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Chloral Hydrate as a Reducer in Silver Impregnation Methods

for the Demonstration of Axons and Neurofibrils

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Bielschowsky type silver impregnation methods are difficult to perform, and there is some difficulty in consistently obtaining good or even adequate results. Bielschowsky's axons and neurofibrils method is cited as being one of the most reliable of the ammoniacal silver impregnation methods. Nonetheless some published procedures carry the following note: "It is common practice with this and other metallic impregnation methods to take several sections from each block. One of these is likely to show rather superior impregnation."

Marsland, *et al.* developed a method for demonstration of axons in formalin fixed paraffin sections of the brain and spinal cord.[1] This method uses excessive silver nitrate (30 mls of 20% silver nitrate) to prepare the ammoniacal silver solution, and reports of poor staining results as well as difficulty in performing the technique are not uncommon.

A search reveals that all other methods follow the same general path; *i.e.*, an initial silver nitrate impregnation, followed by a reducer (developer). Variations do exist; *i.e.*, some techniques require the impregnation and development steps to be repeated in order to reinforce impregnation of fibers and/or cell processes.

Most silver impregnation methods employ formalin or hydroquinone as a reducer. Attempts to find a more reproducible procedure to demonstrate axons and neurofibrils resulted in the following method, presented here in the hope that it will be as useful to your laboratory as it has been to ours.

Fixation: 10% buffered neutral formalin

Microtomy: Paraffin sections

Solutions

10% Silver Nitrate Solution

Silver nitrate	10 g
Distilled water	100 ml

3% Sodium Hydroxide Solution

Sodium hydroxide	3 g
Distilled water	100 ml

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INSIDE THIS ISSUE

Rapid Dieterle Stain with Microwave Heating

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Brinn[1] has successfully utilized microwave heating to drastically reduce the staining times of several metallurgical staining techniques; *i.e.*, Grocott's methenamine silver nitrate, periodic acid methenamine silver, Pascual's modification of the Grimelius argyrophil technique, and the Fontana-Masson argentaffin method. A rapid microwave modification of the Dieterle method was in progress, but not sufficiently reproducible at that time for detailed presentation. The application of heat at 60°C shortened Dieterle's procedure from over 5 hours to 30 minutes.[2]

Dieterle stain is useful in demonstrating spirochetes in syphilis and Lyme arthritis, bacteria in Legionella pneumophilia and in cat scratch lymphadenopathy, Donovan bodies, and melanin. To demonstrate these entities in the past, our laboratory successfully applied a lengthy modification of the Dieterle method.[3]

Utilizing a modification of the Dieterle technique, accelerated by microwave heating, we have reproducibly demonstrated spirochetes in paraffin tissue sections using only 7 minutes of staining time.

The spirochete stain was free of nonspecific silver deposition and compared favorably with the original long procedure, as well as the Warthin-Starry technique.

Fixation: 10% neutral buffered formalin

Microtomy: Section tissue at 5-6 microns.

Equipment

1. Microwave oven. This laboratory uses a Samsung RE 515 D which brings water to a boil in 65 seconds.
2. Plastic Coplin jar with cover.

Solutions

5% Uranyl Nitrate Solution

Uranyl nitrate	5 g
Ethyl alcohol, absolute	100 ml

10% Gum Mastic Solution

Gum mastic	10 g
Ethyl alcohol, absolute	100 ml

Allow gum mastic to dissolve in the alcohol for 2 days and filter before use.

1% Silver Nitrate Solution

Silver nitrate	1 g
Distilled water	100 ml

0.25% Sodium Sulfite Solution

Sodium sulfite	0.25 g
Distilled water	100 ml

Developing Solution

Hydroquinone	1.5 g
Sodium sulfite (0.25%)	60 ml
Distilled water	60 ml
Acetone	10 ml
Formaldehyde, concentrated, 37-40%	10 ml
Pyridine	10 ml

Note: Mix ingredients in above order, gently swirling after each addition. The addition of gum mastic turns the solution milky yellow. When the solution is standing in a well lit area, it becomes brown-streaked and should be gently swirled as the streaks appear. The developer is ready for use in about six hours. Store solution at 0-4°C in brown, stoppered bottle. Discard when solution turns dark.

Staining Procedure

1. Deparaffinize slides and hydrate to distilled water.
2. Place slides in loosely covered plastic Coplin jar containing 5% alcoholic uranyl nitrate solution and heat in microwave oven for 50 seconds.
3. Immediately place slide in distilled water at room temperature for 30 seconds.
4. Rinse slides in 95% alcohol.
5. Place slides in 10% alcoholic gum mastic solution for 3 minutes. The addition of gum mastic turns the solution milky yellow.
6. Rinse slides in 95% alcohol.
7. Rinse slides in distilled water for 1 minute. Shake slides rapidly and wipe remaining water adjacent to section with gauze.
8. Place slides in loosely covered plastic Coplin jar containing 1% silver nitrate solution and heat in microwave oven for 50 seconds.
9. Immediately place slides in distilled water at room temperature until cooled.
10. Dip slides for 5-30 seconds in developer until sections are pale yellow to light tan.
11. Rinse slides in distilled water.
12. 95% Alcohol, 2 dips.
13. Acetone, 2 dips.
14. Xylene, two changes, 15 seconds each.
15. Mount coverglass with synthetic mounting medium.

Results

Spirochetes, some bacteria, melanin and Donovan bodies stain black. Background stains pale tan.

Summary

The Dieterle microwave technique is rapid, simple, reproducible and compares favorably with both the original long procedure as well as the Warthin-Starry technique. Nonspecific background silver deposition is absent or negligible.

References:

1. Brinn, N.T. (1983) Rapid Metallic Histological Staining Using the Microwave Oven, *J. Histotechnology 6*(3), 125.
2. Pickett, J.P. and Roggli, V.L. (1982) Rapid Histological Staining Procedures for Material from Immune-suppressed Patients, *Am. J. Med. Tech. 11*, 893.
3. Van Orden, A.E. and Greer, P.W. (1977) Modification of the Dieterle Spirochetes Stain, *J. Histotechnology 1*(2), 51.
4. Wear, D.J., *et al.* (1983) Cat Scratch Disease: A Bacterial Infection, *Science 221*, 1403.

A Method for the Removal of Adhered Precipitates from Sections

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In tissue processing laboratories, we often experience troublesome adhesion of precipitates or other unknown material on sections during the course of staining, as described in Histo-Logic®, Vol 8, No. 2, April 1978. This short article offers a possible solution for this type of problem.

During a staining process, sections of rat submandibular gland were stained in an old solution of Hansen's hematoxylin. Since the staining solution contained a large amount of precipitates, the sections were contaminated as shown in Figure 1. Interestingly enough, precipitates were seen only on a confined area of the tissue; in this case, on the mucous part of the gland.

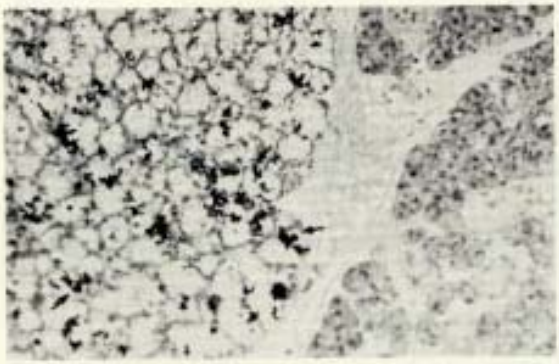

Figure 1 – Precipitate deposits on mucous part of the gland.

All attempts to remove these precipitates were ineffective, except for one procedure.

The effective method was carried out by dropping a small amount of formic acid (98-100%) on the contaminated sections and rinsing them immediately with water, after which almost all of the precipitates were removed.

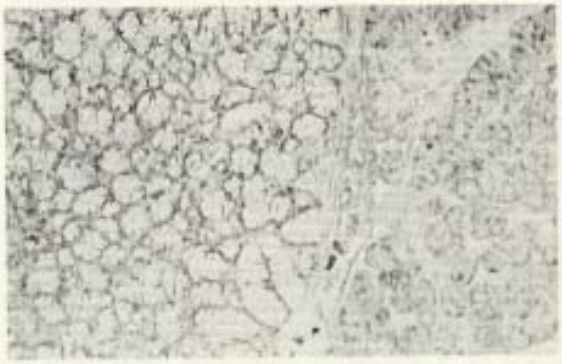

Figure 2 – Section was decolorized due to the action of the formic acid.

A considerable discoloration of hematoxylin occurred at this step, therefore sections were washed in tap water for a short period and restained.

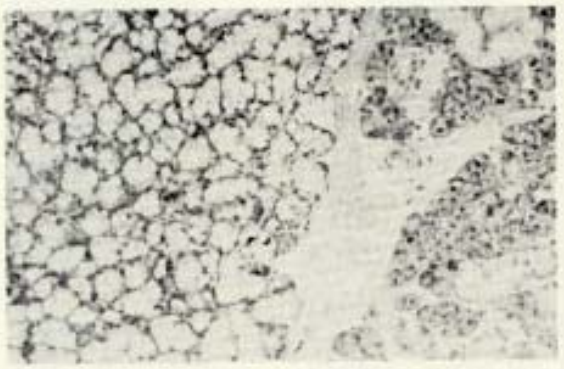

Figure 3 – Section after removal of precipitate and subsequent restaining.

Although this method is quite effective, the vigorous action of formic acid occasionally causes partial detachment of sections from slides, especially if they originally adhered incompletely. At present, I do not know how the method works or the extent of its usefulness for removal of precipitates. Absolute ethanol and other solvents have a similar but considerably weaker effect, but they failed to remove precipitates such as those shown herein.

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64-701-I	ACTH (1-24)—H	rb/ly	0.25 ml
64-702-I	Bombesin—H	rb/ly	0.25 ml
64-703-I	Calcitonin—H	rb/ly	0.25 ml
64-704-I	β Endorphin—H	rb/ly	0.25 ml
64-705-I	Gastrin I—H	rb/ly	0.25 ml
64-706-I	Glucagon—H	rb/ly	0.25 ml
64-707-I	Growth Hormone (Sheep)	rb/ly	0.25 ml
64-708-I	α MSH—H	rb/ly	0.25 ml
64-709-I	Neurophysin II (Porcine)	rb/ly	0.25 ml
64-710-I	Neurotensin—H	rb/ly	0.25 ml
64-711-I	Pancreatic Polypeptide—H	rb/ly	0.25 ml
64-712-I	Prolactin (Sheep)	rb/ly	0.25 ml
64-714-I	Somatostatin—H	rb/ly	0.25 ml
64-715-I	Substance P—H	rb/ly	0.25 ml
64-716-I	TSH (Sheep)	rb/ly	0.25 ml
64-717-I	Vasopressin—Thy	rb/ly	0.25 ml

Thy – Thyroglobulin
H – Hemocyanin

	Typical Titers*	
	PAP	Indirect FITC
ACTH (1-24)—H	1:2000	1:20
Bombesin—H	1:500	1:20
Calcitonin—H	1:2000	1:20
β Endorphin—H	1:2000	1:20
Gastrin I—H	1:500	1:20
Glucagon—H	1:500	1:20
Growth Hormone (Sheep)	1:2000	1:20
α MSH—H	1:1500	1:20
Neurophysin II (Porcine)	1:5000	1:20
Neurotensin—H	1:500	1:20
Pancreatic Polypeptide—H	1:1000	1:40
Prolactin (Sheep)	1:2000	1:20
Somatostatin—H	1:2000	1:20
Substance P—H	1:1000	1:20
TSH (Sheep)	1:2000	1:20
Vasopressin—Thy	1:2000	1:20

*Incubation – 30 minutes at 37°C. Titers may be lower for shorter or room temperature incubations.

Nomenclature

We have adopted several forms and abbreviations. Assume all antisera to immunoglobulins are heavy chain specific unless specified to have (H+L) heavy and light chain activity. (e.g., Anti-Mouse IgM should be understood to be heavy chain specific).

The following are abbreviations commonly appearing on these pages:

ab - antibody
ag - antigen
Affin. - Affinity Purified
Agarose - Agarose Bound
Aggl. - Agglutinating
AP - Alkaline Phosphatase
Conj - Conjugate
Ferr. - Ferritin
FITC - Fluorescein
Frac. - IgG Fraction
Monoform® - Monoclonal
HRP - Peroxidase
RITC - Rhodamine
PBS - phosphate buffered saline
SDS - sodium dodecyl sulfate
PAGE - polyacrylamide gel electrophoresis

lq - liquid
ly - lyophilized
dg - dog
dk - donkey
gp - guinea pig
ft - goat
mo - mouse
rb - rabbit
rt - rat
sh - sheep

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	Typical Titers*	
	PAP	Indirect FITC
Actin	1:50	1:10
Desmin	1:50	1:10
Keratin	1:50	1:10
Myosin	1:50	1:10
Tubulin	1:50	1:10
Also:		
Human Chorionic Gonadotropin (α+β)	1:200	ND
Human Fibronectin	1:200-1:500	1:20
Human Myoglobin	1:200	ND
Human Prostatic Acid Phosphatase	1:200-1:500	1:20-1:50
Porcine Insulin	1:200	ND

*Incubation – 30 minutes at room temperature.
ND – Not Done

Code No.	Antisera to:	Host Form	Size
65-096-1	Actin	rb/lq	1 ml
65-793-1	Desmin	rb/lq	1 ml
65-792-A	Keratin	gp/lq	1 ml
65-791-A	Myosin	rb/lq	1 ml
65-095-1	Tubulin	rb/lq	1 ml
Also Available:			
65-073-1	Human Chorionic Gonadotropin (α+β)	rb/ly	1 ml
64-078-1	Human Fibronectin**	sh/ly	1 ml
65-075-1	Human Myoglobin	rb/ly	2 ml
65-304-1	Human Prostatic Acid Phosphatase	rb/lq	1 ml
65-104-1	Porcine Insulin**	gp/ly	1 ml

** – Broad species cross-reactivity.

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Ammoniacal Silver Solution

To 5 ml of 10% silver nitrate add just enough ammonium hydroxide, drop-by-drop, to redissolve the precipitate which forms. Add 5 ml of the 3% sodium hydroxide solution and with a few drops of ammonium hydroxide redissolve any silver precipitate which may form.

5% Chloral Hydrate Solution

Chloral hydrate	5 g
Distilled water	100 ml

5% Sodium Thiosulfate Solution

Sodium thiosulfate	5 g
Distilled water	100 ml

0.02% Gold Chloride Solution

Gold chloride	0.02 g
Distilled water	100 ml

5% Oxalic Acid Solution

Oxalic acid	5 g
Distilled water	100 ml

Aniline Blue Solution

Aniline blue	0.1 g
Oxalic acid	5 g
Distilled water	500 ml

Staining Procedure

1. Deparaffinize slides to the last absolute alcohol.
2. From this solution, celloidinize slides and harden in 70% alcohol.
   Note: To produce even staining across the entire slide, a smooth even coat of celloidin is essential. To do this, dip slide in celloidin, drain to one corner and air dry for 4-5 seconds before immersing in 70% alcohol.
3. Place slides in 10% silver nitrate solution at room temperature for 1 hour.
   Note: The initial 1 hour in 10% silver nitrate is necessary to successfully stain neurofibrils. If only axons are required, the time can be shortened to 10 minutes. Overnight staining in a less than 10% solution of silver nitrate has not been tried and may result in less background staining. Background staining produced by this method is not excessive, although it would be advantageous to eliminate it.
4. Wash slides well in distilled water.
5. Place slides in ammoniacal silver solution for 2 minutes.
6. Wash slides well in distilled water.
7. Place slides in 5% chloral hydrate solution for 1 minute.
8. Wash slides well in distilled water.
9. Place slides in second change of ammoniacal silver solution for 2 minutes.
10. Wash off ammoniacal silver with 5% chloral hydrate and leave 1 minute.
    Note: Pour enough 5% chloral hydrate on the slide so that it approximately equals the amount of ammoniacal silver on the slide. This solution turns black. Leave for approximately 15 seconds and completely wash off with chloral hydrate.
11. Place slides in 5% sodium thiosulfate solution for 1 minute.
    Note: This technique may have an additional role. While the sodium thiosulfate is needed to remove unreduced silver, it also produces a blackening of the celloidin. However, it is difficult to observe whether or not this blackening contributes to the staining of the axons and neurofibrils.
12. Wash slides well in distilled water and remove celloidin.
13. Place slides in 0.02% gold choride solution for at least 10 minutes.
    Optional: If only axon staining is required.
    Note: Without the toning step, black staining axons on a light brown to brown background are produced. However, to properly delineate neurofibrils, toning is necessary. Stronger gold chloride solutions have a deleterious effect on the clarity of axon and neurofibril staining.
14. Rinse slides with distilled water.
15. Optional: 5% oxalic acid for about 15 minutes. This step reddens the background and produces an intensifying effect on the stained fibers.
16. Optional: Pour off oxalic acid and apply aniline blue solution for 5 minutes, then quickly rinse in tap water. This step is necessary if further staining of the background is required.
17. Dehydrate in alcohol, 95%, absolute, and clear in xylene, three changes each.
18. Mount coverglass with resinous media.

Results

Figure 1

continued on page 238

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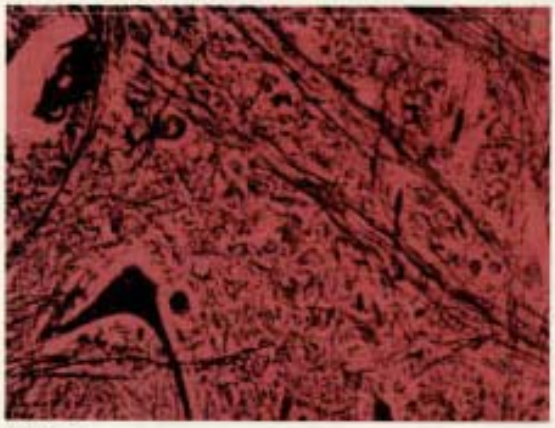

Figure 2

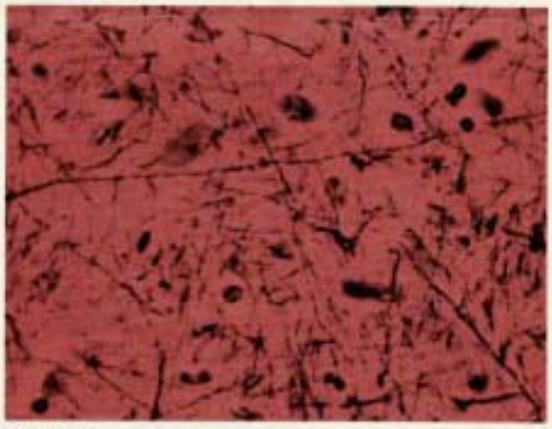

Figure 3

Figures 1, 2 and 3 are representative of the results possible with this method. Note the extensive demonstration of fine, delicate neurofibrils in figure 1 and 3.

Axons and neurofibrils — black
Some connective tissue and elastica — black
Background — reddish or grey

This method produces clean, crisp slides of a nerve fiber stain in a reasonable period of time. The method is consistently reproducible with economical use of silver. We trust this method will be welcomed as a replacement for the procedures you now use.

Reference:

1. Marsland, T.A., *et al.* (1954) Modification of the Glees Silver Impregnation for Paraffin Sections. *J. Neuropathology and Experimental Neurology 13*, 587.

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