Four-hour Wet Lab to Learn H&E Plus 6 Special Stains Procedures

Presented September 26, 2026 at the Biological Stain Commission meeting in San Antonio, TX.

Abstract

This fast-paced wet lab experience is designed to teach histology scientists the way to perform several special stains by hand. Many histology laboratories outsource their special stains or use instrumentation which, although effective, is detrimental to scientists wishing to understand the theory and, more importantly, how to troubleshoot when things go wrong. This laboratory session is open to anyone wishing to expand their special stains knowledge and may be of particular benefit for those persons interested in taking the HT or HTL examinations, those who are new to the histology lab, or those whose laboratories use automated staining instruments exclusively. Stains to be performed by the attendees include hematoxylin and eosin, Verhoeff van Gieson elastic stain, trichrome stain for fibrosis, von Kossa stain for calcium, Wright stain for mast cells and eosinophils, rhodanine stain for copper, and Prussian blue stain for iron. One control slide will be provided for each attendee per stain. However, attendees are welcome to bring their own slides (maximum 2 per stain for a total of 14) if they prefer.

Educational Objectives

- Participants will learn how to perform hematoxylin and eosin, Verhoeff van Gieson elastic stain, trichrome stain, von Kossa stain for calcium, Wright stain for mast cells and eosinophils, rhodanine stain for copper, and Prussian blue stain for iron.
- Participants will learn how to multi-task procedures to optimize time usage.
- Participants will be exposed to differences in staining procedures and will learn how to troubleshoot problems associated with special stains.

Name of Stain: Hematoxylin and Eosin

Reagents Needed:

- Xylene
- 100% ethanol
- 95% ethanol
- DI water
- Harris hematoxylin
- Acid alcohol (0.5 mL concentrated HCl, 50 mL absolute ethanol, 50 mL DI water)
- Bluing reagent
- Eosin

Control slide provided: Multi-tissue microarray. All tissues for these stains were sectioned at 4 µm.

Procedure:

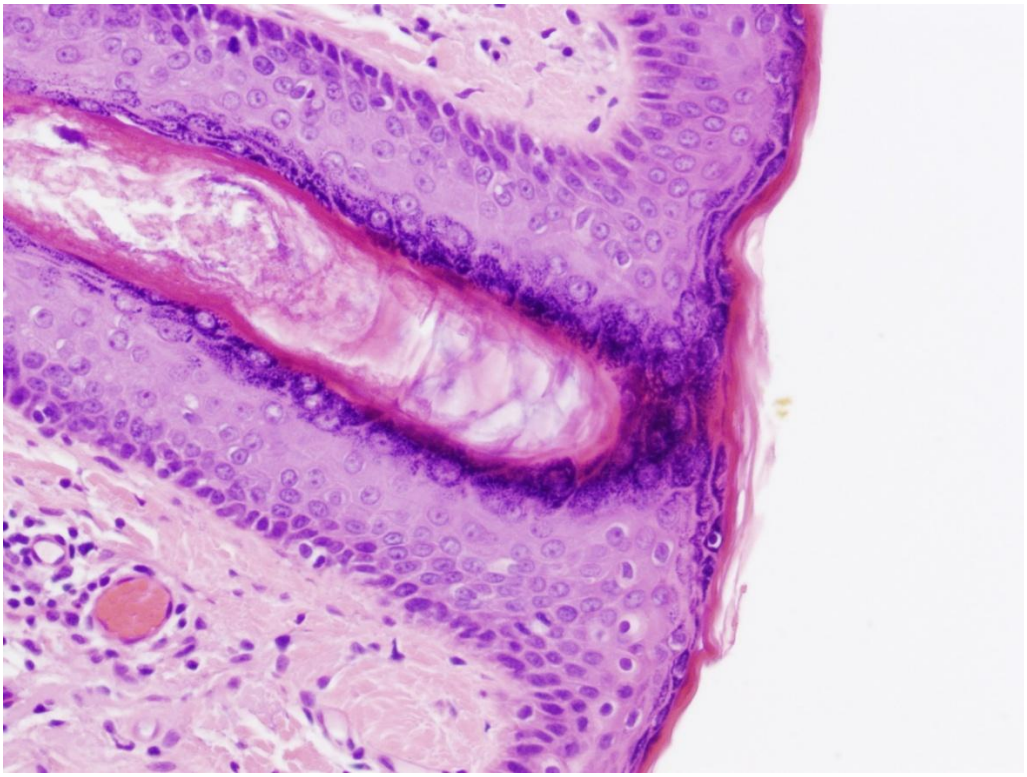
1. Place sections in xylene for at least 1 minute each (3 changes). For each step, agitate slides up and down to ensure good reagent contact.
2. Place sections in absolute ethanol for 10 dips (2 changes)
3. Dip slides in 95% ethanol 10 times
4. Dip in water for 10 seconds
5. Place slides in hematoxylin for 5 minutes
6. Rinse in running DI or tap water for 1 minute. If running water is not available, use at least 3 changes of standing water, but agitate at least 10 times to remove excess hematoxylin.
7. 3-4 quick dips in acid alcohol (0.5% HCl in 50% ethanol) and then place immediately back in running tap water for 1 minute. If running tap water is not available, use at least 5 changes of standing water and dip the slides in each container 10 times.
8. 30 dips in bluing reagent
9. 1 minute in running tap water or 3-5 changes of standing water
10. 10 dips in 95% ethanol
11. 2 minutes 15 seconds in eosin
12. 10 dips in 95% ethanol
13. 10 dips in absolute ethanol (3 changes)
14. 1 minute in xylene (3 changes)
15. Coverslip with resinous mounting medium

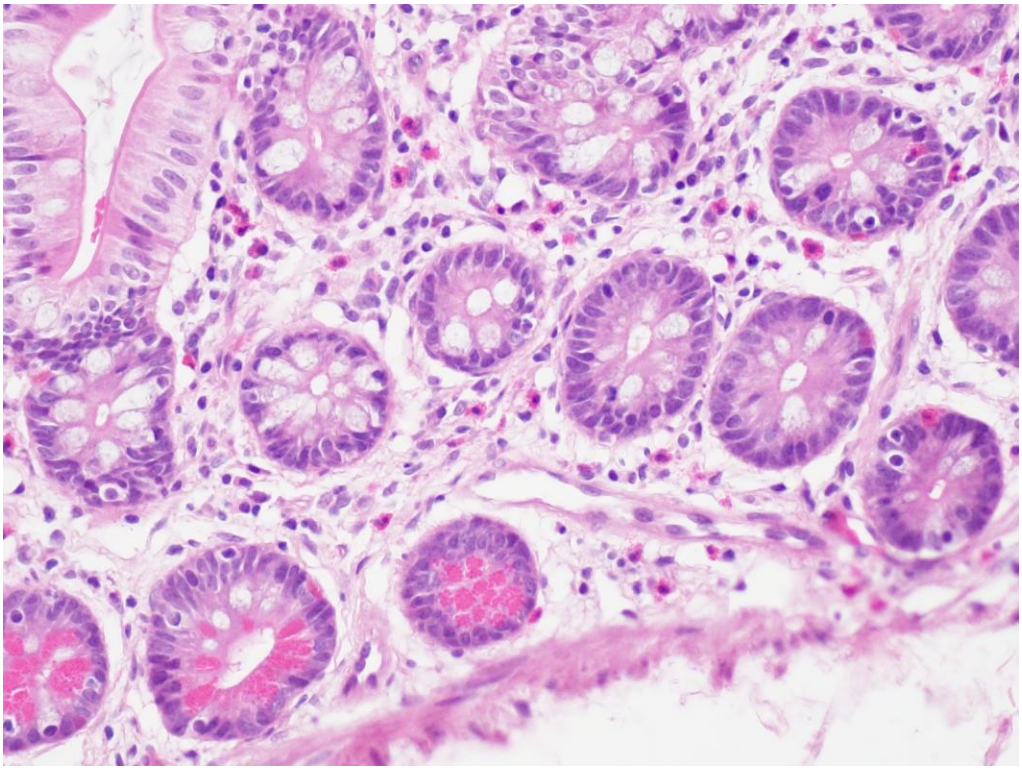
Staining time: 30 minutes

Expected Staining Results:

- Nuclei – Dark purple/blue
- Mucin – Colorless to lavender
- Collagen – Pale pink
- Muscle – Dark pink
- Red blood cells, eosinophils, zymogen and Paneth granules - red

H&E Images (skin and small intestine)





Name of Stain: Prussian Blue

Reagents Needed:

20% Aqueous Solution of Hydrochloric Acid:

- 20 mL hydrochloric acid, concentrated
- 80 mL distilled water

Mix well

10% Aqueous Solution of Potassium Ferrocyanide:

- 10 g potassium ferrocyanide, trihydrate
- 100 mL distilled water

Mix to dissolve

0.1% nuclear fast red solution:

- Dissolve 25 g of aluminum sulfate in 500 ml of distilled water.
- Add 0.5 g of nuclear fast red. Heat solution to boiling or near boiling briefly.

Cool and filter. Solution probably holds up better in opaque container.

Control slide provided: Liver with iron stores

Procedure:

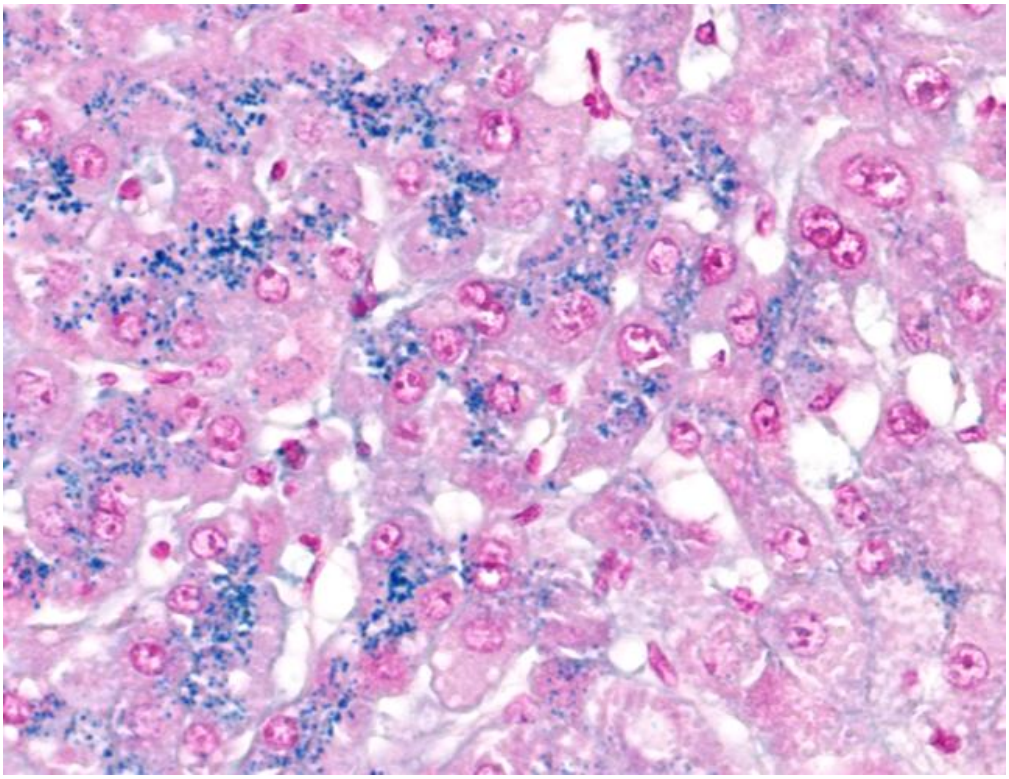
1. Deparaffinize and hydrate sections to distilled water.
2. Mix equal parts of 20% hydrochloric acid and 10% potassium ferrocyanide
3. Immerse slides in this solution for 20 minutes.
4. Wash in running DI or tap water for 1 minute or rinse in several changes of standing water.
5. Counterstain with nuclear fast red for 5 minutes.
6. Rinse in running DI or tap water for 30 seconds or rinse in several changes of standing water.
7. Dehydrate, clear, and coverslip with a resinous mounting medium.

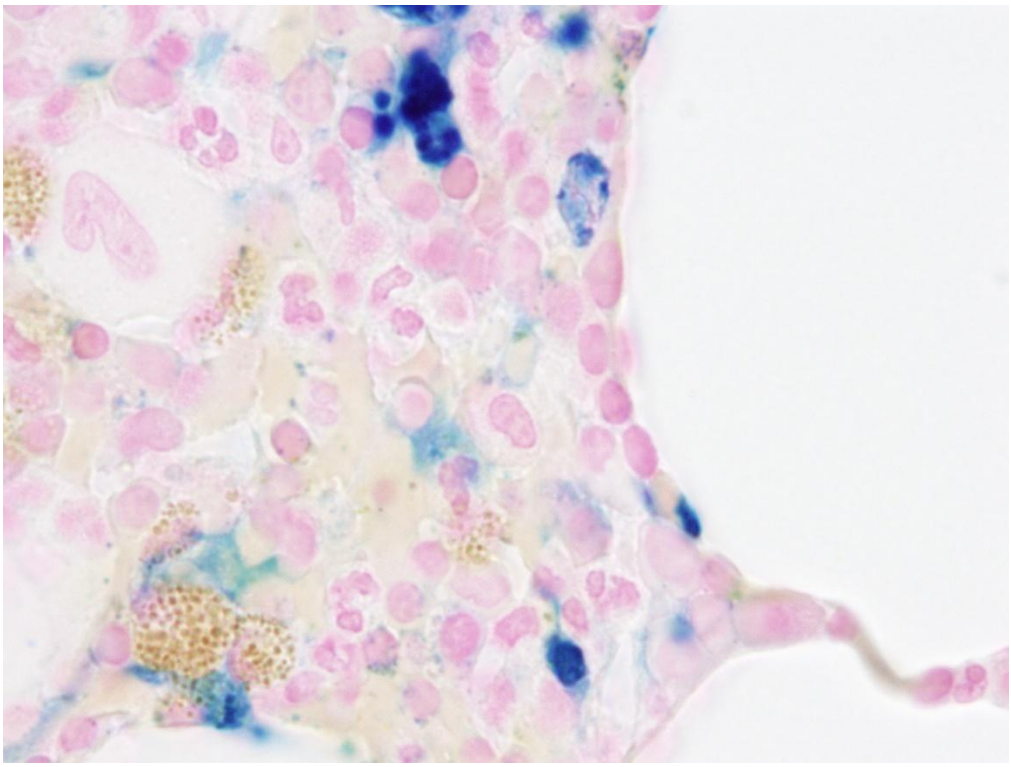
Staining time: 45 minutes

Expected Staining Results:

Iron (ferric form) - bright blue
Nuclei - red
Cytoplasm - pink

Prussian Blue Images (liver and bone marrow)





Name of Stain: Wright

Reagents Needed:

- Wright Stain
- Wright Buffer
- Water

Procedure:

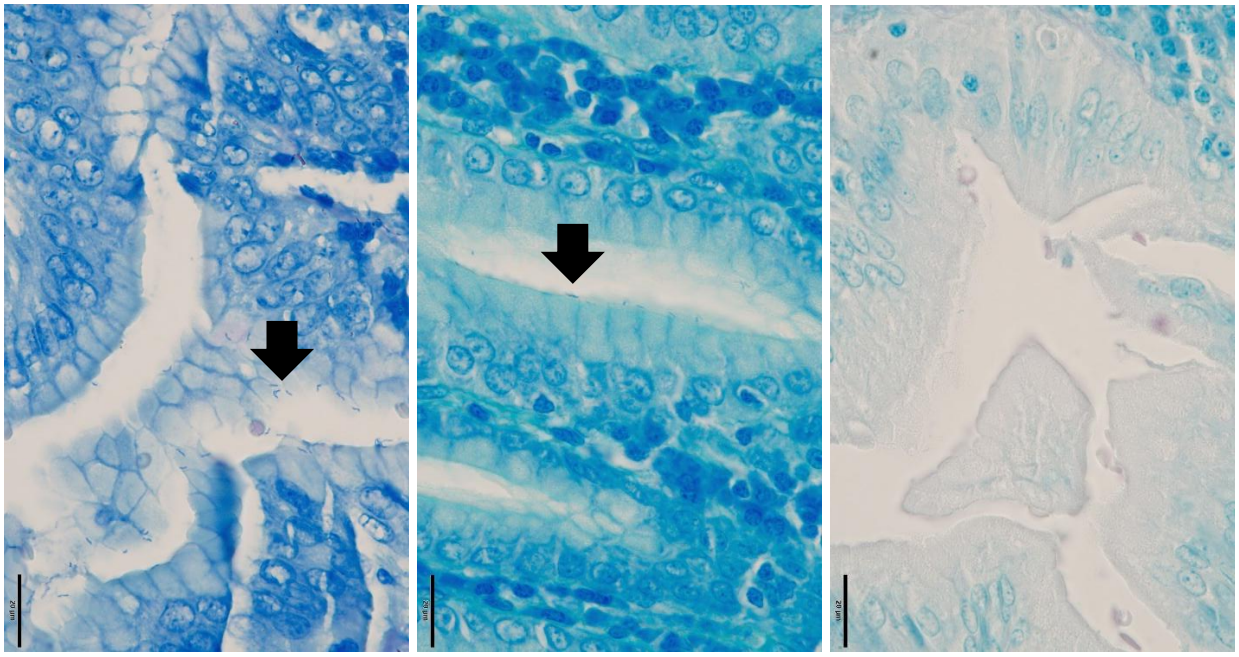
1. Deparaffinize and bring sections to distilled water
2. Stain with Wright stain for 4 minutes
3. Mix 1 part Wright stain with 3 parts Wright buffer
4. Place slides in the freshly-mixed buffered Wright staining solution WITHOUT AGITATING for 3 minutes
5. Rinse 1 minute in water
6. If staining for mast cells, dehydrate using increasing concentrations of alcohol and clear with xylene before coverslipping with resinous mounting medium. If staining for Helicobacter pylori, it is recommended to allow sections to air dry after the last water rinse. The ethanol changes decolorize the blue slightly which makes for better contrast with purple mast cells but makes H. pylori paler and thus easier to overlook. After allowing to completely air dry, dip the tissue section into xylene 10 times and then coverslip.

Control slide provided: Stomach with H. pylori and canine mast cell and non-mast cell tumors

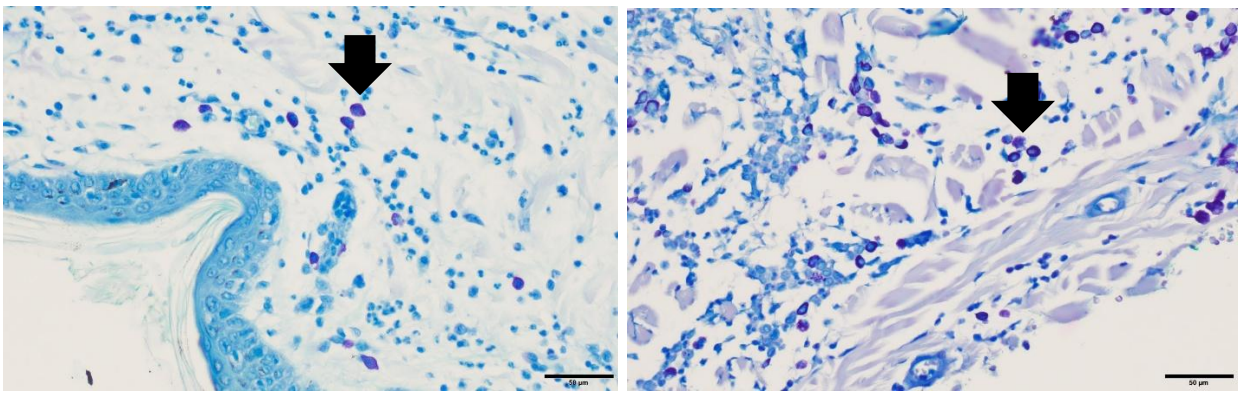
Expected Staining Results:

- Nuclei – blue
- Eosinophils - pink
- Cytoplasm - varying shades of light blue
- Mast cells – purple
- H. pylori - blue

Wright stain images (stomach and skin)



In the first image of stomach, the tissue section was allowed to air dry prior to coverslipping and the dark blue color was retained. In the second image, the section was dehydrated through graded alcohols and has lightened somewhat. It is still acceptable for H. pylori identification but is paler. The third image was left in ethanol rinses too long and is unacceptable for H. pylori evaluation.



The first skin image was dehydrated through ethanols and the lighter blue provides a better contrast to the purple mast cells than the second image which was allowed to air dry.

Name of Stain: Modified Trichrome

Reagents Needed:

Bouin Solution:
- 75 ml picric acid (saturated)
- 25 ml formaldehyde
- 5 ml glacial acetic acid

or

Gram's iodine:
- 0.3 g iodine
- 0.7 g potassium iodide
- 100 ml water

Biebrich Scarlet-Acid Fuchsin Solution:
- 90 ml Biebrich scarlet, 1% aqueous
- 10 ml acid fuchsin, 1% aqueous
- 1 ml acetic acid, glacial

5% Phosphomolybdic Acid Solution:
- 5 g phosphomolybdic acid
- 100 ml water

5% Phosphotungstic acid solution:
- 5 g phosphotungstic acid
- 100 ml water

Aniline Blue Solution:
- 2.5 g aniline blue
- 2 ml acetic acid, glacial
- 100 ml distilled water

Control slide provided: Multi-tissue microarray

Procedure:

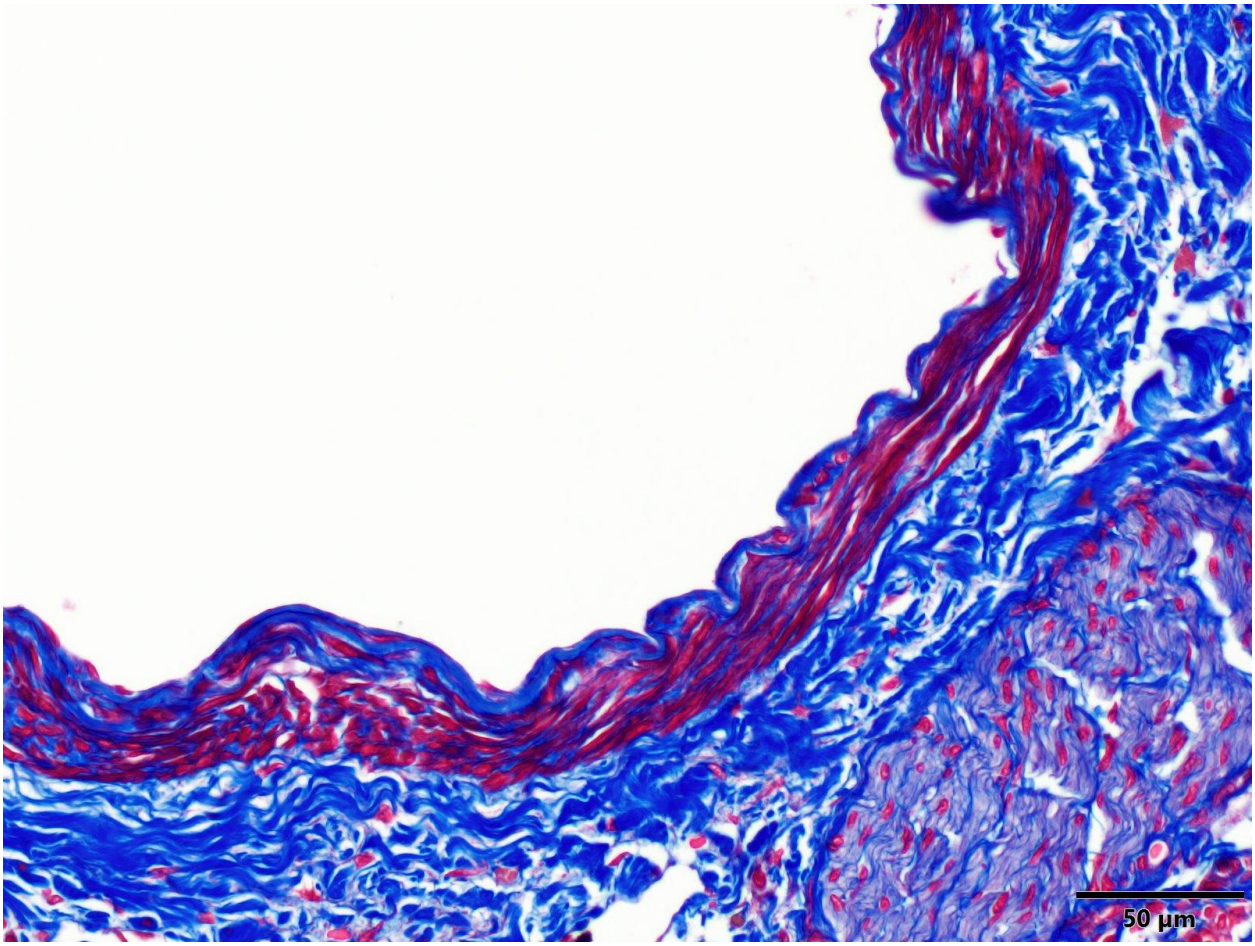
1. Preheat Bouin or Gram's iodine solution to 60 C in oven for 20 minutes or in water bath for 15 minutes.
2. Deparaffinize sections to water.
3. Post-fix sections in Bouin (or Gram's iodine) solution for 15 minutes at 60 C.
4. Rinse in running DI or tap water for 5 minutes to remove the yellow color or rinse for 1 minute if using iodine. If using standing water, rinse in 5 changes.
5. Stain in Biebrich scarlet-acid fuchsin solution for 5 minutes.
6. Wash in DI or tap water for 30 seconds. Alternately, use at least 3 changes of water.
7. Combine equal parts of 5% phosphomolybdic and 5% phosphotungstic acid solutions and differentiate tissue sections for 10 minutes or until collagen is not red. Sections will probably appear pink grossly.
8. Transfer sections to aniline blue solution and stain for 10 minutes.
9. Wash in DI or tap water for 30 seconds. Or rinse for enough changes in standing water for water to remain colorless.

10. Dehydrate through 95% ethyl alcohol, absolute ethyl alcohol (these steps will remove Biebrich scarlet-acid fuchsin and aniline blue reagents if sections remain too long in them) and clear in xylene.
11. Mount with resinous mounting medium.

Expected Staining Results:

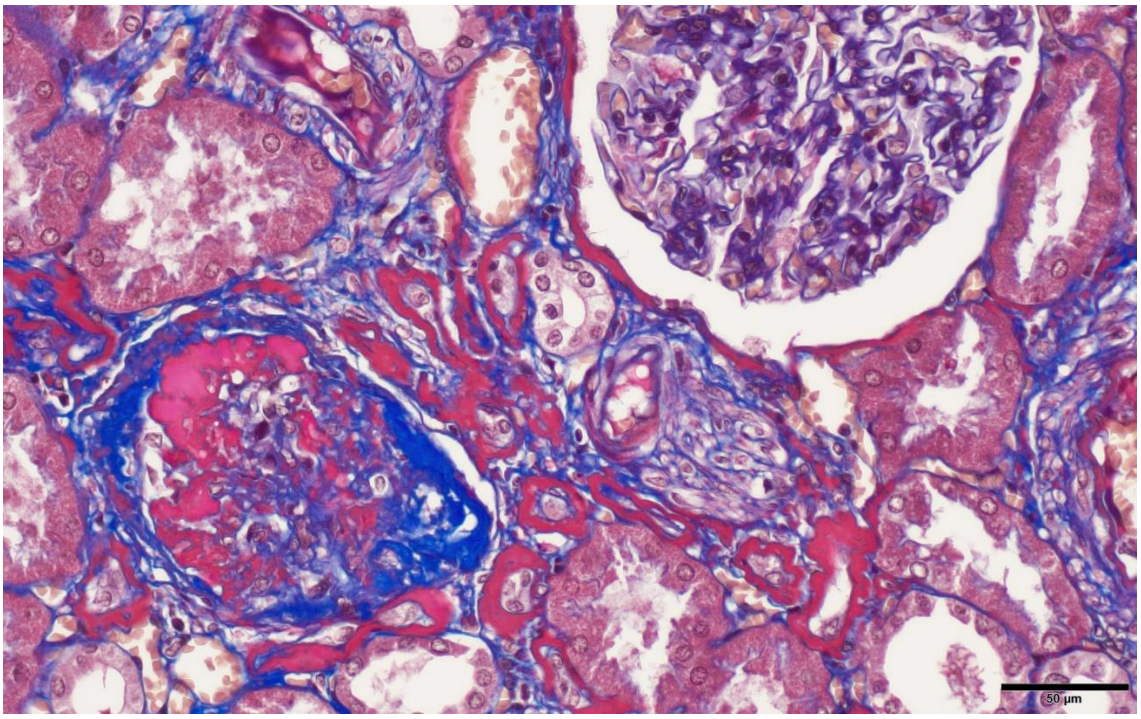
Muscle and cytoplasm – red
Fibrous connective tissue - blue

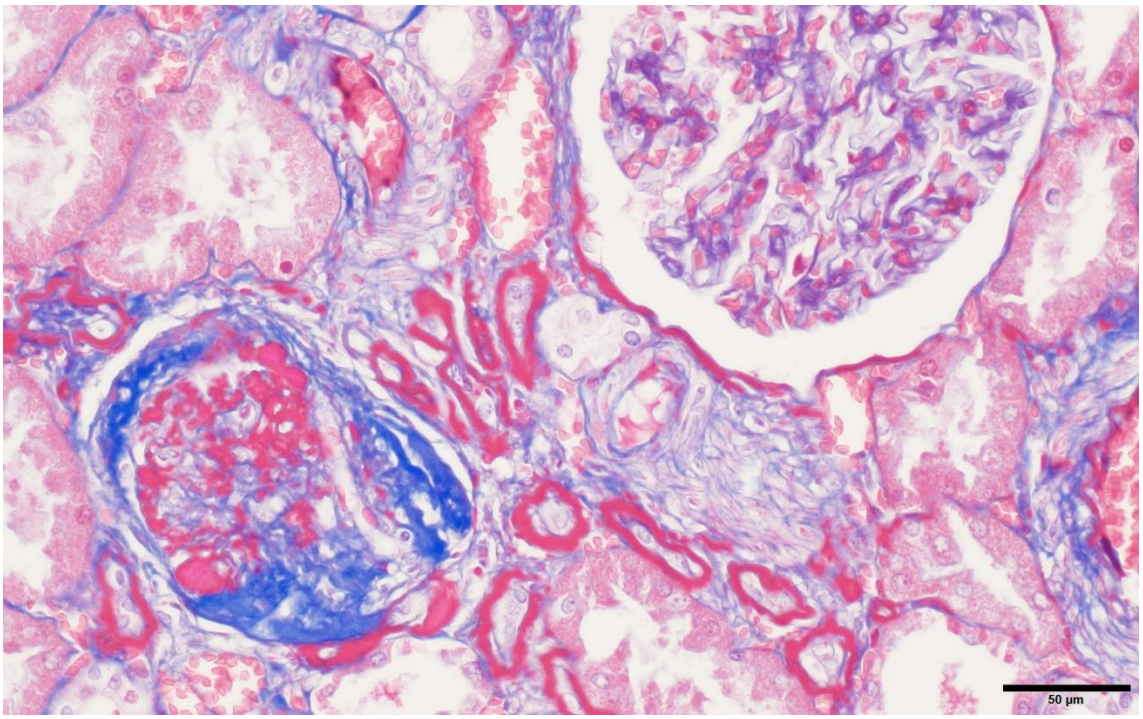
Trichrome images (artery and kidney)



In a well-performed trichrome stain, collagen is dark blue, cells are red, and when the collagen interdigitates with the smooth muscle cells in an arterial wall, on low power, the wall looks almost purple.

In the classic trichrome stain, there is an iron hematoxylin step to stain nuclei after the Bouin or Gram's iodine. However, some laboratories opt to omit the iron hematoxylin step it can dull the red and blue. Because this stain is used to identify collagen, the blue is the important color. See kidney images below without and with iron hematoxylin.





Name of Stain: Von Kossa

Reagents Needed:

1% Aqueous Silver Nitrate Solution

- 1 g silver nitrate
- 100 ml distilled water

5% Sodium Thiosulfate

- 5 g sodium thiosulfate
- 100 ml distilled water

0.1% nuclear fast red solution

- Dissolve 25 g of aluminum sulfate in 500 ml of distilled water.
- Add 0.5 g of nuclear fast red. Heat solution to boiling or near boiling briefly.
- Cool and filter. Solution probably holds up better in opaque container.

Control slide provided: Placenta with calcifications

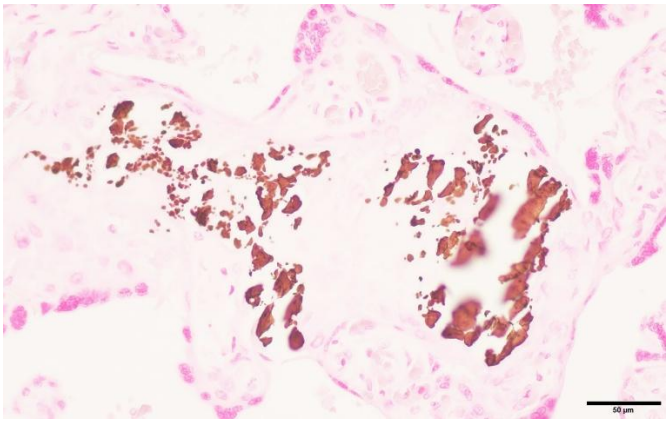
Procedure:

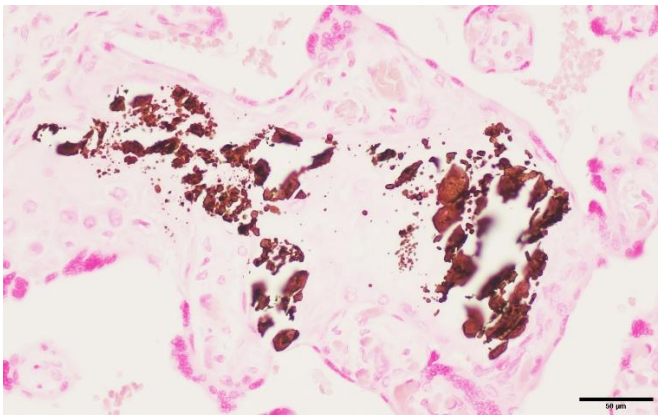
1. Deparaffinize paraffin sections and hydrate to water.
2. For this staining procedure, slides should be laid flat on a staining tray. Wipe away excess water around tissue and draw a hydrophobic barrier. Do not allow the tissue sections to dry. Add water from squirt bottle if there are multiple slides. This stain can also be performed in a glass staining jar or dish, but it must be completely clean with no contamination with metal ions. A metal rack should not be used during the silver nitrate step.
3. After shaking off the water, incubate sections with 1% silver nitrate solution by pipetting the solution onto the surface of the tissue section. Swirl the reagent on the slide to make sure it is getting good contact with the tissue.
4. Sections should be placed under a 60-100 watt light bulb for 30 min if the bulb has the correct color tone and is situated closely to the tissue surface. Lights with warm (yellow) tones develop calcifications more slowly than lamps with blue or ultraviolet waves. If simply letting the tissues incubate under ceiling lights, the incubation time may take up to 3 hours. Note: Calcium deposits begin to show up as tan/beige but will darken to dark brown and then black with longer light exposure.
5. Discard silver solution on slide into a silver waste container and neutralize with saturated sodium chloride solution. Collect filter paper and dispose of as hazardous material.
6. Rinse in running DI or tap water for 1 minute. Or rinse with a squirt bottle for 30 seconds.
7. Remove unreacted silver with 5% sodium thiosulfate for 5 minutes by pipetting reagent onto slide surface.
8. Rinse in distilled water 30 seconds with water bottle.
9. Counterstain with nuclear fast red for 10 minutes. Background should be grossly pink.
10. Rinse in DI or tap water for 1 minute. Or rinse with squirt bottle for 30 seconds.
11. Dehydrate through graded alcohols and clear in xylene.
12. Coverslip using permanent mounting medium.

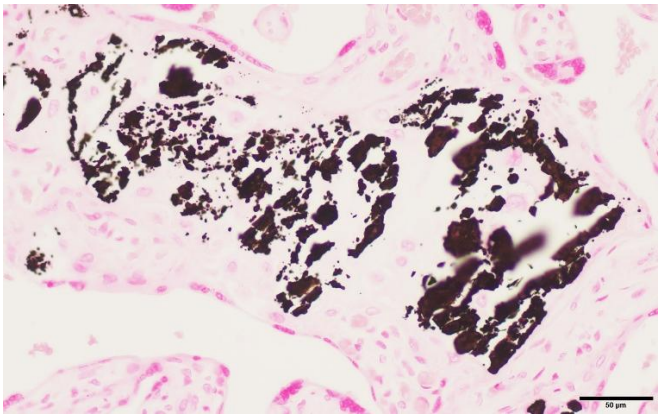
Expected Staining Results:

- Calcium salts - black or brown-black
- Nuclei - red
- Cytoplasm - pink

Von Kossa images (calcifications in placenta). Note the increased darkening of calcifications in proportion to light exposure.







Name of Stain: Rhodanine

Reagents Needed:

1. Rhodanine stock solution
   - dimethylamino benzylidene rhodanine
   - absolute ethanol

   Make a saturated solution (>0.2%) of the rhodanine in alcohol. Store the stock solution at 4°C. Stable for at least 3 months.

2. Rhodanine working solution (prepare fresh before use and shake well)
   Use 1 parts stock solution to 9 parts DI water

3. Hematoxylin (This solution should be Mayers, Gill, Harris, or any other ready-to-use hematoxylin which can be used for H&E staining. This is not an iron hematoxylin.)

Control slide provided: Liver with copper deposits

Procedure:

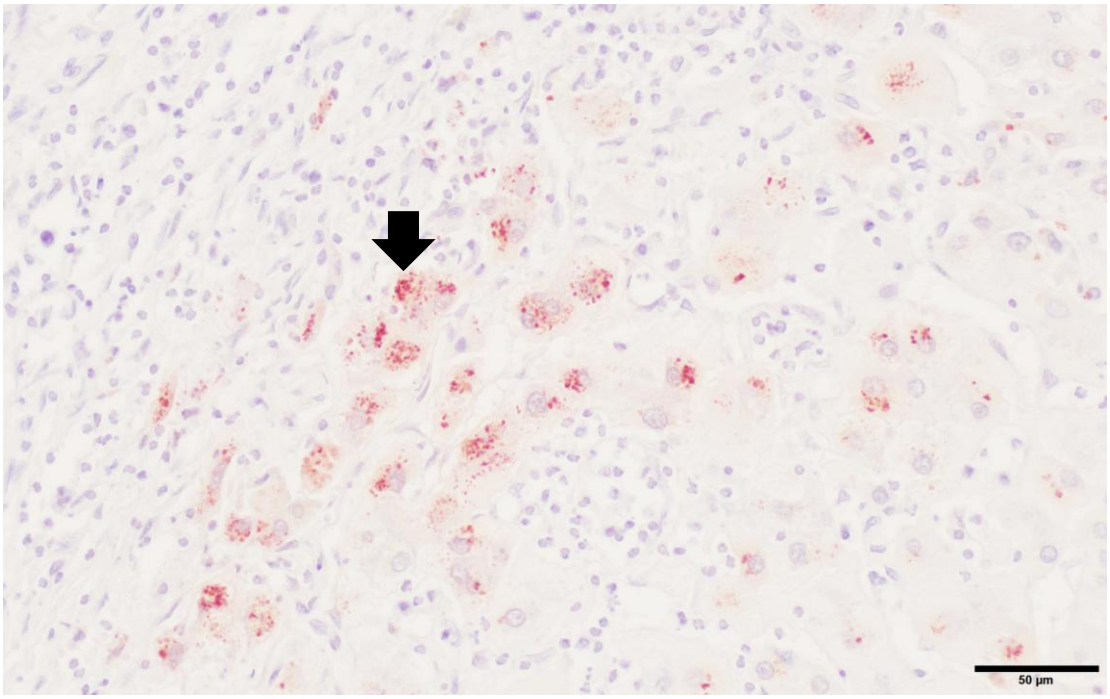
1. Prepare working rhodanine solution by mixing 2 ml from refrigerated stock solution with 18 ml DI water. Mix well. Preheat in 60C oven for 20 minutes or water bath for 15 minutes.
2. Deparaffinize sections to distilled water.
3. Stain in rhodanine working solution at 60°C 45 minutes. Alternately, heat slides in rhodanine copper solution on low power in microwave for 1 minute and let stand 4 minutes. If the tissue exposure time is too short, copper deposits will be pale and may resemble lipofuchsin. If the staining time is too long the copper deposits appear bold but may also show bleeding of chromogen into surrounding cytoplasm.
4. Rinse 1 minute in DI or tap water. Or rinse in at least 3 changes of water.
5. Lightly counterstain with hematoxylin 5-60 secs (timing depends on which hematoxylin is used). Tissue sections should appear pale lavender grossly. If using Harris hematoxylin from H&E staining line, dip twice only in hematoxylin. For Mayers, can leave for 1 minute.
6. Rinse in running tap water for 2 minutes to blue hematoxylin. Alternately, use 5 changes of standing tap water to rinse and blue sections.
7. Dehydrate, clear, and coverslip.

Staining time: 1 hour 15 minutes

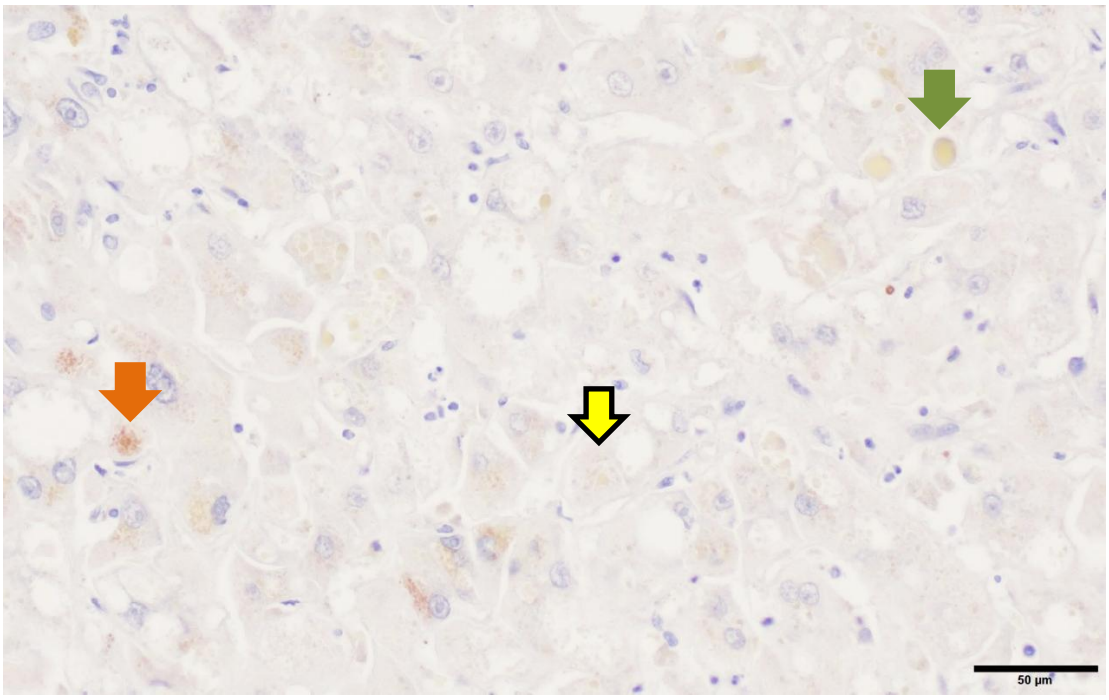
Expected Staining Results:

Copper deposits – brick red or orange-brown
Nuclei and cytoplasm – pale blue

Rhodanine images (liver and liver)



Copper-colored pigment identifies copper.



In this image, the tissue was not incubated long enough in the hot solution and the copper (orange arrow) is difficult to differentiate from lipofuscin (yellow arrow) and bile (green arrow).

Name of Stain: **Verhoeff Van Gieson**

Stock Reagents:

5% Alcoholic Hematoxylin:

- 5 g hematoxylin
- 100 mL absolute alcohol

Dissolve hematoxylin into alcohol with the aid of gentle heat; do not boil. Solution is stable for 1 year.

10% Ferric Chloride:

- 10 g ferric chloride
- 100 mL DI water

Mix well. Solution is stable for 1 year.

Lugol's Iodine:

- 2 g iodine
- 4 g potassium iodide
- 100 mL water

Combine iodine and potassium iodide first. Add 5 mL water. Dissolve, then add remainder of water. Adding all water at once will cause the solution to not dissolve for 1-2 days.

Working Solutions:

Verhoeff's Hematoxylin:

- 20 mL 5% alcoholic hematoxylin
- 8 mL 10% ferric chloride
- 8 mL Lugol's iodine

Add in the order given, mixing between additions.

2% Ferric Chloride:

- 10 mL 10% ferric chloride
- 40 mL Distilled water

  Or
- 2 g ferric chloride dissolved in 100 ml water

5% sodium thiosulfate

- 25 g sodium thiosulfate
- 500 mL water

Van Gieson Solution:

- 5 mL 1% acid fuchsin
- 45 mL picric acid, saturated

Control slide provided: Skin and lung

Procedure:

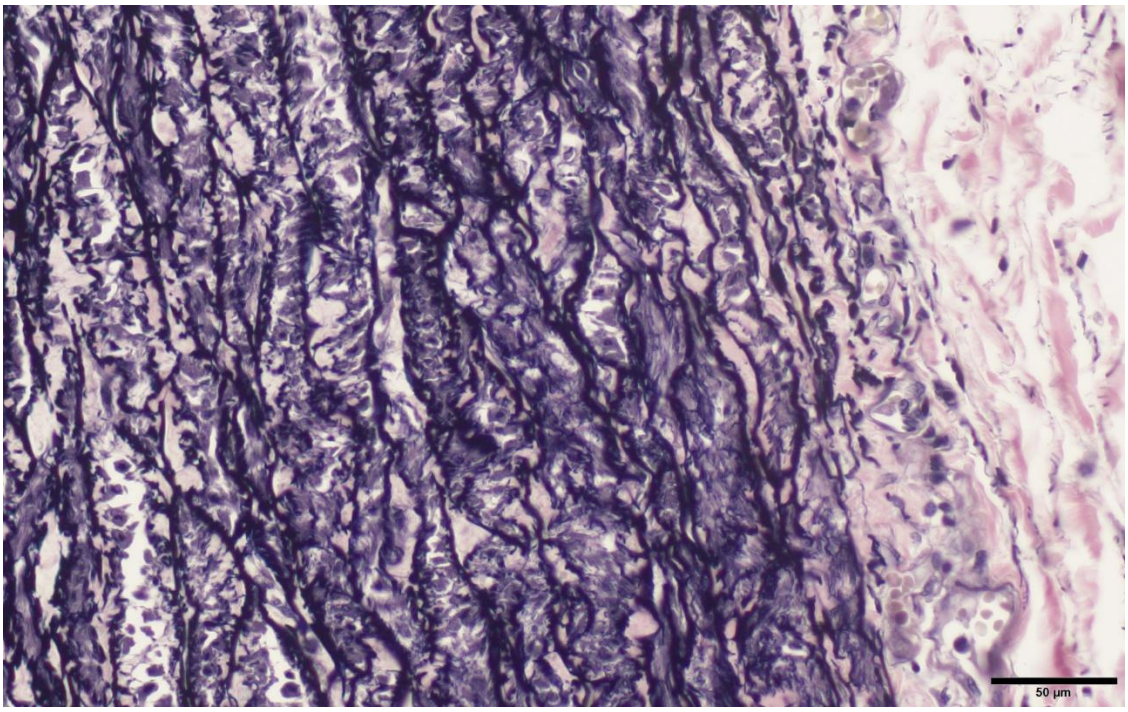
1. Deparaffinize and hydrate sections to distilled water.
2. Prepare Verhoeff's working hematoxylin.
3. Place slides in Verhoeff hematoxylin for 30 minutes (save solution until stain is completed in case sections are inadvertently over decolorized). Sections should be completely black initially, both grossly and microscopically when removed from Verhoeff's hematoxylin. The glass slide itself will also have a gray grungy appearance.
4. Wash in DI or tap water for 30 seconds. Alternately, rinse in several changes of standing water until the black color stops coming off.
5. Differentiate in 2% ferric chloride solution, check microscopically for black fibers on a gray-purple background. Typically, the slide is dipped into ferric chloride just until the glass of the slide becomes clear and colorless again. If you are not sure, it's better to err on the side of underdifferentiation as the subsequent reagents often cause the elastic fibers to pale slightly.
6. Rinse in DI or tap water for 30 seconds. Alternately, use at least 3 changes of standing water.
7. Place sections in 5% sodium thiosulfate for 1 minute to remove unbound iron hematoxylin.
8. Wash in DI or tap water for 30 seconds. Alternately, use at least 3 changes of standing water.
9. Counterstain in van Gieson solution for 5 minutes.
10. Rinse briefly in water. Do not rinse for more than 10 seconds or picric acid may wash away.
11. Dehydrate, clear in xylene, and coverslip.

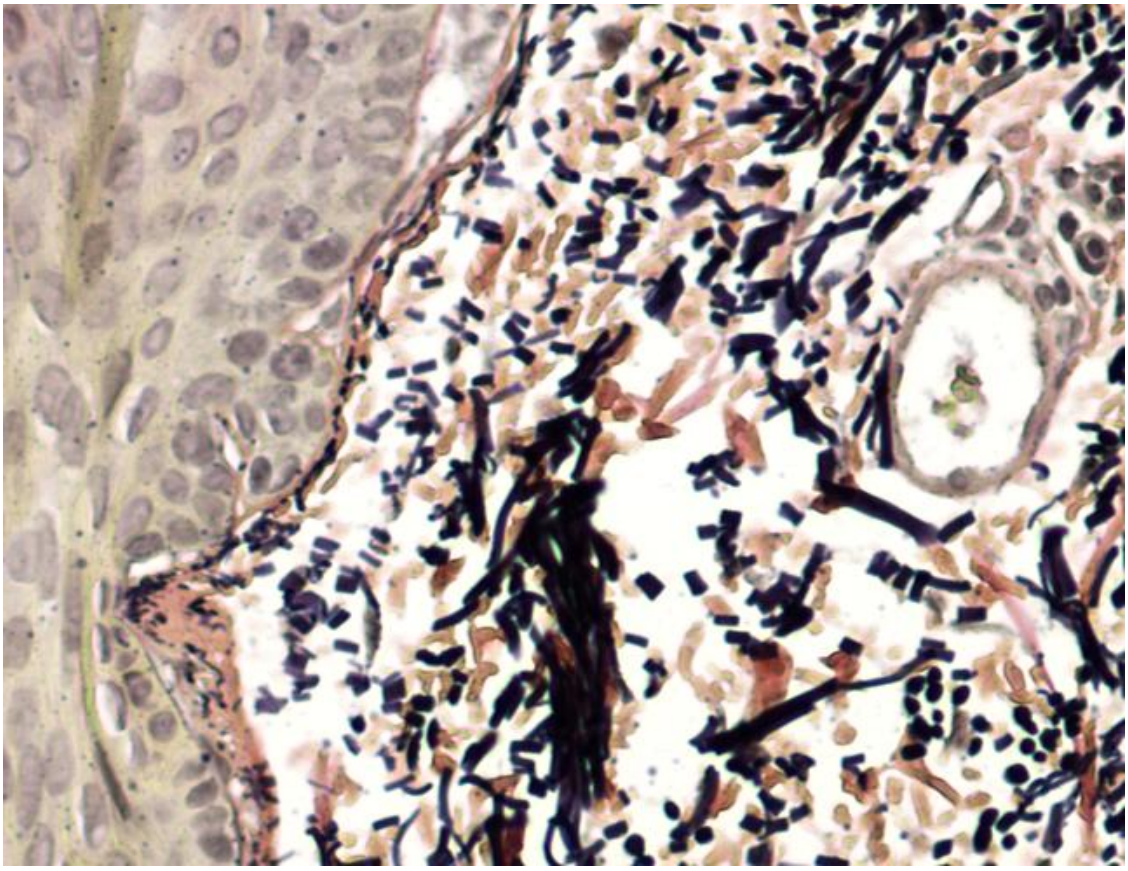
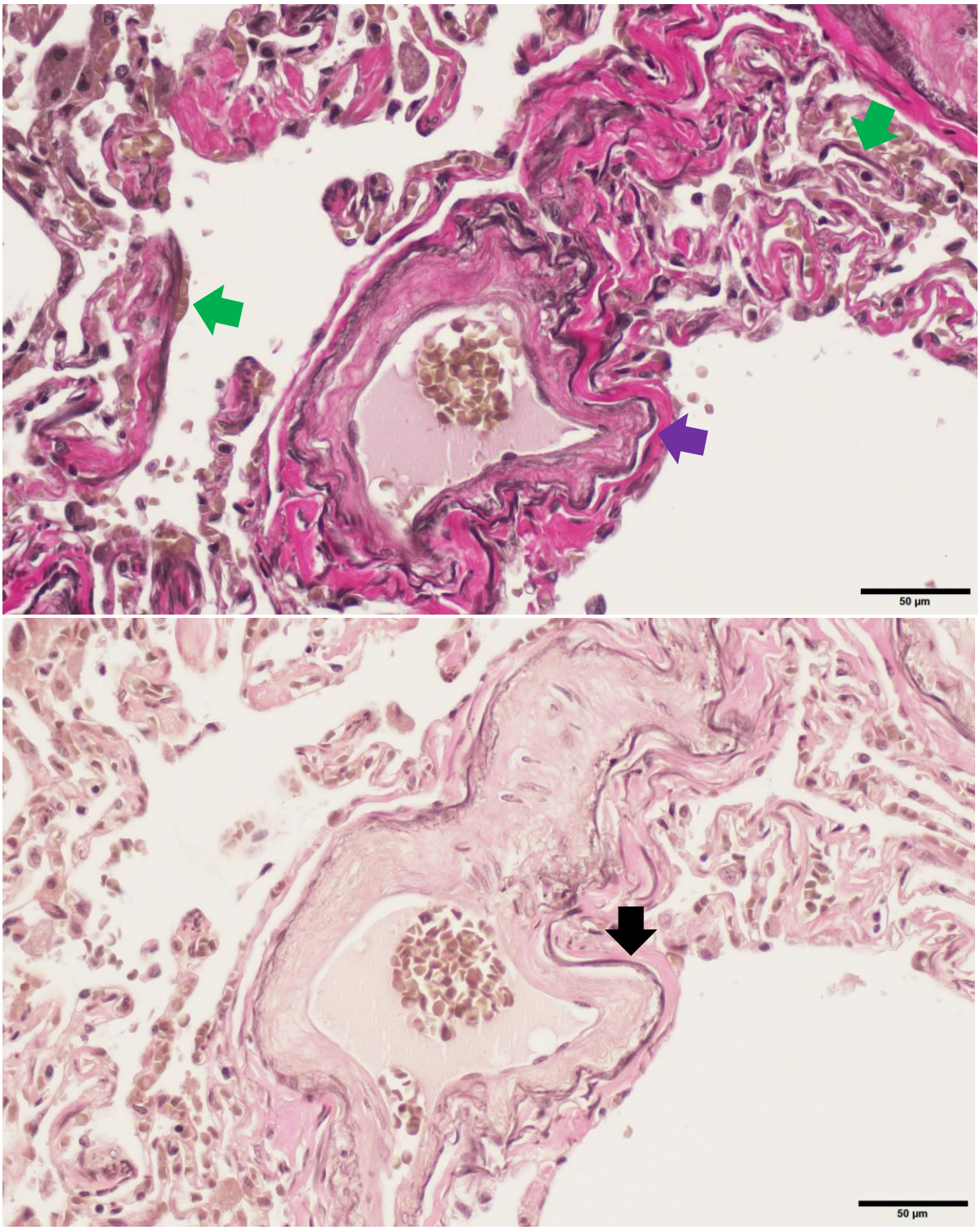
Staining time: 60 minutes

Expected Staining Results:

- Elastic fibers: blue-black to black
- Nuclei: blue to black
- Collagen: red
- Cytoplasm: yellow

Verhoeff van Gieson images (elastic artery, skin, and lung)





In the following images, almost serial sections were taken from the same congested lung. In the top image, the elastic is seen in the wall of an arteriole (purple arrow) and in the walls of the alveolar sacs (green arrows). In the lower image, the iron hematoxylin was overdifferentiated making the elastic fibers (black arrow) difficult to see. Additionally, the counterstain was rinsed too long in water and is also too pale.