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# Haematoxylin – the story of the blues

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> The farther back you can look, the farther forward you are likely to see [1].
>
> – Winston Churchill

Haematoxylin is arguably the most well known of all dyes used in pathology. It remains the primary technique for the demonstration of microscopic nuclear details of cellular and tissue components. However, the story of how this remarkable dye has remained the nuclear dye of choice is a long one and dates back hundreds of years. It is also a story which encompasses many different applications as the dye found value in several spheres of everyday life throughout the centuries, which stretched far beyond the histological study of cells and tissues.

Haematoxylin (etymologically derived from the Greek word hematos: blood and xylos: tree) was originally derived from the tree *Haematoxylon campechianum*, found in the Yucatan peninsula region of Campeche Mexico [2–4]. Although a small village, Campeche soon became a major port of entry to the whole of the Gulf of Mexico and it provided a natural harbour for ships entering and departing the country. History books inform us that this was in the sixteenth century and was documented by the Spanish explorers and conquistadors who landed in Campeche in 1517, the most notable and ruthless of whom was Hernado Cortes (1485–1547). However, the first recorded documentation of these trees was earlier, in 1502. The much-persecuted Mayan civilisation was known to use extracts from the logwood of these trees for a multitude of applications long before this time, as a dye to colour cotton-based fabric also as a treatment for diarrhoea [2,3].

Since dye fabrics were relatively novel in Europe at the time, most common clothing worn in the mid to late fifteenth century was rather drab and lacking vibrant colours. Interest rapidly grew in the use of the logwood extract as a dye fabric across high society throughout Europe. As a trade, it became largely dominated by the Spanish and was increasingly profitable, commanding a high price. As such the Spanish galleons that were exporting large volumes of the logwood back to Spain became the focus of pirates and Buccaneer's alike, including England's Sir Walter Raleigh (1554–1618), who was known to hide his ship off the Spanish Azores awaiting the arrival of the much slower Spanish ships, laden down with heavy cargo, to then swoop down on them and steal the merchandise including the much sort after logwood. The acquisition of this precious logwood made the Spanish the envy of European society. At its height weight for weight the logwood was more precious than nearly all other types of merchandise. A report of an entry by a Captain James in a book entitled British Honduras (1883) discovered that the debarked logwood sold in England for 100 pounds sterling per ton. An average single load of 50 tons of logwood was worth more than an entire year's cargo of any other merchandise [5] (Figures 1 and 2).

It is also worthy of note to recognise the fact that as well as the Spanish the Portuguese explorers were also harvesting another dye wood from the coasts of Central and Southern America. This wood was the Brazil wood, harvested from the *Caesalpinia echinata species*. The extract from these trees was called brazilin. The Portuguese explorers and sailors described the red heartwood of these trees as resembling the glowing embers or coals of a fire, in Portuguese the word to describe this is '*brasa*' hence from this the word came the country's name, Brazil [3]. As well as the Spanish and English, the Dutch and French also expressed interest in acquiring the Haematoxylin logwood. The Spanish naturally claimed a complete monopoly on all logwood sales and this extended to the profits from established logwood plantations. This lead to the seven years' war between the Spanish, French and English (1756–63). The English during this time discovered the Haematoxylin Campechianum trees growing in British Honduras (Belize). Fights over the rights of British to settle and

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Figure 1. Photograph kindly donated from Ms Judy Brincat, showing the *Haematoxylon Campechianum* logwood with the delicate yellow/ white flowers characteristic of the tree. (Taken in Alligator Creek and Townsville, Queensland Australia).

Figure 2. Photograph of a cross section of *Haematoxylon campechianum* logwood showing the rich vibrant colour of the wood used to extract haematin.

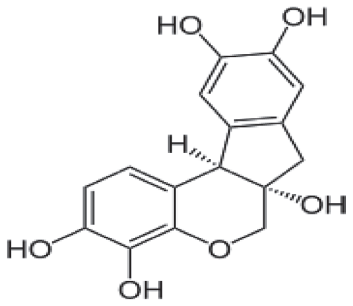
cut logwood in Belize, lead to the Treaty of Paris (1763) which gave rights to the British to cut logwood but gave sovereignty to the Spanish. The British employed woodcutters called 'Baymen'. They sent tons of logwood back to England throughout 1700s and 1800s. Such was their notoriety, to this day the national flag and currency of Belize depicts the Baymen [5]. The haematoxylin continued to find a use as a fabric dye over the centuries and was used to stain the uniforms of both the North and South American soldiers during the American Civil War (1861–65). Similarly, it enjoyed another renaissance during both World Wars during the 1900s [2,3]. At this time access to the German manufactured alternative synthetic aniline dyes, the access to, and cost of which was prohibitive, for rather obvious reasons.

Prior to this time, during the Elizabethan era, early fabric dyers in England found the colours of haematoxylin to be 'fugitive'. Even colourisation of hands and shirt collars was readily removed from those working with the dye. As a result and due to the dye's lack of permanency, an Elizabethan prohibition followed which lasted nearly a century. This paved the way for the introduction of the mordant, which imparted a long-lasting permanency for the dye. The extracted haematoxylin was extracted and subsequently oxidised in boiling water to form hematein. The hematein is a complex phenolic compound similar to flavonoid pigments of flowers. There are two basic procedures which convert the haematoxylin to hematein, natural oxidation ('ripening') by exposure to light and air or alternatively chemical oxidation employing either sodium iodate or mercuric oxide and potassium permanganate. The chemical method is much faster and results in instantaneous oxidation.

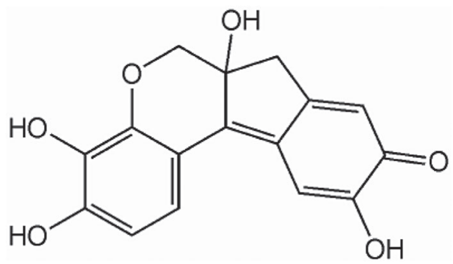
Haematoxylin is a dark blue or violet stain. It is basic and positively charged. It binds to basophilic substances thus it will bind to DNA/RNA, which are conversely acidic in nature and negatively charged. The premise of the interaction between the dyes involves the fact that both DNA and RNA in the nucleus and RNA in ribosomes in the rough endoplasmic reticulum are acidic due to the presence of the phosphate backbones which are integral to the composition of nucleic acids and which are negatively charged. The negatively charged backbones subsequently form salts with basic dyes containing positive charges. Therefore, dyes like haematoxylin will bind to DNA and RNA and stain them violet.

Hematein is anionic with poor affinity for tissue. It therefore requires the presence of a mordant to impart a positive charge to the dye–mordant complex thus enabling binding to anionic tissue components like nuclear chromatin. The word mordant is derived originally from the word *mordere* which is Latin and translated to mean 'to bite'. The French equivalent word is mordre. Mordants are derived from heavy metals such as aluminium, iron, lead, tungsten and molybdenum, etc. [2–4]. They are di/tri valent salts or hydroxides of metals which combine as hydroxides with the dye by displacing a hydrogen atom from the dye (Figure 3). The remaining valences of the mordant serve to attach/bind the dye–mordant complex to the tissue components such as phosphate groups to nucleic acids. The result is a more permanent dye colour [6].

The application of a mordant to the basic oxidised haematin dye extract paved the way for the application of haematoxylin in pathology. It is generally accepted that Waldemeyer is credited with introducing the use of haematoxylin in histological examinations in 1862 [7]. However, debate continues with references to Reichel's work much earlier (1758) [8] with the use of the logwood for microscopic staining, using a simple unmordanted solution to study plant material. Bohmer [9] is generally accepted as the first to introduce the use of an alum mordant in combination with haematoxylin to obtain selective staining in 1864. Other great land marks included the introduction of the iron – alum haematoxylin by Heidenhain (1892) [10], whilst Ehrlich (1886) [11] overcame the instability of haematoxylin and alum by adding glacial acetic acid and thus simultaneously produced his formula for haematoxylin still popularly used to this day.




The molecular structure of haematoxylin prior to 'ripening', via oxidization by natural air and sunlight with boiling water or chemically using either sodium iodate or mercuric oxide and potassium permanganate




The molecular structure of hematein following the oxidization of the haematoxylin molecule.

**Figure 3.** Schematic drawing of the molecular structure of haematoxylin and its subsequent oxidation to form hematein.

It is generally accepted that the hematein forms a complex or 'lake' with the metal iron molecules. The nature of these complexes is still not fully understood. The word 'lake' is in fact derived from the original word 'Lacca'. *Kerria Lacca* is an insect found in India and Thailand, and from which shellac is obtained. The female Lacca insect secrete a deposit on the trees, which is sold as flakes and dissolved in ethanol to make Shellac, popularly used as a wood finish (French polish) and also in nail varnish (chip free). Over time the term lacca or 'lac' has changed to lake and now this is generic term for all dye–mordant complexes [12]. Put simply, a dye lake is a chelate formed from a mordant dye and a metal and is a complex formed between a polyvalent metal ion and dyes.

Haematoxylins are classified according to the mordant they contain and over time there are now a multitude of different types of haematoxylins available, with differing staining qualities for differing tissue types, nearly all named after those who invented or created them. Thus examples include aluminium-based mordant haematoxylins such as Ehrlich's, Mayer's, Harris', Gill's, Delafield's, Cole's and Carazzi's, iron-based mordant haematoxylins such as Weigert's, Heidenhain's, Verhoeff's and Loyez's, tungsten-based mordant haematoxylins such as phosphotungstic acid haematoxylin (PTAH), molybdenum-based mordant haematoxylins including Thomas' and lead-based mordant haematoxylins such as Mallory's.

To this day, haematoxylin remains the most popularly used dye in histology and arguably one of the most influential dyes of all time. Advances in modern day histopathological interpretation have seen the rise of molecular methodology and its influence on the replacement of traditional micro-anatomical histological and cytological studies. Yet it is quite clear that pathological interpretation hinges on the subtleties of the interpretation of the haematoxylin combined with eosin stain (H&E). The main reason that this dye combination has remained so popular within histopathology over time, is that the combination of haematoxylin with eosin produces such a wide range of colour variation which predominantly includes blues, violets and reds.

The name eosin is derived from *eos*, the ancient Greek word for 'dawn' more precisely the Ancient Greek goddess of the dawn. Eosin is a name given to several fluorescent acidic compounds which bind to and form salts which are basic. Compounds like proteins containing amino acid residues such as arginine and lysine will stain dark red or pink as a result of the actions of bromine and fluorescein. There are two main types of eosin. Eosin Y which is the most commonly used form, which produces a slightly yellowish cast and eosin B, which has a slightly bluish cast. Eosin Y is a tetrabromo derivative of fluorescein and eosin B is a dibromo dinito derivative of fluorescein (Figure 4(a) and (b)) [13].

In addition to staining proteins or filaments within the cytoplasm of cells, eosin also stains intracellular membranes as well as staining connective tissue elements such as collagen and muscle. Structures that stain readily with eosin are termed eosinophilic. The combined

**(a)**

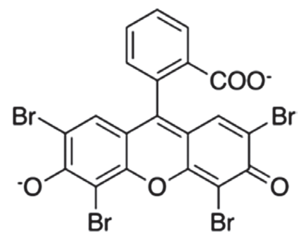



Fig 4a- Eosin Y; a tetrabromo derivative of fluorescein.

**(b)**

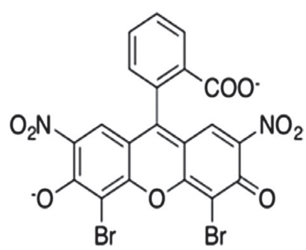



Fig 4b- Eosin B; a dibromo-dinitro derivative of fluorescein

**Figure 4.** Schematic drawings of the molecular structure of eosin Y (a) and eosin B (b).

staining produced is capable of providing a wide range of supportive information including the functional and ultra-structural status of cells. For this reason, H&E staining has remained a gold standard and 'go to stain' for pathologists for many years. The role of improved automation and alternative dye substitutes such as Tango, Newly Blue and Phoenix Blue remains unclear [14]. None however have managed to replace haematoxylin despite the fluctuations and shortages of supply of haematoxylin in the early part of twenty-first century.

Finally, it is worth considering the expanding role of automation within the discipline of tissue sciences. The most popularly employed dye stain combination has readily been adapted for use on automated platforms. This has massively increased the productivity and quality for those laboratories that have embraced these automated procedures. Perhaps more importantly they have enabled the standardisation of staining protocols for routine diagnostic use (Figure 5) [15].

> It is extraordinary that the haematoxylin-eosin stain introduced more than a century ago, has stood the test of time as the standard stain for histologic examination of human tissues. This simple and inexpensive dye combination, if performed well (with a good balance of colours and contrast), can yield enormous amounts of information about the cells their functions or aberrations; otherwise, a lot of diagnostic clues might be missed [16].

This work represents an advance in biomedical science, because it highlights the significant landmarks in the story of how haematoxylin has remained at the forefront of how we assess histopathological tissue.

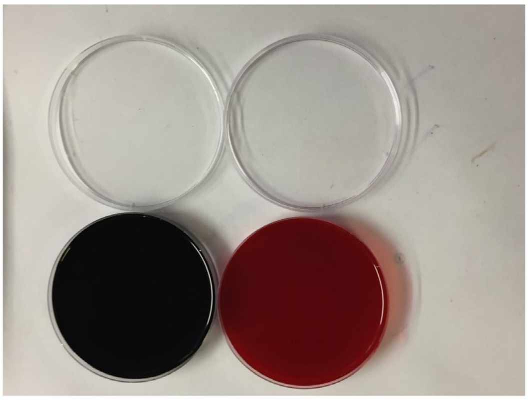


**Figure 5.** Two petri dishes one containing Harris's haematoxylin (left) and eosin Y (right).

## Disclosure statement

No potential conflict of interest was reported by the author.

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