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Modification of Mayer's Hematoxylin-Eosin Method

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Mayer's[1] hematoxylin and eosin method of staining was modified to provide several distinct features: Solutions are more concentrated; chloral hydrate has been added to the hematoxylin solution to act as a preservative; the color acid of eosin is used as the counterstain; the staining is faster, and there is no need to change the solution. When the stain starts evaporating, dilute it to original volume with fresh solution. The staining solution remains stable and can be used constantly without changing for over a year. In contrast to Mayer's solution, which allows slide colors to fade in a short time, this modified solution produces slide colors that remain stable indefinitely. In addition, unlike Lillie's modified solution, it can be used on all fixatives and tissues including cadavers and bone sections, and for special photography studies.

Microtomy:
Cut paraffin sections at 5-10 micra.

Solutions:

Modified Mayer's Hematoxylin

Hematoxylin	4.0 gm
Distilled water	1000.0 ml
Sodium iodate	0.3 gm
Ammonium or potassium alum	50.0 gm
Citric acid	1.5 gm
Chloral hydrate	75.0 gm

Dissolve alum in water (do not use heat); add hematoxylin, iodate, citric acid, and chloral hydrate, in order. Filter hematoxylin solution through coarse filter paper.

Eosin (Color Acid) - stock

Eosin Y (NE 39)	22.0 gm
Distilled water	500.0 ml
Hydrochloric acid, concentrated	12.0 ml

Dissolve eosin completely in distilled water, then add the concentrated hydrochloric acid. Allow to settle overnight and filter. Wash the precipitate on the paper with distilled water until only a pink tint appears in the wash water. Dry the precipitate on the filter paper at 37-40° C for 12-24 hours.

Eosin (Color Acid) - working

Dissolve 15 gm of the dry stain in 1000 ml of 95% alcohol. Let solution settle 2-3 hours, and filter into staining container.

Staining Procedure:

1. Decerate and hydrate to distilled water.
2. Stain slides in hematoxylin for 8-10 seconds.
3. Wash slides with tap water to remove excess stain.
4. Blue slides in saturated sodium bicarbonate for a few seconds.
5. Rinse slides in water to remove all traces of sodium bicarbonate.
6. Stain in eosin for 3-5 seconds.
7. Dehydrate in 3 changes of 95% alcohol, absolute alcohol, and clear in xylene, 3 changes each.
8. Mount coverglass with resinous mounting media.

Result:
A well-differentiated stain with blue nuclei and bright rose background is obtained.

Reference:

1. Armed Forces Institute of Pathology, Manual of Histologic and Special Staining Techniques, 2nd ed., New York, Blakiston Division of McGraw-Hill Book Company, Inc., p. 27, 1960.

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Use of Zinc Chloride in Zenker Type Fixatives

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A great deal has been said and written about the danger from mercury and its compounds when they are released into the environment. A possible source of mercury is the mercuric chloride commonly used in Zenker type fixatives in hospitals and research laboratories. Used fixative is often disposed of by dumping it down the drain and into the ecosystem.

While there are methods of recovering the mercury from used fixative,[1] it is easier and much cheaper to substitute zinc chloride for the mercuric chloride required by the various Zenker type formulas.[2] The main advantage of zinc chloride is that it is less toxic than mercuric chloride. It also does not cause the brown to black mercury precipitated pigment artifact that is found in mercuric chloride fixed tissues. This eliminates the necessity of using iodine and sodium thiosulfate solutions during the staining procedures.

Zenker type fixatives using zinc chloride penetrate the tissue well and coagulate the protoplasmic substances. When used without acetic acid, the cytoplasmic inclusions are well preserved. Sectioned tissues retain their brilliant staining ability because of the mordant action characteristic of Zenker type fixatives. There were no differences in the quality of the slides produced from each type of fixative.

The use of zinc chloride seems to be a reasonable step toward protecting ourselves and our environment from possible mercury poisoning.

References:

1. Spencer, R. W.: Removal of Mercury from Medical Facilities, *New England Journal of Medicine*, 285:971, 1971.
2. Jones, R. M.: *McClung's Handbook of Microscopic Technique*, 3rd edition, p. 56. Hafner Publishing Co., New Jersey, 1964.

Tissue-Talk

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Preparation of Photographic Slides

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A request was received to cut a number of slides of rat tissue for photography. The photographs were to be used in a radiology textbook and needed to be of exceptional quality. The tissue was first cut at 4 microns and mounted, following normal procedures, on regular glass slides with No. 1-1/2 coverglass. The photographer then encountered difficulty in producing first class photographs due to the thickness of the slides.

I then tried picking up the tissue on a No. 0 (zero) coverglass. This glass proved superior. The coverglasses were incubated the same as normal glass slides and stained using Columbia staining jars or petri dishes. The uptake of stain by the tissue and the retention of the tinctorial quality were perfect. The "tissue slip" was then covered with another No. 0 (zero) coverglass and allowed to completely dry.

To label these coverglasses, it was necessary to use two gummed labels which were stuck together leaving a slight opening along one edge. The coverglasses were inserted into this opening and the edges pressed together. This also provided a convenient means of handling the slide without touching the glass.

The photographer found these to be far superior for obtaining publication prints than were the normal glass slides.

Do it Yourself — Terry's Methylene Blue

An Editorial

Due to numerous recent inquiries, the following staining procedure is being presented. It appears that, recently, some laboratories were unable to purchase Terry's polychrome methylene blue from commercial sources. Soon after this, I started receiving inquiries requesting information on possible new sources or instructions on how to make Terry's methylene blue. Procedures for fresh unfixed and fixed tissue are presented. This information was abstracted from the original article.

Terry's Alkaline Polychrome Methylene Blue
A Stat Method
(For fresh unfixed tissue)

Fixation:
Fresh tissue (unfixed)

Microtomy:
Cut frozen sections at 10 microns. (See Step No. 1 Staining Procedure)

Solutions:

1% Methylene Blue (stock)

Methylene Blue	1.0	gm
Distilled water	100.0	ml

1% Potassium Carbonate (stock)

Potassium Carbonate	1.0	gm
Distilled water	100.0	ml

Terry's Methylene Blue (working)

1% Methylene Blue (stock)	6.0	cc
1% Potassium Carbonate (stock)	6.0	cc
Distilled water	18.0	cc

Combine above ingredients and **boil** over a flame for exactly 2-1/2 minutes. Cool solution in running tap water. After the solution has cooled, q.s. to 30 ml to replace any water lost during boiling. Filter solution before use.

Staining Procedure:
1. Float sections onto distilled water.
2. Tease sections onto a glass slide.
3. Drain excess water from slide but do not allow sections to dry.
4. Cover the sections with a small amount of Terry's Working Methylene Blue Solution for 3 to 5 seconds.
5. Drain the stain off the slide and rinse in distilled water.
6. Sections are then remounted on a clean glass slide and examined while still wet.

Results:
Nuclei and epithelial cells — blue
Connective tissue fibrils — red to colorless
Smooth muscle — purple

Remarks:
This stain gives its best results when used on frozen sections of living, unfixed tissue. As soon as the cells die, the staining is unsatisfactory. The section may be coverslipped by employing a drop of water on the under surface of the coverslip. Boiling time of the solution is very important in developing polychrome. If the alkaline stain is boiled longer than 2-1/2 minutes, or if it is slowly cooled; more red is present, more crystalline precipitate forms, and the stain is less intensely active.

Terry's Acid Polychrome Methylene Blue Stat Method
(For fixed tissue)

Fixation:
10% buffered neutral formalin

Microtomy:
Cut frozen sections at 6-10 microns. (See Step No. 1 Staining Procedure)

Solutions:

1% Methylene Blue (stock)
Repeat as above

1% Potassium Carbonate (stock)
Repeat as above

10% Acetic Acid

Glacial Acetic Acid	10.0	ml
Distilled water	90.0	ml

Terry's Acid Polychrome Methylene Blue (working)

1% Methylene Blue (stock)	20.0	ml
1% Potassium Carbonate (stock)	20.0	ml
Distilled water	60.0	ml

Combine and heat to boiling. Boil gently for 2-1/2 minutes. Cool rapidly under running tap water. Add 10 ml of 10% acetic acid to cooled solution. Shake the solution for 1 minute and q.s. up to 100 ml with distilled water to replace any water lost during boiling. Filter solution before use.

Staining Procedure:
1. Float sections onto distilled water.
2. Tease sections onto a glass slide.
3. Drain excess water from slide but do not allow sections to dry.
4. Cover the sections with a small amount of Terry's Working Acid Polychrome Methylene Blue Solution for 3-5 seconds.
5. Drain the stain off the slide and rinse in distilled water.
6. Sections are then remounted on a clean glass slide and examined while still wet.

Results:
Nuclei and epithelial cells — blue
Connective tissue — bright red to colorless
Smooth muscle — purple

Remarks:
If less acetic acid is added to the stain, connective tissue staining will be a brighter red. Conversely, more acetic acid results in pale to colorless staining of connective tissue.

Boiling time of the solution is very important in developing polychrome. Boiling for 2 to 2-1/2 minutes seems to be best, although good acid stains may be obtained from solutions boiled for only 30 seconds or as long as 5 minutes.

Reference:

Terry, B. T.: "The Rapid Preparation of Polychrome Methylene Blue Stains for Frozen Sections of Fresh and Fixed Tissue." *J. Lab. Clin. Med.*, 8: No. 3, 159-164, 1922.

Artifacts can be Deceiving

An Editorial

Following are some examples of artifacts encountered in histotechnology.

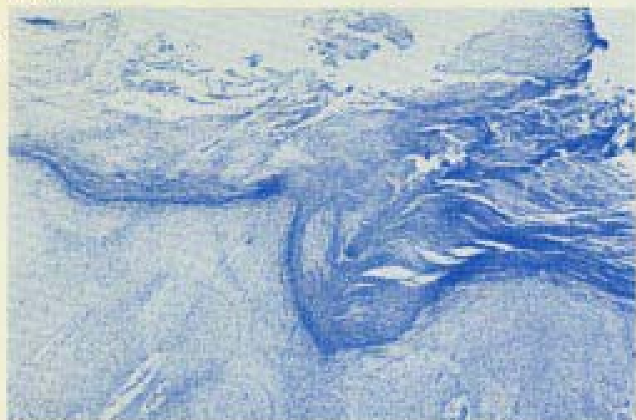


Figure 1:
Section of skin which was stained, dehydrated and cleared in alcohol and xylenes which contained excessive moisture. The round structures are water droplets. H&E. x40.

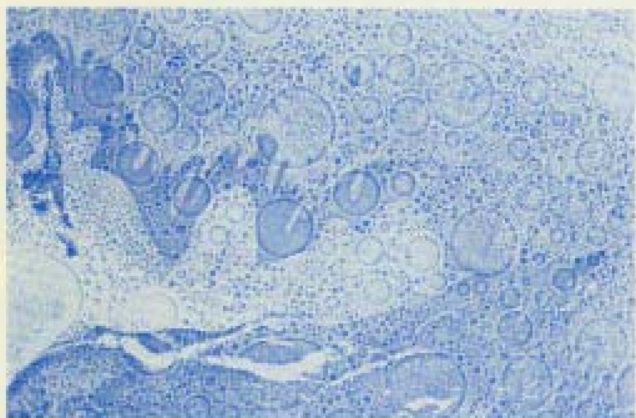


Figure 2:
Same section seen in Figure 1 but under higher power to better illustrate the water droplets and to show that it is necessary to lower the microscope substage in order to see the bubbles distinctly. Without the substage lowered, the section will appear hazy or will have some brown stippling. These effects suggest there is something wrong with the section but the presence of moisture cannot be confirmed unless the substage is lowered. H&E. x120.

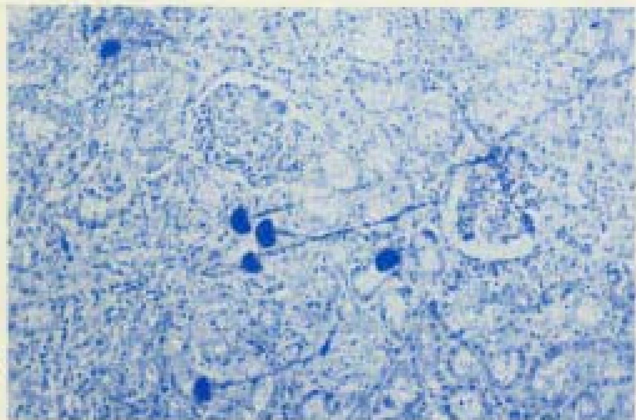


Figure 3:
The structures seen in the middle of the photograph are mold growths occasionally found in hematoxylin staining solutions. H&E. x140.

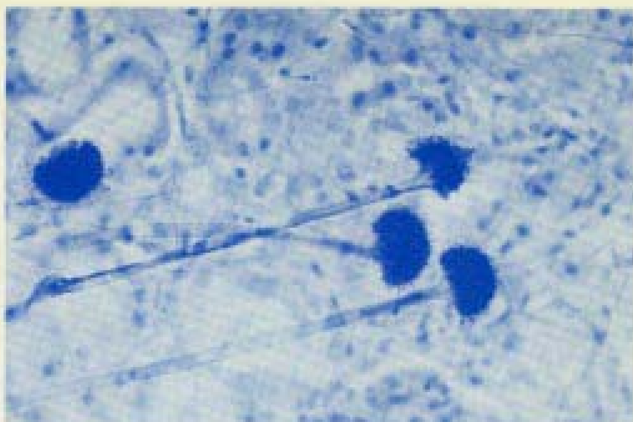


Figure 4:
The section seen in Figure 3 under higher magnification. Note the long stem and budding structures. The growth generally stains the stem light and the bulb stains dark purple. H&E. x400.

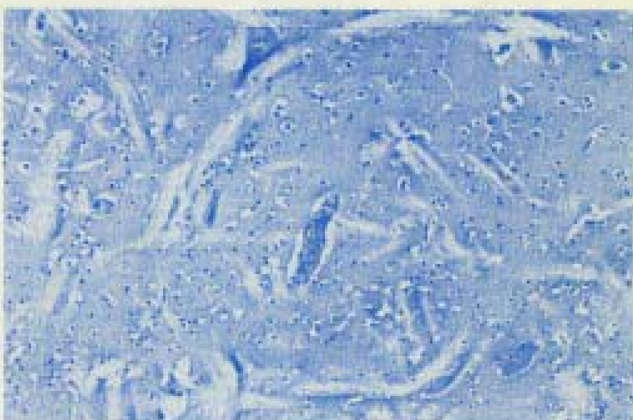


Figure 5:
The bizzare tracks seen throughout this brain section were produced by the use of coarse filter paper to blot the tissue section. Strangely enough, only sections cut at 6 microns exhibited this effect. Those cut at 12 microns (Fig. 6) did not show this artifact. This suggests that one should be careful when using coarse filter paper to blot thin brain sections. (Contributed by Miss Edythe Simpson, AFIP, Washington, D.C.) H&E. x300.

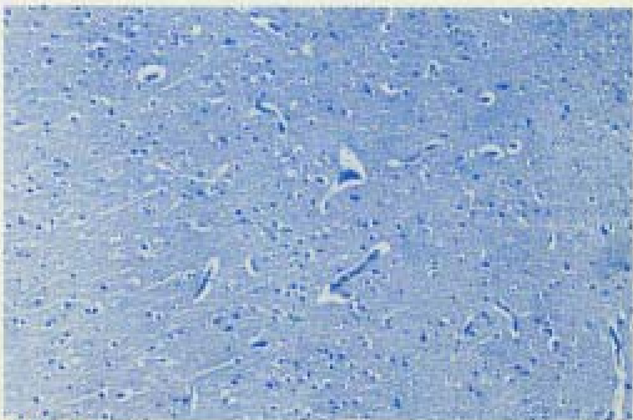
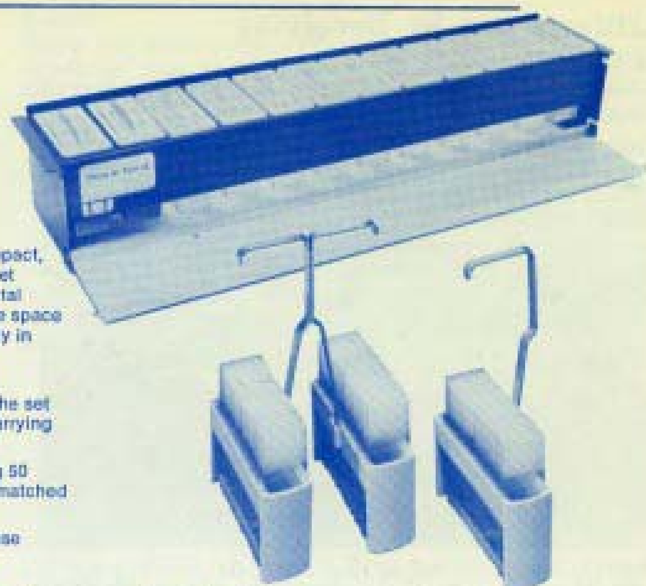


Figure 6:
Section from the same specimen as seen in Figure 5, except this section was cut at 12 microns. Notice the absence of the artifact seen in Figure 5, which was cut at 6 microns and blotted with the same coarse filter paper as Figure 6. H&E. x100.

Space-saving TISSUE TEK® II Slide Staining Sets



Tissue-Tek II Slide Staining Set (No. 4451) provides a space-saving, compact, efficient system for histologic or cytologic slide staining. Each staining set consists of 12 chemically inert plastic staining dishes with lids, and a metal carrier with drain tray/cover. The dishes have been designed to minimize space requirements and solution carry-over. The lids can be stored conveniently in the rear of the tray during staining.

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A Reliable Methyl Green-Pyronin Procedure for RNA and DNA

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The methyl green-pyronin stain has a reputation for being inconsistent and capricious. The procedure presented herein evolved from trial and error and from information provided by Murgatroyd, Mikroskopie, Vol. 18, 1963, p. 285-287. This modified procedure is consistent, reliable, and specific, provided certain criteria are followed:

1. Use Carnoy fixation (formalin will work but is inferior).
2. Never overheat tissue at any time. Use only that temperature necessary to melt embedding media, 57-58° C.
3. Never leave slides in xylene, alcohol, or water for extended periods of time during deceration or hydration.
4. Use dyes of excellent quality and purity. We have used Pyronin GS* (same as Pyronin Y) and Methyl Green GA*, Chroma-Gesellshaft, Schmid & Co. Pyronin B does not work well.
5. It is important to wash the aqueous stock solutions with chloroform. This removes the possibility of diffuse staining characteristics caused by dye impurities.
6. The buffer is of supreme importance. Low pH levels accentuate the pyronin staining of plasma and other acidophilic tissue structures. At higher pH levels (pH 5.0-5.5) the methyl green stains the DNA clearly and brightly, and still allows the RNA to stain brightly without producing a diffuse pink plasma background.

Solutions:

McIlvaine's Buffer (stock)

0.2 M Di-sodium Hydrogen Phosphate (stock)

Di-sodium hydrogen phosphate	28.4 gm
Distilled water	1000.0 ml

0.1 M Citric Acid (stock)

Citric acid	21.0 gm
Distilled water	1000.0 ml

McIlvaine's Buffer (working)

0.2 M Di-sodium hydrogen phosphate (stock)	52.0 ml
0.1 M Citric acid (stock)	48.0 ml

This provides a pH of 5+. Adjust by preference; pH 5.5 appears optimal in my laboratory.

Methyl Green GA (stock)

Methyl green GA	2.0 gm
Distilled water	100.0 ml

Dissolve thoroughly and wash with chloroform in a separatory funnel. (Solution keeps for months.)

Pyronin GS (stock)

Pyronin GS	5.0 gm
Distilled water	100.0 ml

Dissolve thoroughly and wash with chloroform in a separatory funnel. (Solution keeps for months.)

Methyl Green-Pyronin (working)

Pyronin GS (stock)	2.0 ml
Methyl green (stock)	4.0 ml
McIlvaine's buffer (working)	50.0 ml
Distilled water	50.0 ml

Mix well. There is no need to filter solution. Keeps approx. 1 week.

Procedure:

1. Decerate and hydrate slides in distilled water.
2. Stain slides in methyl green-pyronin working solution 30 minutes to 1 hour.
3. Take each slide out of staining solution separately, rinse briefly in distilled water and blot on filter paper. Set aside until all slides are rinsed and blotted.
4. Differentiate by dipping one blotted slide at a time in distilled water and then placing in absolute acetone for 2 to 3 minutes.
5. Transfer to 2 changes of absolute acetone to complete dehydration.
6. Place in 2 changes of 50:50 acetone/xylene for 2 minutes each.
7. Place in 2 changes of xylene, 2 minutes each. This may be done on a larger scale by dipping a basketful of blotted dry slides into a dish of distilled water, placing the basket in acetone, etc.

Remarks:

A control slide using ribonuclease before staining is recommended.

We have noticed no appreciable fading of slides. This procedure can be adapted to cytology, as well as blood and bone marrow smears by shortening staining time and very rapid differentiation.

Because methyl green differs from methyl violet only slightly in chemical structure, it may be necessary to repeat the chloroform wash on the stock solution after several months. The methyl green slowly breaks down, by loss of a methyl group, into methyl violet.

*Roboz Surgical Instrument Co., Washington, D.C.

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The editor wishes to solicit information, questions, and articles relating to histotechnology. Submit these to: Lee G. Luna, Editor, Histo-Logic, P.O. Box 36, Lanham, Maryland 20801. Articles, photographs, etc., will not be returned unless requested in writing when they are submitted.