HISTO-LOGIC®

Lee G. Luna, D. Lit., H.T. (ASCP), Editor A Technical Bulletin for Histotechnology Vol. VI, No. 1 - January, 1976

Technique for Processing Eye Specimens

Edna Prophet, B.A., HT (ASCP)
Washington, D.C. 20306

The paraffin technique used for processing eye sections closely follows the technique used for other tissues, and permits production of completed sections of whole globes ready for diagnosis one week after enucleation.

Use of this technique also makes it possible to perform a greater number of special staining procedures.

Fixation

The enucleated eye should be fixed immediately in 10% formalin which has been buffered with sodium phosphate monobasic and sodium phosphate diabasic (anhydrous) in a volume 15 times that of the eye. Fixation is usually complete in 48 hours and the specimen is ready for processing or shipment as the case may be. (Cutting windows or injecting the fixative into the globe is neither necessary nor desirable.)

The technique after receipt of the globe in the laboratory follows:

1. Wash globe in running water — 16 hours (to remove fixative).
2. Place in 60% ethyl alcohol until ready to gross — 16 hours. (This acts as the first step in dehydration, serves as a good storage solution, and restores normal color. Restoration of color to the globe assists in the identification of abnormal features and structures.)
3. Write a complete description of the abnormal features of the globe including external appearance, measurements, general aspects of globe, and scars of injury and/or operation. Provide gross description giving external appearance, indicating known or suspected lesions and any abnormalities. (Incorporate any special instructions to enable the technician to demonstrate all of the above conditions in relation to the normal structures.)
4. **General Rules for Grossing and Gross Description of the Eyeball**
   - A. **Orientation:**
     - a. **Posterior Ciliary Vessels:** After fixation the posterior ciliary vessels are more prominent on the nasal side (1), see Figure I.
     - b. **The Optic Nerve:** The distance between the optic nerve and the limbus is less on the nasal side (2).
     - c. **Insertion of the Oblique Muscles:** The superior oblique muscle (3) inserts temporally and above the optic nerve. The inferior oblique muscle (4) inserts on the temporal side of the optic nerve, extending inferiorly from the posterior ciliary vessels.
   - B. **Description:** Describe all abnormal features.
   - C. **Diameters:** Measure specimen, anteroposterior, horizontal, and vertical, in mm.

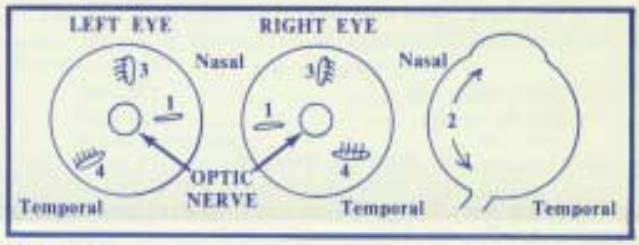

Figure I

D. **Transillumination:** The eye is placed in front of a small intense light, preferably in a small dark box. This demonstrates intraocular shadows which may indicate the presence of a lesion. This will, in turn, determine the plane in which the eye globe is grossed.

E. **Grossing:** When the presence of intraocular foreign bodies is suspected, it is good practice to x-ray the globe before it is opened.

The eye is opened with the aid of a razor blade, see Figure II. Start several millimeters from either side of the optic nerve and pass through the cornea just outside the limbus. During grossing, a right-handed individual holds the eye with the left hand, cornea down against the cutting block. The razor blade is held between the thumb and middle finger of the right hand. With a sawing motion the eye is opened from back to front. The plane of section begins adjacent to the optic nerve and continues through the periphery of the cornea. A description is provided. After the interior of the globe is examined, a second plane of section, parallel to the first, is made, again passing from back to front. During this step the eye is placed flat on its cut surface.

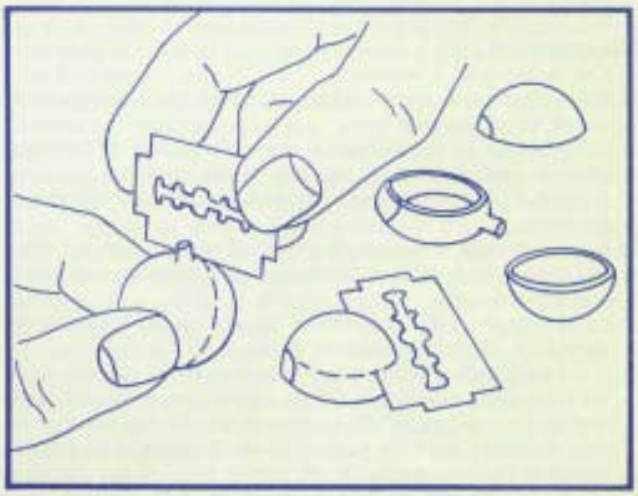

Figure II

COPYRIGHT © 1976, LAB-TEK PRODUCTS, DIVISION MILES LABORATORIES, INC.

F. A thin cross section of the optic nerve results.
G. Sections of the optic nerve are placed with the calottes in 60% alcohol.

5. **Decalcification, Processing and Embedding:** Orientation and embedding instructions should be written on an identification tag which accompanies the specimen through processing. Place in 80% alcohol — to await processing. (Second step of dehydration.)
6. When calcified material is observed on "gross" sectioning, place specimen in decalcifying fluid for several days. (Decalcifying fluid of choice is provided below.)

Decalcifying Fluid
Solution A

Sodium citrate	50 gm
Distilled water	250 ml

Solution B

Formic acid, 90%	125 ml
Distilled water	125 ml

Working Solution
Mix equal parts of Solution A and B

Following use of decalcifying solution, wash specimen in running water for 24 hours.

7. Processing by an automatic processor or by hand, depending upon either the number of specimens or the size, may be accomplished by the following schedule:

Solutions	Overnight Automatic (Conventional Sizes)	Processing by Hand (Large Pieces)
Alcohol, 95%	3 hours	8-24 hours
Alcohol, 95%	2 changes, 1 hour each	(several changes)
Alcohol, 100%	3 changes, 1 hour each	8-24 hours (several changes)
Chloroform	1 hour	2 hours
Chloroform	2 hours	(several changes)
Paraffin (Bioloid* or Peel-A-Way)	2 changes, 1 hour each	5 hours (several changes)
Paraffin	2 hours	
Paraffin (under vacuum)	30-45 minutes	30-45 minutes

8. Embedding is comparable to general embedding procedures using the multiple embedding technique or Tissue-Tek® II Embedding System.

Microtomy:
1. Cut sections at 8 microns.
2. Place block with scleral sides parallel to knife, cornea and nerve on either side.
3. Orient block to get as near a complete section as possible without excessive rough cutting, taking required sections through the nerve and pupil as well as suspected abnormal features.
4. Expose tissue and soak with a piece of cotton saturated with warm tap water. (Minimizes tearing lens, gelatinous exudate and blood.)
5. Chill both block and knife with ice and cut using a smooth slow turn of the microtome wheel.
6. Use two flotation baths: One of distilled water at room temperature upon which the ribbon of sections is placed, and one maintained at 56° C approximately 10 degrees higher than normally used for general tissue flotation. Add gelatin adhesive (3 teaspoonsful of 5% gelatin solution per 1000 ml of water) to this second bath. Individual sections are stretched in the 56° C flotation bath to conform in size and shape with the tissue in the original block.
7. At the close of each day's work, carefully clean with detergent, and dry the flotation bath that contained the gelatin. This insures against bacterial growth adhering to the slides and causing artifacts which would interfere with the diagnosis.

Staining:
After drying, the sections are ready for staining as desired with routine H & E and by special staining techniques.

*VWR Scientific, P. O. Box 3200, San Francisco, California 94119

Routine Tissue Processing Schedule

An Editorial

The processing schedule provided below works well for overnight processing of specimens which do not measure more than 4 mm in thickness.

The schedule can be altered to some extent but several facts should be understood.

1. Expose tissue specimens to the fixative as long as possible, but never less than 6 hours.
2. The time and number of stations used for the chloroform or xylene and paraffins should not be changed. These stages are the most important to insure proper impregnation of tissue by the paraffin. Most of the problems related to inadequate impregnation can be attributed directly to these two stages of processing.
3. The first clearing solution and paraffin should be discarded weekly. The remaining two baths of clearing solution and paraffin should be rotated in the following manner.
   (1) Move the second bath of each solution back so as to make it the first when specimens rotate in the clockwise fashion.
   (2) Move the third bath back so as to make it the second bath.
   (3) Refill the empty bath with fresh solution and place it in the third position of the three bath sequence.

Xylene is not as volatile as chloroform and therefore does not evaporate from the paraffin as rapidly. This generally means that more residual xylene remains in the impregnating paraffins, which in turn retards paraffin impregnation.

Processing Solutions	Time
1. 10% buffered formalin	Allow tissue to remain in fixative solution as long as possible.
2. 80% ethyl alcohol	1 hour
3. 95% ethyl alcohol	1 hour
4. 95% ethyl alcohol	1 hour
5. Absolute ethyl alcohol	1 hour
6. Absolute ethyl alcohol	1 hour
7. Absolute ethyl alcohol	1 hour
8. Chloroform or xylene	1 hour
9. Chloroform or xylene	1 hour
10. Chloroform or xylene	1 hour
11. Paraffin	1 hour
12. Paraffin	1 hour
13. Paraffin	1 hour

Vacuum should be applied to the last paraffin pot.

14. Embed

Stain Technology Control

An Editorial

Listed below are some of the most useful controls for use in stain technology. This will not fulfill all needs for controls in Histotechnology, but it does meet most requirements.

Entity	Source
Amyloid	Tissue containing amyloid
Argentaffin granules	Terminal ileum (small intestine)
Axons, dendrites, myelin, nissl substance and nerve fibers	Cerebrum
Gram positive and negative bacteria	Infected appendix
Basement membrane	Kidney and skin
Bile	Obstructed bile duct with jaundice
Calcium	Bone and calcified lesions
*Chromaffin granules	Adrenal gland medulla
*Cholesterol	Gall bladder with cholesterolosis
Collagen	Lung, kidney, liver
Copper	Wilson's disease
*Cross striations	Skeletal or cardiac muscle
*Desoxyribose nucleic acid (DNA)	Lymph node — plasma cells
*Ribose nucleic acid (RNA)	Normal lymph node — plasma cells
*Elastic fibers	Skin
Fibrin	Blood clot — less than 72 hours old
*Fungi	Any tissue with fungus infection
*Glycogen	Liver
Hyaluronic acid	Skin
Iron	Liver with hemochromatosis
Mast cells	Skin and near blood vessels
Melanin	Skin
Mucin	Small intestine and submaxillary glands
Phospholipids	Normal cerebrum and spinal cord
Plasma cells	Lymph node
Reticulum	Liver and spleen
Uric acid	Gouty joint articulations

The Following Remarks Apply to All Entities Marked with an Asterisk

In order to preserve *Chromaffin* granules, it is essential that tissue be fixed in Orth's Fluid:

Potassium dichromate	2.5 gm
Sodium sulfate	1.0 gm
Distilled water	100.0 ml
Acetic acid, glacial	10.0 ml
Mix and add, concentrated Formaldehyde (37-40%)	10.0 ml

This fixative is not stable and therefore must be prepared just before use.

Fix 3 mm thick specimen for 12 hours. Transfer specimen to 2.5% potassium dichromate for an additional 4 hours.

CHOLESTEROL can only be demonstrated on frozen cut sections. Various fixatives can be used but formalin works quite well. Although the Schultz reaction does not provide permanent slides, in my opinion it is the most specific method for cholesterol.

The CROSS STRIATIONS in skeletal or cardiac muscle are commonly used as control for the PTAH staining procedure. I prefer to use a section of brain. In my experience cardiac or skeletal muscle stains quite well with PTAH solutions which *may not* be reacting to optimum. This may produce the false impression that the staining solution is reacting well. A section of brain works best since a very good working PTAH solution is required to stain the dendrites blue and the cytoplasm of neurons salmon color.

The demonstration of DNA and RNA with the methyl green-pyronin Y method is contingent upon tissue fixation in Carnoy's fluid:

Absolute alcohol	60.0 ml
Chloroform	30.0 ml
Acetic acid, glacial	10.0 ml

Fix 3 mm thick specimen for 5 hours. Specimens may be placed in 95% ethyl alcohol for up to 16 hours if the tissue processor cannot be activated immediately.

I have never seen a good reproducible methyl green-pyronin Y stain on formalin fixed material.

A section of skin is the best control for demonstrating ELASTIC FIBERS since skin contains coarse elastic fibers in the dermis and fine fibers immediately subjacent to the dermis. In my opinion, the demonstration of these fine fibers indicates the staining solution is working to optimum.

HISTOPLASMA CAPSULATUM is one of the most difficult fungi to demonstrate with the use of Grocott's method. It is for this reason that Histoplasma Capsulatum serves as the best control for paraffin embedded tissue sections. Generally speaking, if this fungus stains well, most likely all other types of fungi are going to stain to optimum.

Sections of liver containing GLYCOGEN are universally used as control for the periodic acid Schiff reaction. The best control for the PAS reaction are blood vessel walls which exhibit a bright reddish-purple color only if the reaction worked well. Conversely, glycogen may stain well with Schiff solutions which are considerably below optimum reactive potential.

Important Considerations:

In using normal tissue or tissue structures as controls, remember that normal structures have a tendency to react quicker and better to staining reactions than abnormal structures or entities. For example, mucins from mucin-producing tumors may be more difficult to demonstrate than normally produced mucin in goblet cells. In many instances the granules of carcinoids do not react to silver as well as the normal argentaffin granules of the Kulchitsky cells.

These two examples illustrate the point that good quality control of staining reactions must depend on more than availability of control blocks. The real key to success in stain technology is the discernment of the individual Histotechnologist. I find that most reactions and/or stains provide a tinctorial quality to certain tissue structures that should indicate whether the reaction has worked properly or not. For example, there should always be some silver deposition or reaction on collagen, in a well done Grocott method for fungi. As indicated previously, blood vessels serve as a good control for the periodic acid Schiff reaction. These are built-in controls which can be used to good advantage for controlling staining reactions. To utilize this system successfully one must:

Learn to recognize those structures in tissue which can be used as built-in controls.

Learn to become tinctorially sensitive. That is, learn to recognize when the right shade of color reaction has been applied to a certain tissue structure.

A Technical Bulletin for Histotechnology Vol. VI, No. 1

HISTO-LOGIC®

LTM 21-271 Printed in the U.S.A.

BULK RATE
U.S. POSTAGE
PAID
PERMIT NO. 4954
Chicago, Illinois

Address Correction Requested

Lab-Tek Products
Division Miles Laboratories, Inc.
30W475 N. Aurora Rd., Naperville, Illinois 60540

Two complete Tissue-Tek® systems

paraffin embedding

Tissue-Tek II Tissue Embedding Center provides a paraffin dispenser, warming base mold caddy, hot and cold orientation areas, chiller plate, and cold storage drawer. As an integral part of the system, Tissue-Tek II Vacuum Infiltrator affords complete paraffin infiltration of tissue and can be used for formalin fixation processing. Lab-Tek has developed a complete line of accessories for easy work flow consisting of cassettes, lids, base molds, chuck adapters, process carriers, and storage cabinets.

frozen sections

Tissue-Tek II Microtome/Cryostat and Cryomold® system assures fast freezing of specimens at temperatures between −37°C and −43°C, plus trouble-free cutting of frozen sections. Select the Cryomold suited for the specimen, embed with OCT Compound, freeze and cut tissue with the first or second stroke.

Both of these unique systems are designed for smoother function in preparing top quality tissue slides—saving time and money in the laboratory.

Lab-Tek Products . . . setting the standards by which performance is judged.

Modification of Mallory's Aniline Blue Collagen Stain*

Livia M. Molnar, Department of Orthodontics
University of Washington, Seattle, Washington 98195

Mallory's[1] (1942) Aniline Blue Collagen Stain was modified to obtain good differentiation in a short period of time. The staining time with the original method is 2-3 hours. With the modified stain, time is reduced to 3 minutes. This stain can be applied to any type of tissue and fixative, and never has to be replaced. It stays good, with no special care, for years.

Solutions

Modified Mallory's Stain

Distilled water	600.0 ml
Phosphotungstic acid	6.0 gm
Orange G	3.0 gm
Aniline blue	3.0 gm
Acid fuchsin	3.0 gm

Staining Procedure

1. Decerate and hydrate to distilled water.
2. Stain slides with modified Mallory's, 3 minutes.
3. Differentiate slides in water, 30-60 seconds.
4. Dehydrate in 3 changes of 95% alcohol, absolute alcohol, and clear in xylene 3 changes each.

Results

Nuclei, red; collagen fibrils, blue; ground substance of cartilage, mucin and amyloid, varying shades of blue; erythrocytes and myelin, yellow; elastic fibrils, pale pink, pale yellow, or unstained; teeth and bones, orange to reddish.

1. Mallory, F. B.: Pathological Technique. W. B. Saunders Co., Philadelphia, Penna., p. 153, 1942.

*Prepared under N.I.H. grant RR00166, Research Center, Univ. of Washington.

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 Products, Division Miles Laboratories, Inc., 30W475 N. Aurora Rd., Naperville, Illinois 60540.

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.