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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.

Editor, Lee G. Luna, D. Lit., H.T. (ASCP) | Technical Bulletin for Histotechnology Published: January, April, July, October | Vol. X, No. 1 - January, 1980

Uses of Aerosol OT in the Histology Laboratory

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Aerosol OT* is a reagent which is often used in the photography laboratory. Its usefulness as a wetting agent is due to the fact that even a low concentration can reduce the surface and interfacial tension of aqueous solutions. This ability can be put to good use in several ways in the histology laboratory.

Fixation:

10% buffered formalin will penetrate tissue faster and more effectively if Aerosol OT is added to the fixative before use. A good working solution is obtained by adding 5 ml of a 10% aqueous solution of Aerosol OT to 1 liter of the formalin fixative. This solution is strongly recommended for dense tissue.

Floatation Bath:

By adding a few drops of a 1% aqueous solution of Aerosol OT to the paraffin floatation bath, paraffin ribbons can be stretched out with less wrinkles. Note, however, that too much Aerosol OT in the water bath may cause the paraffin ribbon to sink.

Microtomy:

When sectioning dry specimens (i.e., blood clot, thyroid colloid, etc.), or a very dense specimen (i.e., cervix), cutting may be facilitated by saturating the face of the paraffin block with 1% Aerosol OT for several minutes. This is accomplished by soaking a piece of cotton or gauze with the aerosol and applying it against the "faced" paraffin block. The surface of the block is then cooled with an ice cube, excess moisture wiped off, and sectioned. This procedure can also be of great benefit when sectioning decalcified specimens.

Aerosol OT can be a useful tool in the histology laboratory and is presented here to appraise other technicians of its usefulness.

Note: This is a collaboration of techniques between the author and Mr. Phillip Pickett, Duke University, Durham, North Carolina.

*Aerosol OT is a product of American Cyanamid Co. It is commercially available from Fisher Scientific Co.

Enzyme-Labelled Antibody in Histopathology*

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Introduction:

The introduction of a new concept in methodology into routine histopathology is sufficiently rare to cause considerable interest. Such a concept is the use of an enzyme as an antibody label[9] and the application of these enzyme-labelled antibodies to paraffin sections to identify tissue-associated antigens.[13] Here is a technique with the specificity characteristic of antigen antibody reactions, the accuracy of microscopic localization and a permanence not associated with fluorescent antibody techniques. Most important, it can be used on routinely fixed and paraffin wax-processed material. The choice of horseradish peroxidase (HRP) as the antibody label is primarily due to its relatively low molecular size and the presence of only small quantities of naturally occurring peroxidase in tissues when compared with other possible enzyme labels such as acid phosphatase and nonspecific esterases.

Application Techniques:

There are four basic techniques (Figure 1) for the application of HRP-labelled antibodies.

A. **Direct:** The simplest is the direct technique which uses a HRP-labelled monospecific antiserum ($R_1AH_{(X)}$-HRP) which binds to the antigen sites ($Ag_{(X)}$) within the tissues. The antibody-bound enzyme is then demonstrated using its specific substrate chromogen reagent (DAB).

B. **Indirect:** The indirect technique uses a similar monospecific antiserum, but in this case unlabelled monospecific antiserum is used ($R_1AH_{(X)}$). Sites of antibody binding are then identified by the subsequent addition of second labelled antibody specific for the immunoglobulin of the species used to raise the first antiserum, e.g., HRP-labelled goat anti-rabbit immunoglobulin serum (GAR_1-HRP). The antibody-bound enzyme is demonstrated using its specific substrate chromogen reagent, analogous to the direct techniques. The indirect technique is greater than five times as sensitive as the direct method due to the multiplication of second antibody binding sites (which the first antibody provides) which in turn increases demonstrable enzyme near the antibody binding sites.

C. **Peroxidase Anti-Peroxidase:** The peroxidase anti-peroxidase (PAP) technique further increases the sensitivity; however, it requires a third step. As with the indirect method the tissue antigen sites bind the monospecific rabbit anti-human serum ($R_1AH_{(X)}$). This reagent is antigenic to the goat anti-rabbit immunoglobulin serum (GAR_1) which is next applied to the sections. The goat anti-rabbit immunoglobulin serum is unlabelled and binds the rabbit peroxidase anti-peroxidase reagent (PAP) applied in the third step. The antibody-bound peroxidase is then demonstrated using the substrate chromogen reagent previously mentioned.

Peroxidase anti-peroxidase reagent (PAP) is rabbit anti-peroxidase antibody complexed with its antigen, horseradish peroxidase. PAP rabbit immune complex will primarily bind with goat anti-rabbit immunoglobulin (GAR_1) previously bound to the tissue section resulting in an increase in sensitivity without the subsequent loss of specificity sometimes associated with multi-step techniques.

*Reprinted from "Qualityline," Winter 1978/79, Miles Laboratories.

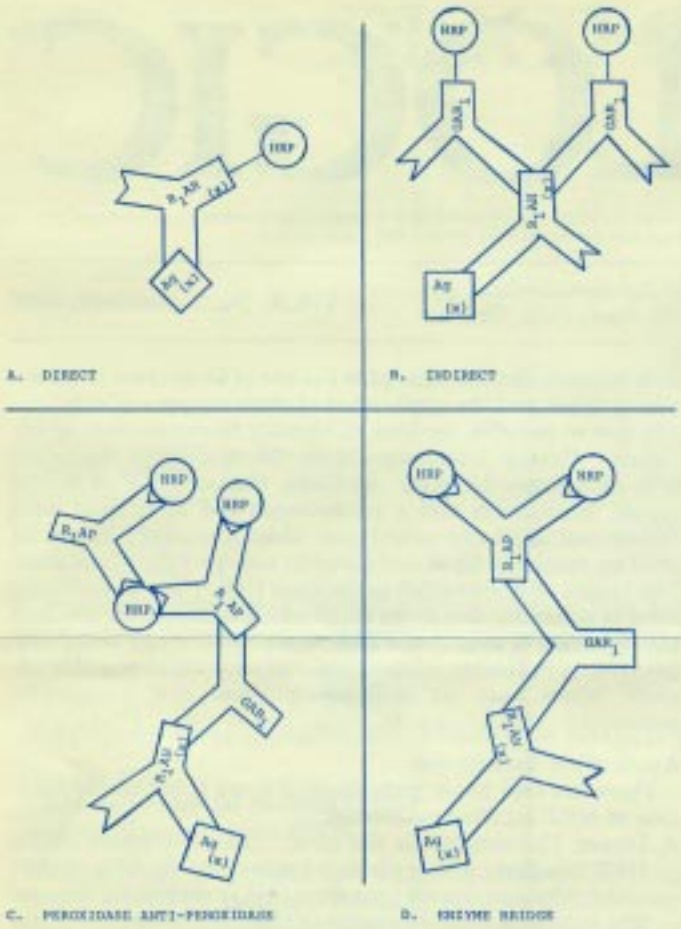

FIGURE 1:
Four basic techniques for the application of HRP-labelled antibodies: A. Direct; B. Indirect; C. Peroxidase Anti-Peroxidase; D. Enzyme Bridge.

D. **Enzyme Bridge:** The enzyme bridge technique is similar to PAP; however, the rabbit anti-peroxidase and its corresponding antigen are applied separately and sequentially and therefore results in a four-step procedure.

E. **General:** With all techniques the antiserum should be used at its optimal dilution to prevent prozoning. The optimal dilution is determined by experimentation, e.g., checkerboard titrations. The dilution range will normally be 1:20 to 1:200 for the first antiserum (R_1AH) but the goat anti-rabbit immunoglobulin (GAR_1) and peroxidase anti-peroxidase (PAP) are normally used at a 1:20 dilution. All dilutions are made in tris-buffered saline (TBS) pH 7.6 (Burns, 1975), and the antisera should be allowed to react for approximately 30 minutes at room temperature. Following each antiserum addition and incubation is a 30-minute wash in TBS using a suitable mixing device. Due to the relative subjectivity of interpretation, care should be taken to standardize methods within the laboratory based on a previously determined standard of measurement and an appropriate number of both positive and negative controls should be run in parallel.

Blocking Techniques:

Naturally occurring or endogenous peroxidase in the tissues will react with the substrate chromogen reagent (DAB) used to demonstrate the antibody-bound enzyme and cause false staining. Blocking of this endogenous peroxidase is carried out before the application of the first antiserum ($R_1AH_{(X)}$) either by producing a colored reaction product different to the color finally produced by the antibody bound HRP, or by specific inhibition.

The Graham a-naphthol pyronin method[5] has been used[13] to produce a red reaction product with endogenous peroxidase, with the diaminobenzidene (DAB) method[3] used to color the antibody-bound enzyme brown. More recently, methanol and hydrogen peroxide mixtures have been used to inhibit the endogenous peroxidase activity.[12]

Nonspecific Reactions:

On occasion, there appears to be some nonspecific reactivity with the PAP technique in particular, which is probably due to Fc binding to basic protein, and is increased with glutaraldehyde fixatives.[1] It can be minimized by using antisera at optimal dilutions and incubating the sections with a 1:5 dilution in TBS of non-immune serum of the second species used (goat in the examples in Figure 1) for 10 minutes. The normal serum is tipped off and not washed before reacting the tissue with the first antiserum (R_1AH).

Binding the antiserum to interstitial IgG can also produce high background staining and apparently nonspecific results. Predigestion of tissue sections with pepsin or trypsin[3] will reduce this effect as will washing in TBS at 4 °C for several hours prior to fixation. Enzyme digestion may also increase reactivity of the antisera allowing greater dilutions to be used, and yet produce an enhanced final result with reacted tissue antigen sites more easily visible.

Substrates for the Demonstration of Peroxidase:

There are approximately 20 substrates for the demonstration of peroxidase, the most commonly used being 3-5' diaminobenzidene (DAB). Oxidative polymerization produces a brown insoluble reaction product which may be further reacted with osmium tetroxide to produce osmium black. DAB is popular as a substrate because the final reaction product has a high color density which is resistant to alcohol and allows dehydration to a mounting medium of high refractive index. The product is also electron dense. DAB, however, is carcinogenic, and a satisfactory alternative has been found — 3,3', 5,5' tetramethyl benzidine (TMB).[6]

a-Naphthol pyronin[5] produces a strong red color which resists alcohol but is not electron dense. Another reagent, 4-chloro 1-naphthol[8] has a slate gray product, and 3-amino 9-ethyl carbazole[7] a strong red product, but both are alcohol soluble and are not electron dense. Seligman[10] suggested a number of substrates but none were electron dense and alcohol resistant. At the present time, 3-amino 9-ethyl carbazole is also a satisfactory alternative to DAB for light microscopy because of the strong red final reaction product and minimal background staining.

Uses for the Technique:

The first applications for the peroxidase-labelled antibody techniques on paraffin sections were to identify plasma cells by the presence of immunoglobulins within the cytoplasm, a technique especially useful for the diagnosis of dysgammaglobulinemias and the analysis of myeloma tissue. Neoplasms tend to be monoclonal whereas a nonspecific response will be polyclonal.

The techniques can also be applied to resin-embedded material, either before or after embedding, for thin section light microscopy and applications requiring electron microscopy, such as virus localization.

The uses in demonstration of intracellular components on formalin saline-fixed, paraffin-processed sections have increased and appear limited only by the ability to produce a specific antiserum (R_1AH) to the tissue-bound antigen.

The techniques have been used to demonstrate autoantibodies on liver cell membranes and the presence of hepatitis B surface antigen (HB_sAg). HB_sAg can also be demonstrated

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Reply to Ganglion Cell Staining Inquiry

Clare S. Kerr
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The January 1979 (Vol. IX, No. 1) issue of *Histo-Logic* contained a request for help concerning the staining of nerve cells in the gut with the Glees-Marsland reduced silver method. Some of the following points may be applicable to this problem.

Fixation should always be for one week or more, in neutral or slightly acid formalin. Control sections of central nervous system should be processed along with the intestinal material. The Glees-Marsland method is difficult and sometimes impossible to do well in parts of the world that have soft tap water. For routine use, one should try either the Bodian[1] or Holmes[1] method. If, however, one wishes to demonstrate the very delicate parasympathetic endings on ganglion cells, some of the methods mentioned in the *Microtomists' Vade-Mecum* (Bolles Lee) 1950 edition, pp. 516-523, edited by J. B. Gaterrby and H. W. Beams, might be useful.

Generally speaking, it is wise to stain some sections with an ordinary cell stain such as hematoxylin and eosin or cresyl violet. This will enable one to make certain that there are nerve cells present before one goes to the trouble of doing a silver preparation.

Reference:

Luna, L.G.: *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3rd Ed., pp. 195-196, McGraw-Hill, New York, 1968.

Can You Help?

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I have had a problem with a new batch of commercial malt diastase. It does not have the ability to digest glycogen. I have found that it is a good idea to check new bottles of malt diastase by running duplicate slides before the older reagent is used up, in order to compare the degree of digestion.

Is there any simple means of testing the quality and/or sensitivity of the diastase without running duplicates through the complete procedure and thus using up precious controls?

If you or any readers of *Histo-Logic* can help me with this problem, it would be greatly appreciated.

Editor's Note: Please forward a copy of any replies to the Editor.

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Printed in U.S.A.

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.