A TECHNIQUE OF SILVER IMPREGNATION FOR GENERAL LABORATORY PURPOSES *

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Anyone who has used silver impregnations over a period of time will have been struck with the possibility of applying them to the demonstration of histological elements other than fibers, and will have been tempted to devise some method that would, at one and the same time, bring out these structures as well as the fibrous elements in a given section. There seems to be no reason why a silver impregnation should not be arranged to fit the purposes of routine tissue examination in the pathological laboratory, a method that would be an improvement over the usual routine stains, inasmuch as it would demonstrate a variety of tissue elements selectively without rendering the use of several stains on several sections necessary.

The following method was designed primarily to demonstrate the finer fibrils in tumors of the melanoma group which elude silver impregnation when the usual methods are employed. They could be shown in frozen sections, but only partially or unsatisfactorily brought out in paraffin material. We therefore experimented with a series of some thirty-five different modes of procedure and discovered that it is possible to obtain even better results in paraffin than in frozen sections. While doing this, we were struck with the general applicability of the method to the demonstration of other tissue components as well. It was found that the preliminary bleach with potassium permanganate and oxalic acid, used in prevailing methods for impregnating reticulum and endoneurium, was the stumbling block that had obstructed successful impregnation of the finer fibrils of our tumors. Further experimentation demonstrated that a preliminary treatment with pyridin and glycerol, in place of the bleach, was practically essential for the attainment of satisfactory results and, in subsequent work, the use of a reducing agent following the gold toning bath (as suggested by Laidlaw's work[1,2])

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was found to be of exceptional value in sharpening the details of the impregnation and converting it essentially into a double impregnation. There are six variants of our procedure chosen from our experimental series of thirty-six variants. The reader need not be alarmed at this large number of variants: it is intended that they shall be used to fit the case in question and ample indication will be afforded for the choice of the proper one, with a tabular view of the results obtainable with each. Unless the best one for a particular purpose be chosen, the results will not be optimal, although any one of the six will give pictures superior to those obtained through methods heretofore used for demonstrating the finest fibrils of the connective tissue. The method is simple, counterstaining is entirely eliminated, and every detail of a given tissue may be brought out sharply.

Technique

Fixation: The finest results obtained were seen in sections made from material fixed in formalin and kept as museum specimens in Kaiserling III for nearly ten years. This fixation, however, is scarcely to be considered practical. The next best fixative is neutral 10 per cent formalin, in which blocks cut thin enough to ensure complete penetration of the fluid should remain for 24 hours at least, longer if possible. If Bouin's fluid is used, the results are comparable to those obtained in the Laidlaw-Bouin method; the nuclei will be unimpregnated, the cytoplasm impregnated in the case of epithelial cells, and mesoblastic cells will be unstained. The resulting pictures are more colorful than those obtained by the Laidlaw procedure.

The method gives very good results if Zenker-fixed tissues are used. They should be fixed for 24 hours, washed in running water for another 24 hours and, after embedding and sectioning, the mercuric chlorid should be removed from the sections with the usual alcoholic iodin solution, and this in turn removed with very weak (1 per cent or less) aqueous sodium thiosulphate. This must then be washed out thoroughly. The oxidation-reduction steps, in which potassium permanganate and oxalic acid are used, *should be omitted* as they produce effects similar to the Bouin fixation. The presence of chromium salts makes no material difference in the subsequent impregnation, except to enhance the impregnation of nervous tissue. On the whole,

formalin fixation gives more colorful results and is, on this account, to be preferred. This does not, however, imply that Zenker fixation is to be eschewed — quite the contrary; it gives very striking pictures in all instances and is well suited to the method.

Embedding: The ordinary routine method of paraffin embedding is used after dehydration of the tissue in ascending percentages of alcohol and in chloroform.

Preliminary Treatment: This is essential in the case of all the variants. The sections are deparaffinized in 2 changes of xylol and absolute alcohol and are then treated from 1 to 24 hours with a mixture of 2 parts pure pyridin to 1 part of pure glycerol. This bath keeps well and may be used repeatedly for many weeks. The sections are transferred directly from this to 2 changes of 95 per cent alcohol, washed in tap water and placed in distilled water.

Impregnating Fluid: This is a simple silver diammino hydroxid solution, depending upon the Kubie and Davidson formula.[3] It is used in all the variants, at full concentration in the first three, at half strength in the last three. To 10 cc. of 10.2 per cent silver nitrate solution in distilled water, strong ammonia is added dropwise until the resulting brown precipitate is just dissolved; 10 cc. of 3.2 per cent pure sodium hydroxid solution in distilled water is added and the reprecipitated silver hydroxid again just dissolved by the addition of a few more drops of ammonia. The solution is then made up to 100 cc. with distilled water that has been heated to about 50°C. Sections are impregnated in this in a closed staining box in the incubator at 37° C, or the paraffin oven at 55° C for 1 hour in the case of Variants 1, 2 and 3, and for 10 minutes in the half-strength solution (5 cc. silver nitrate, 5 cc. sodium hydroxid) in that of the other three variants.

Silver diammino carbonate may be used interchangeably with, and in the place of the hydroxid; it often gives superior results, particularly in those variants in which the tannate mordant is used. It is made up at full strength in all cases; 10 cc. of 10.2 per cent silver nitrate, strong ammonia drop by drop until the precipitate is dissolved, and 10 cc. of 3.1 per cent sodium carbonate in distilled water, instead of the hydroxid. There is no reprecipitation upon adding the carbonate, as the hydrogen ion concentration remains unchanged, and further ammonia is therefore unnecessary. The solution is used in exactly the same manner as the hydroxid.

Reducing Fluid: The developer is a mixture of strong neutral formalin (40 per cent formaldehyd) 1 cc., 1 per cent sodium carbonate in distilled water 3 cc., and distilled water to make 100 cc. Three minutes completes reduction.

Toning and Fixing: The toning bath is a 1:500 solution of Merck's "acid brown" gold chlorid in distilled water. The fixing fluid is the usual 5 per cent sodium thiosulphate ("hypo").

Variant 1

The sections are taken from distilled water, impregnated for 1 hour in the impregnating fluid, washed in 2 changes of distilled water and reduced in the developer for 3 minutes or so. They are then washed in tap water and toned for 3 or more minutes in the gold bath, washed, and fixed in the hypo for 3 or more minutes, after which they are washed and mounted in Canada balsam, after dehydrating in ascending percentages of alcohol and xylol.

Variant 2

This is similar to the preceding formula, except that the Laidlaw oxalic acid (5 per cent) bath is intercalated between the toning and fixing baths, and the fact that toning, redevelopment and fixing are all lengthened to 10 minutes each, to correspond with Laidlaw's directions.

Variant 3 *

In this variant formalin-soda replaces the oxalic acid procedure of its predecessor. It is made up exactly as before (formalin 1 cc., 1 per cent sodium carbonate 3 cc., distilled water to 100 cc.). Used developer should not be employed; it should be made up freshly each time. The treatment with the gold, formalin and hypo is the same as in Variant 2.

Variant 4

In the following three variants a tannic acid mordant is used made up as follows: pure tannic acid 0.2 gm., ammonium bromid 3.5 gm., strong neutral formalin 5 cc., distilled water to make 500 cc.

The sections are mordanted for 15 minutes in the tannic acid bath

* Instead of the soda-formalin solution a solution of 0.5 per cent oxalic acid in 5 per cent neutral formalin has been found to give better results and avoids the danger of precipitates. This was ascertained since the paper was submitted for publication.

TABLE I

Color Variations in Tissues Stained for Silver by 6 Variants

Tissue	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5	Variant 6
Nuclei	Brown	Magenta, slightly brownish	Dull magenta-brown	Brown or black	Black	Sharp brown, reddish or black
Epidermal cytoplasm	Slate brown to brown	Slate violet to magenta	Rose slate, brownish to magenta	Slate brown to fuscous	Violet to violet-brown	Slate blue to slate brown
Glandular cytoplasm	Slate brown	Slate brown to magenta	Magenta-gray	Pinkish gray to brown	Pinkish gray to violet	Violet brown
Erythrocytes	Brown	Magenta	Dark brown to black	Reddish brown	Violet-brown	Brown to seal brown
Collagenous fibers	Lilac to light magenta	Deep magenta to violet	Dull magenta	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Reticular fibers	Black	Black	Dark magenta to black	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Endoneurial fibers Meissner's nevus cells	Red to black	Magenta to black	Magenta to violet or black	Red to black	Magenta to black, finest often carmine	Brick red
Skeletal muscle fibers	Slate brown striæ black	Magenta to dark red, striæ red to brown	Slate pink, striæ red to brown	Pinkish brown striæ black	Violet, striæ deep magenta	Violet to black, striæ indistinct, too intense
Cardiac muscle fibers	Gray	Magenta-gray	Slate pink	Gray, striæ blackish	Violet, striæ magenta	Violet, striæ magenta
Smooth muscle fibers	Gray	Magenta	Rose-gray	Pinkish to brownish gray	Violet	Slate violet
Myelin sheaths	Black	Black	Black	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Nerve trunks	Pink to red	Magenta	Magenta	Brownish pink, epineurium darker	Magenta, epineurium darker	Brick red, epineurium grayish
Melanin	Black	Blue-black	Black	Black	Blue-black	Black

heated to 50° C in the incubator or paraffin oven. They are then treated for ½ to 1 minute with 100 cc. of distilled water to which has been added 3 to 5 drops of strong ammonia. This is the "stop" solution. They are then washed for about 2 minutes in distilled water. The impregnation with silver is complete at the end of 15 minutes instead of 1 hour, as in the preceding variants. After impregnation the sections are washed in distilled water, developed, toned and fixed as in Variant 1.

Variant 5

Proceeding as in the preceding variant, the method changes as soon as the toning bath is reached, to correspond with Variant 2, lengthening the time to 10 minutes and using the 5 per cent oxalic acid-gold developer in exactly the same manner.

Variant 6

This resembles Variant 5 in every particular save one, formalin-soda developer replaces the oxalic acid bath, as in Variant 3.

The formalin-oxalic acid intensifier may be used here, as in Variant 3.

SUMMARY OF STEPS IN THE VARIANTS

1. Neutral formalin or Zenker fixation.
2. Paraffin embedding.
3. Pyridin-glycerol pretreatment for 1 to 24 hours.
4. In Variants 4, 5 and 6; tannic acid mordant for 15 minutes, followed by "stop" solution of ammonia for 30 seconds.
5. (*a*) Variants 1, 2 and 3; impregnation in warm silver diammino hydroxid for 1 hour.

(*b*) Variants 4, 5 and 6; impregnation in this bath at half-strength for 10 minutes.

6. Reduction of silver in formalin-soda developer for 3 minutes.
7. Toning in 1:500 gold chlorid in Variants 1 and 4 for 3 minutes; other variants for 10 minutes.
8. Reduction of gold in Variants 2 and 5 with 5 per cent oxalic acid; Variants 3 and 6 with formalin-soda; in either case for 10 minutes.
9. Fixing in 5 per cent sodium thiosulphate in Variants 1 and 4 for 3 minutes; other variants for 10 minutes.

Note: Thorough washes are indicated between all steps, distilled water being required until the sections have been reduced in Step 6; after that tap water is employed throughout.

Discussion of Results Obtained

After studying many sections from the experimental series used in our work we ran through a set of eleven sections in each variant, the material being taken from ten different organs (heart, two sections of lung, thymus, spleen, liver, kidney, suprarenal, uterus, lymph node and brain) from an autopsy performed almost immediately post-mortem. The color effects of the variants were then tabulated in the appended table. The sixty-six slides of the autopsy series were all impregnated at the same time and therefore represent a standard result.

As will be seen, the intensity of detail progresses through the series up to the fifth variant where it is most marked, and falls off a trifle at the sixth. The use of oxalic acid after toning the sections in gold chlorid develops the partially reduced gold salts that have replaced the silver and thus, by further reduction, "doubles" the impregnation: as a result one sees intensified and predominant magentas and violets, which are "gold colors." The use of a stronger reducer (formalin-soda) changes the picture from a prevalent magenta to a brick red, enhances the nuclear detail, impregnates the cytoplasm less densely, but does not produce as precise a fiber impregnation as does the weaker reducing agent (oxalic acid). This is probably explained empirically by the well known proclivity oxalic acid possesses for reducing gold salts.

It will be noted that the nuclei are listed as being either brown or black in those variants using the tannic acid mordant; there is no transition, they are either the one or the other. This phenomenon doubtless has its significance, occurring as it does in nuclei of the same cell race and apparently similar properties, but just what this may be we do not know. At first it was thought to be an artefact, but this peculiarity has been regularly noted in tannate sections. By referring to the table one may readily gauge the relative merits and drawbacks of the different variants. If it is desired to bring out reticulum selectively, then one of the first three variants should be selected, for the last three are unsuitable as they impregnate collagen

and reticulum exactly alike, magenta or reddish. On the whole, the second and fifth variants will be found to be the best for general use. If the fifth is found to give too intense impregnations, the fourth or the sixth may be substituted. If one desires delicate effects with little or no disturbing cytoplasmic background, then the first variant should be the choice; or the fourth, if a little more cytoplasmic detail, color variety and plasticity are desired. Those variants depending upon the tannic acid mordant will give more colorful pictures, those omitting its use will tend to be monochromatic.

The reader is left to choose the variant that best suits his particular purposes and tastes; we can safely claim that he will find one of them that will fit his needs. Variant 2 gives ideal reticulum impregnation: it is particularly fine in the case of the "Gitterfasern," or reticulum of the liver sinusoids, the lymphoid reticulum and the sheaths of muscles. Variant 5 is particularly suited to the demonstration of muscle striations. With several variants, particularly Nos. 2 and 5, the medulla of the suprarenal is most admirably set off and demonstrated, and the Hassal's corpuscles of the thymus very well brought out because of their metachromatic impregnation.

The four figures in the plate (Figs. 1 to 4) demonstrate the various features of as many typical variants. They were made under exactly similar conditions, except for differences in the time exposure that were contingent upon the density of the impregnation. Four fields, as nearly similar to one another as possible, were chosen in four sections cut from the same block of tissue from a nevus.

It might be well to make some explanation of the different steps used in the six variants. The pyridin-glycerol treatment was originally introduced to increase the definition of the fibrillary structures in nevi, as it was known to be excellent in the case of nerve fibrils and the smaller nevus fibrils were suspected of being such. Whether they are, or not, the pyridin is found to bring them out more clearly than if it is omitted, and to keep down troublesome precipitates. The glycerol was added because it had been noted that Kaiserling III tissue impregnated more colorfully than that fixed in pure formalin. It was found that glycerol did, indeed, increase the metachromasia of the impregnation.

The silver solution is made up equimolar (0.6 molar), which explains the fractions in the "10.2" and "3.2" solutions of silver nitrate and sodium hydroxid. Sodium carbonate is added to the formalin

developer in small quantity to act as a buffer and prevent the formation of formic acid. This promotes complete reduction. Laidlaw specifies tap water in his formalin reducer, but we have used distilled water here in Cincinnati as the tap water has a high chlorin content, owing to the chlorination of the water supply. This point should be borne in mind in other cities where chlorination is the practice and where silver procedures might suffer by having Cl ions present in the water.

The tannic acid mordant is essentially that which is used in the well known Achucarró method for impregnating neuroglia. Its function is to form silver tannate in the sections, thereby shortening the length of time necessary for impregnation and increasing the brown tones in the sections, producing color variety. The ammonium bromid ensures precision and prevents precipitates. The formalin has some effect upon the subsequent impregnation, making it more intense and complete. The "stop" solution of ammonia probably removes excess formalin from the mordanted sections, thus preventing precipitates.

The gold solution replaces the silver in the section with gold. This results in a more pleasing color scheme; without it the sections would resemble Levaditi impregnations, being yellow, brown and black. The oxalic acid, used after toning, reduces the gold that has replaced the silver and intensifies the toning already partially effected in the gold chlorid bath. The use of formalin-soda in place of oxalic acid is attended by similar results, but they are in some ways inferior. The color scheme is more varied, but the details of the finer fibrillary structures are not as clear. On the other hand, it is more suited to the impregnation of the epithelium, for it brings out nuclear detail and impregnates cytoplasm less intensely.

Summary

Six variants of a relatively simple and rapid method for impregnating tissue with silver are described. Although there are a number of steps in the process, they are really very easy to carry out once one has become accustomed to the "schedule." It is not complicated by having six variants, for each of these differs so slightly from its fellows that the change from one to another is a very simple matter, involving little extra time and often materially shortening the total time required. These variants are intended to meet varying needs

and to make the impregnation suitable for general use on almost any tissue. The use of the term "variant" might have been omitted, but it was thought that this clarified the slight variations in the procedure, so we have used that expression in this paper. It is not recommended that the method be used for impregnating brain or spinal cord sections, as there is not enough contrast to make it valuable in that connection.

REFERENCES

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2. Laidlaw, G. F. Silver staining of the endoneurial fibers of the cerebrospinal nerves. *Am. J. Path.*, 1930, **6**, 435.
3. Kubie, L. S., and Davidson, D. The ammoniacal silver solutions used in neuropathology. Their staining properties, chemistry and methods of preparation. *Arch. Neurol. & Psychiat.*, 1929, **19**, 888.

DESCRIPTION OF PLATE

PLATE 38

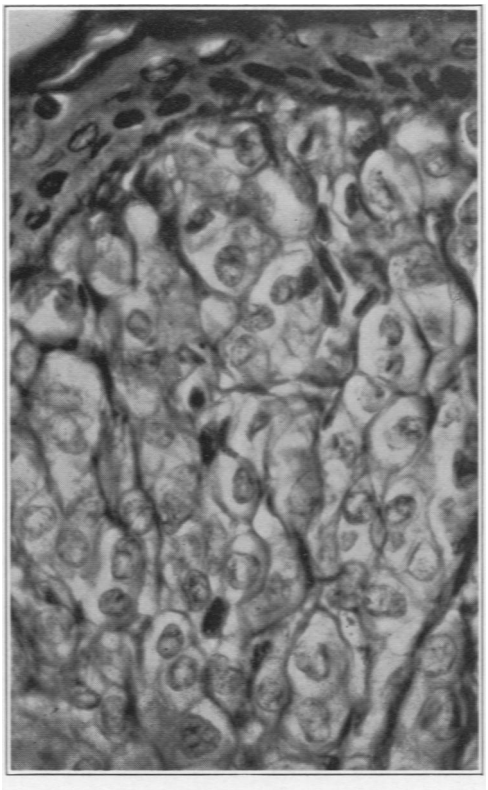
All photomicrographs were taken at about 800 diameters magnification by Mr. Joseph B. Homan of our Department of Medical Art, with the assistance of the authors.

FIG. 1. A field from a pigmented mole, or nevus, impregnated by the first variant. The nevus cells and fibrils are rather pale, the reticulum somewhat darker.

FIG. 2. Similar field impregnated by the second variant. The nevus fibrils are darker, the nuclear detail sharper.

FIG. 3. A third field impregnated by the fourth variant. Nuclei and fibrils still sharper. Note the occasional black nuclei.

FIG. 4. A field slightly deeper in the tumor, but otherwise identical with the preceding. Here the fifth variant was used. The excellent fibril and nuclear detail is at once apparent.



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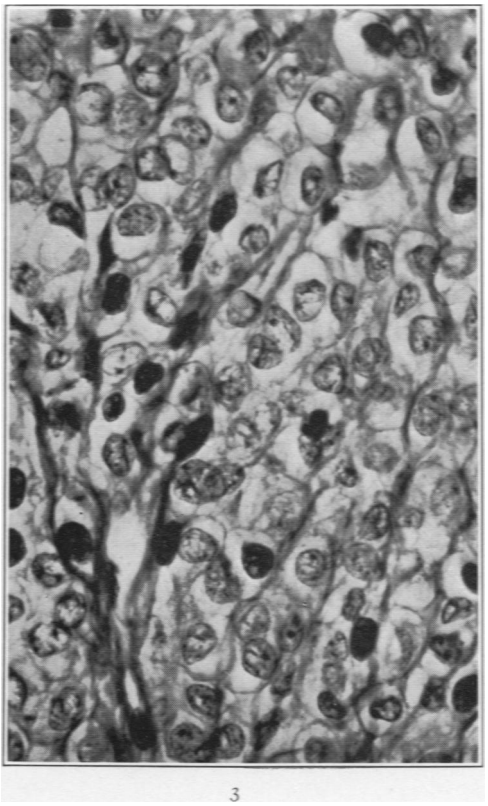

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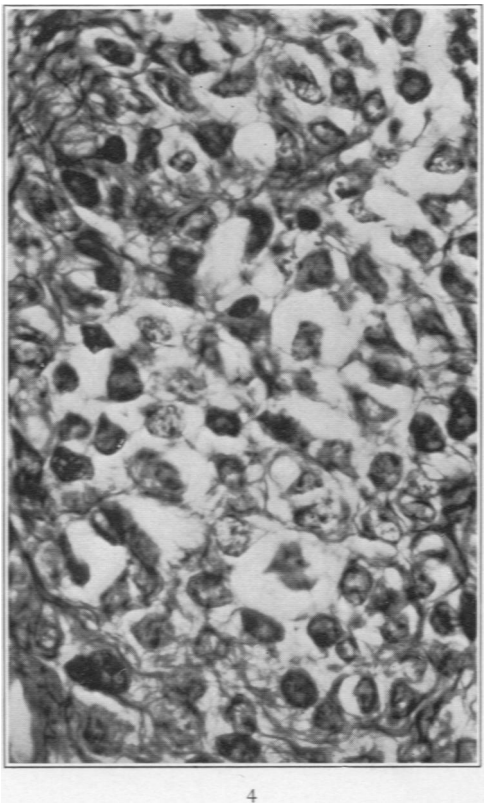

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Foot and Foot

Silver Impregnation for General Purposes