

The Haematoxylins & Eosin

Objectives

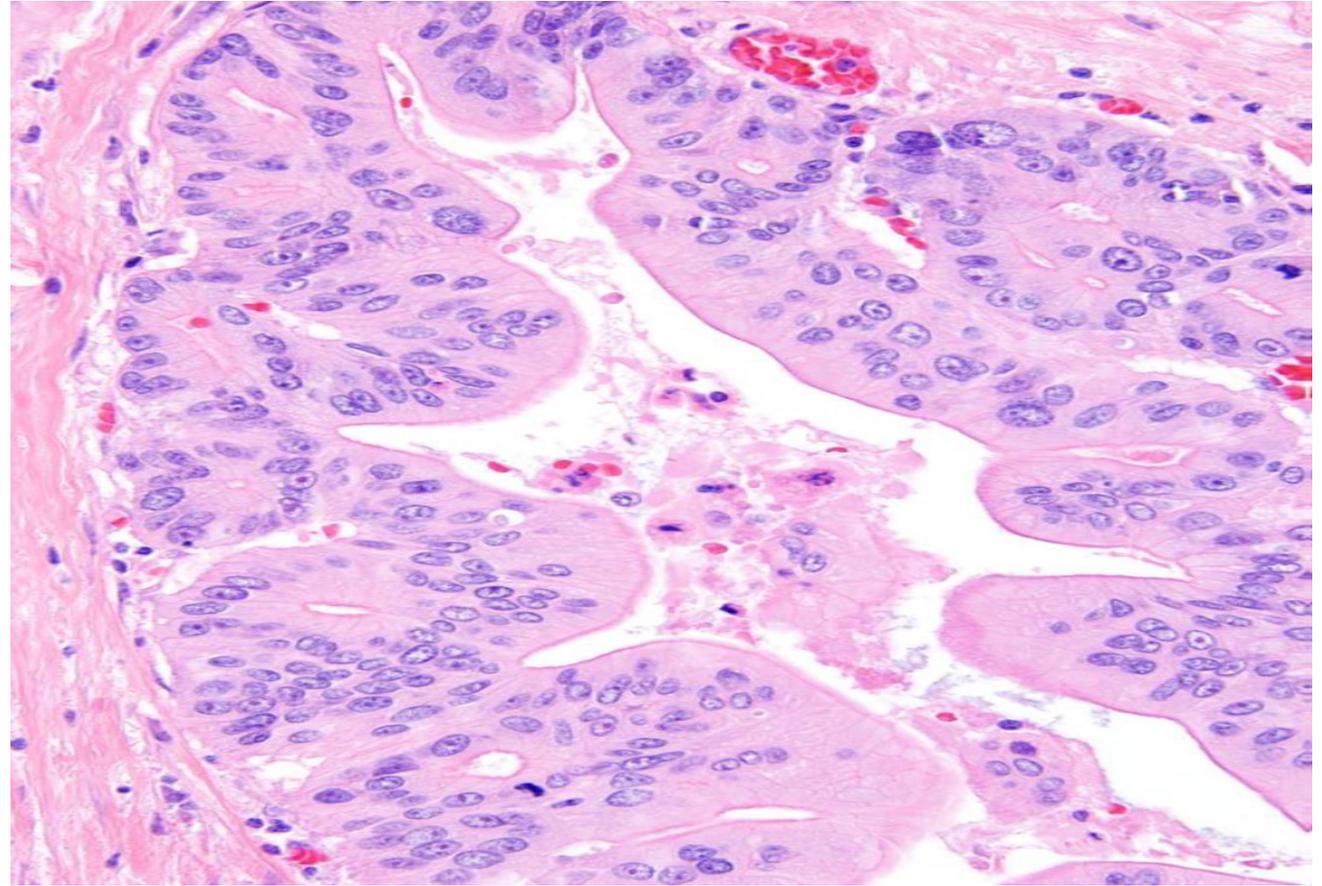
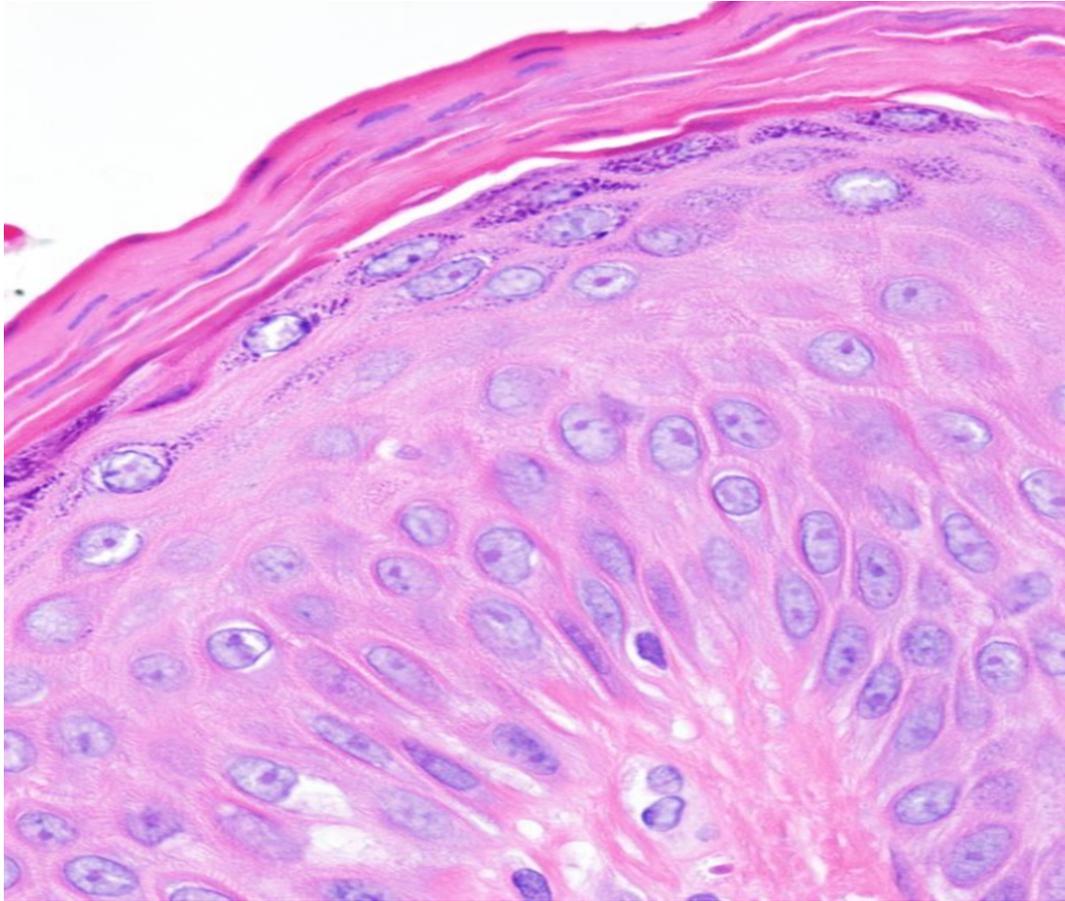
At the end of the lecture, you must be able to:

1. Explain the function of eosin in H &E staining
2. Describe the main properties of haematoxylin and staining using haematoxylin solutions
3. Describe haematoxylin and eosin staining method

Introduction

- H&E stain is the most widely used histological stain.
- Simplicity and ability to demonstrate clearly an enormous number of different tissue structures.
- The haematoxylin component stains the cell nuclei blue-black
- The eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange, and red.

Routine Staining (Hematoxylin & Eosin)



Nuclear detail - Hematoxylin
Contrasting counterstain - Eosin

Eosin Stain

- The eosins are xanthene dyes and have the following types: eosin Y (eosin yellowish, eosin water-soluble), ethyl eosin (eosin S, eosin alcohol-soluble), eosin B (eosin bluish, erythrosin B).
- Eosin Y is the most widely used, and despite its synonym it is also satisfactorily soluble in alcohol.
- Usually used as a 0.5 or 1.0% solution in distilled water (addition of crystal thymol).

Eosin Stain

- Most suitable stain to combine with an alum haematoxylin.
- With proper differentiation, it can distinguish between the cytoplasm of different types of cell, and the different types of connective tissue fibers and matrices, by staining them differing shades of red and pink.

Haematoxylin

- Extracted from the heartwood ('logwood') of the tree *Haematoxylon campechianum*



Haematoxylon campechianum Tree. Insert: leaves on branch.



(A) Logwood, (B) cut end of same logwood and hematoxylin powder.

Hematoxylin..,

- Extracted from logwood with hot water, and then precipitated out from the aqueous solution using urea.
- Haematoxylin itself is not a stain.
- It has to be oxidized, a process referred to as ripening.
- The major oxidization product is hematein, a natural dye that is responsible for the color properties.
- Hematein can be produced from haematoxylin in two ways.

Natural oxidation by exposure to light and air

- Is a slow process (sometimes 3-4 months)
- Resultant solution seems to retain its staining ability for a long time.
- Examples: Ehrlich's and Delafield's haematoxylin solutions.

Chemical oxidation

- Chemical oxidizing agents used are:
 - i. sodium iodate (e.g. Mayer's haematoxylin) or
 - ii. mercuric oxide (e.g. Harris's haematoxylin)
- Converts the haematoxylin to hematein almost instantaneously, so these haematoxylin solutions are ready for use immediately after preparation.

Chemical oxidation..,

- Hematein is anionic, having a poor affinity for tissue, and is inadequate as a nuclear stain without the presence of a mordant.
- The most useful mordants for haematoxylin are salts of aluminum, iron, and tungsten.

Classification

- Haematoxylin solutions can be classified according to which mordant is used:
 - Alum haematoxylin
 - Iron haematoxylin
 - Tungsten haematoxylin
 - Molybdenum haematoxylin
 - Lead haematoxylin

Alum Haematoxylins

- Comprises most of those that are used routinely in the H&E stain, and produce good nuclear staining.
- The mordant is aluminum, usually in the form of 'potash alum' (aluminum potassium sulfate) or 'ammonium alum' (aluminum ammonium sulfate).
- Can be used *regressively* or *progressively* (usually used regressively)

Alum Haematoxylin..,

- All stain the nuclei a red color, which is converted to the familiar blue-black when the section is washed in a weak alkali solution → process called blueing.
- Blueing agents typically are alkaline with a pH range of 7.5-9.0 optimally.
- Blueing is done by the following methods:
 - Running tap water for several minutes
 - Treating the section by Scott's tap water (pH is 8): 2–3 min
 - Ammonium hydroxide (5%): 2–3 min
 - Ammonia vapour: few seconds

Alum Haematoxylin..,

- Examples of alum haematoxylin:
 - Ehrlich`s hematoxylin
 - Delafield`s hematoxylin
 - Cole`s hematoxylin
 - Mayer`s hematoxylin
 - Harris`s hematoxylin
 - Gill`s hematoxylin
 - Carazzi`s hematoxylin

Staining times with alum haematoxylin

Cole's	20–45 min
Delafield's	15–20 min
Ehrlich's (progressive)	20–45 min
Mayer's (progressive)	10–20 min
Mayer's (regressive)	5–10 min
Harris's (progressive in cytology)	4–30 s
Harris's (regressive)	5–15 min
Carazzi's (progressive)	1–2 min
Carazzi's (regressive)	45 s
Carazzi's (frozen sections, see text)	1 min
Gill's I (regressive)	5–15 min

Iron Haematoxylin

- In these hematoxylin solutions, iron salts are used both as the oxidizing agent and as mordant.
- The most commonly used iron salts are ferric chloride and ferric ammonium sulfate, and the most common iron hematoxylin are:
 - Weigert's hematoxylin
 - Heidenhain's hematoxylin
 - Loyez hematoxylin
 - Verhoeff's hematoxylin

Iron Haematoxylin.,

- Over-oxidation is a problem with these stains.
- Mordant/oxidant and haematoxylin solutions are prepared separately and mixed before use.
- Are capable of demonstrating a much wider range of tissue structures, but the techniques are more time-consuming and needs microscopic control for accuracy

Tungsten's haematoxylin

- Widely used Tungsten's haematoxylin is PTAH (Phosphotungstic acid haematoxylin)
- Can be prepared using chemical oxidation or natural ripening of the tungsten hematoxylin solution in light and air.
- Used to demonstrate astrocytes, neuroglia fibers, muscle striations, collagen, reticulin, fibrin
- Widely used as a CNS stain and general tissue structure

Tungsten's haematoxylin

Results

Tissue component	Colour
Muscle striations, neuroglia fibers, fibrin, amoeba	dark blue
Nuclei, cilia, RBC	blue
Myelin	lighter blue
Collagen, osteiod, cartilage, elastic fibers	deep brownish red
Cytoplasm	pale pinkish brown

Lead Haematoxylin

- Incorporate lead salts as mordants.
- Used in the demonstration of the granules in the endocrine cells of the alimentary tract and other regions.
- Also used in the localization of gastrin-secreting cells in stomach.

Table 10.2 The uses of hematoxylin stains

Hematoxylin	Applications	Oxidant	Mordant
Ehrlich	Nuclear stain used with eosin. Stains some mucins	Natural	Alum
DeLafield	Nuclear stain used with eosin	Natural	Alum
Mayer	Nuclear stain used with eosin. Nuclear counterstain	Sodium iodide	Alum
Harris	Nuclear stain used with eosin	Mercuric oxide	Alum
Cole	Nuclear stain used with eosin	Iodine	Alum
Carazzi	Nuclear stain used with eosin (used with frozen sections)	Potassium iodate	Alum
Gill	Nuclear stain used with eosin	Sodium iodate	Alum
Weigert	Nuclear stain used with acid dyes	Natural	Iron
Heidenhain	Intranuclear detail, muscle striations	Natural	Iron
Verhoeff	Elastic fibers	Natural	Iron
Loyez	Myelin	Natural	Iron
Mallory PTAH	Fibrin, muscle striations, glial fibers	Natural	Tungsten
Thomas	Collagen, endocrine cell granules	Hydrogen peroxide	Molybdenum
Solcia	Endocrine cell granules	No oxidant	Lead
Mallory	Iron, copper, lead	No oxidant	No mordant
Weigert-Pal	Myelin (in block preparation)	No oxidant	Chromium- copper

Major Steps in the Routine H&E

1. **De-paraffinisation** – (removal of paraffin wax using xylene)
2. **Rehydration**- (through descending grades of alcohol to water)
3. **Nuclear staining**- using haematoxylin (progressive or regressive)
4. **Wash well in running tap water** (5mins or less)
5. **Differentiation**- acid alcohol (5-10mins)
6. **Bluing**-Ammonia water (10-15mins)
7. **Counterstaining**- using eosin
8. **Wash in running tap water** for 1–5 minutes.
9. **Dehydration**- (application of graded alcohol to 100% alcohol)
10. **Clearing**- xylene (transition from alcohol to non aqueous reagents)

De-paraffinising

N.B The slides where first heated before staining to help bind the tissue to the slide and melt away excess wax.

- However some paraffin still remain and must be removed so that the aqueous solution (e.g. staining solutions) may reach and penetrate tissue components.
- This is accomplished by submersion of slides in xylene which dissolves and removes the excess wax.

Rehydration

N.B. The xylene must be removed since it is also not miscible with aqueous solutions. Its removed by concentrated alcohol (100% absolute alcohol).

- The slides are immersed in descending grades of alcohol and after that in water, until they reach a point where they can enter purely aqueous solutions.
- Most staining techniques move from 100% absolute alcohol to 95% up to 70% alcohol. And then placed in water since alcohol and water are miscible.

Nuclear staining

N.B After rehydration. The slides are able to be stained with the water based hematoxylin solutions, since paraffin was removed and the xylene.

- The slides are immersed in the haematoxylin solution.
- The staining times are established in each lab.

Wash well in running tap water

- Helps to remove excess dye.

Differentiation

N.B If staining is done regressively. This step is important to remove excess stain.

- The slides are immersed in the acidic differentiating solution e.g. 0.3% or 1% acid alcohol.
- The timing of immersion must be established for each lab and procedure so that optimal differentiation is achieved.
- This step is halted by a water rinse.

Bluing

N.B Haematoxylin stains the nuclei a red color, hence this step is important to convert the color to blue-black.

- Bluing is the process of shifting the colour from reddish to blue-black by the application of a weak alkaline solution.
- Ammonia water (ammonium hydroxide and water) is often used, alternatively running tap water (≈ 10 minutes) can be used if pH is suitable.

Counter staining

- Eosin is used to contrast cytoplasm with the nucleus.
- Eosin stains provide contrast to the nuclear stain and shows many cytoplasmic and tissue elements.

Wash in tap water

- To remove excess eosin

Dehydration

N.B Important to remove water since some mounting media are non aqueous.

- This is achieved by using ascending grades of alcohol (e.g. starting from 70% to 100% alcohol).
- Complete dehydration is important to remove any remaining water.
- If incomplete it can result in a cloudy appearance to the final slide, since water is also not miscible with xylene.

Clearing

- Just like in processing xylene is used as a transition between aqueous and non – aqueous.
- Xylene is miscible with the non-aqueous mounting media, but will cause a cloud to form on the final slide if any water is present.
- Xylene is also important at this stage due to its refractive index which produces a clear slide that allows light to pass well under the microscope.
- After clearing mount in a suitable mounting media

Results

- Nuclei blue/black
- Cytoplasm varying shades of pink
- Muscle fibers deep pink/red
- Red blood cells orange/red
- Fibrin deep pink

Troubleshooting in Haematoxylin Staining

Problems	Possible causes	Remedies
Pale-stained nuclei	<ol style="list-style-type: none"> 1. Too much differentiation 2. Too less time in haematoxylin 3. Due to excessive decalcification 4. Haematoxylin is over oxidized 	<ol style="list-style-type: none"> 1. Stain in haematoxylin again 2. Keep in haematoxylin for longer duration 3. Not possible to correct 4. Change the haematoxylin solution
Darkly stained nuclei	<ol style="list-style-type: none"> 1. Too short differentiation 2. Too much time in haematoxylin 3. Thick section 	<ol style="list-style-type: none"> 1. Decolorize and do optimum differentiation 2. Decolorize and give appropriate time in haematoxylin 3. Recut thin section
Nuclei looks reddish brown	<ol style="list-style-type: none"> 1. Insufficient bluing 2. Haematoxylin is degenerating 	<ol style="list-style-type: none"> 1. Restain by giving more time in bluing step 2. Check the oxidation status of haematoxylin
Pale-coloured cytoplasm by eosin	<ol style="list-style-type: none"> 1. Too thin section 2. The eosin solution has pH more than 5 3. Too much dehydration of the section in alcohol 	<ol style="list-style-type: none"> 1. Recut the section properly 2. This may be due to dilution of eosin by the carryover bluing solution. Check pH of eosin solution, and if necessary, adjust pH by adding acetic acid 3. Do not keep the slide in alcohol for a long time
Cytoplasmic staining is very dark	<ol style="list-style-type: none"> 1. Long duration in eosin solution 2. Overconcentrated eosin solution 3. Very quick dehydration in alcohol 	<ol style="list-style-type: none"> 1. Keep the section in eosin for shorter duration 2. Make optimally diluted eosin solution 3. Increase time duration in dehydration
Bluish-black precipitate	It may be due to precipitation of haematoxylin	Filter the haematoxylin staining solution
Staining is irregular and spotty	Improper deparaffinization	Keep the slide in xylene for longer time to remove the paraffin
Dark-blue stain at the edge of the tissue sections	Due to heating artefact for using electrocautery	No solution
Water bubbles in the sections	Incomplete dehydration	Remove the mounting medium and coverslip. Keep the section in absolute alcohol for dehydration. Do several changes and then remount
Milky section after the xylene rinse before putting the coverslip	Incomplete dehydration	Change the alcohol solution. Please dehydrate the section properly before putting in xylene

References

- Bancroft JD & Stevens, **Theory & Practice of Histological Techniques**, 2nd Ed.
- Cook H C, **Manual of Histological Demonstration Techniques**.
- Carson FL, **Histotechnology: A self-Instructional Text**, 3rd Ed.