

## 2-11. SILVER STAINS

- a. **Gridley's Ammoniacal Silver Nitrate For Reticular Fibers**
- b. **Foot's Modification Of Hortega's Silver Carbonate Method For Reticulum**
- c. **Avallone's Modification Of Jones' Silver Methenamine Method For Basement Membranes**
- d. **Jones' Silver Methenamine Method For Basement Membranes**
- e. **Fontana-Masson Silver Nitrate Method**

### Staining Procedures For Plastic Embedded Tissue

*Verified at the Applications Laboratory of the Biomedical Division, Sorvall Microtomes*

NOTE: When preparing a solution for or performing any silver staining procedures, it is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chlorine-free water.

- a. **Gridley's Ammoniacal Silver Nitrate for Reticular Fibers (Ref 4)**

#### SOLUTIONS:

##### SOAmmoniacal Silver Nitrate Solution

20 drops	Sodium hydroxide, 10%
20ml	Silver nitrate, 5%
a few drops	Ammonia water, 50%
30-40ml	Distilled water

NOTE: All glassware should be chemically clean. This solution must always be freshly prepared. Add 20 drops of sodium hydroxide to 20ml of silver nitrate. Add 50% ammonia water drop by drop until there is a layer of granules left on the bottom of the cylinder. The ammonia water should be fresh and only a minimal amount used. Add distilled water to make 60ml. Prepare just before use.

##### 1% Periodic Acid

1.0g Periodic acid

100.0ml Distilled water

##### 2% Silver Nitrate

2.0g Silver nitrate

100.0ml Distilled water

##### Formalin Solution

30.0ml Stock formalin

70.0ml Distilled water  
0.2% Gold Chloride  
10.0ml Gold chloride, 1%  
40.0ml Distilled water  
5% Sodium Thiosulfate  
5.0g Sodium thiosulfate  
100.0ml Distilled water

#### **STAINING PROCEDURE:**

NOTE: It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chlorine-free water.

1. Heat slides on 60°C to 80°C hot plate for 10 minutes.
2. Cool.
3. Treat in periodic acid for 15 minutes.
4. Rinse in distilled water.
5. Stain in silver nitrate for 30 minutes.
6. Rinse in distilled water.

If sections appear to loosen, blow dry and refix on hot plate. If sections loosen later, refix longer.

7. Stain in ammoniacal silver nitrate for 15 minutes.
8. Rinse quickly in distilled water.
9. Fix in formalin solution for 5 minutes.

Wash in distilled water for 3 changes.

Tone in gold chloride for 2-5 minutes.

Wash in distilled water.

Bleach in sodium thiosulfate for 5 minutes.

Wash in distilled water for 10 minutes.

Blow dry.

Mount.

#### **RESULTS:**

Fine reticular fibers are stained black on a light taupe background.

## b. Foot's Modification Of Hortege's Silver Carbonate Method For Reticulum (ref 6)

### SOLUTIONS:

#### Silver Carbonate Solution

10ml Silver nitrate, 10%  
10ml Lithium carbonate, saturated (1.25%)  
approx. 150ml Distilled water  
10-20 drops Ammonium hydroxide, concentrated  
60-Ethyl alcohol, 95%

NOTE: All glassware should be chemically clean. This solution must always be freshly prepared.

- Place 10ml of the silver nitrate solution in a 100ml capacity graduated cylinder.
- Add 10ml of a saturated solution of lithium carbonate.
- Wash the white precipitate three times with distilled water. To do this, simply add approximately 30 to 40ml of distilled water to the silver carbonate mixture in the cylinder. Shake the cylinder vigorously, allow precipitate to settle to bottom, and carefully decant the supernatant fluid. Do this three times.
- Add 25ml of the distilled water to the cylinder. Almost dissolve the precipitate with ammonium hydroxide added drop by drop (approximately 6 to 15 drops) while shaking the container vigorously. Avoid adding too much ammonium hydroxide; it is better to leave a few grains of precipitate than to add too much.
- Bring the solution up to 100ml with 95% ethyl alcohol. Pour solution into a small flask (250ml capacity) for easier handling. A precipitate will form. Dissolve precipitate by adding a few more drops of ammonium hydroxide.
- Filter this alcoholic solution, then warm it for 20 minutes in a paraffin oven at 56°C to 58°C.

#### 0.25% Potassium Permanganate

0.25g Potassium permanganate  
100.0ml Distilled water

#### 5% Oxalic Acid Solution

5.0g Oxalic acid  
100.0ml Distilled water

#### 20% Neutral Formalin

20.0ml Neutral formalin  
80.0ml Distilled water

#### 0.2% Gold Chloride Solution

1.0g Gold chloride  
500.0ml Distilled water

## STAINING PROCEDURE:

Note: It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chlorine-free water.

1. Heat slides on 60°C to 80°C hot plate for 10 minutes.
2. Cool.
3. Oxidize in potassium permanganate for 5 minutes.
4. Rinse in distilled water.
5. Treat in oxalic acid for 10 minutes.
6. Wash in distilled water for 5 minutes.

If sections appear to loosen, blow dry and refix on hot plate; cool, then proceed.

7. Stain in warmed silver carbonate solution in 40°C oven for 30 minutes.
8. Rinse in distilled water.
9. Fix in neutral formalin for 5 minutes.

Wash in distilled water for 10 minutes.

Tone in gold chloride for 5 minutes.

Wash in distilled water.

Bleach in sodium thiosulfate for 2 minutes.

Wash in distilled water for 5 minutes.

Counterstain if desired with eosin, or hematoxylin and Van Gieson's stain (2-12).

Rinse in distilled water.

Blow dry.

Mount.

## RESULTS:

Coarse connective tissue fibers -- brownish pink; reticulum -- black; nuclei -- black.

### c. Avallone's Modification Of Jones' Silver Methenamine Method For Basement Membranes (Ref 4)

## SOLUTIONS:

1% Periodic Acid

1.0g Periodic acid  
100.0ml Distilled water

3% Methenamine

3.0g Hexamethylene tetramine (methenamine)  
100.0ml Distilled water

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5% Silver Nitrate

5.0g Silver nitrate  
100.0ml Distilled water

0.05 M Sodium Borate

19.07g Sodium borate (H<sub>3</sub>BO<sub>3</sub>) (decahydrate)  
qs to 1000.0ml Distilled water

0.05 M Boric Acid

3.0g Boric acid  
qs to 1000.0ml Distilled water

0.2% Gold Chloride

10.0ml Gold chloride, 1%  
40.0ml Distilled water

Solution is stable for approximately 50 slides.

5% Sodium Thiosulfate

5.0g Sodium thiosulfate  
100.0ml Distilled water

Methenamine Silver Solution

42.5ml Methenamine, 3%  
2.5ml Silver nitrate, 5%  
7.0ml Sodium borate, 0.05 M

to adjust pH

to 9.15-9.2 Boric acid, 0.05 M

Harris' Hematoxylin and Eosin

See procedure 2-7, b

**STAINING PROCEDURE:**

NOTE: It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chlorine-free water.

1. Heat slides on 60°C to 80°C hot plate for 10 minutes
2. Cool.
3. Treat in periodic acid for 11 minutes.
4. Rinse well in 2-3 changes of chloride-free distilled water for 20 seconds each.
5. Place slides in the Coplin jar in heated distilled water (68°C).
6. Pour 35ml of the methenamine silver solution into a 250ml Erlenmeyer flask and swirl it around in the heated water bath. Pour the distilled water from the Coplin jar previously filled with slides. Replace with hot methenamine silver solution.
7. Rinse the slides in hot distilled water before examining and before returning the slide to the hot methenamine silver solution.

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After 12 minutes of exposure to methanamine silver, check the slide microscopically at 1-minute intervals. When the capsule basement membrane is a fine black line, remove the section from methenamine silver and place in distilled water. If sections appear to loosen, blow dry and refix on hot plate, cool, and proceed.

8. Tone in the working gold chloride solution for 10-15 seconds or until background is clear. Check each slide under the microscope. Do not overtone.
9. Rinse well in distilled water.

Treat in sodium thiosulfate for 2-3 minutes. If sections were overdone in gold chloride, only treat in sodium thiosulfate for 1-2 minutes.

Wash in running tap water.

Rinse in distilled water.

Counterstain with Harris' hematoxylin and eosin ( 2-7, b).

Rinse in distilled water.

Blow dry.

Mount.

#### **RESULTS:**

Basement membrane	black
reticular fibers	black
nuclei	blue
cytoplasm, red blood cells, collagen and connective tissue	pink to orange.

#### **d. Jones' Silver Methenamine Method For Basement Membranes (Ref 5)**

#### **SOLUTIONS:**

##### 0.5% Periodic Solution

0.5g Periodic acid  
100.0ml Distilled water

##### 3% Methenamine Solution

3.0g Hexamethylene tetramine (methenamine)  
100.0ml Distilled water

##### 5% Silver Nitrate Solution

5.0g Silver nitrate  
100.0ml Distilled water

##### Borate Buffer Solutions (Stock)

Solution A: 0.2 M Boric Acid

12.36g Boric acid  
1000.0ml Distilled water

Solution B: 0.05 M Sodium Borate

19.07g Sodium borate  
1000.0ml Distilled water

##### Borate Buffer Solution, pH 8.2 (Working)

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13.0ml Solution A  
7.0ml Solution B  
0.2% Gold Chloride  
10.0ml Gold chloride, 1%  
40.0ml Distilled water

Solution is stable for approximately 100 slides.

3% Sodium Thiosulfate (Hypo) Solution  
3.0g Sodium thiosulfate  
100.0ml Distilled water

Methenamine Silver Solution, pH 8.2 (Working)  
42.5ml Methanamine, 3%  
2.5ml Silver nitrate, 5%  
12.0ml Borate buffer, pH 8.2

Prepare fresh just before use and filter. This solution is stable for approximately 60-75 minutes. After this time, there is a breaking down process which produces a black precipitate and is picked up on the slides.

Harris' Hematoxylin and Eosin  
See procedure 2-7, b.

### **STAINING PROCEDURE:**

NOTE: It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chlorine-free water.

1. Treat in periodic acid for 11 minutes.
2. Rinse in distilled water.
3. Filter freshly prepared methenamine silver solution into Coplin jar.
4. Place slides into methenamine silver then place Coplin jar in prewarmed 70°C water bath. Start timing at this point, approximately 30-40 minutes. Check under microscope every 10 minutes for medium brown color. Before checking under the microscope, rinse slides in 70°C chlorine-free distilled water. Check for proper staining with microscope, then return to hot water rinse before the hot staining solution. If destaining is desired, the section may be dipped 1 or 2 times in very dilute potassium ferricyanide.
5. Wash in distilled water.
6. Dip in gold chloride until grey.
7. Rinse in distilled water.
8. Treat with sodium thiosulfate for 30 seconds.
9. Rinse in running tap water for 10 minutes.

Wash in distilled water for 5 minutes.  
Perform Harris' hematoxylin-eosin stain procedure ( 2-7, b).  
Dip in 95% ethyl alcohol for 2-3 dips.

Blow dry.  
Mount.

**RESULTS:**

Basement membranes	black
reticulum fibers	black
nuclei	blue
cytoplasm, collagen, and connective tissue	pink to orange

**e. Fontana-Masson Silver Nitrate Method (Ref 6)**

**SOLUTIONS:**

Silver Nitrate Solution (Fontana)

100ml	Silver nitrate, 11%
200-400ml	Distilled water
50-70	Ammonium hydroxide, concentrated drops

NOTE: All glassware should be chemically clean. This solution must always be freshly prepared.

To 95ml of silver nitrate solution, add 50-60 drops of ammonium hydroxide until the solution is clear with no precipitate. Add, drop by drop, approximately 5 to 6 drops of silver nitrate to cause the solution to become slightly cloudy. Let the solution stand overnight. When ready to use, dilute each 25ml of the silver solution with 25 to 75ml of distilled water, then filter. Cover staining dish with foil to reduce the effects of light. (Freshly prepared, undiluted solutions may be used immediately.)

0.2% Gold Chloride Solution

10.0ml	Gold chloride, 1%
40.0ml	Distilled water

5.0% Sodium Thiosulfate (Hypo) Solution

5.0g	Sodium thiosulfate
100.0ml	Distilled water

**STAINING PROCEDURE:**

NOTE: It is absolutely essential that all glassware be acid cleaned with concentrated nitric acid and rinsed in several changes of chlorine-free water.

1. Heat slides on 60°C to 80°C hot plate for 10 minutes.
2. Cool.
3. Put slides into silver nitrate solution and place in 56°C to 60°C oven until sections are light brown; 60-75 minutes. Or, leave slides in undiluted room temperature solution in the dark for 24 hours.

4. Rinse in distilled water.
5. Tone in gold chloride for 10 minutes.
6. Rinse in distilled water for 3 changes.
7. Bleach in sodium thiosulfate for 5 minutes.
8. Rinse in distilled water.
9. Counterstain in nuclear fast red, 0.1% safranin or eosin-phloxine for 5 minutes.

Rinse in distilled water.

Dip in 95% ethyl alcohol to remove background stain for 1-2 quick dips.

Blow dry.

Mount.

#### **RESULTS:**

Silver reducing substances -- gray or black, including melanin and formalin pigments, argentaffin and paneth cell granules.

**Warning:** Some of the chemicals used for the staining procedures given in this section may be hazardous if misused. For this reason, read and observe all warnings and cautions provided by the manufacturer for each chemical before proceeding with a staining procedure.

**Note:** In order to prevent sections from loosening from the slides during staining, all sections should be heat-fixed (60°C to 100°C) to the slides for a minimum of 2-5 minutes prior to staining, preferably at the time the sections are mounted on the slides.