

HISTO-LOGIC[®]

No reader should utilize materials and/or procedures discussed in HISTO-LOGIC articles unless the reader, by reason of education, training and experience, has a complete understanding of the chemical and physical properties of all materials to be utilized and the function of each material by which any procedure is accomplished.

Editor, Lee G. Luna, D. Litt., H.T. (ASCP)

Technical Bulletin for Histotechnology
Published: January, April, July, October

Vol. XI, No. 4 - October, 1981

Hematoxylin Eosin/Phloxine Stain

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Comment:

We have used this procedure for the past few years. We previously used denatured ethyl alcohol and this did not clear well with xylene. The isopropyl alcohol works very well. We use the acid water instead of acid alcohol because a bluish film remained on the slides with acid alcohol, and the decolorization is also quicker with the acid water.

Fixation:

10% neutral buffered formalin

Technique:

Cut paraffin sections 5-6 micrometers

Solutions:

Stock Eosin Solution	
Eosin Y water soluble	1.0 gm
Distilled water	100.0 gm

Stock Phloxine Solution	
Phloxine B	1.0 gm
Distilled water	100.0 gm

Eosin-Phloxine Working Solution	
Stock eosin solution	100.0 ml
Stock phloxine solution	10.0 ml
95% ethyl alcohol	780.0 ml
Glacial acetic acid	4.0 ml

Make up working solution as needed. Working solution should be changed at least once a week.

Acid Water	
Hydrochloric acid	2.0 ml
Distilled water	98.0 ml

Ammonium Water	
Ammonium hydroxide (concentrated 58%)	1.5 ml
Distilled water	1000.0 ml

Hematoxylin, Gill III*

Staining Procedure:

1. Deparaffinize in xylene for 2 minutes.
2. Deparaffinize in xylene for 2 minutes.
3. Dip slide 8 times in 100% ethyl alcohol.
4. Dip slide 8 times in 95% ethyl alcohol.
5. Dip slide 8 times in 70% ethyl alcohol.
6. Place slides in tap water for 2 minutes.
7. Stain slides in Gill III hematoxylin for 3 minutes.
8. Rinse slides in tap water.
9. Three quick dips in acid water.
10. Place slides in running tap water for 2 minutes.
11. Blue slides in ammonium water until blue.
12. Place slides in running tap water for 2 minutes.
13. Counterstain slides in eosin/phloxine for 2 minutes.
14. Dip slides 8 times in 95% ethyl alcohol.
15. Dip slides 8 times in 95% ethyl alcohol.
16. Dip slides 8 times in isopropyl alcohol.
17. Dip slides 8 times in isopropyl alcohol.

18. Dip slides 8 times in isopropyl alcohol.

19. Clear in xylene, and mount coverglass with resinous media.

Results:

Nuclei — blue

Cytoplasm — pink

Reference:

Luna, L.G.: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 3rd Ed., McGraw-Hill, New York, 1968.

*Lerner Laboratories, 17 James Street, New Haven, CT 06513

Liquid Coverslipping Media

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In our laboratory we spend a great deal of time coverslipping Pap and other microscopic slides. Because of this, we have instituted the use of a liquid coverslipping media.* Following are some suggestions on its use and advantages.

Advantages:

1. Liquid coverslipping media is a high viscosity fluid with a refractive index of 1.4951 ± 0.005.
2. It is colorless and transparent, and can be applied as thick or thin as necessary on Pap smears of uneven thickness.
3. No coverglass is necessary and bubbles are easily eliminated.
4. It comes in a convenient applicator can, and requires no dilution.
5. The media contains an antioxidant to inhibit stain fading.
6. Approximately 300 Pap smears can be covered with an 8-ounce can.

Application on Pap Smears:

When applying liquid coverslip, the slides should be held horizontally. After the media has been applied, the smear should be put in a rack and placed on a level surface to "set-up" for at least 20 minutes. Xylene can be used to clean overflows. Clean the spout of the applicator can with xylene to avoid clogging. The fumes from liquid coverslip are potent; therefore, cover and dry slides in a well ventilated area. We also use liquid coverslipping media to affix coverglasses to tissue sections.

We do not substitute liquid coverslipping media for a glass coverslip on tissue sections, since we feel that a coverslip provides better quality mounting. In addition, coverslipped slides are also ready to read immediately after covering.

Drying:

Liquid coverslip will dry sufficiently for screening purposes in about 20 minutes after application. However, nothing should be laid on top of the slides until they have been allowed to dry overnight. Smears can be filed safely after air drying for one week.

Cleaning the Surface:

The liquid coverslip media is soluble in xylene. When "dotting" the slide we recommend the use of water soluble ink. The removal of non-aqueous ink dots with xylene will dissolve the liquid coverslip surface. Immersion oil can be blotted off, and the surface rinsed in warm water. Dust can also be washed off a dried smear with warm water.

*Permoslip (Albon Scientific), and Flo-Texx (Lerner Laboratories)



A Modification of Southgate's Mucicarmine Method

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Introduction:

Using my academically obtained knowledge of chemistry and my laboratory experience acquired over a period of years, I have modified the technique for making mucicarmine staining solution for histologic purposes. My modification is based upon Southgate's technique of 1927, which was an alternate method of the original Mayer's technique of the 1800's.

Carmine, a mixture of chemicals, has a basic component of carminic acid which is a complex, hydroxylated, aromatic molecule. When this acid is dissolved in a solvent, it dissociates into positive and negative ions; the positive ions are hydrogen ions, and the negative ions are the actual staining portion of the molecule. Carminic acid dissolves best in a solvent of alcohol and water mixed as compared to pure alcohol or pure water. If this mixture is warmed, it dissolves even better.¹ As with many acids, carminic acid is more soluble in a neutral or slightly alkaline solvent than in an acidic solvent. For this reason alone, I felt it necessary to increase the amount of aluminum hydroxide to further neutralize the acidic effect of aluminum chloride and to stress slow and gradual addition of aluminum chloride so as to prevent precipitation of the already dissolved carminic acid by too much acidity.

It is suggested that a sensitive balance be used to make proper weight measurements. However, for places that do not have such elaborate equipment, cautious estimations should yield satisfactory results. It is paramount that as little variation as possible is allowed.

The instructions in this paper have been written in such manner that a beginning laboratory technician as well as the professional should be capable of successfully following them. For this reason, to some, certain points may appear to be redundant.

Materials Needed:

1. 1000-ml Erlenmeyer Flask (Pyrex)
2. 50-ml Pyrex test tube, 12.0 cm x 2.5 cm
3. One long-handled test tube holder
4. Two small, dry, stainless steel spatulas
5. One 25-cm glass stirring rod
6. One 100-ml glass graduated cylinder
7. Boiling water bath, set up under ventilation hood or in a well ventilated room
8. Mettler Balance, model PN1210, or equivalent balance capable of accurately measuring to 1/100 gm
9. Normal colonic mucosa (human) as control tissue

Solutions Needed:

1. Weigert's iron hematoxylin solution²
2. 0.25% Metanil yellow solution³
3. Mucicarmine solution (as described below)

Preparation of Mucicarmine Solution:

Using the materials listed above, follow this procedure precisely; small degrees of variation can be critical to the results you obtain from the staining solution.

Carmine (Aluminum calcium lake)	4.25 gm
Aluminum hydroxide ($\text{Al}(\text{OH})_3 \cdot \text{nH}_2\text{O}$)	4.45 gm
Ethyl alcohol, 50%	375 ml
Ethyl alcohol, 25%	25 ml
Aluminum chloride anhydrous (AlCl_3)	2.05 gm

Thoroughly mix the dry carmine and aluminum hydroxide in the 50-ml test tube. Add the 25 ml of 25% ethyl alcohol to the test tube, and using the glass stirring rod, thoroughly mix until as much of the dry mixture as possible is into solution. Using the test tube holder, warm the solution by lowering the tube intermittently into the water bath, stirring continuously with the glass rod. Warming should be for no more than one minute. Do not allow solution to boil or be contaminated with the boiling water. Using the premeasured 375 ml of 50% ethyl alcohol, rinse the entire contents of the test tube into the 1000-ml Pyrex flask, stirring with the glass rod each time so that the mucicarmine mixture is removed from both the glass rod and the inside of the test tube. Using the dry stainless steel spatula, slowly and gradually add the aluminum chloride to the solution in the flask, swirling after each addition. Do not breathe the HCl vapors! After addition of all the aluminum chloride, immediately place the flask into the boiling water bath and watch closely for signs of boiling inside the flask. Boil for exactly 2 1/2 minutes. Promptly remove from the water bath and allow to cool. Seal the flask with "Parafilm" and allow to sit under refrigeration (do not freeze) for 24 hours. Remove the flask from the refrigerator, maintaining its seal, and allow the contents to reach room temperature, agitating periodically. Filter once with standard laboratory filter paper to obtain stock solution. To obtain the working solution, dilute one part stock to four parts distilled water (1:4 ratio).

Keep the stock solution in a sealed container and under refrigeration. The stock should yield good results for a minimum of 5 months.

The average staining time is 120 minutes (2 hours). Each new stock should be tested at 30, 60, 90 and 120 minutes, since the working solution dilution ratio may have to be adjusted slightly.

Fixation:

10% buffered neutral formalin

Microtomy:

Cut paraffin sections at 6 micrometers.

Staining Procedure:

1. Deparaffinize and hydrate to distilled water.
2. Weigert's iron hematoxylin, 7 minutes.
3. Wash in running water for 10 minutes.
4. Metanil yellow, 1 minute.
5. Rinse quickly and remove excess water before next step.
6. Working mucicarmine solution, 120 minutes.
7. Rinse in 3 changes of 95% ethyl alcohol, dehydrate and clear in xylene.
8. Mount coverglass with resinous media.

References:

1. Merck Index, An Encyclopedia of Chemicals and Drugs, 8th Ed., p. 211.
2. Luna, L.G.: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 3rd Ed., McGraw-Hill, New York, 1968, p. 35.
3. Luna, L.G.: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 3rd Ed., McGraw-Hill, New York, 1968, p. 78.

*Article was submitted in June 1979 when Mr. Payne was a Navy HM3 employed at the Naval Regional Medical Center, Portsmouth, Va. He is now attending Old Dominion University.

A Word of Caution

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The following incident recently occurred in our laboratory. We would like this published in *Histo-Logic*, so histotechnologists can be aware that there is a definite problem with this particular procedure.

On February 25, 1981, histology technician Tamela Bird was attempting to prepare a solution for the softening of keratin described on page 11 (see procedure below) of the *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, Third Edition, when the alcohol burst into flames. The procedure was followed in the order printed, with the exception of the concentrated nitric acid, which was omitted. Alcohol spilled over the counter, floor, a stool and the garbage can, all of which caught fire. Courier Charles Turner was in the department at the time, found a large container of water and immediately drowned the fire with a large quantity of water. A fire extinguisher was brought into the department by Cindy Spencer but was not needed. There were no injuries or damage to the department.

Preventative measures taken included:

1. Reviewed procedure for softening keratin and omitted its use in the future.
2. Acquired a new fire extinguisher for the histology department.
3. Everyone was alerted to the possible dangers of using water on a chemical fire.
4. A report of the incident was sent to the editor of the *AFIP Manual* and the *Histo-Logic* newsletter.

Keratin and Chitin Softening Procedures*

There are no highly satisfactory procedures for softening keratin and/or chitin which would result in both rapid softening and subsequent good section staining. By use of concentrated sulfuric acid with the aid of heat, keratin can be completely dissolved from the tissue section. However, much tissue destruction also will occur.

For the softening of chitin, the following procedure is found to give a satisfactory result.

1. Fix specimens in a fixative of choice.
2. Place specimens in the following solution until completely de-chitinized.
Change solution every 2 days for best results.

Mercuric chloride	4.0 gm
Chromic acid	0.5 gm
Nitric acid, concentrated	10.0 ml
Ethylalcohol, 95%	50.0 ml
Distilled water	200.0 ml
3. Wash in running water for 3 hours.
4. Dehydrate, clear and impregnate with paraffin or process as desired.

EDITORIAL NOTE: Printing of the procedure herein in no way condones its use, but is printed to show readers the method Ms. Bird makes reference to. In the Editor's hands the procedure has worked well, but the various ingredients must be added after the alcohol and distilled water are mixed. In other words, mix alcohol and water first, then add other ingredients slowly. It is strongly suggested, however, that this method be used only when absolutely necessary and with utmost care. We thank Ms. Bird for calling this problem to our attention.

*Luna, L.G.: *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3rd Ed., McGraw-Hill, New York, 1968, p. 11.

Methyl Green Pyronine

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Fixation:

Formalin, Zenker's or Helly's

Microtomy:

Cut paraffin sections at 6 microns.

Solutions:

0.5 M Sodium Phosphate (Stock)
Sodium phosphate, dibasic (use powder form) . . . 70.5 gm
Distilled water . . . 1000.0 ml
Dissolve with low heat.

0.1 M Citric Acid (Stock)
Citric acid . . . 21.7 gm
Distilled water . . . 1000.0 ml

Methyl Green-Pyronine Solution*
0.5 M Sodium phosphate (stock) . . . 52.5 ml
0.1 M Citric acid (stock) . . . 47.5 ml
Methyl Green75 gm
Pyronine Y25 gm

Mix — then add:

0.5% Phenol (aqueous)5 ml
1% Resorcinol (aqueous)2.5 ml
(Phenol and Resorcinol should be made fresh)

Staining Procedure:

1. Deparaffinize slides and hydrate to distilled water.
2. Stain slides in methyl green-pyronine for 1 minute.
3. Rinse slides in distilled water.
4. Blot slides gently and dehydrate in 100% alcohol and clear in xylene.
5. Mount coverslip with resinous media.

Results:

RNA — pink to red
DNA — green to bluish-green

*This solution requires 2-3 days to ripen.

Appointment to Biological Stain Commission

The Editor of *Histo-Logic* (Lee G. Luna) was recently elected to membership in the Biological Stain Commission, by action taken by the Trustees of the Commission at their annual meeting, June, 1981. The field of histotechnology should be very much involved with the activities of this Commission since we use many dyes in our daily laboratory activities. The Commission's five major goals are:

1. To assure continued availability of dyes and best use of dyes in biomedical application.
2. To promote optimal cooperation among makers, vendors, and users of dyes in biomedical application.
3. To assure the quality of dyes by testing them and certifying only those which meet prescribed standards.
4. To educate dye users about sources of reliable dyes and their best use.
5. Publish new knowledge about such dyes.

Notice of my appointment to this Commission is provided herein, primarily to offer any assistance or information relative to problems involving the five goals. Address inquiries and/or information to: Lee G. Luna, Chief, Histopathology Laboratories, Armed Forces Institute of Pathology, Washington, DC 20306.



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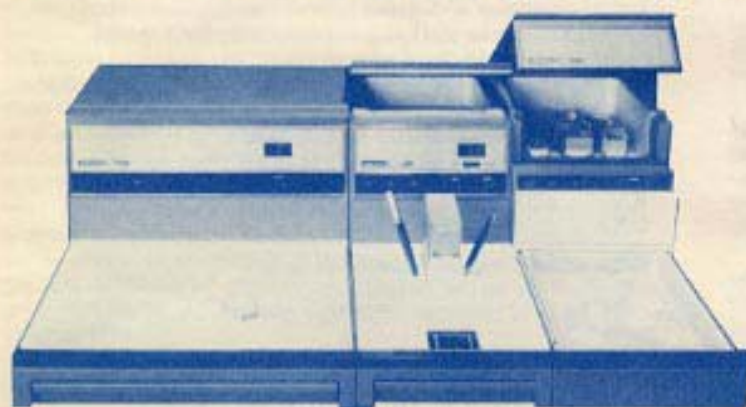
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The histology systems source

Stain-Technology "Wet" Workshop and Seminar, March 7-12, 1982

Presented by: Lee G. Luna, H.T. (ASCP)
The Center for Histotechnology Training

This five-day extensive wet workshop and seminar will afford registrants the opportunity to utilize 20 special stains demonstrating more than 25 pathologic entities.

In addition to the practical special staining aspects, lectures will be presented daily (to include evenings) on chemistry of staining; staining mechanisms; tissue identification for histotechnologists; shelf life of solutions; preferred controls; introduction to immunoperoxidase; decalcification; and various other subjects directly related to the production of high-quality microscopic slides.

Workshop Objectives:

Upon completion of this course, the registrant will be able to: (1) efficiently and effectively perform 20 special stains; (2) understand the mechanism and chemistry of staining reactions; (3) gain a working knowledge of general and specific pitfalls regarding special stains; (4) gain necessary expertise to determine the quality of stained slides; (5) acquire extensive knowledge for determining shelf life of staining solutions; (6) in addition to the above, registrant will learn how to identify various tissue structures. The latter subject is most important since it allows one to identify the staining qualities of a given structure or entity.

For program and related information, contact: Registrar, Center for Histotechnology Training, P.O. Box 2453, Rockville, MD 20852.

To receive your own personal copy of HISTO-LOGIC, or to have an associate added to the mailing list, submit home address to: Lab-Tek Division, Miles Laboratories, Inc., 30W475 North Aurora Rd., Naperville, Illinois 60540.

Printed in U.S.A.

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 20706. Articles, photographs, etc., will not be returned unless requested in writing when they are submitted.