

molecule; whenever a dye of large molecular weight is unable to penetrate, it will always do so at the expense of the smaller molecule.

Information concerning the molecular sizes of dyes is not generally available, although use may be made of molecular weights to obtain an indication of relative size. However, in aqueous solution many dyes consist of aggregates of dye molecules, e.g. methyl blue solution may be expected to be particulate even under high power light microscopy. A few dyes appear in aqueous solution as discrete molecules, e.g. Eosin Y which, although having a molecular weight of 692, penetrates and stains red cells with ease.

Heat

Heat increases the rate of staining and also enables the less porous elements to be coloured by large molecule dyes; this may be due to the breaking down of conglomerates of dye molecules.

pH

In order to achieve adequate and even staining of connective tissue fibres, trichrome techniques require that dyes are in low pH solutions, probably in the range pH 1.5 to pH 3.0. The acidity may be obtained by using acetic acid.

Nuclear stains for trichrome staining

Due to the acidity of dye solutions used in the differential staining of connective tissue fibres, standard alum haematoxylin is decolourised in the subsequent treatment. Iron haematoxylin is more resistant to acid solutions and are prescribed in most of the techniques.

An improved and resistant stain can be achieved by sequential staining with celestin blue or solochrome prune in iron alum solution, followed by a conventional alum haematoxylin (for details see Ch. 7).

Fixation for trichrome staining

Zenker's solution, formal mercury and picromercuric alcohol are the most satisfactory fixatives for

trichrome techniques. It is probable that most fixatives giving enhanced acidophilic staining are satisfactory. Buffered formalin and alcohol are less satisfactory, producing less intense and precise staining of connective tissue fibres. Treatment of formalin-fixed sections overnight with picromercuric alcohol solution, followed by removal of mercury pigments, is said to give enhanced connective tissue staining.

Trichrome stains are improved by the degreasing technique of Lendrum, in which sections are treated in a sealed container of trichlorethylene for 24 to 48 hours following dewaxing in xylene.

Van Gieson technique (1899) *Connective Tissue Sections*

Paraffin. For celloidin or LVN sections see p. 338, and Notes d and e below.

Solution

| | |
|--|---------------------|
| Saturated aqueous picric acid solution | 50 cm ³ |
| 1 per cent aqueous acid fuchsin solution | 9.0 cm ³ |
| Distilled water | 50 cm ³ |

Method

1. Dewax sections and bring to water.
2. Stain nuclei by the celestine blue-haemalum sequence (see p. 113).
3. Wash in tap water.
4. Differentiate in acid alcohol. *4 sec*
5. Wash well in tap water.
6. Stain in van Gieson solution 3 minutes.
7. Blot.
8. Dehydrate through alcohols. *10 sec. each*
9. Clear in xylene and mount in DPX.

Results

Nuclei — *blue/black*
 Collagen — *red*
 Other tissues — *yellow*

Notes

- a. Fixation is not critical, buffered formalin being satisfactory.

- b. Washing in water after van Gieson solution should be avoided, the colour balance being impaired.
- c. Nuclear staining should be intense before application of van Gieson solution; the picric acid will act as a differentiator.
- d. Celloidin sections are washed in distilled water after van Gieson solution.
- e. Celestin blue may stain the celloidin intensely. In such circumstances, Weigerts iron haematoxylin should be used.

Role of phosphotungstic and phosphomolybdic acids

Although phosphomolybdic and phosphotungstic acids do not give identical reactions in trichrome staining techniques, their properties are similar. Experimental work indicates that the principles involved are identical, but it is not suggested that the two substances are interchangeable in any given technique.

Throughout the literature on trichrome staining, reference is frequently made to mordanting in phosphotungstic or phosphomolybdic acids. There remains some controversy about the precise roles of these two substances, but it is unlikely that they act as mordants towards the anionic dyes used in these techniques.

Everett and Miller (1974) have shown that treatment of formalin-fixed sections with PMA or PTA greatly reduces staining of all tissue components, other than collagen fibres, with aniline blue and other similar anionic dyes. Blocking towards smaller dye molecules such as Biebrich scarlet was shown to be less complete. Binding of PTA to epithelium and connective tissue fibres was demonstrated by the quenching of autofluorescence and by the reduction of the bound PTA to a blue colour by means of titanium trichloride solution. These workers postulated that the differential staining by the trichrome methods occurs by binding of aniline blue to basic residues in the connective tissues not already blocked by PTA.

Baker (1958) stated that PMA acts as a 'colourless acid dye' of large molecular size, and hence slowly diffusing.

Practical uses of PMA and PTA

In trichrome staining, there are three stages at which the PMA or PTA may be used.

1. Before treatment with the small molecule dye
2. Combined in solution with the small molecule dye
3. Before treatment with the large molecule dye

Any combination of these techniques is possible.

If a section is first treated with PMA or PTA solution and then with a low concentration of 'levelling' dye in the same solution, the levelling dye will colour nothing but the erythrocytes. In practice, the first treatment with the PMA or PTA is frequently omitted without detriment to the final results.

When a section is first treated with a levelling dye or other suitable small molecule anionic dye and then with PMA or PTA solution, the PMA or PTA competes with the dye and gains access to the collagen easily, expelling the dye in the process. If treatment is stopped at the right moment only collagen will be free to stain when treated with a 'milling' or other large molecule dye. If treatment with the large molecule dye is greatly prolonged, some staining of muscle and cytoplasm may take place.

In addition to the rather complex role played by PMA and PTA in connective tissue staining, it must not be forgotten that both are quite capable of acting simply as conventional acidifying agents. A 10 per cent solution of PTA having a pH of less than one, indeed PTA is unstable at a pH greater than about two.

Two further large molecule polyacids, silicotungstic acid and borotungstic acid may be found useful in trichrome techniques, although no published work has been found.

Identification of dyes

There is little standardisation between manufacturers in the naming of dyes, consequently the same chemically identical dye may be obtained from different suppliers under a wide range of names (see Ch. 6 and Table 8.1).

the ferric salt in the prepared celestine blue solution strengthens the bond between the nucleus and the alum haematoxylin to provide a strong nuclear stain which is reasonably resistant to acid.

The celestine blue-alum haematoxylin procedure

Preparation of solution

Celestine blue solution

| | |
|--------------------------|---------------------|
| Celestine blue B | 2.5 g |
| Ferric ammonium sulphate | 25 g |
| Glycerin | 70 cm ³ |
| Distilled water | 500 cm ³ |

The ferric ammonium sulphate is dissolved in the cold distilled water with stirring, the celestine blue is added to this solution, and the mixture is boiled for a few minutes. After cooling the stain is filtered and glycerine is added. The final stain should be usable for over 6 months.

Method

1. Dewax sections in xylol, hydrate through graded alcohols to water.
 2. Stain in Celestine blue solution — 5 min.
 3. Rinse in distilled water.
 4. Stain in an alum haematoxylin (e.g. Mayer's or Cole's) — 5 min. *OR Haematoxylin 2min*
 5. Wash in water till blue.
- 5 using haem 2min after washing*
Proceed with required staining technique.

*put in lithium carb for 2 sec
wash in tap H₂O*

Eosin

Eosin is the most suitable stain to combine with an alum haematoxylin to demonstrate the general histological architecture of a tissue. Its particular value is its ability, with proper differentiation, to distinguish between the cytoplasm of different types of cells, and between the different types of connective tissue fibres and matrices, by staining them differing shades of red and pink.

The eosins are xanthene dyes and the following types are easily obtainable commercially:

Eosin Y (eosin yellowish, eosin water soluble) CI No 45380 (CI Acid Red 87)

Ethyl eosin (eosin S, eosin alcohol-soluble) CI No 45386 (CI Solvent Red 45)

Eosin B (eosin bluish, erythrosin B) CI No 45400 (CI Acid Red 91).

Of these, eosin Y is much the most widely used, and despite its synonym it is also satisfactorily soluble in alcohol; thus it is sometimes sold as 'water and alcohol soluble'. As a cytoplasmic stain it is usually used as a 0.5 or 1.0 per cent solution in distilled water, with a crystal of thymol added to inhibit the growth of fungi. The addition of a little acetic acid (0.5 cm³ to 1000 cm³ stain) is said to sharpen the staining. Differentiation of the eosin staining occurs in the subsequent tap water wash, and a little further differentiation occurs during the dehydration through the alcohols. The intensity of eosin staining, and the degree of differentiation required, is largely a matter of the individual pathologist's taste. Suitable photomicrographs of HE stained tissues are easier to obtain when the eosin staining is intense and the differentiation slight.

Ethyl eosin and eosin B are now rarely used although occasional old methods specify their use, e.g. the Harris stain for Negri bodies.

Alternative red dyes have been suggested as substitutes for eosin, e.g. phloxine, Biebrich scarlet etc., but although these substitutes often give a more intense red colour to the tissues, they are rarely as amenable to subtle differentiation as eosin and are generally less valuable.

Under certain circumstances eosin staining is intense and difficulty may be experienced in obtaining adequate differentiation; this may occur after mercuric fixation.

Over-differentiation of the eosin may be continued until only the red blood cells and granules of eosinophil polymorphs are stained red; this manoeuvre is occasionally used to facilitate the location and identification of eosinophils.

Staining procedures using alum haematoxylin

Standard haematoxylin and eosin stain for paraffin sections

1. Dewax sections in xylol, hydrate through graded alcohols to water.