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No reader should utilize materials and/or undertake 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.

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Problems with Aldehyde Fuchsin Staining

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A much used method for demonstrating the granules of pancreatic B cells and most pituitary basophils is Gomori's[2] aldehyde fuchsin (AF) stain. It will also stain elastic fibers and is useful for demonstrating mast cells.[3] More recently it has been found valuable in staining hepatitis B surface antigen in paraffin sections of liver biopsies.[8, 9]

Unfortunately, there are problems which occur with the AF stain and with its many modifications. In a recent paper Mowry[7] indicates that there are at least seven factors which may affect the staining properties of AF. Four of these factors, which account for the majority of the problems with AF staining, are as follows:

1. **The Basic Fuchsin (BF).** In the past, BF consisted mainly of rosanilin (C.I. 42510) and pararosanilin (C.I. 42500) in varying proportions. For some time, BF's made in the United States have been mainly either rosanilin or pararosanilin, depending on the method of dye synthesis.[4] To further complicate the matter some dyes designated as pararosanilins by some companies have been found, after retesting by the Biological Stain Commission, to be rosanilin. Also, some dyes designated as rosanilins have been found to be pararosanilins.

Studies done by members of the Biological Stain Commission have shown that AF made with rosanilin will not work in most AF methods. AF made with pararosanilin will give satisfactory staining in all AF methods. According to Charles Willis (personal communication), the Technical Director of the Biological Stain Commission, one can tell if a BF is rosanilin or pararosanilin by the dye content indicated on the label. BF's which have a high dye content (97-99%) are likely to be primarily pararosanilin, and BF's which have a lower dye content (88-93%) are likely to be rosanilin, regardless of the C.I. numbers on the labels.

2. **The Aldehyde.** Usually 1% of the AF stain is paraldehyde. The AF of Hartroft and Wrenshall[3] and Mowry[7] contains 3% paraldehyde. Mowry recommends using enough paraldehyde so that 3% of the AF is paraldehyde and claims that it stains pancreatic B cells more strongly. It is important to use fresh paraldehyde in making AF as AF made with deteriorated paraldehyde may stain weakly or not at all. I use 10 ml ampules of U.S.P. paraldehyde obtained from the hospital pharmacy and stored in the refrigerator to make a 3% solution.

3. **Temperature and Duration of Ripening.** Most reports agree that ripening at room temperature requires at least two or three days. For formalin fixed tissues, Mowry[7] recommends ripening for five days at room temperature. Ripening is accelerated by heat. The AF of Hartroft and Wrenshall[3] contained 3% paraldehyde instead of 1% and was ripened overnight at 40-45° C. Elftman's[1] AF was ripened for twenty-four hours at 37° C. We have found that AF ripened for five days at room temperature gives consistently good results.

4. **Useful Life of AF.** Gomori[2] indicated that aged AF which no longer stained pancreatic B cells did stain elastic fibers adequately. Nearly all reports mention that once AF is ripened, staining potency declines with time. The useful shelf-life of AF differs according to its intended use. Its staining capacity for pituitary thyrotropes diminishes faster than its capacity to stain pancreatic B cells. Aged AF (6-8 weeks) will stain elastic fibers and hepatitis B surface antigen. It will also stain pancreatic B cells if the staining time is greatly increased (several hours to overnight). Because its staining properties change with age it is important to always have on hand AF which is relatively fresh in order to avoid delay caused by preparation and ripening. It is recommended that fresh AF be prepared about once a month and when adequately ripened the previous batch discarded.

The majority of problems encountered with AF staining can be remedied by following these four simple rules:

1. Use only basic fuchsin, which is pararosanilin, when making AF.
2. In preparing AF always use fresh paraldehyde, preferably from an ampule which has been kept refrigerated.
3. Properly ripen AF before use.
4. Use AF within its shelf-life.

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Sectioning of Paraffin Embedded Brain Tissues An Alternate Method

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Sectioning of paraffin embedded brain tissues can be a difficult and time consuming task. Problems most frequently encountered are wrinkling and compression of sections, coupled with the tendency of sections to fold over or slip off the slides during the staining process. Obtaining recuts from old blocks present more of a challenge. Striation and shattering, especially when there is dried blood in the tissue, are common frustrations. These problems prompted some experimentation in the histology laboratory at the University of Chicago Hospitals and Clinics.

Striation, shattering of blood clots while cutting, and rapid separation of the tissues from the paraffin when placed on the water bath all seemed to be associated with the brittleness of old tissue in blocks which had been stored for weeks to several months. In short, they seemed "dry." If moisture or processing volatile chemicals had evaporated from the tissues during storage, it seemed reasonable to assume that reintroduction of moisture might assist in forming a firmer matrix. Certain absorbent methods have been utilized in histology laboratories to facilitate the cutting of difficult tissues; i.e., rapid decalcification of bones with 5% aqueous HCl[1] and soap or glycerin for softening bones or uterus[2] These methods are employed at the cut surface of the block prior to final sectioning.

Since recuts involve that portion of the tissue near the block's surface, a similar absorbent method was tried. Deionized water was used in the manner described in Method I, below. After the blocks were hydrated, rather than placing them on ice before cutting as is routinely done, they were cut at room temperature with the aid of a **cold knife.** This was most effective since the cold knife provided more control over the sectioning of 10 micron sections, than was provided by the temperature of the block. Sections were cut with ease, did not separate from the paraffin on the water bath, and produced no problems during the staining process.

In our laboratory, additional problems arose with previously uncut blocks. It was noticed that most of the technicians trimmed all the blocks from an autopsy case at the same time. Brain blocks were trimmed and set aside until they had accumulated a number sufficient to fill at least one staining rack with slides — usually 10 or more blocks. At times, these blocks remained as long as two weeks in a trimmed state before final sectioning.

This prompted more experimentation. A previously uncut block was trimmed and placed on a scale. It lost moisture so rapidly that the weight indicator was in slow, constant motion, and the scale reading was lower each minute. This seemed to indicate that most of the moisture loss took place soon after the tissue was exposed to air.

Therefore, Method II was adopted for previously uncut blocks and final sectioning was done immediately after trimming, using a **cold knife** at room temperature.

Additional data was needed to support the assumption that brittleness in the tissue was due to excessive dehydration and that the longer a cut block was exposed to air, the more moisture it lost. Ten blocks were weighed after sectioning by the usual method. These blocks were then stored in a cardboard box. A block of pure paraffin was used as a control and it too was stored as above. Eight weeks later, the blocks were weighed again. The results are shown in the Table below. The blocks were then handled as indicated in Method I and sectioned. These stained slides, along with the original slides, were shown to several resident pathologists. They reported essentially no appreciable difference, but when pushed to make a decision, preferred the hydrated sections.

Consequently, both of these methods are used now by the University of Chicago technicians, either routinely or as alternate approaches to troublesome brain blocks.

Method I
Old Blocks:

1. Align blocks on microtome and trim, as little tissue as possible, but insure full section.
2. Place blocks, cut surface down, in shallow pan containing about 1/8 inch room temperature tap or deionized water.
3. Check frequently for change in color from yellowish-brown to whitish-gray. This varies with each block and may take only 5 minutes or as long as 10-20 minutes. When this change occurs, block is ready to be cut. (It should not be hydrated more, or tissue will begin to expand out of block. Should this happen, allow the block to remain at room temperature and it will retract into its original position in the paraffin block.
4. Place block on microtome with knife in place for first section. Make sure knife is free of paraffin particles by wiping front and back with xylene-dipped gauze.
5. Hold ice cube on knife for 5-10 seconds and section immediately at 8-10 microns. A good ribbon should form.

Method II
New Blocks (Previously Uncut Blocks):

1. Trim block at room temperature. If trimmed sections are smooth and artifact free, continue final sectioning on the same area of the knife.
2. Make sure knife is free of paraffin particles by wiping quickly front and back with xylene-dipped gauze.
3. Hold ice cube on knife for 5-10 seconds and section immediately at 8-10 microns. Always clean and re-ice knife before attempting additional ribbons.

Moisture Loss

Block	Gm. wt. after final sectioning	Gm. wt. 8 wks. later	Net moisture loss in mg. wt.
Br 2	10.249	10.239	10
Br 4	10.096	10.090	6
Br 7	10.398	10.392	6
Br 9	9.622	9.608	14
Br 10	9.353	9.330	23
Br 11b	10.568	10.558	10
Br 13	10.666	10.644	22
Br 15	10.356	10.348	8
Br 16	10.652	10.637	15
Br 17	10.272	10.264	8
Paraffin control	11.133	11.133	0

Losses range 6-23 mg. Average 12 mg.

References:

1. Culling, C.F.A., and Barkoczy, B.C.: Paraffin Embedding of Bony Tissues followed by Decalcification of the Layer to be Sectioned. Stain Tech., 219-220, Nov., 1971.
2. Preece, A.: A Manual for Histologic Technicians, Third Edition. Little, Brown and Company, Boston, 1972.

The Masson Trichrome Some Useful Information

An Editorial

Next year the Masson[1] trichrome will be celebrating its 50th year of service to the field of pathology (Masson, P.J.: J. Techn. Methods, 12:75-90, 1929). This technique has withstood the challenges of many modifications and new techniques for the demonstration of connective tissues. The long tenure of this procedure proves that Dr. Masson's development was a monumental accomplishment, particularly since most laboratories continue to use this stain on a daily basis. While this is a time proven procedure, there are two aspects which, in my mind, continue to plague the final results. (1) **Uneven staining of the collagen fibers** and (2) **overly stained muscle fibers.**

By uneven staining I refer to those instances in which not all collagen fibers stain with the aniline blue dye. Microscopically the fiber color ranges from a deep blue to a deep red. In between these two extremes one sees various shades of color. This problem is produced by denatured collagen fibers. The denaturing of collagen takes place when paraffin blocks are sectioned and one fails to seal the cut surface of the block, leaving the tissue exposed to the atmosphere. The degree of denaturization, and therefore the effect it has on the staining reaction, depend on how long the tissue is exposed to the atmosphere before being resectioned.

This problem occurs only on paraffin blocks which are recut (for additional sections), some days after the initial cutting. It should be understood that this seldom occurs on sections obtained for the first time. Denaturization of tissue also occurs when tissue is allowed to dry, or when it is exposed to excessively hot paraffin for an extended period. In addition, it occurs when sections are cut and

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Note: The first four issues (Vol. I No. 1 & No. 2, Vol. II No. 1 & No. 2) were not numbered when published. For this index, we have numbered these issues as follows: Vol. I No. 1 pages 1-4, Vol. I No. 2 pages 5-8, Vol. II No. 1 pages 9-12, Vol. II No. 2 pages 13-16. To effectively use these indices you should number these issues to correspond with the above.

SUBJECT INDEX

Vols I-VIII
1971-1978

stored at room temperature for long periods of time without being exposed to a drying oven or slide warmer soon after microtomy.

Another factor which produces varied staining results is attributable to the mordanting of sections with Bouin's solution. If the mordanting is not complete, one can expect uneven staining. To prevent this problem, sections should be mordanted in Bouin's solution overnight or, alternatively, sections should be placed in preheated Bouin's solution before being placed in a 60° C oven for an hour. Section should then be allowed to cool for 30 minutes before continuing with the water wash.

The problem of overly stained muscle fibers has bothered me for some time and here again, some steps should be taken to eliminate this deficiency in technique. By the muscle being too dark, I refer to those instances in which the muscle bundle is stained so intense that microscopically it appears as a solid mass of cytoplasm. I believe that a good Masson should demonstrate muscle strongly but delicate enough to allow the microscopic demonstration of fine detail.

One may select one of several ways to correct this problem: Reduce the time in the major staining solutions. For example, the following time changes can be made if you are using the Masson procedure presented in the AFIP Staining Manual.[1] Step 7, biebrich scarlet-acid fuchsin solution, reduce staining time from 2 to 1 minute. Step 9, phosphomolybdic-phosphotungstic acid solution, reduce the time from 10 to 5 minutes. Step 10, aniline blue solution, reduce the time from 5 to 2 minutes. Additional applicable information to resolve this problem is provided by Lillie and Fullmer[2]: "Phosphotungstic acid tends to intensify the plasma (cytoplasm) stain; phosphomolybdic, the fiber stain; and alcohol weakens the plasma stain. Pretreatment with hot Bouin's solution intensifies staining of muscle and plasma. Water washing extracts plasma stains more than fiber stains, whereas acetic acid rinsing makes the preparation more transparent without altering the color balance."

As indicated previously, the Masson trichrome is a time-tested procedure. However, it can be made more selective by introducing some minor changes. This coupled with the information regarding the staining alterations resulting from denatured tissue can be most useful if applied to the performance of this most useful procedure.

References

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An Intriguing Question in Search of an Answer

An Editorial

There are many unexplainable happenings in histotechnology which sometimes boggle the imagination. For example, wrinkles, bubbles, knife lines, etc., are often found in the precise areas one wishes to photograph. Sections fall off the slide during staining on that one case the pathologist wants to see right away. If one is going to misplace or lose a specimen, it will be the most important one. Undoubtedly you could add to the list from your own personal experiences. There is one phenomenon which should be studied and attempts made to find a solution; i.e., **what causes the strong bond between floating debris and the tissue section or glass slide?**

For example, Figure 1 shows debris which has deposited on the tissue section. The staining solution was aldehyde fuchsin. Undoubtedly this material developed in the solution and was transferred to the section during the staining process. Thompson and Luna illustrate many such problems in their book "An Atlas of Artifacts Encountered in the Preparation of Microscopic Tissue Sections," Charles C. Thomas publisher, Springfield, IL. The production and/or development and deposition of numerous types of debris on tissue sections is not difficult to understand. What is difficult to understand, is what produces such a strong bond between the debris and the section.

That there is a strong bond can be proven by the fact that it is impossible to remove any type of artifactual debris from tissue sections even though the section is exposed to the force of strong running tap water. On many occasions I have recognized debris on the section during the staining process and have attempted to dislodge it by various means, including holding the section under the force of wide open faucet water. Attempts to dislodge some of this material with a fine camel hair brush often result in producing a hole in the section instead of dislodgement of the debris. All sorts of theories concerning this debris/section attachment have crossed my mind, but none have proven valid. There must be some sort of a strong adhesive attraction involved at submicroscopic levels, but what is it?

Is it chemical, physical, a catalytic process, or is it simply an adhesive affect produced by protein or section adhesives such as gelatin or egg albumen. I believe the answer and solution to this perplexing problem would prevent many periods of consternation for both the Histotechnologist and Pathologist.

Any concrete answers to this problem should be sent to the editor. All valid and logical answers will be published in a future issue of **Histo-Logic®.**

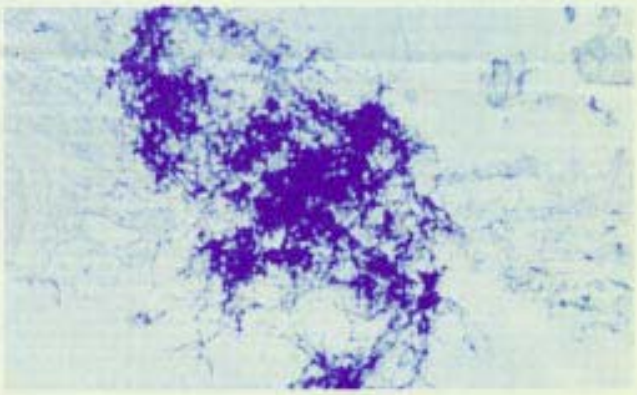

FIGURE 1:
Debris deposited on tissue section during the staining process. Aldehyde fuchsin for pancreatic islet cells, X300.

Request for Help from Australia

An Editorial

The following communication was received by Miles Laboratories, Australia PTY. LTD., last July. It is being presented here to solicit your assistance in providing an answer to this Histotechnologist from Australia. Send replies to the editor who will in turn publish the information in a future issue of Histo-Logic. A photograph illustrating the staining reaction of the gut nerve cell bodies would be desirable.

"Dear Sir,

Replying to your last technical bulletin, I am indeed very keen to keep receiving copies of Histo-Logic and also to take part in your planned column.*

At the moment I have no particular information to submit, but I hope to be able to shortly, mainly in the field of histochemistry.

One problem I have recently come across is with silver staining of nerve cell bodies in gut sections. I have had very little success with the Glees-Marshland Method and I wonder if someone may have an alternative which they have found successful.

Thank you for promoting histology amongst histologists.

Yours truly,

David M. Bloom, B. App. Sc."

*Editor's Note:
This refers to an upcoming global histology column planned for future issues of Histo-Logic.

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Technically Speaking

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Lab-Tek Division, Naperville, IL

At periodic intervals during the hectic processing schedules in a histology laboratory, it becomes necessary to identify a tissue cassette to facilitate "Stat" processing, sectioning and staining.

At present, metal and plastic cassettes are identified by tying a string to the cassette cover, specific placement in the processing basket or by using color-coded paper tags. These techniques are time consuming and at best, hit or miss.

Lab-Tek has recently introduced an extension of the Tissue-Tek® II Process/Embedding Cassette line. Color-Coded Process/Embedding Cassettes are now available in four colors: green, blue, yellow and pink.

Product code numbers are:

4179 Tissue-Tek II Process/Embedding Cassette (yellow)
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4183 Tissue-Tek II Process/Embedding Cassette (green)
4184 Tissue-Tek II Process/Embedding Cassette (blue)

How can color coding help you and how can color coding suit your laboratory's needs? Here are a few helpful suggestions color coding can provide:

1. Segregate surgicals from autopsies.
2. Separate "Stat" tissue from routine work.
3. Segregate bone specimens to the end of sectioning process to prevent early nicking of the blade.
4. Segregate special procedures.
5. Separate research projects.
6. Readily identify satellite laboratories or hospitals.
7. Segregate work load by year (1978-green, 1979-blue, etc.).

These are but a few of the many uses of color coding. Application is only limited by your imagination.

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.
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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.