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Evaluation of Paraffins

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An evaluation of 13 commercially available paraffins was conducted to determine if paraffins in any way affect microtomy (tissue sectioning). The paraffins were Paraplast™, Gurr's paraffin wax, Tissuemat™, Baker's paraffin wax, Fisher's Bath Wax, Arthur Thomas' paraffin, Blue Ribbon Paraffin™, Gurr's Paramat™*, Peelaway™, and Bioloid™; also Tissuemat™ with dimethylsulfoxide. Esso paraffin wax was included in the study as a control.

Tissues from fourteen organs were obtained from an autopsy performed shortly after death. Tissues were macro-sectioned to a 4 mm. thickness, then fixed in 10% buffered neutral formalin for 72 hours to insure proper fixation. Specimens were then washed in running water for one hour to remove as much fixative as possible. An automatic tissue processor was used for dehydrating, clearing and paraffin impregnation according to the schedule below.

Processing Schedule

1. Ethyl alcohol, 80% - 2 hours.
2. Ethyl alcohol, 95% - 3 changes, 1 hour each change.
3. Ethyl alcohol, 100% - 2 changes, 1 hour each change.
4. Ethyl alcohol, 100% and chloroform (equal parts), - 1 hour.
5. Chloroform - 2 hours.
6. Paraffin (being evaluated) - 3 changes, 2 hours each change.

NOTE: Tissues were placed under vacuum for one hour prior to embedding.

Microtomy (tissue sectioning) was done by three technicians with varying degrees of experience. They were asked to compare the paraffins for speed of impregnation, ribboning qualities, paraffin cracking when cooled with ice, tissue shattering during rough cutting, ability of sections to form ribbons and spreading quality of paraffins when placed on a 42° C. tissue flotation bath. The ease with which wrinkles could be teased from a section while on the water was also studied.

Following staining with hematoxylin and eosin, sections were examined microscopically to determine if any of the paraffins affected the staining reactions or tissue structures. A microscopic check was made for residual paraffin material left on slides similar to that seen when beeswax is used.

Results of Evaluation

The results of this study suggest that Paraplast™ impregnates tissue slightly faster than other paraffins. However, this was only an impression and should not be accepted as factual.

This study also substantiates earlier reports by Ballou[1] and Prophet[2] that Bioloid™ is the best available paraffin for impregnating and embedding eye globes. It also confirms their finding that Paraplast™ will not allow proper stretching of the globe section while on the flotation bath.

The most significant finding was the identical performance of all paraffins with the exception of the control. It must be concluded that all paraffins presently used in histologic technique are good. In the final analysis, the production of poor slides cannot be attributed to the paraffins but instead to other facets of histotechnology such as inadequate fixation, processing and embedding. More frequently, poor sections are the result of inadequate knife sharpening and poor microtomy.

*British product

References

1. Ballou, E.F.: A paraffin technique for processing eyes. *Amer. J. Med. Techn., 22:* No. 5, 287-291, 1966.
2. Prophet, E.B.: Chief, Ophthalmic Laboratory Branch, Histopathology Laboratories Division, A.F.I.P., Washington, D.C., *personal communication.*

Special Precautions for All Silver Nitrate Procedures

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1. All glassware used for preparation of solutions must be chemically cleaned with potassium-sulfuric acid solution, washed thoroughly in running water and rinsed in three changes of glass-distilled water.
2. All chemicals used for the technique must be reagent grade and all solutions must be made up with either double-distilled water or water for injection.
3. Solutions of silver nitrate must be kept in amber bottles and the lips of the bottles kept free of contaminants.
4. When heating solutions, be very careful not to exceed author's recommendations as to temperature and time. The use of a good flotation water bath is preferable to an oven which may fluctuate in temperature.
5. Solutions must be covered at all times and kept in a dust free atmosphere. Cork stoppers must never be used. Discard silver nitrate solution if a precipitate forms. The containers must be chemically cleaned and rinsed before reusing.
6. Forceps for handling slides must be teflon tipped or coated with clean, filtered paraffin. Never use metal staining trays or allow any metal to come in contact with these solutions.
7. Slides must not be touched with fingers once they have been deparaffinized. While doing the technique, avoid areas in which fumes of formalin, ammonia, hydrochloric acid, etc., are present.
8. Prepare all solutions as fresh as possible. Stewart-Smith[1] and Wallington[2] warn that ammoniacal silver solutions can become explosive if stored too long. They also recommend that all glassware used for preparing silver solutions be immediately acid cleaned to prevent any explosions.

References

1. Stewart-Smith: *J. Path. Bact., 55:* 227, 1954.
2. Wallington, A.E.: *J. Med. Lab. Tech., 22:* 220, 1965.

Artifacts Produced During Tissue Fixation

An Editorial

Calcium Carbonate Crystals

Calcium carbonate (marble chips) is frequently used as a neutralizer in formalin fixative solutions. But it can create problems, because the fixation process often causes some of the calcium carbonate to crystallize. These crystals of varying sizes and shapes can deposit and subsequently grow within tissue specimens.

Generally, the crystals will grow radially from a central point in a laminated pattern. The real problem posed by the formation of these crystals is that they resemble an *in vivo* reaction. However, these crystals can be identified by the following procedure:

> Crystals polarize well and are slightly soluble in pure water. Because of their conversion to the more soluble hydrogen carbonate, they dissolve readily in water containing dissolved carbon dioxide. This conversion occurs when the deparaffinized slides are placed in tap water containing small pieces of dry ice (CO_2) for 30 minutes. Slides are then stained with hematoxylin and eosin.

The use of calcium carbonate for neutralizing formalin fixative solutions is not recommended as it is the least useful of the neutralizing fixative agents. This opinion is based on the following reasons:

1. The so-called formalin crystals will develop and staining results will be adversely affected after several weeks of tissue storage.
2. This method of neutralizing gives a false sense of security since tissue is in a neutralized state only during its contact with the solution. Tissues will lose their neutrality soon after removal from formalin containing calcium carbonate.
3. Calcium carbonate produces crystals in tissue sections.

Furthermore, by comparison, the ease with which Lillie's buffered neutral formalin can be made, and the problems it prevents, afford little reason for using neutralized or unbuffered formalin fixatives.

Buffered Neutral Formalin Solution

37 - 40% formalin	100.0 ml
Distilled water	900.0 ml
Sodium phosphate monobasic	4.0 gm
Sodium phosphate dibasic (anhydrous)	6.5 gm

One can simplify the compounding of the above fixative solution by the use of a marked container to indicate the levels of distilled water and formaldehyde. The sodium phosphate monobasic and dibasic salts can be premeasured in sufficient quantities to last for months and placed in moisture-free plastic containers until ready for use.

Artifactual Iron Deposition

Metal lids used on fixative containers are subject to corrosion, especially when used with Zenker's- or Bouin's-type fixatives. Unbuffered formalin-type fixatives also act to form corrosion very rapidly. The corrosive action takes place more frequently if the container's metal lid is loose or if the diaphragm is missing. Corrosive contaminate in the fixative solution can deposit on tissue specimens and produce artifactual staining results when iron stains are performed. These artifactual results are most significant in specimens such as liver, spleen and bone marrow. To eliminate this problem, plastic containers are recommended for all phases of fixation.

Pseudocalcification

In 1965, Luna and Gross[1] described several examples of pseudocalcium in tissue sections fixed in 10% formalin neutralized with calcium acetate. Since calcium acetate continues to be used to neutralize formalin type fixatives, it is felt that a reminder of its potential problems is in order.

These deposits are difficult to distinguish from those produced during true calcification. Pseudocalcification can be mistaken for true calcification because their staining properties with the various calcium stains or reactions are identical. Pseudocalcium can be distinguished from true calcification by the use of one of the mucosaccharide staining techniques such as Alcian blue or Mowry's colloidal iron. The true calcification will produce mucosaccharides on the peripheral margins while pseudocalcium will not.

Reference

1. Luna, L. G., Gross, M. A.: Artifacts Simulating Calcium Associated with Improper Buffering of Formalin Fixative. *Amer. J. Med. Techn., 31:* No. 6, 412-416, 1965.

Methods for More Specific Differentiation of Melanin Pigment

An Editorial

Numerous methods can be found for the removal of melanin from tissue sections. In the laboratories of the Armed Forces Institute of Pathology, the potassium permanganate and oxalic acid procedure is preferred for the following reasons:

1. The procedure removes melanin completely if the treatment period is adequate. Most melanins, including the dense ocular melanin, will be removed in thirty minutes.
2. Potassium permanganate will not dissolve formalin pigment. Some of the superficial crystals may be removed, making the remaining crystals more difficult to see microscopically. However, their polarizing properties remain. The polarizing properties of formalin crystals assist in their differentiation from melanin, iron and lipofuscin since the latter substances do not polarize.
3. Potassium permanganate and oxalic acid treatment can be used for the differentiation of melanin from iron. Iron is only slightly affected but not removed by this treatment and subsequently can be demonstrated by using one of the conventional iron stains.
4. Lipofuscin is also slightly affected by this treatment and can be differentiated from melanin by subsequent staining with the acid fast method found on page 186 of the AFIP Staining Manual. The differentiation between melanin and these two substances is important since they appear similar microscopically. In addition, all three pigments reduce silver nitrate. Therefore, they cannot be differentiated on the basis of silver preparations, which are the methods presently used for demonstrating melanin. It is also significant that melanin sometimes has a positive reaction with iron stains.

Our preference for removing melanin with potassium permanganate and oxalic acid does not mean that other removal methods react adversely.

Staining After Permanganate Treatment

Since iron, lipofuscin, and formalin pigment remain after potassium permanganate treatment, it is desirable to demonstrate them (or any other pigment) clearly. For this reason, it is important that a weak hematoxylin be used for nuclear staining since it will not obscure small pigment deposits. A weak Mayer's hematoxylin serves this purpose. Alternatively, a borax methylene blue solution or a nuclear fast red (Kernechtrot) solution can be used. An eosin-type stain should never be employed after melanin bleach as most pigments are yellowish-brown and are normally found in the cytoplasm. Some of the smaller pigment deposits may be difficult to see microscopically since there is little color differentiation between them and the counterstain.

Weak Mayer's Hematoxylin Solution

Hematoxylin crystals	0.5 gm
Distilled water	1000.0 ml
Sodium iodate	0.1 gm
Ammonium or potassium alum	25.0 gm
Citric acid	0.5 gm
Chloral hydrate	25.0 gm

Dissolve the alum in water without heat; add and dissolve the hematoxylin in this solution. Add the sodium iodate, citric acid and the chloral hydrate. Shake until all components are in complete solution. The final color of the stain is reddish violet. Stain keeps well for months.

Staining Procedure

Melanin is removed from tissue sections by the use of Method One found on page 42 of the AFIP Staining Manual. Sections are subsequently stained with weak hematoxylin for 15 mintues, followed by a 10 minute wash in running tap water to blue nuclei. Dehydrate, clear and coverslip.

Borax Methylene Blue Solution

Methylene blue	1.0 gm
Borax	1.0 gm
Distilled water	100.0 ml

Staining Procedure

After melanin removal, sections are stained as follows:

1. Rinse in distilled water.
2. Stain in borax methylene blue solution for 2 minutes or longer. Staining results will vary depending upon the length of exposure to potassium permanganate.
3. Rinse in distilled water.
4. Dehydrate, clear and coverslip.

The third possible choice of a nuclear stain for melanin bleached sections is Kernechtrot.

Nuclear Fast Red (Kernechtrot) Solution

Aluminum sulfate	5.0 gm
Distilled water	100.0 ml
Kernechtrot	0.1 gm

Dissolve aluminum sulfate in distilled water prior to adding Kernechtrot. Add Kernechtrot to solution with the aid of heat. Cool, filter, and add a grain of thymol as a preservative.

Staining Procedure

After melanin removal, sections are stained as follows:

1. Rinse in distilled water.
2. Place slides in Kernechtrot solution for 5 minutes.
3. Rinse in distilled water – at least three changes.
4. Dehydrate, clear and coverslip. If slides turn cloudy, they were not rinsed sufficiently in distilled water.

The use of a specific nuclear stain following the potassium permanganate oxalic acid treatment is dictated by the results desired. Weak Mayer's hematoxylin should be used routinely. Kernechtrot should be used when one desires to overshadow the light gray color remaining after melanin removal. Borax methylene blue is suggested for a better demonstration of the pigments remaining such as iron, lipofuscin and formalin, since these pigments appear brighter microscopically after this nuclear stain.

Hematoxylin and Eosin Staining Problems and Solutions

An Editorial

Small, clear crystals often seen throughout sections may be crystals of aluminum potassium sulfate which is used as a mordant in many hematoxylin solutions. These crystals indicate that the hematoxylin solution was not mixed properly resulting in the aluminum potassium sulfate reconverting to its original crystalline form. These crystals can be seen easily under polarized microscopy. When crystals are observed, the hematoxylin solution should be discarded. If the solution is not discarded, the deterioration will continue and a black pigment will appear throughout the section. This pigment is identified as hematein (the coloring agent of hematoxylin) which precipitates due to the absence of the mordant described above. This breakdown of hematoxylin results in poor section staining. Mixing of any solution requires good agitation, the best of which is a magnetic stirrer.

To prevent uneven staining of tissue sections, agitate slides several times in staining solution before allowing them to remain for the recommended time period. This problem is more acute in H&E preparations where microscopically one sees muddy, grayish-pink, uneven staining cells.

Inadequate dehydration of microscopic slides is characterized by a brownish granular appearance of the tissue section. This artifact can be seen easily when the substage on the microscope is lowered.

Tissue slides should not be allowed to dry during the application of the coverslip. An artifact with two distinct features can be produced: (A) sections may exhibit brown stippling which resembles pigment, or (B) trapped air may be seen in the nucleus as a distinct glossy black structure. These features are seen clearly by lowering the substage of the microscope. These artifacts can only be removed from sections by the following process:

1. Place slides in xylene to remove coverslip.
2. Absolute alcohol – 2 changes, 5 minutes each.
3. 95% alcohol - 2 changes, 5 minutes each.
4. Running tap water 15 minutes.
5. Check microscopically to be sure problems have been eliminated. If not, allow slides to remain in running tap water for an additional 10 minutes.
6. Restain as desired.

Excessive use of mounting media or that which is allowed to thicken will result in a foggy appearance when the section is observed under high power microscopy (X450 or more).

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Editor's Corner

"Quotes"

"HISTO-LOGIC" seems like a marvelous new idea!!!!

Mrs. Elaine Kurtz
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"I have received the first edition of "Histo-Logic" this past week. I would like to comment at this time that I think this is a good idea and I hope it will continue to be published. A publication such as this, with the cooperation of everyone in this field could really prove to be informative."

Catherine E. Miller
Bethlehem, Pennsylvania 18015

"Congratulations and good luck with Histo-Logic!"

Jules Elias
Stony Brook, New York 11790

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Can you help?

"The problem I have recently encountered is the need for ascertaining rather minutely whether or not tumor lies at the peripheral and deep margins of some fairly extensive skin resections for a plastic surgeon who has just newly come to our community. The surgeon often skin grafts by a flap and will go back and remove a small segment from the margin if tumor remains at some point. Short of imbedding each piece separately with a number, I have struck out by trying to mark with India ink, slit the undersurface and make other approaches to this problem. Could you tell me if there are any dyes or ways of marking margins or tissues selectively so that they can be recognized after they have been imbedded and sectioned and are examined by me on the microscopic slide?"

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