Counterstaining Hemalum

Bryan D. Llewellyn

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Yesterday I spoke about alum hematoxylin staining but I did not comment on the counterstain usually used, *i.e.* eosin. As an acid dye, eosin staining has much in common with other acid dyes, such as those used for single solution and multi-step trichrome methods and the various quad stains. The basic underlying principles are very similar, merely the application being different.

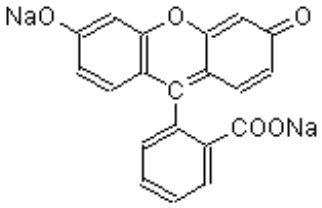
Acid dyes as a group are quite diverse, ranging from picric acid, which has a molecular weight of 229, to the large molecular weight dyes such as fast green FCF at 809. In fact, this difference in molecular weights is one of the features of these dyes that enables us to manipulate them so successfully.

So what is an acid dye? Simply put, it is an organic salt in which the anion is intensely coloured, and which will attach to cations while retaining its colour. That definition gives a clear indication as to the chemical basis for this staining, that of ionic interaction between dye anions and tissue cations. The former are carboxyl, hydroxyl and sulphonic groups predominantly, while the latter are nearly always amino groups. In most cases it involves plain ionic bonds.

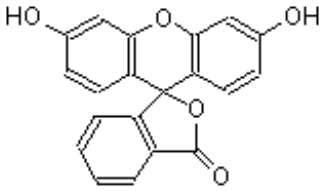
The Eosins

Eosin belongs to a group of dyes called fluorones, which are derived from fluorescien(Horobin and Kiernan 2002.). The sodium salt of fluorescien is called uranin. It is not used histologically, but its relationship to eosin is quite apparent when the formulae are compared. These dyes are fluorescien homologues halogenated with bromine, iodine or chlorine. Eosin Y ws itself is tetrabromofluorescien, for instance, *i.e.* fluorescien with four bromine atoms attached.

The free anion of eosin Y is called eosinol Y, and its relationship to fluorescien is also apparent. Eosinol, and similar compounds from the other dyes related to eosin such as phloxine and erythrosine, can be used to stain tissues.



Uranin



Fluorescien

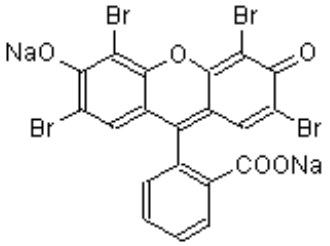
Eosin is commonly applied as a solution in hard tap water, from 0.5% - 2%. It is applied for a couple of minutes, the excess is then washed out until it is stained the way we want, then dehydrated, cleared and mounted. This works well in London, England. It happens that the water there is rich in calcium and magnesium ions and is slightly alkaline. It turns out that water with those characteristics is just about ideal for eosin staining, and for gaining the maximum variation in staining intensity with that.

For London it is a very fortuitous circumstance as, due to the local water quality, an H&E stained section in London can show considerable variation in intensity between the various types of tissue, with deep pink erythrocytes, paler muscle and even paler collagen.

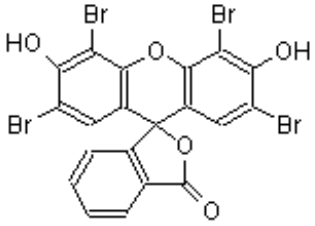
Unfortunately for eosin staining elsewhere, not everyone in the world has access to London tap water, although I understand that the a big cola company tried to remedy that by bottling it and selling it as a purified water for a long time.

Fortunately eosin Y can also be used in alcoholic solution. I became familiar with this very early in my training as the hospital in which I worked as a student, in London as it happened, used a 0.1% solution in 80% industrial methylated spirits. It was applied for 15 seconds, followed by a slow rinse with absolute methylated spirits and clearing with xylene. The results very closely approximated those obtained with aqueous staining.

I have used this method extensively in Canada with ethanol rather than methylated spirits and have always obtained quite satisfactory results for about 40 years.



Eosin Y ws



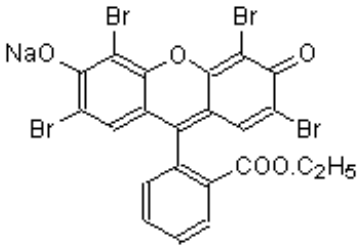
Eosinol Y

To use eosinol instead of eosin it must first be made since it is rarely, if ever, listed by dye vendors. Make a strong solution of eosin Y ws in water (10%). Slowly add concentrated hydrochloric acid little by little. A precipitate will form. Keep adding hydrochloric acid until no more precipitate forms. Filter out the precipitate using a filter paper. The precipitate is the eosinol. It is insoluble in water, so it can be washed with distilled water to remove any residual acid, then dried at room temperature. When dry it may be ground into a powder and stored in a jar like any other dye. It is quite stable at room temperature.

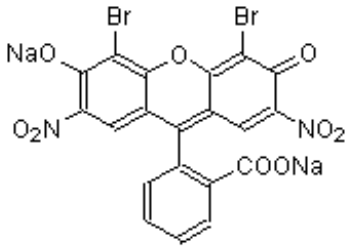
For use, a small amount is dissolved into the final dehydrating ethanol or into the first clearing xylene. The sections are allowed to remain in it until the staining is at the required depth, then clearing is completed and the section coverslipped. The staining is somewhat homogenous, but this procedure enables material to be stained that otherwise does not take up any eosin. It may be useful for some necrotic, decayed or fetal tissues which are causing problems. It is not recommended as a regular procedure.

There is a variant of eosin, ethyl eosin, which is sold as alcohol soluble eosin, but this is quite redundant. It has the same formula as eosin Y but with an ethyl group replacing the sodium. Ordinary eosin Y is ethanol soluble at sufficient concentration to make it satisfactory as a stain from ethanol solution.

There is also an eosin with a blue cast, eosin B, which is rarely used but stains essentially the same as eosin Y but looks cold. Neither of these dyes is particularly popular.

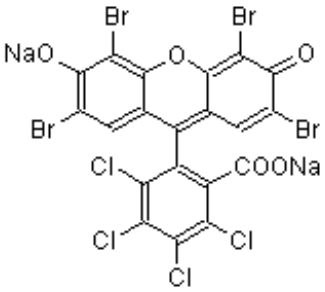


Ethyl eosin

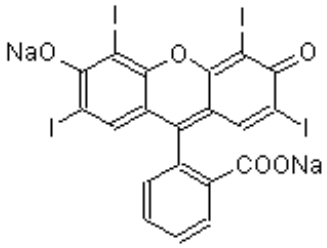


Eosin B

There are other dyes related to eosin Y which are sometimes added to the simple ethanolic solution in order to increase differential staining. These are phloxine B and erythrosine B, but most commonly phloxine. Erythrosine B is used as a food colouring in many countries. The intense rosy red colour of cherries in canned fruit salad may be because they have been coloured with erythrosine. In fact, I was once contacted by a food chemist from a company getting ready to market a canned fruit salad as to how much erythrosine to use and how to stabilise the colour.



Phloxine B



Erythrosine B

Mixed dyes

Adding small amounts of other dyes to eosin Y is quite prevalent in the United States. It comes about in an attempt to increase the intensity of staining in a part of the world where water quality varies greatly in different places. In some parts of the country the water may be soft and acidic periodically, during the spring snow melt, for instance. In other places acetic acid is added to the eosin in order to increase staining intensity deliberately. Applying an acid dye from an acid medium does increase staining intensity, but usually also results in a "flattening" of staining, that is, the differential effect obtained with eosin Y and London tap water is lost and the staining becomes homogenous.

Due to this, some laboratories add a small amount of phloxine B to the eosin. This usually results in more intensely stained erythrocytes, in effect putting back some differential effect. It is quite surprising how little phloxine needs to be added to accomplish this.

Typical solutions would resemble the first two formulae below:

Acidified Eosin	
Ethanol	800 mL
Distilled water	200 mL
Eosin Y ws	2.0 g
Glacial acetic acid	5 mL

Acidified Eosin with Phloxine	
Ethanol	800 mL
Distilled water	200 mL
Eosin Y ws	2.0 g
Phloxine	1.0 g
Glacial acetic acid	5 mL

Eosin Trichrome ingredients	
Ethanol	Eosin Y ws
Isopropanol	Phloxine B
Methanol	Orange G
Acetic acid	

The solution would be applied for a minute or so, then washed well with 80% ethanol, then absolute ethanol and cleared. Sometimes mixtures of alcohols may be used, such as iso-propanol, methanol, ethanol, but the effect is the same.

In an attempt to get even more differentiation into the counterstain, sometimes the small molecular weight dye, Orange G is added. This gives an orange cast to some structures and the picture is not unpleasing. Surgipath sells a solution like this under the name Eosin Trichrome. Their Material Safety Data Sheet lists the ingredients in the third column above.

Perhaps the most unusual mixture of dyes used as a hemalum counterstain is Meter's Eosin, which I understand is quite popular in some laboratories: The biebrich scarlet has much the same function as the orange G, but with a darker and redder colour.

Meter's Eosin	
Distilled water	800 mL
95% ethanol	200 mL
Biebrich scarlet	0.4 g
Eosin Y ws	5.0 g
Phloxine B	2.1 g

HPS

Yet another approach to counterstaining hemalum was taken by others. In the past this method was quite popular in one very large hospital in Ontario, Canada. In North America it is known as the HPS or Hematoxylin phloxine saffron. Other places may know it better as the HES, or hematoxylin erythrosine saffron. The technique requires an aqueous solution of phloxine or erythrosine and a completely water free alcoholic solution of saffron. As the world's supply of saffron is now much less than formerly, its role as a dye having been replaced by synthetic alternatives, the price has skyrocketed and the cost of doing this method makes it prohibitive. I should note that cheaper saffrons do not work well in this technique, although Indian saffron as used in Indian cooking is usually satisfactory and is not too badly priced.

The procedure is to stain nuclei with hemalum and blue, apply the phloxine or erythosine, dehydrate thoroughly, then soak the section in water-free alcoholic saffron until collagen has stained yellow but muscle is still pink. The end point is determined visually.

The saffron is extracted into the absolute ethanol in an incubator over a few days, and even trace amounts of water cause the staining to fail. Needless to say, the procedure never became very popular, but it does give a nice looking section with differential staining. Alternatives using synthetic dyes have been suggested, but the extra attention required in busy laboratories make using them unattractive as a routine method, although they retain their usefulness as special stains.

All of these procedures are interesting from another point of view, quite apart from their value as counterstains for hemalum. They all attempt to bring about some form of differential staining whether it be by preferentially removing some of the dye by washing it out, mixing together different dyes and letting them preferentially stain different components from a single solution, or applying dyes sequentially so that the second can replace the first as with phloxine and saffron.

My point is that in other staining methods these same practices have been used to bring about the one-step and multi-step trichrome methods as well as the methods based on the phloxine tartrazine technique, which itself depends on the same principles as the HPS.

References

Horobin, R. W., and Kiernan, J.A., (2002). *Conn's Biological Stains*, 10 ed., Biological Stain Commission. BIOS, Scientific Publishers, Oxford, UK

Lee F. W., (1969). *Eosinol counterstain for routine paraffin embedded tissue.* Journal of Medical Laboratory Technology, **v. 26** (Jan), pp. 36-7