

An Easily Controlled Method for Staining Mitochondria

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THE great disadvantages of the standard staining methods for mitochondria are that they are very complicated and cannot be applied to different tissues until suitable times for the action of the various stains have been worked out by a long process of trial and error.

The following method is easy to control and apply. Destaining and restaining are quickly carried out, times may be worked out on a single section, and, as each reagent is used for a single purpose (not, for example, as a simultaneous differentiator and stain), each stage may be controlled separately. Mitochondria are red, cytoplasm is pale blue or colourless, nuclei are medium blue, and plasmosomes (nucleoli) pink or red.

1. Fix small pieces in Helly's fluid for 6 hours.
2. Postchrome for 48 hours at 37° C. in a saturated aqueous solution of potassium dichromate.
3. Wash overnight in running water.
4. Embed in paraffin wax and cut sections of a suitable thickness (about 3 μ). Bring the sections down to water, treating with iodine ($\frac{1}{2}$ in 70 per cent. alcohol) and then sodium thiosulphate (5 per cent. aqueous solution) on the way.
5. Dry a slide except where the sections are, flood with acid fuchsine in aniline-water, and heat gently until 'steaming', in order to overstain, exactly as for Altmann's technique. (For detailed instructions see Baker, 1945, p. 190.)
6. Wash off the acid fuchsine with distilled water, observe the section under the high power of the microscope, and irrigate with an alkaline solution. One drop of saturated aqueous sodium carbonate solution in 10 c.c. of distilled water gives a fairly rapid differentiation, taking 30 sec. to 1 $\frac{1}{2}$ minutes.
7. To stop differentiation and brighten the acid fuchsine, dip the slide into 1 per cent. hydrochloric acid. For the criteria of differentiation see below. If brightening is undesirable because extraction of the dye from the cytoplasm is difficult, wash in distilled water instead of dipping into the acid.
8. When the slide has been correctly differentiated, wash it in distilled water, then counterstain progressively in a $\frac{1}{2}$ per cent. aqueous solution of water-soluble methyl blue. The stain 'Soluble Blue' (not 'soluble blue, crystals AS') sold by B.D.H. is suitable. For a discussion of methyl blues see below. Wash off the stain with distilled water, dip in 1 per cent.

acid for 3 seconds only, wash with distilled water, go through the alcohols (in no great hurry), and mount in Canada balsam.

After the acid fuchsin has been applied, the section is brilliant red throughout, though it may be possible to see that the mitochondria are darker than the rest of the cytoplasm. Differentiation should proceed until the mitochondria are dark red against pink cytoplasm. Some cells (e.g. nephridial cells of the leech *Glossiphonia complanata* (L.)) will give up all the acid fuchsin from the cytoplasm before the mitochondria begin to pale. Others (e.g. intestinal epithelium of the mouse) retain a certain quantity, but it is easily masked by the methyl blue. For such cells as the former, comparatively thick sections (5–10 μ) may be used, but for the latter they should be thin (3 or 2 μ). If differentiation is carried too far, the section can be restained in acid fuchsin.

When the staining and differentiation of the acid fuchsin are satisfactory, the nucleus and cytoplasm are counterstained with a methyl blue dye. This dye, being acid, does not interfere with the differentiation of the acid fuchsin. Many basic dyes or dye-lakes can be used to differentiate acid dyes. For example, Kull (1914) used toluidine blue to differentiate acid fuchsin, but as the blue had overstained the section before completing differentiation, he used aurantia to differentiate the differentiator, thereby producing his well-known trichrome method.

The trouble with such procedures is that the times of suitable differentiation and suitable staining by a combined stain and differentiator are unlikely to be the same, and it is difficult to control a differentiation proceeding in a coloured solution. Trichrome methods give beautiful results when properly carried out, but dichrome methods are perfectly satisfactory for almost all purposes, and with a colourless differentiator are far easier to control. Quite good preparations can be made from mouse small-intestine epithelium (and probably from other tissues) by overstaining in acid fuchsin, and staining and differentiating simultaneously with Ehrlich's haematoxylin, tap-water (which is not sufficiently alkaline to disturb the acid fuchsin) being used for blueing. A suitable time in the haematoxylin for 3 μ sections of this tissue is 7 minutes. But the cytoplasm is not very clear and the contrast with the red in the mitochondria is rather poor. Methyl green (a basic dye) may be used as in Bensley's method (1911) but it is exceedingly rapidly removed by alcohol and very sensitive to acid balsam. Methyl blue (water soluble) when acting as an acid dye is superior because differentiation of the acid fuchsin can be carried out separately and it will not interfere, it can be made to stain nuclei well, it gives good colour-contrast with the mitochondria, and it is not sensitive to alcohols and acid balsam. (It is removed by strong alcohol, but, as with acid fuchsin, only after periods of some hours.) If overstaining occurs it can be removed, but much more rapidly, by the same differentiator as was used for acid fuchsin, followed by 70 per cent. alcohol.

The group of dyes usually called methyl blue (excluding methyl blue SS) are closely related acid triphenylmethane dyes. They can behave as acid or as

basic dyes according to the circumstances of their preparation and the pH of the media in which they are dissolved. In alkaline solutions they are dull blue, rather slowly staining, acid dyes, rather easily removed by alcohol. In acid solution they are intensely blue basic dyes fast to alcohol. When dissolved in distilled water they may behave as acid or as alkaline dyes according to the circumstances of preparation. Various samples were examined, and it was found that those that are sold in the form of crystals with a reddish-bronze lustre and give intensely reddish-blue solutions in distilled water behaved as basic dyes, and those sold as dark-blue powders giving a less intense and rather dull-blue solution in distilled water behaved as acid dyes. B.D.H. Soluble Blue is an example of this class. An acid dye is essential to avoid further differentiation of the acid fuch sine, which is rapidly removed by the basic methyl blue dyes. Use of a basic methyl blue in an alkaline solution cannot be recommended because, if the solution is sufficiently alkaline for the dye to be acidic, then it will itself differentiate the acid fuch sine. It is therefore essential to use an acid dye in distilled water. As the colour produced by it is purplish and rather feeble, the dye can be made much more intense and less purple in tone by dipping the slide into 1 per cent. hydrochloric acid for a few seconds only. If the slide is left in it for longer, the methyl blue, now basic, will attack the acid fuch sine. Intensification is complete in 2 or 3 seconds, and the slide is immediately removed and freed from acid by washing with distilled water.

Methyl blues are fairly readily removed by 70 per cent. alcohol when in the alkalized state, that is, when behaving as acid dyes. If a valuable slide has been overstained with methyl blue, it can be destained by a minute's treatment with sodium carbonate solution followed by a quarter of an hour in 70 per cent. alcohol, and may then be restained with acid fuch sine and methyl blue.

The method described has given satisfactory results with the intestine, liver, and kidney of the mouse, and nephridial cells, muscle-fibres, and gut epithelium of the leech *Glossiphonia*. Kidney should be cut if possible at $2\ \mu$, as the mitochondria are clustered so thickly together. Suitable times for differentiation and counterstaining are best worked out on the first slide of each batch, and then applied to the rest. The time for methyl blue varies greatly with the thickness of the section, thicker sections requiring much less staining than thinner ones. $3\ \mu$ sections of mouse intestine required 1 minute, $10\ \mu$ sections only 30 seconds.

SUMMARY

A method for staining mitochondria is described in which sections are overstained with acid fuch sine, differentiated in sodium carbonate solution, and counterstained with a methyl blue acting as an acid dye.

REFERENCES

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