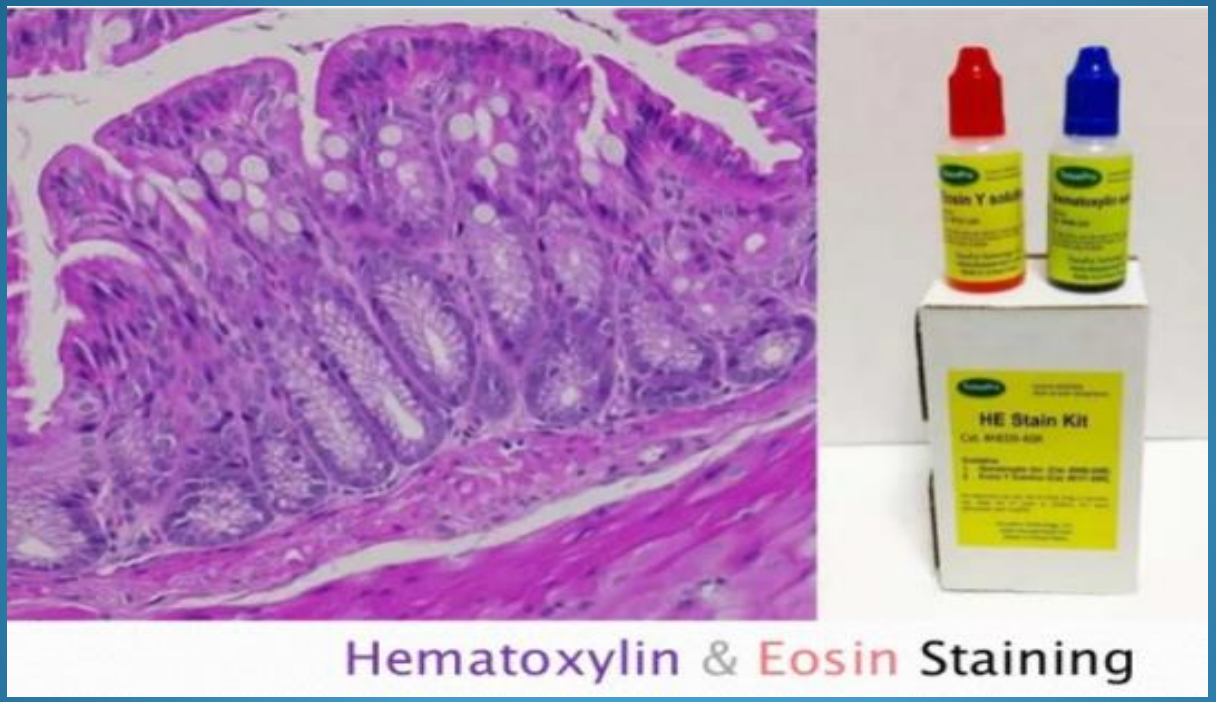


BY-
DR. SAUMITRA MAHENDRA
MD PATHOLOGY

Introduction

The Hematoxylin and Eosin stain (H&E) is the most widely used histological stain because :

- comparative simplicity
- Ability to demonstrate clearly an enormous number of different tissue structures.
- Hematoxylin stains cell nuclei blue black → shows good intranuclear detail.
- Eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange, and red.

Basics of Staining

- Stains → chemical substances used to achieve visible color contrast in the microscopic picture of a prepared tissue

- Staining → treating tissues or cells with a series of reagents so that it acquires a color; no particles of dye are seen and the stained element is transparent.

Purpose of Staining

- Outlines tissues and cellular components.
- Identification of tissues.
- Establishes the presence or absence of disease processes.

Common Staining Methods

- Most commonly used staining methods are –
- **Hematoxylin and Eosin staining in Histopathology**
- **Gram's Stain and Ziehl–Neelson staining in Microbiology.**
- **Romanowsky staining in Hematology.**
- **Papanicoloau staining in Cytology**

Dyes

- Essentially Aromatic benzene ring compounds or derivatives that possess the twin properties of color and ability to bind to tissue.

Dyes & its classification

Categories based on	Examples
Origins	Natural (Hematoxylin & Carmine) Synthetic Aniline
Physiochemical Properties	Fluorescent (acridine orange) Leuco (Leuco methylene blue) Metachromatic (toluidiene blue) Neutral (azure-eosinate)
Structure	Azo (orange) Metal complex (Al or Fe complexes of hematein) Xanthene (pyronine Y)
Use in biological staining	Fat (oil red O) Flourescent probe(YOYO-1) Mucin (alcian blue)
Use in textile dyeing	Acid (eosin) Basic (safrarine) Direct (Congo Red)
Supposed mode of action of dye	Mordant (Gallacyarine chrome alum Reactive(mercury orange)

Dyes & its classification

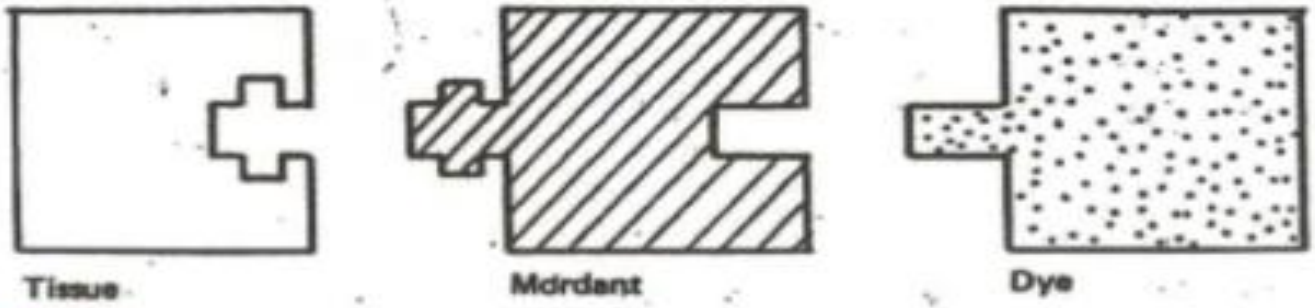
Acidic Dyes	**Stains basic components such as cytoplasm, acidiophil granules, etc** **Eosin, Acid Fuchsin**
Basic Dyes	Stains acidic components such as nucleus, basophil granules,etc. Hematoxylin, Basic Fuchsin, Methylene Blue.
Neutral Dyes	Consists of mixtures of basic and acidic dyes. Both cations and anions contain chromophoric groups and both have colored radicals. Romanowsky dyes formed by interaction of polychrome methylene blue and eosin

Mordant

- A polyvalent metal ion which forms coordination complexes with certain dyes.
- A substance which acts as an intermediary between dye and tissue, thus increasing the affinity between them.
- Strictly applicable to salts and hydroxides of divalent and trivalent metals.
- Should not be used to indicate any substance that improves in staining in some other manner (accentulators and accelerators)

Mordant

- The mordant dye complex 'lake' combines with tissue to form tissue-mordant-dye complex, which is insoluble.



Mordant- Dye Applications

- Mordant is applied first, followed by the dye.

e.g Heidenhain's iron hematoxylin

- Mordant and dye are mixed together and then applied.

Commonly done in histotechnology

e.g Alum hematoxylin solutions

- Dye applied first, followed by the mordant.

Hardly done in histotechnology.

Accentuators

Accentuators:

- Substances which increase the staining power of dye.
- They increase the intensity & selectivity of stain.

e.g KOH in Lofflers methylene blue
phenol in carbol fuschin & carbol thionin.

Accelerators

- Accentuators used in metallic impregnation technique for the nervous system.

e.g chloral hydrate

Trapping agents

- Agents which holds dye combination with tissue or bacteria .

e.g tannic acid/iodine

Types of Staining reactions

- Absorption or direct staining – tissue penetrated by dye solution.
- Indirect staining – using intermediate treatment with mordant
- Physical staining – simple solubility of dye in element of tissue.
- Chemical staining – formation of new substance e.g. PAS
- Adsorption phenomenon – accumulation on the surface of the compound.

Types of Staining Methods

- Vital
- Routine
- Special

- Regressive
- Progressive

Types of Staining Methods

Vital Staining	**Applied to living tissue** **Accomplished by injecting staining solution into some part of the body** **Mixing of stain with living cells** **Primarily used for research.**
Routine Staining	Stains tissues with minimal differentiation except between nucleus and cytoplasm. Demonstrates general relationship among cells, tissues and organs. e.g Hematoxylin and Eosin stains
Special Staining	Demonstrates selective features of tissues : Particular cell products. Microscopic intracellular and intercellular structure. e.g PAS stain for mucopolysaccharide.

Types of Staining Methods

Regressive Staining	·Tissue is initially overstained and then partially decolorized (differentiated) until the proper endpoint is reached. · Sharper degree of differentiation is obtained ·The differentiation is controlled visually by microscopic examination. ·Faster and more convenient .
Progressive Staining	•Tissue is stained for a predetermined time for adequate staining of the nuclei and leaves the background tissue relatively unstained. •Once the dye is taken up by the tissues, it is not removed. •The tissue is left in the dye solution until it retains the desired amount of coloration. •The differentiation solely relies on the selective affinity of dyes for different tissue elements.

Differentiation

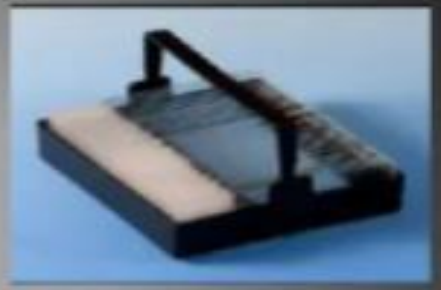
- Removal or washing out of excess stain until the color is retained only by the tissue component that is to be studied.
- Done with acid alcohol or ethyl alcohol
- Exposure to air may oxidize and improve the process.

Requirements for Staining

- All glassware should be thoroughly cleaned.
- Correct solvent should be used.
- Silver and osmic acid solutions should be kept in dark bottles.
- Solutions like dilute ammonia should be freshly prepared.
- Constituents of stain dissolved should follow the formula.
- Alcoholic solutions of the stain should be kept in dark stoppered bottles.
- All dyes should be filtered before use.

Armamentarium in staining

- Specially designated bench
- Staining bench should be facing the window.
- Slide washing tray made of stainless steel.
- Bunsen burner to heat up the stain.
- Thermostatically controlled hot place to melt the wax.
- Microscope to control staining reaction.



Armamentarium in staining

Slides are stained using –

- Using staining dishes
  - Small grooved coplin jars with lids.
  - Large staining troughs

- Using staining racks
  - 2 pieces of stout rods 2-4 cm apart.

- Using staining machine
  - Same as processing machine but carry slide racks.

Hematoxylin

- The word Hematoxylin is derived from old Greek word Haimato meaning blood & Xylon meaning wood.
- A natural dye extracted from the core or heartwood of tree Haematoxylon campechianum.
- The hematoxylin is extracted from logwood with hot water, and then precipitated out from the aqueous solution using urea.
- The major oxidization product of hematoxylin is hematein, a natural dye that is responsible for the color properties.

The History of Hematoxylin

- Waldeyer firmly established the use of hematoxylin in histology in 1862.
- Bohmer combined hematoxylin with alum as a mordant and obtained more specific staining in 1864.
- Heidenhan introduced his classical Iron-Alum- Hematoxylin method used as a standard technique in cytology.
- Ehrlich overcame the instability of hematoxylin and alum by adding glacial acteic acid and simultaneously produced his formula for hematoxylin used today.

Ripening

- The process of oxidation of hematoxylin.
- The major oxidization product is hematein, a natural dye that is responsible for the color properties.
- It is a poor dye but metallic mordant and forms the most powerful stain.
- Carried out in 2 ways –
  - Natural oxidation.
  - Chemical oxidation.

Ripening

Natural Oxidation

- Carried out by exposure to light and air
- Slow process – takes about 3–4 months
- Resultant solutions seem to retain its staining ability for a long time.

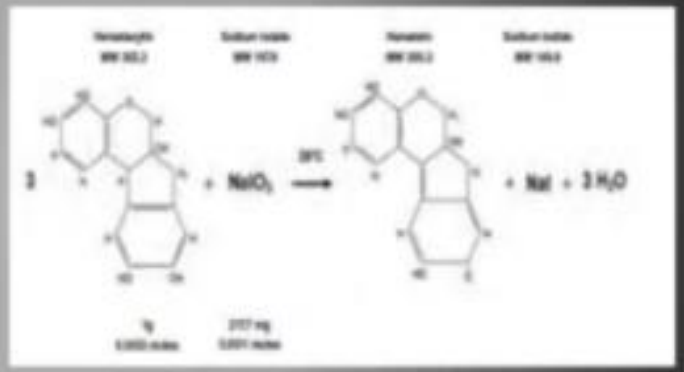
Advantages	Disadvantage
Once oxidation has reached an acceptable level, the staining solution may be used and it lasts for longer.	•Requires a considerable period of time. •Two batches of naturally ripened product may not produce the same staining qualities.

e.g Ehrlich's & Delafield's hematoxylin solutions

Ripening

Chemical Oxidation

- Achieved by addition of oxidizing agents such as mercuric oxide, sodium iodate and potassium permanganate.
- This process converts the hematoxylin → haematin almost instantaneously, so these hematoxylin solutions are ready for use after preparation.



Ripening

Properties of chemically oxidized hematoxylin

- Shorter useful life than the naturally oxidized haematoxylins.
- The process of over-oxidation of hematoxylin has established that the production of oxyhaematein inhibits successful staining.
- Glycerol has been incorporated in many formulas as it's a stabiliser to prevent over-oxidation and prevent evaporation.

Ripening

Properties of chemically oxidized hematoxylin

- Haematein is anionic, having a poor affinity for tissue, and is inadequate as a nuclear stain without the presence of a mordant.
- The mordant/metal cation confers a net positive charge to the dye-mordant complex and enables it to bind to anionic tissue sites, such as nuclear chromatin.
- The type of mordant used influences strongly the type of tissue components stained and their final color.
- The most useful mordants for hematoxylin are salts of aluminum, iron, and tungsten, although hematoxylin solutions using lead as a mordant are occasionally used in the demonstration of argyrophil cells).

CONT.....