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Automatic Staining Versus Hand Staining

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With advances in research and improvement in surgical procedures, a larger work load and a demand for faster results follow.

Through the years, equipment for use in histology has become more efficient and easier to operate. This modern equipment has accelerated the process of slide production.

However, there is still a great deal of wasted time in staining. If this procedure could be automated, it would relieve the technician to perform other laboratory functions.

A number of staining procedures were compared to determine the feasibility of automatic staining.

The stains were performed on the Autotechnicon-Mono, using the one hour timing clock. All beakers, except those used for xylene, were partially filled with 1-1/2 inches of paraffin to conserve solutions. A running tap water wash was made by boring two holes in a plastic beaker. The inlet hole is 1 inch from the bottom and the outlet hole is located at the water line. (A paraffin bath was coated with an inactive plastic to prevent any reaction between the metal and the solutions.) Three hematoxylin and eosin methods were performed. The traditional technique, with Harris' hematoxylin, was performed manually. The Technicon-brand hematoxylin and eosin procedure was accomplished manually and mechanically.

Manual procedure using Harris' hematoxylin:

1. Decerate in xylene and hydrate to water.
2. Stain in Harris' hematoxylin for 5 minutes.
3. Rinse in running tap water for 1 minute.
4. Decolorize in 1% acid alcohol, 8-10 dips.
5. Rinse in water.
6. Place in ammonia water until sections are blue.
7. Wash in running water, 1-3 minutes.
8. Stain in 1% alcoholic eosin for 1 minute.
9. Dehydrate with 2 changes each of 95% and absolute alcohol.
10. Clear in xylene, 2 changes.
11. Mount with a synthetic resin.

Manual procedure using Technicon reagents:

1. Decerate in Paraway* for 2 minutes.
2. Hydrate in 2 changes of S-29* to distilled water.
3. Stain in Technicon's hematoxylin for 5 minutes.
4. Wash in distilled water for 1 minute.
5. Blue in 0.01 N lithium carbonate solution.
6. Wash in distilled water for 1 minute.
7. Stain in Technicon's eosin for 2 minutes.
8. Rinse in distilled water.
9. Dehydrate with 2 changes of S-29.
10. Clear and mount with a synthetic resin.

*Technicon's proprietary names for xylene and alcohol substitutes, respectively.

The automated procedure was:

1. Paraway for 2 minutes.
2. S-29 for 2 minutes.
3. S-29 for 2 minutes.
4. Distilled water for 2 minutes.
5. Technicon's hematoxylin for 5 minutes.
6. Running water for 2 minutes.
7. Lithium carbonate for 2 minutes.
8. Distilled water for 2 minutes.
9. Technicon's eosin for 2 minutes.
10. Distilled water for 2 minutes.
11. S-29 for 2 minutes.
12. S-29 for 2 minutes.
13. Remove slides from the Mono, clear in xylene, and mount with a synthetic resin.

Microscopically there was no difference in the staining quality of the tissue sections using the three procedures.

In addition to the three hematoxylin and eosin methods, some special stain comparisons were made.

Two different Van Gieson stains were performed; a procedure marketed by a commercial firm, and the Van Gieson method in the AFIP Staining Manual.[1] Each procedure was performed manually and automatically.

The main difference between the procedures was the type of hematoxylin used. An alum hematoxylin cannot be used with the Van Gieson procedure. Picric acid in the Van Gieson solution will decolorize an alum hematoxylin. Little or no difference was observed in the results between the manual and automated stains.

Gomori's one-step trichrome:

1. Decerate in two changes of Paraway, 2 changes of S-29, and 1 of distilled water.
2. Mordant in Bouin's solution for 1 hour at 56° C. A plastic coated paraffin bath set at 56° C was used for mordanting in Bouin's. (Switch the Mono to manual and after one hour, return it to automatic.)
3. Wash in running tap water for 10 minutes.
4. Stain in Weigert's hematoxylin for 10 minutes.
5. Rinse in water for 2 minutes.
6. Stain in Gomori's trichrome stain for 20 minutes.
7. Rinse in water for 2 minutes.
8. Dehydrate in two changes of S-29, 2 minutes each.
9. Remove from the Mono, clear in xylene, and mount with a synthetic resin.

The technician is required to momentarily shift his attention from the Mono to manual and again back to automatic.

The procedure for the manual method is described in the AFIP Staining Manual. There is little difference in the results of the two methods.

Conclusion

It is the feeling of the author that a laboratory processing large numbers of H & E slides daily will find automation to be a time saver. The most successful automated stains are those which are progressive, since they do not require differentiation. For example, it would be difficult to automate Verhoeff's elastic stain due to the requirement for precise differentiation. The stain selected for automation should be composed of solutions which are relatively stable.

Although these procedures were performed on the Technicon Mono automatic tissue processor, there is no reason to be-

lieve they cannot be performed on any automatic stainer. Solutions easily contaminated by metals cannot be used in automated systems unless steps have been taken to eliminate contact with all metals, i.e., staining rack and/or staining dish.

Reference

1. Luna, L.G.: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 3rd ed., New York, McGraw-Hill Book Co., Blakiston Division, 1968.

Differential Staining Methods for Cells in the Central Nervous System

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Cells in the nervous system stained with hematoxylin and eosin (H&E) are commonly classified according to three criteria: (1) size, (2) shape, and (3) intensity of nuclear basophilia. Because of variations in thickness and/or plane of sectioning, identification of cell types in the central nervous system (CNS) through these criteria becomes increasingly inaccurate and misleading. Although specific staining methods for each cell type in the CNS (Neurons, astrocytes, oligodendrocytes and microglia) are available,[1,2] most of these methods require frozen sections which are impractical when working with large tissue samples. Moreover, the interrelationships between different cell types cannot be visualized in a single section. A staining method is being developed in our laboratory to make different cell types, in the CNS, readily discernible.

The Feulgen reaction of mild acid hydrolysis at a high temperature (1.0 N HCl at 60° C for 12 minutes) is a well recognized staining method specific for nuclear DNA.[3] Acid hydrolysis has been attempted previously with strong acids at room temperature, however, prolonged exposure was required to achieve satisfactory results.[4] In our laboratory, the process of acid hydrolysis has been accomplished with concentrated hydrochloric acid (12.4N) at room temperature. Hydrolysis can be reduced to one minute and still yield nuclear aldehyde groups which will subsequently react with the Schiff reagent to give a magenta color. Morphologic alterations, including clumping of nuclear chromatin and shrinkage of tissue, become evident only if the tissue sections are hydrolyzed in the concentrated acid for more than two minutes. After one minute of hydrolysis at room temperature, the Feulgen reaction is visually very satisfactory. Astrocyte and microglia nuclei are stained pale violet-red. Oligodendrocytes are more intensely red because of the dense chromatin pattern. Neuronal nuclei, due to the great dispersion of chromatin material, are stained only very faintly.

A second dye can be used to stain the protein moiety of the nucleic acid so that the differential effect of the cell types can be accentuated. Dyes such as Toluidine Blue O, Azure A, Fast Green FCF, and Naphthol Yellow S were attempted. Toluidine Blue O and Naphthol Yellow S failed to give satisfactory color differentiation at pH's between 3.0 and 7.0, with concentrations of 0.01% - 0.5%. However, Fast Green FCF and Azure A did permit cell type differentiation following the Feulgen reaction.

With the Feulgen-Fast Green staining method (Table I), the neurons had bluish-purple nuclei and a green cytoplasm, while the astrocytes and oligodendrocytes had purplish and violet-red nuclei, respectively. Cytoplasmic staining of the glial cells was minimal and was undetectable against the pale green background.

The Feulgen-Azure A staining method (Table II) provided a clear background through which the neuronal nuclei remained a very faint violet color (from Feulgen reaction). The astrocytes and oligodendrocytes possessed distinct red and blue colors, respectively. No cytoplasmic staining was achieved with this method. Microglia represent a negligible population in the normal CNS. They are stained like the oligodendrocytes by the described methods, and can be readily recognized by their thin, rod-like shape.

Potential applications of color-differentiating stains include more accurate definition of glial cells reacting to injury (Satellitosis, gliosis, etc.), classification and differential counts of neurons, and glial cells in common anatomic malformations.

TABLE I
Feulgen Reaction Counterstained with Fast Green FCF*

Brain tissue fixed in phosphate-buffered 10% neutral formalin, embedded in paraffin and cut at 10 micra.

Staining Procedure

1. Decerate sections and hydrate to distilled water.
2. Place sections in 12.4 N HCl for 1 minute, room temperature.
3. Rinse well in distilled water.
4. Place sections in Schiff's reagent for 10 minutes, prepared in traditional manner.[1]
5. Rinse 3 times, 1 minute each, with 0.05% sodium bisulfite, then rinse well in distilled water.
6. Place sections in 0.1% Fast Green FCF, pH 6.2, for 5 minutes.
7. Dehydrate in 95% alcohol (quick dip), absolute alcohol, clear in xylene and mount with a synthetic resin.

*Fast Green FCF: Allied Chemical, C.I. No. 42053, total dye content: 93%

TABLE II
Feulgen Reaction Counterstained with Azure A*

Brain tissue fixed in phosphate-buffered 10% neutral formalin, embedded in paraffin and cut at 10 micra.

Staining Procedure

1. Decerate sections and hydrate to distilled water.
2. Place sections in 12.4 N HCl for 1 minute at room temperature.
3. Rinse well in distilled water.
4. Place sections in Schiff's reagent for 10 minutes, prepared in traditional manner.[1]
5. Rinse 3 times, 1 minute each, with 0.05% sodium bisulfite, then rinse well in distilled water.
6. Place sections in 0.1% Azure A, pH 4.0, for 1 minute.
7. Rinse in distilled water, dehydrate in 95% alcohol, absolute alcohol, clear in xylene and mount with a synthetic resin.

*Azure A: Allied Chemical, C.I. No. 52005, total dye content: 89%

References

1. Lillie, R.D.: Histopathologic Technic and Practical Histochemistry. New York, McGraw-Hill Book Co., 605-625, 1957.
2. Thompson, S.W.: Selected Histochemical and Histopathological Methods. Charles C. Thomas, Springfield, Illinois, 773-780, 1966.
3. Feulgen, R., and Rossenbeck, H.: Mikroskopischchemischer Nachweis einer Nucleinsaure von Typus der Thymonucleinsaure und die darauf beruhende elektive Farbung von Zelkernen in mikroskopischen Preparaten, Ztschr. f. physio. Chem., 135: 203-248, 1924.

4. Itikawa, O. and Ogura, Y.: The Feulgen Reaction after Hydrolysis at Room Temperature. *Stain Technol.*, **29**: 13-16, 1954.

Improved Mucin Staining

An Editorial

The demonstration of mucin with Mayer's or Southgate's methods can be improved tinctorially by the following suggestions:

1. Decolorize the hematoxylin in 1% acid alcohol until the cytoplasm is completely colorless and only the nuclear elements are distinct.
2. Ensure that metanil yellow is not applied heavily.

Failure to remove excessive hematoxylin from sections will result in incomplete staining of mucin. A fine microscopic sheen of hematoxylin will remain over the mucin. The sheen prevents the carmine solution from staining mucin completely. Excessive metanil yellow will also overshadow mucin deposits. The combination of these two factors produces sections that look thick and have a brown appearance when viewed macroscopically and microscopically. A good mucicarmine preparation will demonstrate clear crisp nuclei, yellow (not light brown) cytoplasm, and bright reddish-pink mucin.

Urea Polymers

An Editorial

Urea polymers are formed in tissue, which has been placed in a Zenker-type fixative containing urea, and subsequently immersed in formalin for storage. Urea is added to a fixative to prevent hemolysis. Microscopically the polymer is a round, clear-to-gray crystalline structure in hematoxylin and eosin preparations. It can be differentiated from the Zenker crystal since it does not react (turn black) by exposure to 20% ammonium water. The polymer polarizes easily. Zenker crystals do not polarize after the ammonia water treatment. In addition, Zenker crystals appear to be slightly above the tissue surface while the urea crystalline polymer is embedded in the tissue and appears as a flat structure.

Time-Saving Idea for Special Stains

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The task of consolidating the different ingredients for numerous special stains was always dreaded. Although the staff attempted to maintain the solutions in a central area, it often became impossible. Invariably, Weigert's iron hematoxylin would be missing from the Trichrome area and used for another special stain. The periodic acid would be removed from the refrigerator and stored with silver solutions. Valuable time was spent searching for reagents and solutions.

The following procedure was devised for ending the chaos and confusion. A dozen 10" X 13" trays, similar to those used by the Food Service of many hospitals, were labeled with the names of the procedures performed most frequently. All the working solutions (stock solutions, if fresh working solutions are required) are placed on the same tray. The procedure, typed on a 5" X 8" card, is located in the center of the tray.

For example, the acid-fast tray contains a bottle of carbolfuchsin, a bottle of peanut oil, one bottle of 1% acid alcohol, a coplin jar of working methylene blue, one empty coplin jar, a funnel, and three coplin jars filled with peanut oil and numbered 1, 2, and 3. Two 5" X 8" cards provide instructions for the Ziehl-Neelsen and Fite-Faraco methods.

The periodic acid-Schiff tray is stored in the refrigerator, but all other trays are stored in cabinets, well labeled, with the instruction card in place.

Effects of Unbuffered Formalin on Staining Reactions

An Editorial

It must be recognized that staining reactions and tissue elements are altered and others obliterated by the deleterious and insidious effects of unbuffered formalin fixative. Formalin is oxidized to formic acid, and the following ruinous effects are produced: (a) The so-called formalin pigment will frequently react during the staining procedure to mask or, in some cases, simulate microorganisms, pigments, or other elements suggesting disease. Formalin pigment has a notorious reputation for reducing silver in procedures for staining fungi (Grocott's methenamine-silver), melanin (Fontana-Masson), spirochetes (Warthin-Starry), and reticulum procedures. (b) Unbuffered formalin gradually destroys nuclear basophilia if tissue is exposed longer than three weeks. (c) Unbuffered formalin dissolves copper, iron, and calcium during the process of fixation. (d) It alters the staining of some cell granules. (e) It will also inhibit the aldehyde-fuchsin reaction for demonstrating Paget's cells. These are only a few of many examples but should be convincing evidence that unbuffered formalin should not be used.

Neutralized formalin (not to be confused with buffered formalin) creates a false sense of security. When tissue is placed in a neutralized fixative, the pH is lowered rapidly from the formation of formic acid, and some of the irreversible alterations mentioned occur.

Unbuffered or neutralized formalins should not be used for routine fixation and storage of tissue. The number and magnitude of alterations cannot be predicted or estimated.

Histopathological Technique Symposium

October 1-5, 1973, are the dates established for the "Ninth Annual Symposium on Histopathologic Techniques." Arrangements are presently being made to include several facets of histologic technique not previously presented. This year's program, as in the past, will include workshops all day Monday and Tuesday, and Wednesday evening, consisting of basic and advanced cryotomy, to include enzyme histochemistry, microtomy, special stains, interpersonnel communications, management for the histology supervisor, etc.

The scientific sessions will consist of two and a half days of lectures covering many facets of histology. Special effort is being made to provide lectures dealing with practical, useful information. Friday afternoon will be a unique experience for all. This period, of approximately two hours, will be called "Two Minute Histoquickies," and will consist of numerous presentations by registrants desiring to share their histological technique "nuggets" with their fellow histotechnologists.

Plans are to have the manufacturers and/or their representatives exhibit again as they have for the last several years. In addition, plans are being made to have an outstanding speaker for the banquet on Thursday evening.

The Symposium will be held at the Sheraton and Holiday Inn, Silver Spring, Maryland. This promises to be the best Symposium ever, so make your plans early. The Symposium program will appear in the July issue of HISTO-LOGIC.

PLEASE NOTE: Future inquiries regarding this Symposium should be made to Roberta Mosedale, Registrar, P. O. Box 36, Lanham, Maryland 20801.

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Editor's Corner

Southwestern Pennsylvania Seminar

The Southwestern Pennsylvania Histology Society is presenting a seminar on May 23, 24, and 25. For information contact: Dolores Van Halt, 7268 Travella Boulevard, Pittsburgh, Pennsylvania 15235.

St. Louis Conference

The St. Louis Society of Histology Technicians is presenting their first annual conference on Saturday, May 12, 1973, from 9:00 a.m. until 5:00 p.m. The conference will be held in the Excelsior Miramar Room of the Holiday Inn Midtown, 4483 Lindell Boulevard, St. Louis, Missouri. For information contact: Ms. Chris Clark, 4643 Carrie Avenue, St. Louis, Missouri 63115.

ERRATUM

In HISTO-LOGIC, Vol. III, No. 1, January, 1973, "Further Information on Eosin Preparation," the name of a famous pathologist was misspelled: Dr. Arthur Perdu Stough should have read Dr. Arthur Purdy Stout. Our apologies!

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.

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