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Histology Special Stain - Martius Yellow, Brilliant Crystal Scarlet, Aniline Blue (MSB) - Royal Oak

Document Type: Procedure

I. PURPOSE AND OBJECTIVE:

The purpose of this document is to provide a procedure for the demonstration of fibrin, connective tissue and muscle. It is used to demonstrate deposits of fibrin. Fibrin is a specific protein formed as a result of the clotting process, and is a prominent feature in renal transplant rejections and tissues that have received a physical trauma.

II. PRINCIPLE:

The reaction is based on the acid/base (+/- charges) and the hardness/softness of the tissue and the dyes. The negative charged dyes will be attracted to the positively charged tissue components. Hard dyes will be attracted to hard tissue components, while soft dyes will be attracted to soft tissue components. Potassium dichromate changes some of the hardness of some of the components. Weigert hematoxylin stains the nuclei. Martius yellow stains the red blood cells. Brilliant crystal scarlet (or other medium size red dyes) stains the muscle and fibrin. The phosphotungstic acid is taken up by the collagen. The martius yellow and brilliant crystal scarlet are subsequently bound to the phosphotungstic acid. The aniline blue (or other large size blue or green dyes) stains the collagen.

III. SPECIMEN COLLECTION AND HANDLING:

A. Fixation

1. Any well-fixed tissue. Mercuric fixative preferred.

B. Processing

1. Standard, overnight processing.

C. Section Thickness
    1. Routine specimens 5µ.

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

E. Type of Slide
    1. Plain slides.

IV. REAGENTS:

A. **Saturated Aqueous Picric Acid**
Purchased Pre-Made

B. **Bouin Fluid**

Saturated aqueous picric acid	**150.0 mL**
Formaldehyde, 37-40%(HCHO)	**50.0 Ml**
Acetic acid, conc. (CH_3COOH)	**10.0 mL**

Mix together, adding the formaldehyde and acetic acid slowly, as not to splash. Store at room temperature; stable for months.
SEE CAUTION UNDER SPECIAL SAFETY PRECAUTIONS CONCERNING PICRIC ACID.

C. **1% Alcoholic Hematoxylin – Solution A**

Hematoxylin powder	**1.0 gm**
Distilled water	**5.0 gm**
Absolute alcohol, reagent	**95.0 mL**

Dissolve hematoxylin in distilled water. Add absolute alcohol and mix. Store at room temperature in a dark brown bottle;
stable for months.

D. **29% Ferric Chloride**

Ferric chloride	**29.0 gm**
Distilled water	**100.0 mL**

Dissolve together. Store at room temperature; stable for months.

E. **Iron Chloride – Solution B**

29% ferric chloride	**4.0 mL**
Distilled water	**95.0 mL**
Hydrochloric acid, concentrated (HCL)	**1.0 mL**

Mix together 29% ferric chloride and distilled water. Slowly add hydrochloric acid, drop by drop, with stirring, to solution.
Store at room temperature; stable for months.

F. **Working Weigert Hematoxylin**

1% alcoholic hematoxylin (Solution A)	**20.0 mL**
Iron chloride (Solution B)	**20.0 mL**

Mix together. Store at room temperature; stable for 2-3 days.

G. **Martius Yellow (Acid Yellow 24) Solution**

Phosphotungstic acid	**2.0 gm**
95% ethanol, reagent	**100.0 mL**
Martius yellow	**0.25 gm**

Dissolve together martius yellow and 95% ethanol. Add the phosphotungstic acid. Dissolve together with the aid of low heat.
Store at room temperature; stable for several months. Filter before use; may be reused until weak.
CAUTION: Heating ethanol is dangerous; use only low heat. Do NOT leave unattended.

H. **Chromotrope 2R Solution**

Chromotrope 2R	**1.0 gm**
Distilled water	**97.5 mL**
Acetic acid, concentrated	**2.5 mL**

Dissolve together. Store at room temperature; stable for months. Filter before use; may be reused until weak.

I. **1% Phosphotungstic Acid**

Phosphotungstic acid	**1.0 gm**
Distilled water	**100.0 mL**

Dissolve together. Store at room temperature; stable for several months.

J. **Aniline Blue Solution**

Aniline Blue (CI 42755)	**0.5 gm**
Distilled water	**99.0 mL**

Dissolve together. Store at room temperature; stable for several months. Filter before use; may be reused until weak.

V. EQUIPMENT:

A. Balance
B. 60°C degree oven
C. Magnetic stirrer

VI. SUPPLIES:

A. Erlenmeyer flasks
B. Graduated cylinders
C. Funnel
D. Filter paper
E. Coplin jars

VII. QUALITY CONTROL:

Tissue with fibrin.

VIII. SPECIAL SAFETY PRECAUTIONS:

A. Picric Acid
    1. Is toxic, highly explosive (4) and an extreme fire hazard (4).
    2. Keep picric acid moist at all times.
    3. If dry around the tops of the jar, wash off dry particles before opening.
    4. Store in an explosion-proof cabinet.

B. Formaldehyde
    1. Is a poison.
    2. May be fatal or cause blindness if swallowed.
    3. Cannot be made non-poisonous.
    4. Possible cancer hazard.
    5. Irritating to eyes, skin and respiratory tract.
    6. Can cause severe eye burns.

C. Acetic Acid
    1. Is an acid.
    2. Add slowly, drop by drop, to solution.
    3. May cause skin and eye burns.

D. Hematoxylin
    1. Is incompatible with oxidizers and alkalies.
    2. Store separate.

E. Ferric Chloride
    1. Is a corrosive.
    2. May cause skin and eye burns.
    3. Can be irritating to respiratory tract.

F. Martius Yellow
    1. Is and irritant.

G. Phosphotungstic Acid
    1. Is a corrosive.
    2. May cause skin and eye irritation.

H. Chromotrope 2 R
    1. Is an irritant.

I. Aniline Blue

1. Is highly toxic.
2. Is a suspected carcinogen.

IX. PROCEDURE:

Step	Action	Time	Notes
1	Deparaffinize and hydrate slides through graded alcohol to distilled water.		
2	Place in working Bouin's solution in 60°C oven.	45-60 minutes	Steps 2-4 can be skipped if tissue was fixed in a mercuric fixative (B5). If Bouin's is not available, a potassium dichromate-hydrochloric acid solution can be used. In place of step 2 Bouin's, place slides in the “Post-Mordant” solution at room temperature for 5 minutes, then wash in running water until color disappears (1-5 minutes). Then proceed to step 5, Weigert Hematoxylin.
3	Remove from oven, and place slides in a different coplin jar containing water.		
4	Wash in running water until all color disappears from the tissue.	1-5 minutes	
5	Place in working Weigert hematoxylin.	7 minutes	
6	Rinse in running tap water.	1 minute	
7	Rinse in 95% ethanol, 1-2 changes each.	5-10 seconds	
8	Stain in Martius Yellow solution.	2 minutes	
9	Rinse in 95% ethanol, 1-2 changes each.	5-10 seconds	
10	Rinse in distilled water, 1-2 changes each.	5-10 seconds	
11	Stain in chromotrope 2R solution.	10 minutes	Any medium red dye molecule may be substituted.
12	Rinse in distilled water, 1-2 changes each.	5-10 seconds	
13	Place in 1% phosphotungstic acid, jiggling slides occasionally.	5 minutes	
14	Rinse in distilled water, 1-2	5-10	

	changes each.	seconds	
15	Stain in aniline blue.	1-10 minutes	Check the staining of the aniline blue with a microscope. The intensity of the red and blue should be equal. Do NOT check constantly, as this seems to make the color muddy. Any large molecule dyes may be substituted.
16	Rinse quickly in distilled water.	5-10 seconds	
17	Dehydrate through graded alcohols, clear with xylene.		
18	Coverslip using a synthetic mounting media.		

X. LIMITATIONS:

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

1. Tissue must either be fixed in a mercuric fixative (B5)

   OR

2. Must be post-mordanted in the Bouin's solution. Failure to do so will result in muddy colors - the collagen will be reddish-blue and the muscle will be bluish-red.
3. An iron hematoxylin-like Weigert must be used. The acid dyes in the trichrome solution decrease the intensity of nuclear stains. If an alum hematoxylin is used, no nuclear staining will be seen. Nuclear staining may be poor even with the iron hematoxylin.

XI. RESULTS:

A. Fibrin - **red**
(early fibrin may be yellow; very old fibrin may be blue)

B. Collagen - **blue**

C. Muscle fibers - **red**

D. Nuclei - **blue**

E. Red Blood Cells - **yellow**

XII. REFERENCES:

A. Bancroft JD, Stevens A: Theory and Practice of Histological Techniques, 3rd edition. New York, NY, Churchill Livingstone, 1990

B. Karyn Abrans-Engeman, HTL(ASCP): “The Milligan Trichrome: An Easy, Reliable Staining Procedure”. Surgipath Micro-views, volume 7, Issue Number 2, Fall 1991

Approval Signatures

Step Description	Approver	Date
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