are exactly the same for the patient and control specimens during the staining process. Control specimens can be precut before cutting the patient specimen. If precut patient slides are used then the multitissue control block can be cut and placed on the patient slide. When cutting controls, the end of the slide that is for the patient should not be placed into the water bath, this can reduce the charge of the slide.

### Negative controls

In most laboratories, negative controls are run by substituting for the primary antibody either nonimmune serum from the same species as the primary antibody or the diluent used for the primary antibody. If diluent buffer is used, the negative control will not detect nonspecific binding of animal serum components to the tissue, and any staining observed will be the result of either endogenous peroxidase activity or binding of other antibody reagents to the specimen [Bourne 1989].

## Antibody evaluation & validation

### Antibody specification sheet

The antibody specification sheet provided with a commercial antibody contains valuable information regarding the antibody or antiserum. The immunohistochemist should be familiar with the specification sheet for many reasons.

The following are typically included, but are not always available:

- a monoclonal antibody will list the clone and the isotope (IgG or IgM) to ensure that the secondary antibody will bind to the primary antibody
- the expiration date under recommended storage conditions
- long term or short term storage temperatures; some antibodies require long term storage at  $-20^{\circ}\text{C}$ ; measures to ensure that the antibody storage condition is optimal will assist with antibody stability and maximum reactivity for the life of the antibody
- the protein concentration and suggested working dilution ranges
- pretreatment (antigen retrieval) solution, pH, and/or method, if recommended
- expected positive and negative tissue types, including normal and tumor.
- reference to publications and applications for the antibody; these are useful if additional staining methods are needed
- antibody type: in vitro (IVD), research use only (RUO), or analyte specific reagent (ASR)

### Prediluted & concentrated antibodies

Prediluted antibody refers to a commercially available "ready-to-use" solution. The vendor has already taken a concentrated antibody and diluted it down to an "optimal" dilution. Because it has been diluted, the shelf-life is routinely shorter and it cannot be frozen to extend it, thus prediluted antibodies are less economical if not used before the date of expiration. Prediluted antibodies must be validated for reactivity before use on patient tissue. The laboratory cannot assume that the antibody is ready to use with that laboratory's method and must perform serial dilutions (1:2, 1:4, 1:8, and 1:16) to validate. The exception is a Food and Drug Administration (FDA) approved antibody test, which mandates the antibody concentration in addition to times, temperature, and detection reagents. This is usually manufactured and distributed as an FDA approved kit or the manufacturer provides specific protocols to follow.

A concentrated antibody is one that is supplied in an "undiluted" form. Dilutions must be performed to find the optimal dilution for antigen detection, and the user has more control of fine tuning the dilution to be compatible with the laboratory's fixation and processing protocols. These undiluted antibodies are more economical because they allow for the modification of



titers used in staining. In addition, they routinely have a longer shelf-life when stored properly; most concentrated antibodies can be “snap” frozen using liquid nitrogen and stored at  $-70^{\circ}\text{C}$  indefinitely.

If undiluted antibodies come in solution form, it is best to dilute only the amount of antibody needed for the current diagnostic slides or the smallest amount feasible when very high dilutions are needed. Dilutions are expressed as ratios, as in a 1:20 dilution. This is equivalent to 1 part antibody solution in a total of 20 parts, or 1 part antibody plus 19 parts of diluent. Pipettes capable of measuring in microliters ( $\mu\text{L}$ ) are a must in the immunohistochemistry laboratory. Microliter ( $\mu\text{L}$ ) and lambda ( $\lambda$ ) are used interchangeably, with both equal to 0.001 mL. Pipettes that will cover a range, such as 0.5–10  $\mu\text{L}$ , 10–100  $\mu\text{L}$ , and 100–1,000  $\mu\text{L}$ , are the most useful. For higher dilutions, it is best to prepare a more concentrated stock dilution, and then prepare further dilutions from this stock dilution.

A basic formula to use to make 1 mL (1,000  $\mu\text{L}$ ) of a 1:x dilution is [Chlipala 1985]:

$$\frac{1,000}{x} = \mu\text{L of raw antisera required}$$

$$1,000 - \frac{1,000}{x} = \mu\text{L of antibody dilution buffer required}$$

This formula can be changed easily by changing 1,000  $\mu\text{L}$  (1 mL) to whatever volume is required.

## Antibody validation

Routinely a standard staining protocol will be used for most formalin fixed tissue samples. This will also assist with laboratory reproducibility and consistency. Although it will be necessary to have unique protocols, the number of antibodies requiring unique detection, special pretreatments, and incubation times should be kept to a minimum.

An example of a standard laboratory protocol is as follows:

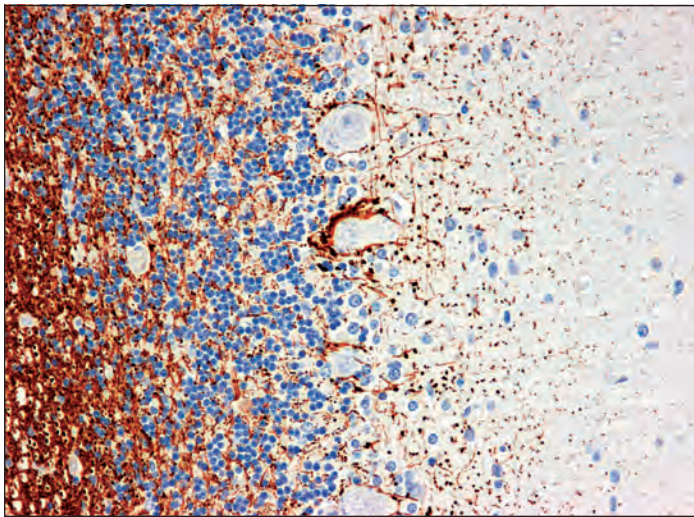
1. Retrieval: EDTA, pH 8.0
2. Heating method: modified pressure cooker ( $115^{\circ}\text{C}$  for 25 minutes or  $124^{\circ}\text{C}$  for 40 minutes). Combination of time and temperature are based on the stringency of retrieval needed.
3. Peroxidase quenching: 3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), 10 minutes
4. Detection system: universal polymer, HRP, DAB
5. Staining times: antibody 30 minutes, polymer 10 minutes, and chromogen 5 minutes
6. Counterstain: Mayer hematoxylin, 3 minutes

There are 2 approaches to new antibody validation, the first using no retrieval, multiple retrieval solutions, and multiple enzyme pretreatments; this approach may be necessary when validating a “homegrown” antibody or an antibody that has no previously documented use in the clinical laboratory. The second approach is researching the antibody and planning a validation protocol using the wealth of information available. Resources for a starting point are the antibody or detection vendor, antibody specification, journal articles, and text books [Dabbs 2006, Shi 2000]. These resources provide basic methods before staining, thus knowledge is gained of the recommended retrieval solution, pH and method, type of detection used, and positive and negative tissue types. Knowing expected staining patterns and location of expression, such as nuclear or cytoplasmic, can assist when reviewing staining results.

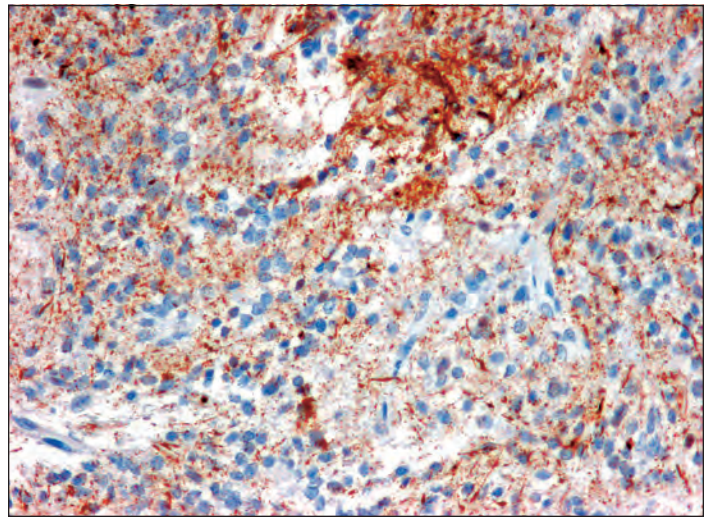
Upon receiving the new undiluted antibody, the laboratory will typically begin with a series of testing variables. First perform serial dilutions (1:100, 1:200, 1:400, and 1:800) of the antibody using a known positive and negative tissue control. Usually the manufacturer will indicate an approximate dilution; however, several different dilutions should be used with the standard laboratory protocol to determine the correct dilution for your laboratory. It is important to use a diluent that contains carrier proteins and the proper pH for antibody stability.

Review the results for an optimal dilution looking for the best signal-to-noise ratio. If the 1:100 targeted cells are staining faintly, with no nonspecific staining, then stain further with more concentrated dilutions of 1:25, 1:50, and 1:100. To ensure no errors in dilution preparation and reproducibility, always repeat in the new series the previous dilution that is closest to optimal. If the 1:800 appears overstained with high signal and high background, then start with 1:800, 1:1,600, and 1:3,200 to further validate. If the initial staining yields negative or suboptimal results, then the laboratory should begin with changing only 1 variable at a time. Routinely changing the pH of the retrieval solution is first; if unsuccessful, the introduction of an enzyme step omitting the retrieval solution is recommended. The introduction of an enzyme will require not only a redetermination of the antibody concentration but also an evaluation of the time in the enzyme solution. It is recommended that when an enzyme is introduced, times of 5, 10, 20, and 30 minutes in the enzyme solution be evaluated to ensure that adequate retrieval is performed. Some antibodies do not require any type of retrieval, and using retrieval may introduce false positive staining [i12.10](#), [i12.11](#), [i12.12](#).

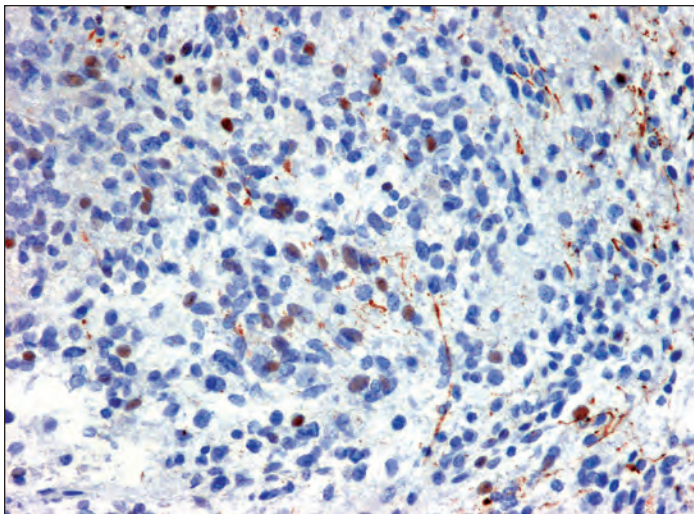
Once the appropriate protocol and dilution are determined for a given antibody, at least 10 positive and 10 negative neoplasms should be tested to ensure the targeted cells are staining appropriately [CAP 2013] to validate sensitivity, specificity, accuracy, and reproducibility before using on patient tissue. After

**i12.10**

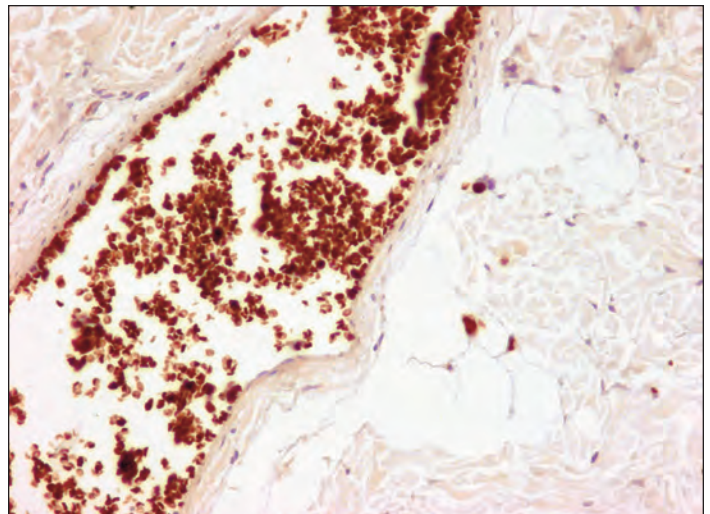
Expected staining patterns with SMI-31 antibody are seen on this positive control tissue from the cerebellum.

**i12.12**

A brain biopsy specimen stained with SMI-31 antibody demonstrating expected staining patterns after no pretreatment of the sections. Compare with **i12.11**.

**i12.11**

A brain biopsy specimen stained with SMI-31 antibody that demonstrates false nuclear staining because of unnecessary heat-induced epitope retrieval.

**i12.13**

Red blood cells demonstrating positive staining after insufficiently blocked peroxidase activity.

complete validation, the slides should be retained in a permanent file for reference. A written and/or electronic record of optimum antibody dilution and retrieval procedure, if applicable, should be maintained for each antibody **f12.4**. Antibodies that fall under the category of “predictive markers” are required to follow a different validation process.

### Storage of antibodies

Both the storage container and the temperature at which the antibody is stored are very important. Polypropylene, polycarbonate, or borosilicate glass is recommended for storage because these materials have low protein absorptive properties. If the antibody solution contains very low concentrations of protein, 0.1%-1.0% bovine serum albumin may be added to

reduce loss through polymerization and absorption on the container [Boenisch 2001].

The recommendations of the manufacturer as to the storage temperature should be carefully followed. Most prediluted antibodies and kits are stored at 4°C-8°C to avoid repeated freezing and thawing. Once lyophilized antibodies have been diluted, they are usually divided into aliquots and stored at -70°C.

### Blocking reactions

Most immunoperoxidase methods include 2 blocking reactions. The first is the use of hydrogen peroxide, usually prepared in absolute methanol, to block tissue endogenous peroxidase activity. If the tissue contains many red blood cells, this blocking step is essential **i12.13**.



## Validation Form for Antibodies &amp; Tissue Controls

Date requested: \_\_\_\_\_

Purpose of development: \_\_\_\_\_

Antibody validation: \_\_\_\_\_ Positive tissue control \_\_\_\_\_ Negative tissue control \_\_\_\_\_

Retrieval instrument: (circle one) Modified pressure cooker (125°) or (115°), waterbath, microwave, steamer, or other \_\_\_\_\_

Retrieval solution: \_\_\_\_\_

Lot# \_\_\_\_\_ Expiration \_\_\_\_\_ pH: \_\_\_\_\_

Enzyme pretreatment solution: \_\_\_\_\_

Lot# \_\_\_\_\_ Expiration \_\_\_\_\_ Time: \_\_\_\_\_

Detection: \_\_\_\_\_ Lot# \_\_\_\_\_ Expiration \_\_\_\_\_

Chromogen: \_\_\_\_\_ Lot# \_\_\_\_\_ Expiration \_\_\_\_\_

Automated: y/n (if yes) instrumentation: \_\_\_\_\_

| Antibody name | Antibody lot# | Antibody exp date | Tissue type | Control block ID | Dilution | Date stained | Comments/results |
|---------------|---------------|-------------------|-------------|------------------|----------|--------------|------------------|
|               |               |                   |             |                  |          |              |                  |
|               |               |                   |             |                  |          |              |                  |
|               |               |                   |             |                  |          |              |                  |
|               |               |                   |             |                  |          |              |                  |

Reviewed by (Medical Director or Designee) \_\_\_\_\_ Final approval date: \_\_\_\_\_

f12.4

Antibody &amp; tissue control validation form



The second block is for nonspecific background staining that may occur as a result of antibody (protein) attachment to highly charged collagen and connective tissue elements [Bourne 1989]. If the first protein solution applied to the tissue is the primary antibody, nonspecific binding can occur. The secondary antibody will bind to this nonspecifically bound primary antibody, and react with the substrate-chromogen, resulting in nonspecific positive staining. The best way to prevent this type of nonspecific staining is to add an innocuous protein solution to the tissue before the primary antibody is applied. The protein will bind to the charged sites and then nonspecific binding of the primary will not occur. Nonimmune serum from the same animal species in which the secondary antibody is produced is the most common source of the blocking reagent. The nonimmune serum is applied just before the primary antibody. After 10-20 minutes of incubation, the excess is tapped off (do not rinse off) and the primary antibody is applied. Casein and nondry fat milk have also been used as a blocking agent.

### Multilink biotinylated secondary antisera

Secondary antibodies that are typically called “multilink” or “universal” refer to cocktails of antibodies raised in different species. These secondary antibody mixtures are commonly biotinylated antimouse IgG and IgM and antirabbit IgG. Other species such as goat can also be added. The laboratory technician should know the species in the secondary antibody mixture to ensure that the targeted antibody is compatible. One advantage of this type of secondary antibody is that it avoids the problem of having to stock various specific link antibodies (eg, biotinylated goat antirabbit or goat antimouse IgG and IgM).

### DAB reaction product intensification

Several methods have been used to intensify the DAB reaction product. Heavy metals can be used for intensification of the DAB reaction product by supplementing, or following, the incubation medium with reagents such as 1% cobalt chloride. However, there is a risk of increasing background staining with heavy metal intensification, and some of the metals should not be used with the immunohistochemical detection of nuclear antigens [Elias 1990], especially if weak hematoxylin counterstaining is used.

Imidazole is an effective intensification reagent. Elias [1990] states that the addition of 0.01M imidazole to the DAB incubation medium at pH 7.6 is superior to standard DAB and DAB-cobalt chloride-nickel sulfate or DAB-cobalt chloride procedures, both in sensitivity and detection efficiency. Imidazole also significantly inhibits the pseudoperoxidase activity of hemoglobin.

Osmium tetroxide may also be used to intensify the DAB reaction product, but unlike the other reagents mentioned, it is always used after the DAB reaction. The DAB reaction product is osmiophilic and will be darkened by mild poststaining osmification. This also helps prevent fading of the final reaction product

during long term storage; however, unless carefully done, this reagent has the possibility of darkening any background staining present as well as intensifying the reaction product.

Elias [1990] warns that increased sensitivity is not always commensurate with enhancement of staining intensity. For example, post-treatment osmium boosts staining intensity without increasing detection efficiency.

### Buffer solutions

For reproducible results, the pH of the buffer used during staining must be maintained and checked often. For each new lot of commercially prepared buffer, or each time a buffer is prepared, the immunohistochemist should check and document its pH before use. Typically, 2 buffers are used during the staining process: PBS and tris-base solution (TBS). Both can be used with an HRP detection system, but only TBS should be used with an alkaline phosphatase detection system. The addition of a wetting agent will assist with even distribution of the reagents during staining and improve rinsing steps; at the same time, it will slow down bacterial growth. Common wetting agents used are Tween-20, Triton-X, and brij-35. The buffer should be used at room temperature during staining.

## Commonly used antibodies & their applications

Antibody stains are routinely used by the pathologist to identify a neoplasm and define whether it is benign or malignant, and whether it is metastatic or primary. The pathologist identifies the extent to which cells in a neoplasm resemble normal cells (differentiation) both morphologically and functionally. The type of neoplasm is defined by the germ cell layer (endoderm, mesoderm, ectoderm) from which it derives. Types of neoplasms include carcinomas, sarcomas, lymphomas, melanomas, mesotheliomas, neuroendocrine tumors, gliomas, and germ cell tumors.

### Neoplastic terminology

**Neoplasm:** A new growth of tissue in which cell multiplication is uncontrolled and progressive; can be either benign or malignant.

**Tumor:** Swelling; one of the cardinal signs of inflammation and morbid enlargement. Often used to mean “neoplasm.”

**Anaplasia:** Lack of differentiation. Some features include variation in size and shape of cells and nuclei, dark nuclei, giant cells, and disturbed orientation.

**Differentiation:** The distinguishing of 1 thing from another and the extent to which neoplastic cells resemble comparable normal cells, both morphologically and functionally.

**Benign:** Shows a lesser degree of anaplasia than malignant tumors; often surrounded by a fibrous capsule; does not produce metastasis or significant invasion.

**t12.1 Anaplastic tumor workup (typical results)**

|                   | Cytokeratin AE1/AE3 | Cam 5.2 | CD45RB LCA | HMB45/MART1 | GFAP | Vimentin |
|-------------------|---------------------|---------|------------|-------------|------|----------|
| Carcinoma         | +                   | +       | –          | –           | –    | ±        |
| Lymphoma          | –                   | –       | +          | –           | –    | ±        |
| Melanoma          | –                   | –       | –          | +           | –    | +        |
| Sarcoma           | –                   | –       | –          | –           | –    | +        |
| High grade glioma | +                   | –       | –          | –           | +    | +        |

LCA, leukocyte common antigen; GFAP, glial fibrillary acidic protein

**t12.2 Small cell neoplasm panel**

|                      | Keratin (HMW) | NSE | Vimentin | LCA | Neurofilament | Desmin | WT1                | CD56 |
|----------------------|---------------|-----|----------|-----|---------------|--------|--------------------|------|
| Small cell carcinoma | +             | +   | –        | –   | –             | –      | – (except ovarian) | +    |
| Lymphoma             | –             | –   | –        | +   | –             | –      | –                  | –    |
| Neuroblastoma        | –             | –   | –        | –   | +             | –      | –                  | +    |
| Rhabdomyosarcoma     | –             | +   | +        | –   | –             | +      | –                  | +    |
| Wilms tumor          | +             | –   | +        | –   | –             | +      | +                  | ±    |

HMW, high molecular weight; LCA, leukocyte common antigen; NSE, neuron specific enolase

**t12.3 Adenocarcinoma subtypes**

|                       | CK7 | CK20 | TTF1 |
|-----------------------|-----|------|------|
| Lung                  | +   | –    | +    |
| Breast                | +   | –    | –    |
| Ovarian (nonmucinous) | +   | –    | –    |
| Ovarian (mucinous)    | +   | +    | –    |
| Thyroid               | +   | –    | +    |
| Pancreas              | +   | –    | –    |
| Colorectal            | –   | +    | –    |
| Renal cell            | –   | –    | –    |

CK, cytokeratin

**t12.4 Prostate carcinoma vs adenosis (benign hyperplasia)**

|             | PSA | PSAP | P63           | Racemase P504s* | HMW keratin, CK903 |
|-------------|-----|------|---------------|-----------------|--------------------|
| Carcinoma   | +   | +    | –             | +               | –                  |
| Hyperplasia | +   | +    | + basal layer | –               | + basal layer      |

HMW, high molecular weight; PSA, prostate specific antigen; PSAP, prostate specific alkaline phosphatase

\* $\alpha$ -methylacryl-coA racemase (AMACR, P504s)

**Malignant:** A neoplasm that invades adjunct tissue and has the capacity to metastasize; typically shows degrees of anaplasia.

**Primary:** The site at which a neoplasm first arises.

**Metastasis:** Transfer of disease from 1 organ or part of the body to another not directly connected with it, because of transfer of either pathogenic microorganisms or cells (eg, malignant tumor cells).

The most common approach to the use of immunohistochemistry in diagnosis involves the use of panels of antibodies to differentiate 1 neoplasm from others under consideration. Examples of common panels are given in **t12.1**, **t12.2**, **t12.3**, and **t12.4**. Single antibodies used for tumor identification and differentiation are given in **t12.5**.

**t12.5 Antibodies used for the differentiation and identification of tumors\***

| <b>Antibody</b>                             | <b>Clones mAb, rmAb, or polyclonal pAb<sup>†</sup></b>                                                                       | <b>Neoplasm identification &amp; diagnostic differentiation</b> |
|---------------------------------------------|------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|
| bcl2                                        | mAb: 124, 100/D5, bcl-2/100/D5, 100                                                                                          | Follicular lymphoma                                             |
| CD117                                       | mAb: T595<br>rmAb: YR125, 9.7<br>pAb: A4502, RB-9038-P                                                                       | Gastrointestinal stromal tumor                                  |
| CD15                                        | mAb: MMA, C3D-1, Carb-3, BY87, H198                                                                                          | Hodgkin lymphoma                                                |
| CD20                                        | mAb: L26, 7D1 or MJ1<br>rmAb: MJ1                                                                                            | B cell lymphoma                                                 |
| CD3                                         | mAb: F7.2.38, LN10, PS1,<br>rmAb: EP41, EP449E, SP7, 2GV6, EP272 MAD-000325QD, MRQ-39 103R<br>pAb: A0542, IR503/IS503, N1580 | T cell lymphoma                                                 |
| CK20                                        | mAb: Ks20.8, PW31<br>rmAb: EP23, SP33<br>pAb: E16444                                                                         | Colon carcinoma                                                 |
| Cytokeratin (pankeratin)                    | mAb: AE1/AE3, KL1, OSCAR                                                                                                     | Carcinoma                                                       |
| Desmin                                      | mAb: D33, DE-R-11, ZC18<br>pAb: RB-9014-P                                                                                    | Leiomyoma, leiomyosarcoma                                       |
| Estrogen receptor                           | mAb: 1D5, 6F11<br>rmAb: EP1, SP1                                                                                             | Breast carcinoma                                                |
| Glial fibrillary acidic protein (GFAP)      | mAb: 6F2, GA-5, SP2/AG14<br>rmAb: EP6724<br>pAb: Z0334, 760-2516                                                             | Glioblastoma multiforme                                         |
| Gross cystic disease fluid protein GCDFP-15 | mAb: 23A3, D6, SPM135<br>rmAb: EP1582Y, EP95                                                                                 | Breast ductal carcinoma                                         |
| Her2                                        | mAb: CB11<br>rmAb: 4B5, EP1045Y, SP3<br>pAb: A0485                                                                           | Breast carcinoma                                                |
| HMB-45                                      | mAb: HMB-45                                                                                                                  | Melanoma                                                        |
| CD45, leukocyte common antigen              | mAb: 2B11+PD7/26, X16/99, RP2/18                                                                                             | Lymphoma                                                        |
| Melan-A                                     | mAb: A-103, HMB45 + MC-7C10+M2-9E3 + T31, M2-7C10                                                                            | Melanoma                                                        |
| Progesterone receptor                       | mAb: 16, 1A6, PgR 636, PgR 1294<br>rmAb: Ep2, SP2, SP42, Y85                                                                 | Breast ductal carcinoma                                         |
| Prostate specific antigen                   | mAb: ER-PR8, 35H9<br>pAb: A0562, RB-084, IS514/IR514, N1517, 760-2506                                                        | Prostate adenocarcinoma                                         |
| S100                                        | mAb: 4c4.9, 12E2E2<br>rmAb: EP32<br>pAb: Z0311, NCL-S100p, RB-9018p, E031, 760-2523, IR/IS504, PA0900                        | Schwannoma                                                      |
| Thyroid transcription factor 1 (TTF1)       | mAb: 8G7G3/1, SPT24<br>rmAb: EP1584Y, SP141                                                                                  | Lung adenocarcinoma                                             |
| Vimentin                                    | mAb: V9, Vim 3B4<br>rmAb: SP20                                                                                               | Seminoma                                                        |

\*From [NordiQC <http://www.nordiqc.org>; ISBN 978-0443102790]

<sup>†</sup>Monoclonal antibody (mAb), rabbit monoclonal antibody (rmAb), or polyclonal antibody name (pAb).

Combinations of antibody expression, both positive and negative assist the pathologist when working up an anaplastic tumor for differential diagnosis. Consideration of cellular morphology, tissue source and patient history are key factors for a pathological diagnosis. Antibodies listed in this table are not used individually for diagnosis and are not all inclusive.

Development of new clones and antibodies are constant. Clones can be combined together to create cocktails that may increase the sensitivity or specificity of the test; cocktails were included in this table only when a combination of clones was the standard for the antibody, as with CD45.



## Quality control

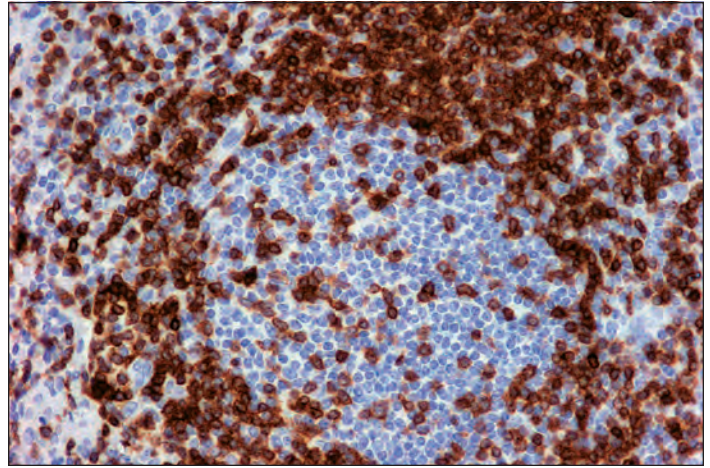
Measures of QC and thorough documentation are fundamental in a diagnostic laboratory. QC measures serve many roles in the laboratory and are especially useful for troubleshooting. Antibody and control tissue validation must be documented and maintained. Each laboratory should define a QC system that enables the immunohistochemist to have easy access to previous validation results and final protocols, especially in a laboratory where manual staining is performed. Tracking of lot numbers, expiration dates, dilutions, protocols, pH monitoring, and validation of controls are basic QC in the immunohistochemistry laboratory. Laboratory accreditation agencies also have specific QC and documentation requirements. Both paper and electronic documentation are useful, but if a final protocol can be maintained electronically it assures the immunohistochemist that the most current information is saved and retrievable.

### Recommended QC steps for an antibody

1. Verify and document on the bottle and the specification sheet the antibody name, receipt date, and expiration date
2. Check storage temperature and conditions upon receipt
3. Use a form to plan initial validation to include pretreatment, incubation times, detection system, control tissue, dilutions and date **i12.4**
4. Document the date validation was performed and who approved the final procedure, such as the medical director
5. Make a final copy of the procedure that can be accessed by the technician
6. Keep all records and document and date any modifications to the procedure; historical records may be useful for troubleshooting or revalidating an existing antibody
7. Maintain a separate slide file for each antibody lot and dilution validation; the date on the slides should correlate with the antibody records

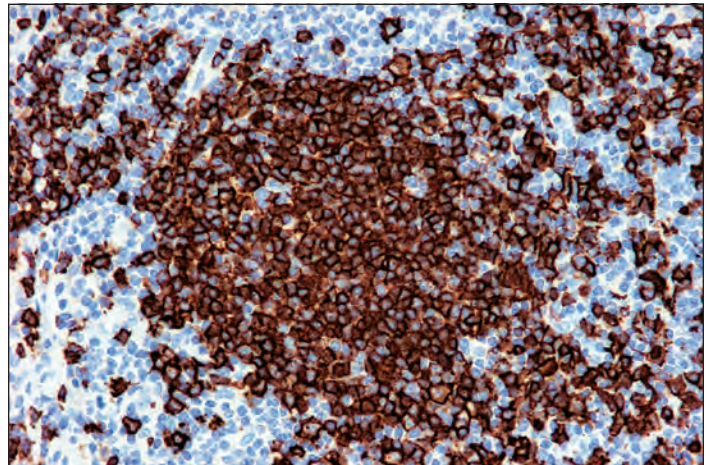
### Positive & negative tissue controls

Positive controls are those tissues that express the antigen in either normal tissue components or tumor cells **i12.14**, **i12.15**. Negative tissue controls are either normal tissue or tumor cells that do not contain that antigen. When validating an antibody, it is important to optimize the dilution using both positive and negative tissue types. This ensures that a low expressing tumor will not be missed and a false positive result is not misinterpreted. Tissue blocks that contain several different normal and tumor tissues exhibiting positive and negative expression are very helpful to decrease the cost of validation. New technology such as the tissue microarray is making a constructive impact on QC measures with antibody validation **i12.16**, **i12.17**, **i12.18**.



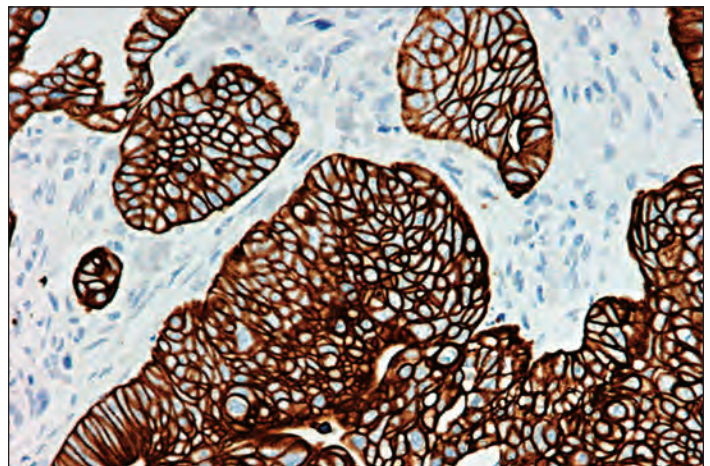
**i12.14**

CD3 T cell staining pattern is demonstrated in a normal lymph node control.



**i12.15**

CD20 B cell staining pattern is demonstrated in a normal lymph node control.



**i12.16**

Cam 5.2 antibody staining of a colon adenocarcinoma control specimen.