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Area	Laboratory-Histology
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Histology Special Stain - Steiner and Steiner - Royal Oak

Document Type: Procedure

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I. PURPOSE AND OBJECTIVE:

The purpose of this document is to provide a procedure for the demonstration of spirochetes such as Treponema (syphilis) and Borrelia (Lyme disease). It will also non-differentially stain all bacteria and is used to demonstrate Helicobacter and Legionella.

II. PRINCIPLE:

The stain is an argyrophilic silver reaction. The microorganisms will adsorb silver from the silver nitrate but will not be visible. Hydroquinone will reduce, or "develop" the silver to a visible metallic form. Gum mastic coats the organisms, and slows down the rate of reduction, so there is less non-specific staining. Uranyl nitrate is the sensitizer. The silver is deposited onto the bacteria and spirochetes, increasing their size, and allowing them to be seen with a microscope.

III. SPECIMEN COLLECTION AND HANDLING:

A. Fixation
    1. Any well-fixed tissue.
    2. 10% neutral buffered formalin preferred.
    3. Avoid any chromate or mercuric fixatives.

B. Processing
    1. Standard, overnight processing.

C. Section Thickness

1. Cut paraffin section at 5µ.
2. For minimal deposits, cut at 7µ.

D. Slide Drying
    1. 30 minutes at 60°C.

E. Type of Slide
    1. Plain slides

IV. REAGENTS:

A. **1% Uranyl Nitrate**

Uranyl nitrate	**1.0 gm**
Distilled water	**100.0 mL**

Dissolve together. Store in refrigerator (3°C.); may be reused until weak, about 2 weeks, if used daily.

B. **1% Silver Nitrate**

Silver nitrate	**1.0 gm**
Distilled water	**100.0 mL**

Dissolve together in acid clean glassware. Store in a dark glass bottle in the refrigerator (3°C.); stable for several months.

C. **10% Gum Mastic**

Gum Mastic	**20.0 gm**
Absolute ethanol	**200.0 mL**

Dissolve together using magnetic stirrer for several hours. Filter. Store in refrigerator (3°C); stable for several months.

D. **2.5% Gum Mastic**

10% Gum mastic	**50.0 mL**
Absolute ethanol	**150.0 mL**

Mix together well. Store in the refrigerator (3°C.); stable for several months.

E. **0.04% Silver Nitrate**

1% Silver nitrate	**2.0 mL**
Distilled water	**28.0 mL**

Mix together in acid clean glassware. Store in refrigerator (3°C.); stable for several months.

F. **2% Hydroquinone**

Hydroquinone	**1.0 gm**
Distilled water	**50.0 mL**

JUST BEFORE USED, dissolve together; must be completely dissolved.

G. **Reducing Solution**

2% Hydroquinone	**50.0 mL (25.0 mL)**
2.5% Gum mastic	**20.0 mL (10.0 mL)**
Absolute ethanol	**10.0 mL (5.0 mL)**

JUST BEFORE USE, mix together. Filter.
Then add:

0.04% Silver nitrate	**5.0 mL (2.5 mL)**

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V. EQUIPMENT:

A. Balance
B. Microwave oven or 60°C water bath
C. Magnetic stirrer

VI. SUPPLIES:

A. Erlenmeyer flasks
B. Graduated cylinders
C. Funnel, filter paper
D. Coplin jars
E. Non-metal forceps

VII. QUALITY CONTROL (QC):

A. Tissue with spirochetes for demonstration of spirochetes.
B. Tissue with gram + and gram – bacteria for demonstration of Helicobacter.
C. Tissue with Legionella bacteria for demonstration of Legionella.

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VIII. SPECIAL SAFETY PRECAUTIONS:

A. Uranyl Nitrate
    1. Is and oxidizer.
    2. Store separately from other material.

B. Silver Nitrate
    1. Is an oxidizer.
    2. Store separately from other material.
    3. Is poisonous and may be fatal if swallowed.
    4. Causes skin and eye burns.
    5. Is an irritant to the respiratory system.

C. Gum Mastic
    1. Not a hazardous substance or mixture.

D. Hydroquinone
    1. Is an irritant to eye, skin, and respiratory system.

IX. PROCEDURE:

Step	Action	Time	Notes

1	Deparaffinize and hydrate sections through graded alcohol to distilled water.		
2	Sensitize sections in 1% uranyl nitrate, in a 60°C. water bath.	15 minutes	Or 30 seconds on high in microwave oven, swirl then 30 seconds on high, swirl and allow it to sit at room temperature for 5 minutes
3	Rinse in distilled water, 3 changes.	10 seconds each	
4	Place in 1% silver nitrate in 60°C. water bath.	1 hour	Or 30 seconds on high in microwave oven, swirl then 30 seconds on high, swirl then 10 seconds on high, swirl and allow it to sit at room temperature for 5 minutes.
5	Rinse in distilled water, 3 changes.	10 seconds each	
6	Dehydrate in 95% ethanol, 2 changes.	10 seconds each	
7	Dehydrate in absolute ethanol, 2 changes.	10 seconds each	
8	Place in 2.5% gum mastic.	5 minutes	
9	Allow sections to air dry.	2 minutes	The section should appear a milky-white. Allow to air-dry completely before proceeding with the next step.
10	Rinse in distilled water, 2 changes.	10 seconds each	
11	Place sections in FRESHLY PREPARED reducing solution.	Bacteria and Helicobacter 2-15 minutes Spirochete 20-60 minutes	Reducing solution MUST be made up fresh, just before use. Sections may be developed at room temperature, or solution may be warmed in a water bath (45-60°C.) for a few minutes first. Check with microscope. During development, large bacteria will develop first followed by smaller bacteria, Legionella bacteria, and finally spirochetes. Check development with the microscope.
12	Rinse in distilled water 3 changes.	10 seconds	
13	Dehydrate through graded alcohols and clear in xylene.		
14	Coverslip.		

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X. LIMITATIONS:

A. The following may influence the validity of test results:

1. Use non-metal forceps. This will prevent non-specific silver precipitation.
2. Use acid clean glassware. This will help prevent non-specific silver precipitation.
3. Uranyl nitrate (Step 2) can be omitted in staining for bacteria, since they will always stain with silver nitrate. However, the staining may be variable, and there may be an increase in background staining. This step MUST be used for spirochetes.
4. Gum mastic can be filtered into two containers. One container can be reused for several months for coating the slides. The other container can be used for the developing solution. This will save on the amount of gum mastic being used.
5. 1 gram hydroquinone powder can be placed in test tubes in advance, for easier access.
6. To save on reducing solution, filter into two coplin jars. When ready to reduce, add 2.5 mL of 0.04% silver nitrate to one of the coplin jars. Use this to develop the slides. The other coplin jar of reducing solution WITHOUT the silver nitrate can be stored in the refrigerator, even overnight, for the next batch of slides. When ready to use the second coplin jar, add 2.5 mL of 0.04% silver nitrate, and develop as usual. Discard reducing solution if the solution is no longer white.
7. Helicobacter is a gram-negative bacterium. Make certain that both the gram positive and negative bacteria are stained in the control, especially the smaller bacteria.
8. Spirochetes located near nuclei or melanin may not be as sharply demonstrated, due to the greater affinity of nuclei and melanin for the silver. Prolonged development may be needed.
9. If spirochetes and bacteria appear under-stained after coverslipping, remove coverslip, go back through xylene, and hydrate through graded alcohols to distilled water. Place slides back into the same developing solution, until the correct color.
10. Cut spirochete control slides will NOT stain if older than 2 months. You may go back to the same block and cut new sections, and these will stain. Therefore, cut only enough spirochete control slides to last 2 months. Write the date cut on the control slides when they are being cut, and discard if more than 2 months old.
11. Hydroquinone must be made up fresh, just before use.
12. As all microwave ovens vary, the microwave times stated may need to be adjusted.

XI. RESULTS:

A. Spirochetes - **dark brown/black**
B. Donovan bodies - **dark brown/black**
C. Melanin - **dark brown/black**
D. Chromatin, formalin pigment - **dark brown/black**
E. Foreign materials in macrophages - **dark brown/black**
F. Nuclei - **dark brown/black**
G. Background - **yellow/light tan**

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XII. REFERENCES:

A. Garvey W, Fathi A, and Bigelow F: Modified Steiner for the Demonstration of Spirochetes. Journal of Histotechnology, 8(1), March 1985.

B. Carson FL: Histotechnology: A Self-Instructional Text. Chicago, IL, ASCP Press, 1990.

C. Sheehan DC, Hrapchak BB: Theory and Practice of Histotechnology, 2nd edition. Columbus, Ohio, Battelle Press, 1980.

D. Microwave oven modification developed by the Histology Laboratory at William Beaumont Hospital, Royal Oak, MI.

Approval Signatures

Step Description	Approver	Date
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Applicability

Royal Oak