

Review

Hematoxylin Eosin Stain: An Overview

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ABSTRACT

Introduction:

The most widely used stain in histopathology is hematoxylin-eosin (H&E). Though this stain is more than a century old, no other stain has been able to replace it and it is still the most popular stain amongst histopathologists due to its ease of use and excellent contrast it imparts to tissues. Artefacts are appearances that are created falsely and have the capability to interfere with diagnosis if not identified.

Source of Data:

Textbooks of histological techniques along with PubMed database were thoroughly reviewed to obtain all literature pertaining to H & E stain and its associated artefacts.

Growing Points:

This review gives an insight into the general principles of haematoxylin and eosin staining procedure and the common artifacts encountered during staining with their remedy.

KEYWORDS: Mordant, Blueing, Hematoxylin, Eosin, Artefacts

INTRODUCTION

Microscopic examination of tissue is the mainstay for histopathological diagnosis of any disease. Microscopy requires colour contrast for differentiating between tissues. Tissue sections that are unstained can hamper confirmatory diagnosis. Thus, the need for agents that can impart different colours to tissues becomes mandatory. These

agents are called as stains. These staining techniques are based on different chemical reactions that are dependent on the ionic composition of tissues and thus form the basis of staining.

Hematoxylin and Eosin has emerged as a popular stain amongst histopathologists due to its excellent compatibility with a variety of fixatives other than the routinely used

formalin. Another advantage is its capacity to stain the nuclei and cytoplasm in contrasting colours of blue-black and pink respectively. The collagen, extracellular matrix, cytoplasm of cells and red blood cells are stained in varying intensities of pink to orange^{2,3}.

Routinely used stains are usually water soluble and so is H & E. The primary requisite for staining tissue sections with H & E is to rehydrate them as all the water of tissues is removed during processing¹

hematoxylin - Hematein. The production of this Hematein can be achieved by either natural oxidation which is also referred to as ripening as the solution has to be exposed to sunlight for a long time before it becomes suitable as a stain. They have an advantage of longer shelf life, but take a long time to be made and hence good amount of planning is required. The other method of hematein production is by chemical oxidation which as the name suggests utilizes chemical that bring about the conversion very quickly. Chemically oxidised hematoxylin's

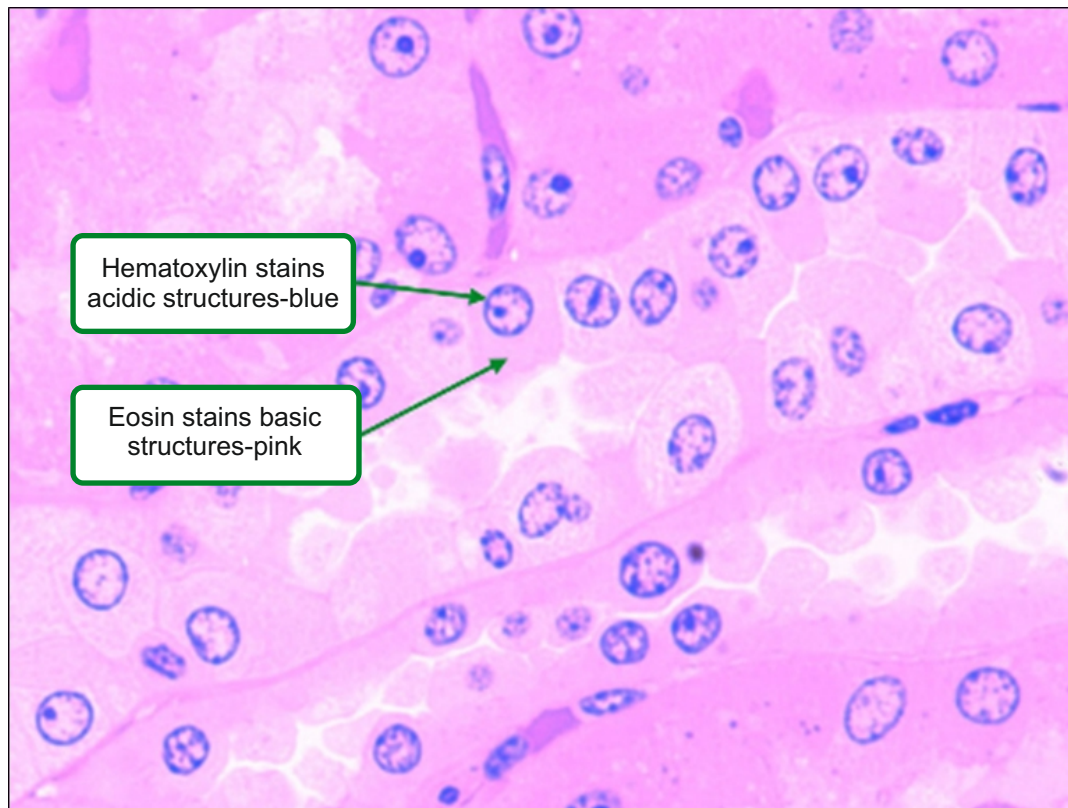


Figure 1: Hematoxylin and Eosin Staining

HEMATOXYLIN

Hematoxylin has affinity for the acidic tissues and hence acts as a nuclear stain. It is obtained from a tree *Haematoxylum campechianum* (*H. campechianum*). The word Hematoxylin is derived from the Greek word “*hematos* meaning blood” and “*xyl*o meaning wood”⁴.

The isolation of hematoxylin crystals from this tree has been attributed to Michel Eugene Chevreul, a French Chemist. He boiled the wood chips in water which gave an orange-red solution which turned black on cooling. This solution was evaporated to obtain the crystals⁵.

The actual staining is achieved by the oxidation product of

use different oxidising agents like sodium iodate (Mayer's hematoxylin), mercuric oxide (Harris's). The chemically oxidised hematoxylin's have a shorter life as they are continuously oxidised by light and air.

Another problem with hematein is its anionic nature, due to which it has low affinity towards tissues and hence further requires the addition of an agent called a “Mordant”. Mordants are substances that help the stain to bind to tissues. These are usually either salts or hydroxides of metals⁶. The most commonly used mordant are salts of aluminium, iron, molybdenum, lead and tungsten. The hematoxylin will always be named according to the mordant it contains.

Blueing

The process of converting the reddish colour imparted to the tissue by hematein into blue colour by changing the pH of the tissue is called blueing. This conversion from the acidic nature of hematein to the basic nature is essential for removing the extra hematein thereby creating space for the counterstain molecules. Tap water is alkaline in nature and hence sufficient to bring about this conversion. Conversely alkalis like lithium carbonate ammonia or "Scott's tap water" can also be used. The process of blueing if done excessively will result in loss of hematein even from the nucleus. Hence proper monitoring is essential for optimum blueing.

Progressive and Regressive Staining

When the hematoxylin and eosin are used sequentially for an optimum period of time so that only selective tissues that have affinity for the stain take up the stain, example Mayer's and Delafied's, Harris' and Gill's Hematoxylin. On the other hand, regressive staining is a technique in which overstaining of tissues is achieved by hematoxylin and then the excess colour is removed using a weak acid. The tissues that have low affinity for hematoxylin lose the stain while those that have strong affinity retain the stain. This is followed by water wash to remove any acid followed by the cytoplasmic stain that is eosin which is taken up by tissues that are free of any stain. E.g., Ehrlich's, Harris' and Delafield's Hematoxylin³. Progressive staining has the advantage that better contrast is achieved but the disadvantage that it has to be very closely monitored and over staining is the most common pitfall. Hence regressive staining is preferred by most histologists.

Commonly used variants of Alum Hematoxylin are Harris, Mayer, Ehrlich, Cole, Gilland Delafield.

Ehrlich's & Delafield Hematoxylin – These are naturally ripened hematoxylin. It deploys the regressive staining schedule. The differentiation is achieved by 1% glacial acetic acid in alcohol. The usual staining time ranges between 10-30 minutes and is primarily dependant on the type of fixative used and the extent of oxidation achieved. More the oxidation less is the time required for staining^{8,9}.

Harris Hematoxylin – This is chemically activated hematoxylin utilising alum as a mordant. It is one of the most commonly used hematoxylin and can be used regressively or progressively¹⁰.

Mayer's Hematoxylin – It is also a chemically oxidised hematoxylin and uses alum as a mordant. It is considered to have high affinity for the nucleus making it more widely chosen compared to the naturally ripened Ehrlich's haematoxylin. It can be used progressively. Staining time of 5-10 minutes is sufficient. It fails to stain mucopolysaccharides and hence is often used as a counter stain while demonstrating the presence of glycogen, amyloid and Mucicarmine. It is also used for

visualization of microfilaria and amoebae¹¹.

Carazzi's Hematoxylin – This employs alum as a mordant and is chemically ripened with the help of potassium iodate. It is also used progressively, but has the disadvantage of the stain fading quickly hence not a very popular stain¹².

Cole's Hematoxylin – It is a chemically ripened hematoxylin utilizing iodine for the ripening. Advantage lies in the fact that it can be used immediately. Its major disadvantages are that it is incompatible for use in immunofluorescence, stain fades very quickly and staining process is time consuming¹³.

Gill's Hematoxylin – This is also an alum hematoxylin. It uses sodium iodate for its chemical ripening. It is more stable than Harries hematoxylin and prevent auto oxidation thereby having a longer shelf life. Its primary drawback is its tendency to show prominent background staining due to its affinity for slide adhesive¹⁴.

EOSIN

It is a fluorescein derived counterstain along with hematoxylin, due to its excellent colour contrast. It is a xanthene dye, acidophilic in nature and has affinity for basic tissues like cytoplasm. It stains collagen, cell cytoplasm and muscle tissue. The colour imparted by eosin ranges from pink to red based on the nature of the tissues.

Two types of Eosins are available based on the technique used for derivation. Eosin Y is also called as eosin Yellow and is obtained by bromination of Fluorescein while Eosin B, known as Eosin Blue as it imparts a bluish tint to the Eosin and is a bromo dinitro derivative of fluorescein^{3,15}.

HEMATOXYLIN AND EOSIN STAINING ARTIFACTS

Artifacts

These are appearances that are artificially produced in tissue sections due to alterations in routine technique. They are very important as they can confuse an inexperienced histopathologist and interfere with correct diagnosis. Artifacts can develop any time from taking of biopsy to staining of the slide. We will be only discussing about artefacts related to H & E Staining¹⁶.

Artifacts related to Residual Wax

The tissue after processing is embedded in paraffin wax for sectioning. If this wax with which the tissue is impregnated is not completely removed it will hamper the dye penetration into the tissue and thus that part of the tissue will prevent proper staining. This part tends to appear cloudy and details of cells will not be appreciable [Figure 2]¹⁷.

Solution: Dip the slide in xylene which is a solvent for xylene and restart the staining again.

Artifact due to Contaminated Staining Solution

Dust particles, gauze fibers and other foreign material can get incorporated into the staining solution which can obscure fine details of tissues by getting superimposed on them.

Solution – Filter staining solution from time to time using a filter paper. Keep the stain in a tightly screw capped bottle [Figure 3]¹⁷.

Artefact related to Acetic Acid

Acetic acid is an integral component of H & E staining and helps to remove hematoxylin from the lower affinity areas of tissue like cytoplasm and collagen. This allows for attachment of eosin to tissues. If the time of exposure of tissue to acetic acid is increased it causes removal of hematoxylin from the high affinity areas like nucleus also. This results in the tissue

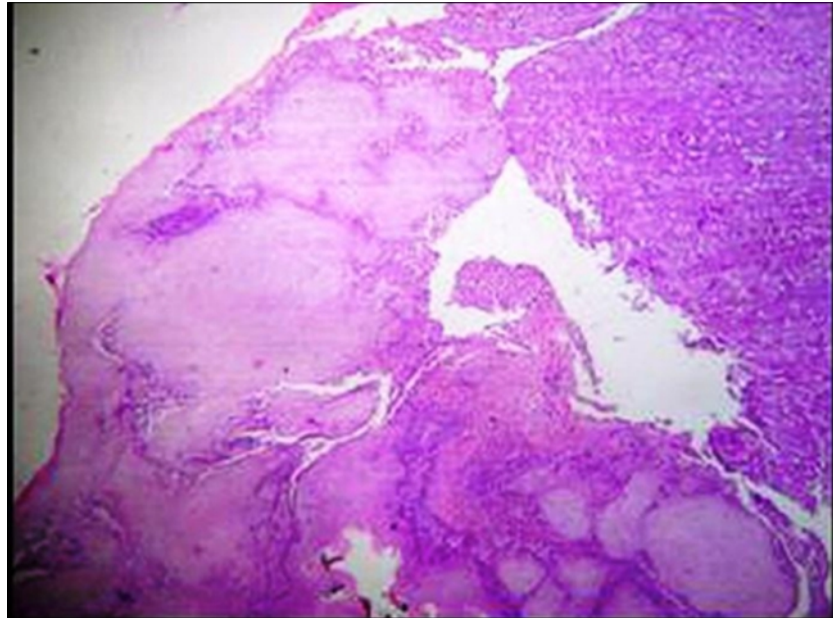


Figure 2: Artefact due to Residual Wax

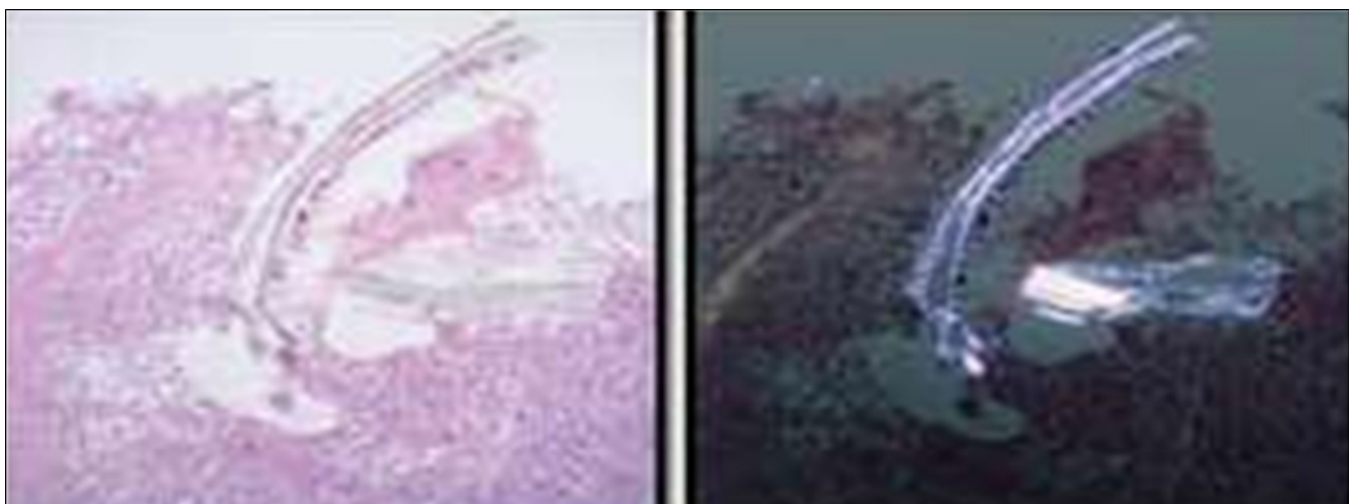


Figure 3: Artefact due to Contaminated Staining Solution under Routine Light Microscopy and Polarized Microscope

appearing as a structure less mass and may be confused as hyaline or amyloid both of which are structure less components of tissues in disease¹⁸.

Solution – Reduce the time for which the tissue is dipped in acetic acid.

Artifact due to Mordant of Hematoxylin

Many Hematoxylin contain aluminium potassium sulphate as a mordent. This aluminium potassium sulphate tends to crystallize if the solution is not mixed properly before staining. These crystals then get deposited on the section while staining and can be confused with the pigment hematin.

Solution – Mixing of haematoxylin regularly followed by filtering, prior to use.

Artifact due to Fluorescent Sheen of Hematoxylin

The hematoxylin stain sometimes develops a metallic sheen on the surface of the stain. This is due to the oxidation of the stain. If the stain is not discarded these oxide particles will deposit on the tissue [Figure 4]^{19,20}.

Solution: Discard the solution and use fresh solution.

CONCLUSION

The Haematoxylin and Eosin staining is the most popular staining technique used in histopathology. Its ease of staining, and excellent contrast makes the visualization of minute details possible. Having a detailed knowledge about the stain, its technique and environmental factors that can interfere with it will help the pathologist identify alterations in the stain and also differentiate staining related artefacts from others.

CONFLICT OF INTEREST: None

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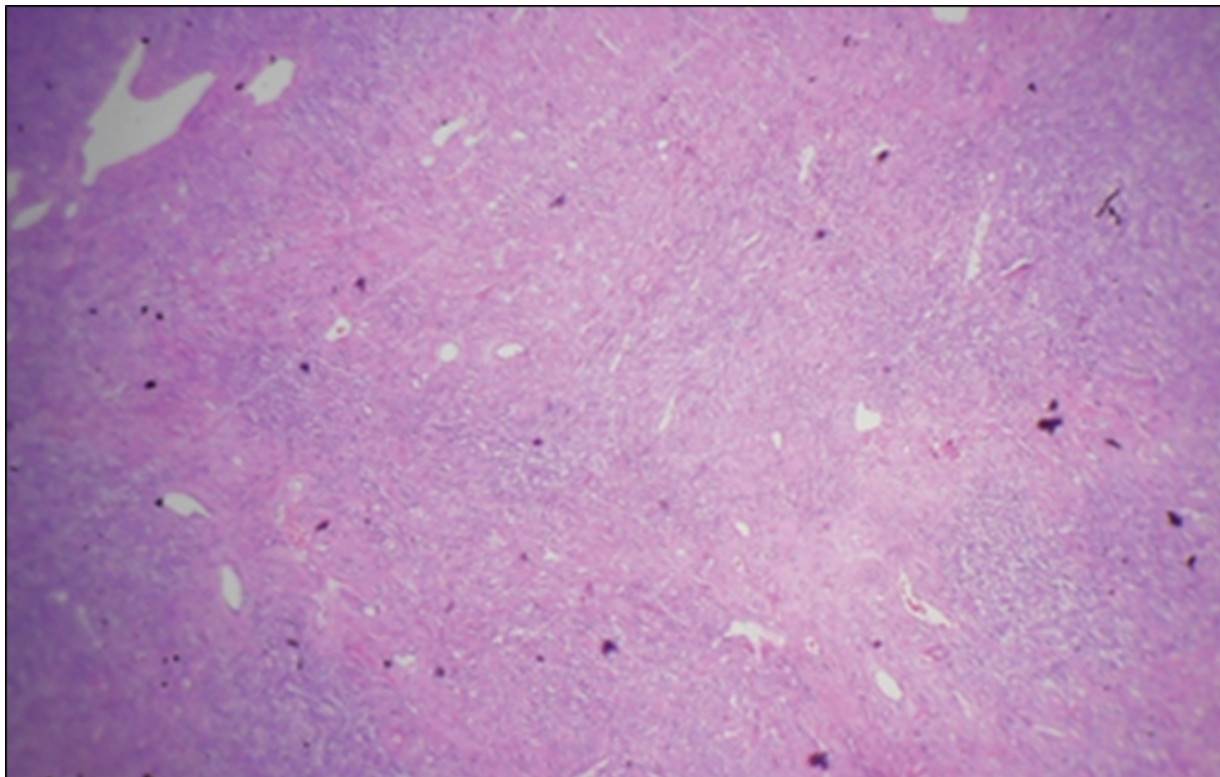


Figure 4: Stain Deposit due to Formation of Fluorescent Sheen in Hematoxylin Solution

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