Henry Ford Hospital Medical Journal

Volume 15 | Number 1 Article 9

3-1967

A Comparison Of Light Microscopy Staining Methods Applied To A Polyester And Three Epoxy Resins

Valerie Bryant

John H. L. Watson

Henry Ford Hosp. Med. Bull.
Vol. 15, March 1967

A COMPARISON OF LIGHT MICROSCOPY STAINING METHODS APPLIED TO A POLYESTER AND THREE EPOXY RESINS

VALERIE BRYANT AND JOHN H. L. WATSON
Edsel B. Ford Institute for Medical Research
Detroit, Michigan

INTRODUCTION

Tissue sections, cut relatively thin (0.5-1.5 microns) from plastic embedment for viewing in the light microscope are valuable aids for the orientation and interpretation of ultrathin serial sections which are subsequently cut about 350 AU thick for observation with the electron microscope. Resolution in the light microscope is improved with such sections, but could be even better if these plastics were dissolved in the manner in which paraffin is dissolved in xylene. This method is ineffective with resin embedments, and other attempts to remove the resins have caused unnecessary distortion in the tissue structures. The results achieved with routine staining of the plastic embedment specimens for light microscopy are discussed here, using a polyester (Vestopal W) and three epoxy resins (Epon, Maraglas and Araldite) as examples. Each of these is widely used by electron microscopists for embedding and subsequently staining tissue specimens (Schantz and Shecter, 1965, Grimley, 1964). Particular emphasis is directed toward the manner in which the fixations (osmium tetroxide, or glutaraldehyde post-fixed with osmium tetroxide) and their buffers affect the intensity of staining for light microscopy.

MATERIAL AND METHODS

Human kidney, thyroid, bronchial mucosa and lung, taken as surgical biopsies, as well as salamander pituitary and dog liver, were used to determine the affinity for staining after the different fixation and embedment procedures.

All the tissue was cut into 1 mm pieces and fixed in either cold (0-5°C), unbuffered 3% glutaraldehyde, or in 3% glutaraldehyde buffered with either phosphate or cacodylate, post-fixed in 1% osmium buffered with either phosphate, cacodylate or s-collidine (Sabatini, Bensch and Barnett, 1963). The pH range of the fixatives was 7.0 - 7.4. Some of the tissue was also fixed in 1% osmium buffered with veronal acetate, phosphate or s-collidine at 0-5°C and at pH of 7.3 - 7.4. A 0.4M solution of sucrose

was added to all the fixatives except the cacodylate buffered glutaraldehyde. Between the glutaraldehyde fixation and the post-fixation with osmium, a 0.2M solution of sucrose was added to 0-5°C phosphate or cacodylate wash at pH 7.2.

Dehydration at room temperature followed. Tissue to be embedded in the polyester was dehydrated in various grades of acetone up to and including 100%, followed by infiltration with the acetone-diluted vestopal. The vestopal to acetone concentrations were 1:3, 1:1, and 3:1 mixtures, and the tissues were finally placed in capsuled, catalysed vestopal for polymerization at 60°C for forty-eight hours.

Dehydration and infiltration in each of the epoxy resins Araldite, Epon, using Luft's methods (1961), and in Maraglas (Lockwood, 1964) were done similarly. Dehydration was achieved by using ethanols graded from 50 to 100%. Two fifteen minute changes in propylene oxide were followed by overnight infiltration with the resin, mixed at a 1:1 proportion with propylene oxide. Polymerization in capsules at 60°C for forty-eight hours produced the hardened block for sectioning.

All sections, whether for light microscopy or electron microscopy, were cut on an LKB ultramicrotome with glass knives. The sections for light microscope observation were placed on albumen-coated glass slides, stained appropriately, coverslipped and put on a hot plate. Flotation-fluid staining (Bennett and Radimska, 1966) is also very effective. Color photomicrographs were taken with a Zeiss photomicroscope on both 35 millimeter Kodachrome and Ektachrome film.

STAINING PROCEDURES APPLICABLE TO POLYESTER RESINS

Periodic acid Schiff stain, (Coleman, 1938)

1. Immerse slide in 0 5% periodic acid for 10 minutes.
2. Wash in water.
3. Schiff's reagent for 30 to 45 minutes.
   Add 1 gram basic fuchsin to 200 ml. boiling distilled water, filter, cool and add 2 grams potassium metabisulfite and 10 mls. 1N hydrochloric acid. Let bleach for 24 hours in stoppered bottle. Add 0.5 grams activated charcoal (moist), shake for about 1 minute and filter through coarse paper. Keep refrigerated.
4. Wash in water.
5. Rinse in peracetic acid* for a few dips, or immerse for 30 seconds. Add 130 mls. hydrogen peroxide (30%) to 48 mls. glacial acetic acid. Add 1.1 ml. sulfuric acid (concentrated). Mix well, and let stand for 1 to 3 days at room temperature. Add 20 mg. dibasic sodium phosphate (hydrated) as stabilizer.
6. Wash in water for 2 minutes.

*Peracetic acid, oxidizing reagent, (Lillie, 1952).

Hemotoxylin stain, (Corrington, 1941)

7. Stain with hematoxylin for 30 to 45 minutes.
   (Absolute alcohol 32.00 mls., hematoxylin 0.67 gm., glacial acetic 3.00 mls., glycerol 32.00 mls., potassium alum 3.00 gms., distilled water 32.00 mls.)
   Heat distilled water, potassium alum, and hematoxylin together, cool, and then add alcohol, glycerol and glacial acetic acid.
   To ripen: Either let this stain stand for about 2 weeks in an unstoppered bottle, or add 20 mg. potassium iodate.
8. Wash in tap water.
9. Dip in lithium carbonate for one minute (5 drops of saturated $LiCO_3$ to 60 cc. water).
10. Rinse in water.

Counterstains orange G and eosin Y

11a. Dip in 1% eosin in alcohol for 15 to 30 minutes, *OR*

11b. Stain with acidified 2% orange G in water (add a ½ cc. of O.1N HCl for every 50 cc. of the stain to intensify).

12. Rinse in alcohol rinses (95% and 100%), clear in xylene, mount and coverslip.

STAINING PROCEDURES APPLICABLE TO EPOXY RESINS

Methylene blue azure II, (Richardson, 1960)

1. Stain with methylene blue azure II for 30 to 60 minutes. Heat flooded slide to 60°C on hot plate for 2 to 5 minutes. Mix equal volumes of 1% azure II in distilled water and 1% methylene blue in a 1% borax solution. Heat gently. Filter.
2. Wash in water.
3. Dry on hot plate.
4. Clear in xylene, mount and coverslip.

Toluidine blue, (Trump, 1961)

1. Flood the slide with freshly prepared, filtered, aqueous solution of 0.1% toluidine blue in 2.5% sodium carbonate (pH 11.1) for 30 to 120 minutes.
2. Remove excess stain with filter paper.
3. Wash in water.
4. Rinse in alcohol rinses (90% and 100%), clear in xylene, mount and coverslip.

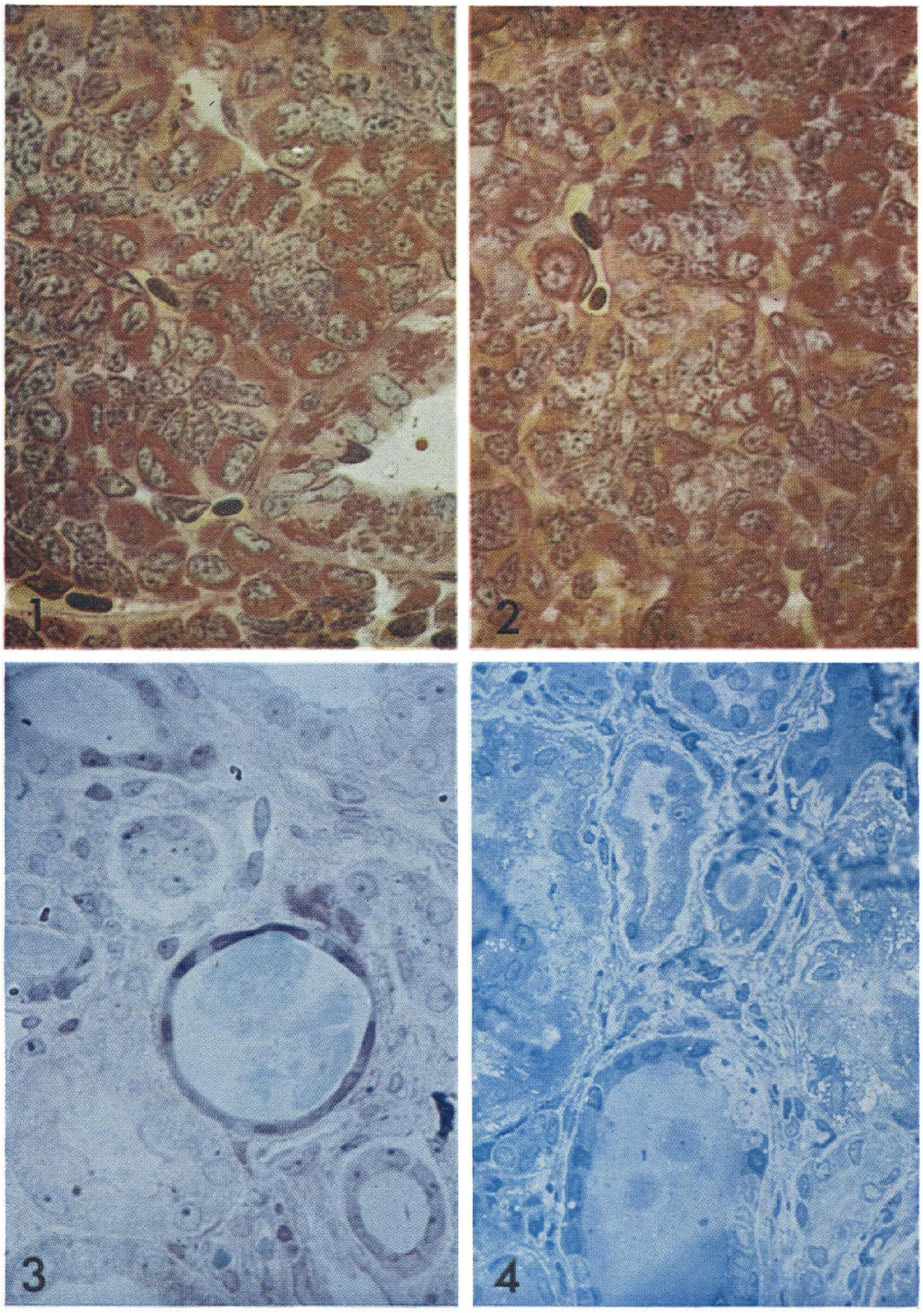

1
2
3
4

PLATE 1 Figures 1-4

COMPARISON OF THE INTENSITY OF DIFFERENT STAINING TECHNIQUES USED WITH TISSUES FIXED IN OSMIUM, BUFFERED WITH EITHER VERONAL ACETATE OR PHOSPHATE, AND EMBEDDED IN EITHER A POLYESTER OR AN EPOXY RESIN.

1. Salamander pituitary gland fixed in osmium buffered with veronal acetate, embedded in Vestopal. The nuclei are stained with hematoxylin, the basophils are PAS-positive, and the acidophils and blood cells are orange-G positive. X 1,900.

2. Salamander pituitary gland fixed in osmium buffered with phosphate, embedded in Vestopal. The staining procedure is the same as in figure 1. The staining affinity for phosphate buffer is greater, causing the stains to be more intense. X 1,900.

3. Human kidney fixed in osmium buffered with veronal acetate, embedded in Epon. The glomerulus, blood cells and cell cytoplasm are stained with methylene blue azure II. X 3,600.

4. Human kidney fixed in osmium buffered with phosphate, embedded in Epon. The staining procedure is the same as in figure 2. Methylene blue azure II appears to stain more intensely when used in combination with this buffer. X 1,900.

Discussion

When tissues in polyesters and epoxy resins are stained for light microscopy, there is a definite trend for the polyesters to be more permeable to *acid* stains, and for the epoxy resins to be more permeable to the *basic* ones. Tissue embedded in Vestopal is stained intensely with acid aqueous and alcohol solutions because the polyester resin is observed to absorb both water and the alcohol somewhat more slowly than does the tissue. Tissues embedded in Epon, Araldite or Maraglas stain well with basic aqueous stains such as methylene blue azure II, toluidine blue, and basic fuchsin (Winkelstein, J., Memefee, M. G., and Bell, A.); they stain poorly, or not at all with acid aqueous stains such as Ehrlich's hematoxylin and orange-G, and poorly, if at all with acid alcohol stains, since both the resin and the tissue absorb the dyes equally well to yield poor differentiation.

The fixation of the tissue is another factor which influences the intensity of the staining. Figures 1 and 2 compare the effects which differently buffered osmium fixatives have on routine light microscopy stains used on the salamander pituitary gland. The tissue in figure 1 has been fixed in veronal acetate buffered osmium, and the tissue in figure 2 in phosphate buffered osmium. Both are embedded in Vestopal. The nuclei are stained with Ehrlich's hematoxylin, the basophils are PAS-positive, and the acidophils and blood cells are orange-G positive. The comparison made in these two figures demonstrates the more intense staining reaction which occurs when a phosphate buffer is used in combination with osmium over that when the veronal acetate buffer is used. Figures 3 and 4 of human kidney stained with methylene blue azure II demonstrate similar staining reaction with the epoxy resins. Figure 3 demonstrates stained tissue fixed in osmium buffered with veronal acetate, while the tissue in figure 4 has been fixed in phosphate buffered osmium.

This strong staining reaction to phosphate buffered fixatives is further demonstrated in tissue that has been double fixed. Figure 5 shows a dog liver sample that has been fixed in glutaraldehyde buffered with phosphate, post-fixed with phosphate buffered osmium and embedded in Araldite. This tissue has been stained with toluidine blue, and this especially strong metachromatic stain has given the lipid droplets a green and the nuclei a blue appearance.

Thus, the kind of buffer used in combination with any fixative and in any embedment is important in staining. This is demonstrated again in figure 6, where the salamander pituitary gland has been embedded in Vestopal, fixed in unbuffered glutaraldehyde without post-osmication and stained with PAS. hematoxylin and orange-G. Here, the cell components appear intensely stained, but the cytoplasm of the acidophils and the limiting cell membranes are not distinct with this preparation.

Comparison of tissue sections shows that image contrast is higher in the polyester than in the epoxy embedments. The stains demonstrated in sections of polyester embedment usually show better differentiation, which results in a "crisper" appearance

than in epoxy resins. This is evident when one compares the Vestopal embedded tissue in figures 1 and 2 with the Epon embedded tissue in figures 3 and 4. Tissues in both types of embedments have been fixed comparably.

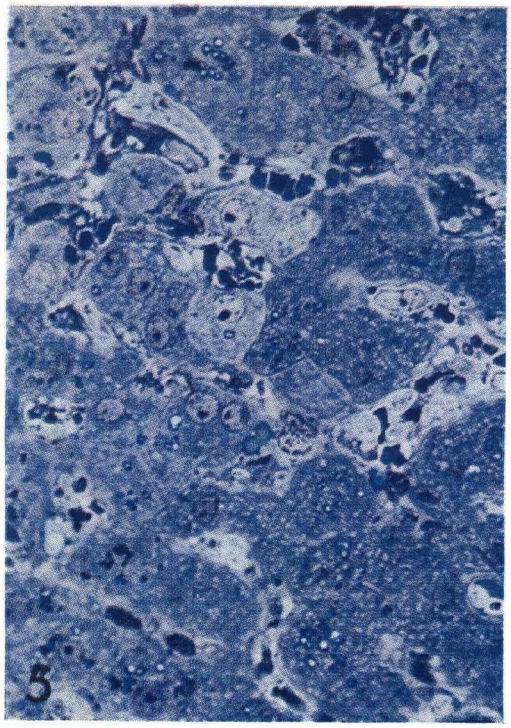
One disadvantage of staining cell nuclei in tissue which has been fixed, or even post-fixed in osmium tetroxide is the lack of affinity of this organelle for routine hematoxylin stains after oxmium fixation. The application of an oxidant to the section, prior to staining it with hematoxylin, allows the hematoxylin to stain with polyesters, but not with epoxy resins. Apparently the oxidant is able to break the osmium fixation bonds in the nucleus with polyester embedment. As a result, peracetic acid used on Vestopal, or on methacrylate-embedded tissue (Munger, 1961), gives satisfactory results, while other oxidants either destroy important cytoplasmic structures or do not produce a sufficiently intense stain. Figures 7 and 8 demonstrate the effects of peracetic acid on the nucleus. In figure 7 after fixation in osmium and embedment in Vestapol, and without prior treatment with peracetic acid, the nuclei are colorless with hematoxylin, whereas in figures 8 after application of peracetic acid for two minutes prior to the hematoxylin the nuclei are colored bluish purple. This procedure does little to improve nuclear staining by hematoxylin for tissue embedded in epoxy resins. However, methylene blue azure II, toluidine blue and basic fuchsin are all effective as stains for both the polyester and the epoxy resins.

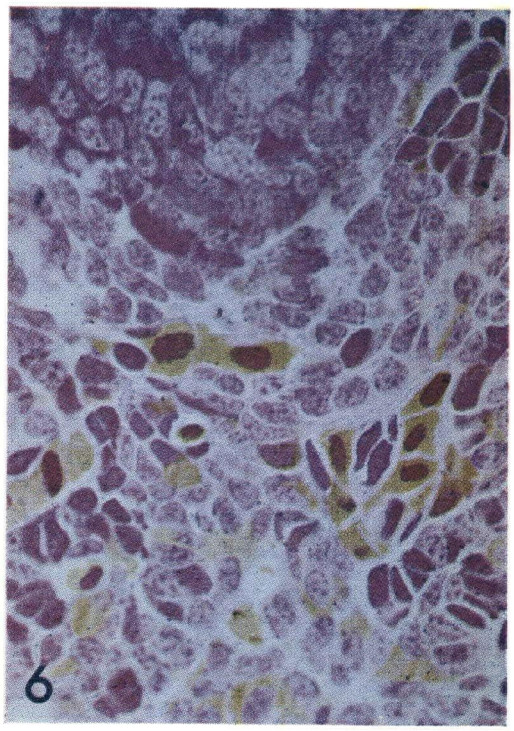
Conclusions

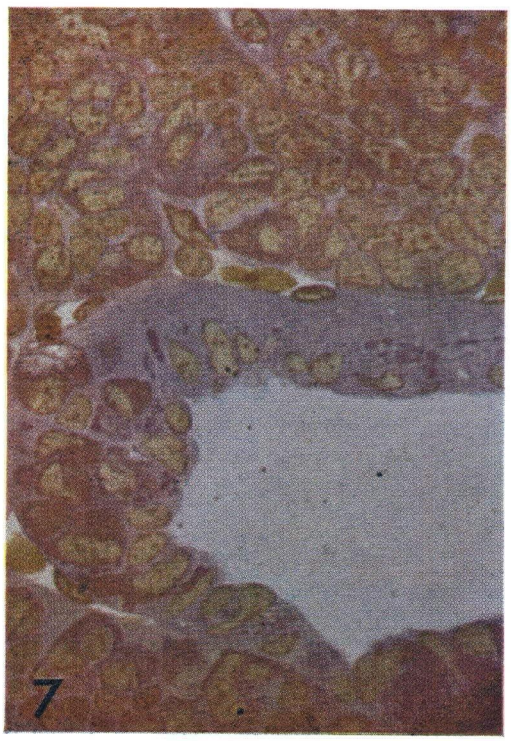
1) Tissue embedded in the polyester Vestopal is permeable to a variety of acid stains and is stained also with basic dyes.

2) Tissue embedded in epoxies, (Epon, Araldite and Maraglas) stain well with basic metachromatic stains, and a few cell components, such as the mucopolysaccharides will stain well with PAS if preoxidized.

3) It is confirmed that phosphate buffered glutaraldehyde, and phosphate buffered osmium (whether used alone or in post-fixation to glutaraldehyde) produce a strong staining intensity. The use of veronal acetate or cacodylate as a buffer results in less intense staining with both fixatives.


5


6


7

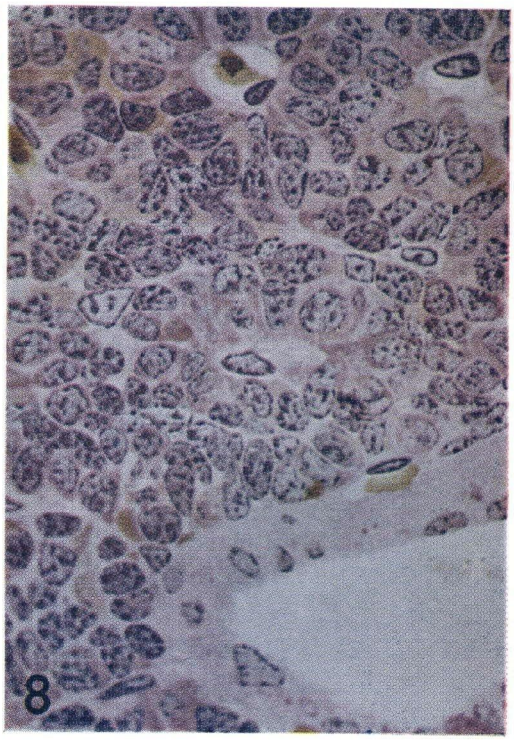

8

PLATE 2 Figures 5-6

5. Dog liver fixed in phosphate buffered glutaraldehyde, post-fixed in phosphate buffered osmium, embedded in Araldite and stained with toluidine blue. This metachromatic dye gives the lipid droplets a green and the nuclei a reddish-blue appearance. X 2,600.

6. Salamander pituitary gland fixed in glutaraldehyde only, embedded in vestopal and stained with hematoxylin, PAS and orange-G. The nuclei, the blood cells and the cells of the pars nervosa appear intensely stained. However, the cytoplasm of the acidophils and the limiting cell membranes are not distinct with this preparation. X 1,900.

Figures 7-8

STAINING TECHNIQUES USED WITH THE POLYESTER VESTOPAL W, FOR INTENSIFYING HEMATOXYLIN STAIN.

7. Salamander pituitary gland fixed in osmium and embedded in Vestopal, stained with hematoxylin, PAS and orange-G *without* peracetic acid oxidation prior to hematoxylin staining. The nuclei appear colorless. X 1,900.

8. Salamander pituitary gland fixed, embedded and stained the same way as figure 7, after immersing the tissue in peracetic acid for two minutes before hematoxylin staining. The nuclei are readily colored bluish purple by the stain. X 1,900.

REFERENCES

Bennett, Dorothea, and Radimska, Olga. 1966. Flotation-fluid staining. Toluidine blue applied to Maraglas sections. Stain. Techn., 41:349-351.

Coleman, L. C. 1938. Preparation of leuco basic fuchsin for use in the Feulgen reaction. Stain Techn., 13:123-124.

Corrington, J. D. 1941. Working with the Microscope. McGraw-Hill Book Co., Inc., New York.

Grimley, Philip M. 1964. A tribasic stain for thin sections of plastic-embedded OsO_4-fixed tissues. Stain Techn., 39:229-235.

Lillie, R. D. 1952. Ethylenic reaction of ceroid performic acid and Schiff reagent. Stain Techn., 27:37-45.

Lockwood, W. R. 1964. A reliable and easily sectioned epoxy embedding medium. Anat. Rec., 150:129-140.

Luft, John H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. and Biochem. Cyt., 9:409-414.

Munger, B. L. 1961. Staining methods applicable to sections of osmium-fixed tissue for light microscopy. J. Biophys. and Biochem. Cytol., 11:502-506.

Richardson, K. C. 1960. Staining epoxy resins for light microscopy. Stain Techn., 35:319.

Sabatini, D. D., Bensch, K., and Barnett, R. J. 1963. The preservation of cellular structure and enzymatic activity by aldehyde fixation. J. Cell Biol., 17:19-58.

Schantz, Arthur, and Schecter, Arnold. 1965. Iron-hematoxylin and Safranin-0 as a polychrome stain for Epon sections. Stain. Techn., 40:279-283.

Trump, B. F., Smuckler, E. A., and Benditt, E. P. 1961. A method for staining epoxy sections for light microscopy. J. Ultrastruct. Res., 5:343-348.

Winklestein, J., Memefee, M. G., and Bell, A. 1963. Basic Fuchsin as a stain for osmium fixed, epon-embedded tissue. Stain. Techn., 38:202-204.