NUCLEAR AND CYTOPLASMIC STAINING

Presented by:
Jacqueline Brooks
MB, HTL, SCT (ASCP) Q IHC, CM IAC
Cunningham Pathology
jbrooks@cunninghampathology.com
Housekeeping

To receive credits you must complete the following:

- View complete presentation
- Pass the Quiz with a 70% or higher score
- Fill out the Evaluation Form for the presentation
- Sign the hard copy roster provided by your laboratory manager

Visit www.auroradx.com/edutraining to access this presentation again, download a PDF of it, take your quiz and fill out your evaluation form as well as obtain instructions on how to report credits to NSH

Encourage your teammates to participate and become the top-notch facility in the Aurora Diagnostics family to show case the best well-trained laboratorians and recognized for excellence in performance.
Cell Morphology
Cell Membrane

The outer boundary of the cell. The cell membrane helps control what substances enter or exit the cell. It is composed mainly of protein and lipid molecules.
Endoplasmic Reticulum

**Endoplasmic reticulum**
The Endoplasmic reticulum is a network of membranes in eukaryotic cells which helps in control of protein synthesis and cellular organization. A network of interconnected membranes forming sacs and canals. Transports materials within the cell, provides attachment for ribosomes, and synthesizes lipids.
Golgi apparatus

Intracellular stack of membrane bounded vesicles in which glycosylation and packaging of secreted proteins takes place.
Ribosomes

Particles composed of protein and RNA molecules. Function to synthesize proteins.
Mitochondria

A small intracellular organelle which is responsible for energy production and cellular respiration.
Lysosomes

A class of morphologically heterogeneous cytoplasmic particles in animal and plant tissues latency of these enzymes. The intracellular functions of lysosomes is characterised by their content of hydrolytic enzymes and the structure-linked is depend on their lytic potential. The single unit membrane of the lysosome acts as a barrier between the enzymes enclosed in the lysosome and the external substrate. The activity of the enzymes contained in lysosomes is limited or nil unless the vesicle in which they are enclosed is ruptured. Such rupture is supposed to be under metabolic (hormonal) control.
Peroxisomes

LYSOSOMES AND peroxisomes, THE CELL'S "DIGESTIVE SYSTEM" When a white blood cell engulfs a bacterium and destroys it, the white cell's lysosomes do most of ..."
Centrosome

An organelle located in the cytoplasm of all animal cells and many plants, fungi, and protozoa that controls the polymerization, position, and polar orientation of many of the cell's microtubules throughout the cell cycle.
Cilia and Flagella

The flagella is a tail like appendage that propels the cell. Both the *cilia* and *flagella* are attached to the outside of the cell.
Microfilaments and Microtubules

The cytoskeleton (also CSK) is a cellular "scaffolding" or "skeleton" contained within the cytoplasm and is made out of protein. The cytoskeleton is present in all cells; it was once thought to be unique to eukaryotes, but recent research has identified the prokaryotic cytoskeleton. It has structures such as flagella, cilia and lamellipodia and plays important roles in both intracellular transport (the movement of vesicles and organelles, for example) and cellular division.
nuclear envelope The double membrane that separates the nucleoplasm (see nucleus) of a cell from the cytoplasm. The membranes consist of lipid bilayers that are separated by a perinuclear space (or compartment). The outer membrane is continuous with the rough endoplasmic reticulum and is structurally and functionally distinct from the inner membrane. The envelope is perforated at intervals by nuclear pores, which provide a channel for the selective transfer of water-soluble molecules between the nucleus and the cytoplasm. Each nuclear pore is surrounded by a disc-shaped structure (nuclear pore complex) consisting of an octagonal arrangement of eight protein granules.
Nucleolus

A small dense body (sub organelle) within the nucleus of eukaryotic cells, visible by phase contrast and interference microscopy in live cells throughout interphase. Contains RNA and protein and is the site of synthesis of ribosomal RNA. The nucleolus surrounds a region of one or more chromosomes (the nucleolar organiser) in which are repeated copies of the DNA coding for ribosomal RNA.
Chromatin

Stainable material of interphase nucleus consisting of nucleic acid and associated histone protein packed into nucleosomes. Euchromatin is loosely packed and accessible to RNA polymerases, whereas heterochromatin is highly condensed and probably transcriptionally inactive.
Atypical adenomatous hyperplasia of the prostate

Loss of structural differentiation within a cell or group of cells often with increased capacity for multiplication, as in a malignant tumor.
Metastasis
Cause or Causes of Cancer
H&E Stain

1. Fixation
2. Processing
3. Embedding
4. Microtomy
5. Slide Drying
6. Deparaffinization
7. Hydration
8. Staining (Hematoxylin, differentiation, bluing, Eosin)
9. Dehydration
10. Clearing
11. Coverslipping
Skin
Skin

keratinized squamous epithelium
Skin

Nucleus open chromatin
STAINING MECHANISMS

Most staining reactions involve both physical and chemical factors. The fat stain is an example of a purely physical stain, with the dye absorbed (soaked up) by, and dissolved in, the lipid.

Chemical bonding of the dye to the tissue:

Ionic or electrostatic bonding occurs when the dye and the substance to be dyed develop different charges and thus become attracted to each other.

Hydrogen bonding occurs when covalently bonded hydrogen is attracted to atoms that have a strong electronegative charge.

Covalent bonding occurs when atoms share electrons.

Van der Waals forces are caused by the electrostatic attraction of a molecule to the electrons of its neighboring molecules.
Skin / RBC’s
Prostate Bx High power

Nucleus open chromatin
NUCLEAR STAINING

(DNA in the nucleus gives it a net negative charge)

Nuclear staining is not fully understood, but apparently occurs through 2 different mechanisms.

• Staining done with the basic (cationic or positively charged) dyes

• Staining done with dyes combined with, or followed by, metal mordants

Basophilic – An acidic (anionic, negatively charged) substance that is easily stainable with basic (cationic, positively charged) dyes
Prostate Bx low power

Open gland

Open gland
Cytoplasmic Staining

- (proteins are in the cytoplasm giving it a net positive charge)
- Acidophilic – A basic (cationic, positively-charged) substance that is easily stainable with acid dyes. An example is cell cytoplasm, which is readily stainable with the acid (anionic, negatively charged) dye eosin.
- If the eosin solution is not below pH 6, the eosin will not attach to the cytoplasmic proteins because above pH 6, the proteins will have a net negative charge.
- IEP (isoelectric point) (the point where the positive and negative charges are equal) The IEP of proteins is approximately pH 6; below the IEP or below pH 6, the net charge on the nonnuclear proteins is positive and the attraction is for an anionic dye above the IEP, the net charge is negative and the attraction is for a cationic dye.
Prostate bx low power normal
The Dyes

- **Chromophore**—The chemical grouping that bestows the property of color on a compound

- **Chromagen** – A benzene derivative containing a color-bearing group, or chromophore

- **Auxochrome** – The chemical group present in a dye that causes it to bind to certain tissue elements. This group can develop a charge (+ or -) and thus bind to oppositely-charged groups present in the tissue. The amino (-NH2) and the carboxyl (-COOH) groups are frequently occurring auxochromes.

- **Amphoteric** – Describes a substance that is capable of acting as either a base or an acid, depending on the pH of the solution.
Breast tissue
Poorly Diff breast ductal CA

- Stroma
- Crowded nuclei
- Cells filling duct
Poorly Diff Ductal CA / breast

Irregular cells filling duct
Factors Affecting Dye Binding

• pH
• temperature
• concentration
• salt content
• fixative (formalin reacts with the NH2 group, because this is the primary group for binding eosin, tissue fixed in formalin will bind less eosin than when fixed in some of the other solutions.)
Gallbladder

- Lumen
- RBC’s
- Glandular cells
- Smooth muscle
Gallbladder

- RBC's
- Glandular Cell
- Lumen
Gallbladder low power

- Serosal surface
- Mucosal surface
- RBC’s
- Smooth muscle
- Lumen
The Nuclear Dyes

- Hematoxylin – the most widely used nuclear stain, it is extracted from logwood a tree indigenous to Central America. The freshly cut wood is colorless but becomes dark reddish brown when exposed to atmospheric oxidation; the oxidized dye is hematein. Synthetic hematoxylin is also available but most laboratories still use solutions prepared from the natural product.

- It is very important to remember that hematoxylin is not a dye; hematein, the oxidation product of hematoxylin, is a weak anionic dye. Oxidation of hematoxylin is necessary and may be achieved naturally by exposing the solution to atmospheric oxygen, or by using oxidizing agents such as sodium iodate, mercuric oxide, and potassium permanganate; this oxidation process is also called ripening. Solutions should always contain some un-oxidized hematoxylin because the process of ripening continues with atmospheric oxidation, and complete oxidation or over oxidation lead to a breakdown of the solution and the loss of good staining.

- Oxidized hematoxylin (hematein) has little affinity for tissue but becomes a strong dye with a particular affinity for nuclei when combined with a metallic mordant.

- The mordant-dye combination is called a lake, and the most commonly used hematoxylin lakes are combinations of hematein with either aluminum or iron.
Hematein vs Hematin

• Hematein – The oxidation product of hematoxlin and the active staining ingredient in hematoxylin solutions. Hematein is the active dye formed by the action of either light and air or a chemical oxidizing agent on hematoxylin.

• Hematin- Granular, brownish-black crystalline deposit occurring in tissue. Hematin is the term commonly used to denote formalin pigment, formed by the action of acidic formaldehyde on blood-rich tissue, but malarial pigment and acid hematin are also hematins.
Appendix low power

- Loose Connective Tissue
- Serosal Surface
- Mucosal surface
- Lymphocytes
Appendix / lymphocytes

Different stages of maturation

Mature lymphocyte

Immature lymphocyte
Types of Hematoxylin

**Harris Hematoxylin**
- Hematoxylin
- Absolute ethyl alcohol
- Ammonium aluminum sulfate (mordant)
- Distilled water
- Mercuric oxide or sodium iodate (oxidizer)

**Delafield Hematoxylin** (oxidation occurs naturally)
- Solution A
- Ammonium Aluminum sulfate (mordant)
- Distilled water
- Solution B
- Hematoxylin
- Alcohol, 95%

- Glycerol
Tonsil / lymphoid tissue

- Mature lymphocyte
- Immature lymphocyte
Types of Hematoxylin

**Mayer Hematoxylin**
- Hematoxylin
- Distilled water
- Sodium iodate (oxidizer)
- Ammonium or potassium aluminum sulfate (mordant)
- Citric acid
- Chloral hydrate

**Ehrlich Hematoxylin** (oxidation occurs naturally)
- Hematoxylin
- Alcohol, 95%
- Distilled water
- Glycerol
- Ammonium or potassium aluminum sulfate (mordant)
- Glacial acetic acid
Umbilical Cord / RBC’s

- Smooth muscle
- Red Blood Cells
- Lumen
Types of Hematoxylin

Gill Hematoxylin
- Distilled water
- Ethylene glycol
- Hematoxylin, anhydrous
- Sodium iodate (oxidizer)
- Aluminum sulfate (mordant)
- Glacial acetic acid
- Note: Marketed commercially in 3 different strengths and mucin, especially in goblet cells, will be stained by Gill hematoxylin, but not by the other hematoxylin solutions.

Weigert Hematoxylin (Iron Hematoxylin)
- Solution A
- Ferric chloride, 29% (mordant and oxidizer)
- Distilled water
- Hydrochloric acid, concentrated
  - Solution B
- Hematoxylin
- Alcohol
- Note: Not used in the routine H&E stain but used as a nuclear stain in many of the non-routine techniques because it resists de-colorization in acidic staining solutions.
Placenta / RBC’s

Red blood cells or erythrocytes

Open chromatin
Celestine Blue

- Ferric ammonium sulfate (mordant)
- Distilled water
- Note: Celestine blue may be substituted for hematoxylin in the H&E procedure and gives identical results.
POC

lymphocyte

Active Cell
Differentiation

Progresively stained slides do not require differentiation

Regressively stained slides are over stained then excessive stain is removed (differentiation)
Tonsil / knife mark

- RBC’s
- Lymphocytes
- Knife mark
Bluing

After nuclear staining with hematoxylin solutions and differentiation if indicated, the sections are blued. This is done with solutions that are weakly alkaline. The change in pH induced by the bluing agent changes the solubility of the dye lake. The aluminum-hematein complex is red and soluble below pH 5; bluing converts the red, soluble complex to a more desirable blue lake that is insoluble in the usual staining solutions.

- Dilute lithium carbonate
- Ammonium hydroxide
- Scot solution (magnesium sulfate, sodium bicarbonate, tap water)
Plasma Stains

The plasma stains are most frequently anionic, or negatively charged, dyes that combine with very cationic, or positively charged, tissue groups. The basic amino acids, such as arginine, histidine, and lysine, are common sites for dye binding.

Note: Eosin is the most widely used counter-stain in the routine staining of sections. The best staining with eosin will occur at a pH of approximately 4.6 to 5. Used properly, at least 3 shades of pink can be obtained with eosin alone.

- Erythrocytes (deepest shade of pink)
- Collagen
- Cytoplasm of muscle or epithelial cells

All the above should stain different intensities of pink. Whether the collagen or muscle/epithelial cells are the intermediate shade of pink is determined by choice of fixative, duration of fixation, heat during processing, stain formulation, differentiation step and the nature of the collagen itself.
Colon Polyp

- Red blood cells
- Lymphocytes
- Glandular cells
Eosin

Eosin Counter-stain
• Eosin Y (1% aqueous solution)
• Ethyl alcohol, 95%
• Acetic acid, glacial

Eosin-Phloxine B Counter-stain
• Eosin Y (1% aqueous solution)
• Phloxine B (1% aqueous solution)
• Alcohol, 95%
• Acetic acid, glacial
• Note: Some laboratories prefer an eosin-phloxine B solution, because the pink shades are more vivid but must be careful not to over-stain.
Colon Polyp / Eosinophil

- Glandular cells
- Eosinophil
- Lymphocyte
### H&E Progressive Stain

1. Xylene, 3 changes  
   - 2 minutes each
2. Absolute alcohol  
   - 10 dips each
3. Alcohol, 95%, 2 changes  
   - 10 dips each
4. Tap water runs off evenly
5. Hematoxylin, Mayer  
   - 15 minutes
6. Or acidified Harris  
   - 1-3 minutes
7. Tap water, 2 changes  
   - 10 dips each
8. Ammonia water, 0.25% or until blue
9. Lithium carbonate, 0.5%
10. Tap water, 2 changes  
    - 10 dips each
11. Eosin  
    - 10-20 dips
12. Or Eosin – phloxine  
    - 1-3 minutes
13. Alcohol, 70%  
    - 10-15 dips
14. Alcohol, 95%  
    - 10-15 dips
15. Absolute alcohol, 3 changes  
    - 10-15 dips each
16. Xylene, 3 changes  
    - 10-15 dips each

- Let slides remain in last container of xylene until a coverslip is applied.

---

**ILLUMINATING THE PATH TO BETTER HEALTH**

58
H&E Regressive Stain

1. Xylene, 3 changes
2. Absolute alcohol
3. Alcohol, 95%, 2 changes
4. Tap water
5. Hematoxylin, Delafield, Ehrlich, or Harris without acid
6. Tap water, 2 changes
7. Hydrochloric acid 1% in 70% alcohol
8. Running water
9. Ammonia water, 0.25%, or lithium carbonate, 0.5%
10. Tap water, 2 changes
11. Eosin or eosin-phloxine
12. Alcohol, 70%
13. Alcohol, 95%
14. Absolute alcohol 3 changes
15. Xylene, 3 changes
16. Let slides remain in last container of xylene until a coverslip is applied.

RESULTS
- Nuclei: Blue
- Erythrocytes and eosinophilic granules: Bright pink to red
- Cytoplasm and other tissue elements: Pink shades
Cervical Bx with HPV effect

The Koilocyte displays a large perinuclear halo with irregular clear-cut edges (HPV)
Cervical Bx High grade

Irregular cell with nucleoli

Irregular nuclear border
Cervical Bx High grade

- Nuclei
- Irregular nuclear border
- Intranuclear cytoplasmic inclusion
- Clumpy chromatin
Pap smear low grade with HPV
Pap/low grade with HPV

- Keratinized cytoplasm
- Perinuclear halo
Pap smear high grade

- Hyperchromatic nuclei
- Increased N/C ratio
Pap smear positive adenocarcinoma

Adenocarcinoma cells

Normal squamous cell
Hints to Help Achieve Good H&E Staining

• Microscopically check control slide (small intestine) prior to running patient samples
• Do not allow sections to dry at any point during staining
• Keep solutions covered while not in use. Make sure solutions completely cover slides. If any precipitate is noted at the top of the hematoxylin container, filter the solution into a clean dry container.
• Develop a routine schedule for changing solutions based on the number of slides stained each day. Record and document.
• After applying the bluing solution wash the sections very well; any carry over of the bluing solution will change the pH of the eosin and cytoplasmic staining will be lacking.
• Do not pass the slides through the dehydrating solutions too quickly, because dehydrating solutions also serve to differentiate. Keep in mind that the more dilute the alcohol, the more eosin that will be removed.
Hints to Help Achieve Good H&E Staining

• Tissues that have been fixed for longer than normal may require increased staining times.
• Staining times also may need to be adjusted according to the fixative used, the time in hematoxylin may need to be increased after fixation in Helly, Zenker, or B-5 fixatives, and the time in eosin will frequently need to be decreased.
• If using a xylene substitute, the manufacturer’s recommendations must be closely followed.
• Daily check and record the pH of the running tap water. Some tap water may not be acceptable before or after hematoxylin. Iron, sulfur, and chlorine will produce weak nuclear staining. Chlorine content, which varies seasonally, will also cause staining variability. Highly alkaline or hard water may serve as an excellent bluing agent but may create dark nuclear or background staining.
• Remember that what appears to be a staining problem is not always a staining problem. If the problem cannot be identified easily as a staining problem, cut and stain sections from a previous day’s workload in which the staining was excellent. If the staining is still excellent on the previous material, then the source of the problem must be in some other area. The duration of fixation, the use of heat during processing, and the possible carryover of formalin or water into the clearing and infiltration reagents are areas that should be examined when an apparent staining problem is proved not to be so.
Restoring Tissue Basophilia

Staining properties of markedly over-decalcified bony tissues cannot be restored, so proper initial decalcification is very important.

Method I
- Place deparaffinized slides from tissue overexposed to Bouin solution in 5% aqueous lithium carbonate solution for 1 hour.
- Wash in running tap water for 10 minutes, and stain using desired method.

Method II
- Place deparaffinized slides in a 5% aqueous sodium bicarbonate solution for 3 hours (4 hours for tissues overexposed to Zenker solution).
- Wash in tap water for 5 minutes, and stain using desired method.

Method III
- Place deparaffinized slides in 5% aqueous periodic acid for 30 minutes.
- Rinse in 3 changes of distilled water, and stain using desired method.
Frozen Section Staining

• Cut the frozen section and fix in 37% to 40% formaldehyde for 20 seconds (I prefer using alcoholic formalin)
• Rinse the section very well in at least 3 changes of tap water (if using alcoholic formalin one water rinse)
• Stain in Harris hematoxylin with acetic acid for 1 to 1 ½ minutes
• Rinse in 2 two changes of tap water
• Place slide in 0.25% ammonia water or another bluing agent, and leave until blue.
• Rinse in 2 changes of tap water
• Stain in eosin with 15 to 20 dips or until the desired intensity is achieved.
• Dehydrate with 95% alcohol and absolute alcohol – 10 dips in 2 changes of each alcohol
• Clear the sections with xylene – 10 dips in 3 changes
• Mount with synthetic resin
• Results:
  • Nuclei Blue
  • Cytoplasm and other tissue elements Shades of pink
Notes on Frozen Section Staining

• Fix cut sections immediately; do not allow the slides to air-dry or morphologic preservation will be poor
• Can stain with rapid H&E stain or use metachromatic dyes such as toluidine blue O or polychrome solutions (purchased commercially)
• Solutions should be changed on a regular basis depending on usage
• Alcoholic formalin may be used instead of concentrated formaldehyde
TROUBLESHOOTING THE H&E STAIN

Incomplete Deparaffinization
• Dry sections properly before beginning deparaffinization; if improper drying is the cause, slides can be treated with absolute alcohol to remove the water, and then re-treated with absolute alcohol to remove the water, and then re-treated with xylene to remove the paraffin; if incomplete drying is severe, the sections may loosen from the slides
• Allow sufficient time in xylene for complete deparaffinization; if this is the cause, return to xylene for a longer time
• Avoid contaminated xylene; change the solution if necessary
• If the slides have been stained, decolorize and restain

Nuclear staining is not crisp
• Fix tissue specimen completely
• Dehydrate and clear tissues completely before infiltrating with paraffin
• Do not use heat on the processor except for the paraffins
• Do not leave tissue in melted paraffin for a prolonged period
• Dry microscopic slides at the correct temperature (60-70 degrees C) and for the shortest time possible that ensures complete drying
Troubleshooting H&E stain

Pale Nuclear staining
• Not leaving in hematoxylin long enough
• Staining with over oxidized or depleted hematoxylin
• Over differentiating the hematoxylin
• Note: pale nuclei in bone sections may result from over-decalcification

Dark Nuclear Staining
• Sections left too long in hematoxylin
• Sections too thick
• Differentiation step too short

Red or Red-Brown Nuclei
• Ensure that sections are blued properly; it is not possible to over-blue the sections
• Check oxidation status of hematoxylin as given in sections following “Delafield hematoxylin”
Troubleshooting H&E Stain

Pale Cytoplasmic staining
• Check eosin solution pH; adjust with acetic acid if necessary
• Completely remove bluing reagent before transferring the slide to eosin
• Do not allow stained slides to stand in the lower concentrations of alcohols after the eosin; the more water in the alcohol, the more eosin that will be removed
• Ensure that sections are not too thin

Dark cytoplasmic staining
• Avoid over concentrated eosin solution, especially if phloxine is present; if necessary, dilute the eosin solution
• Do not leave section in eosin too long
• Allow sufficient time in dehydrating solutions, especially 70% alcohol, to allow good eosin differentiation
• Check section for proper thickness
Eosin not properly differentiated

If 3 shades of eosin are not apparent

• Timely and complete fixation
• Good dehydration and clearing during processing
• Eosin-stained section remains in the lower dilutions of alcohol for proper differentiation; adequate time in 70% alcohol will give the best differentiation of the eosin
• Eosin is at the correct pH
Troubleshooting H&E Stain

Blue-Black Precipitate on top of sections
• Filter hematoxylin
• Hazy or Milky water and slides
• When the slides and water turn milky following the rehydrating alcohols, it indicates the presence of xylene on the slides
• When slides appear hazy or milky in the last xylene used in clearing, it indicates that water is still present on the slides and that dehydration is not complete.

Uneven H&E staining
• May be caused by water or fixative in the infiltrating paraffin, or by contamination of reagents in closed tissue processors because of equipment malfunction or absorption of atmospheric water by the dehydrating alcohols on the open processors.
• Use toluene instead of xylene in areas of high humidity if using open processors; toluene is more water tolerant than xylene
• Check equipment for malfunction
Troubleshooting H&E Stain

Dark Basophilic staining of nuclei and cytoplasm, especially around tissue edges
  • Laser and electro-cautery techniques denature macromolecules and produce heat artifact, generally marked by dark basophilic staining in nuclei and cytoplasm. There is no remedy for this artifact

Poor contrast between nucleus and cytoplasm
  • The nucleus is too pale to contrast well with the cytoplasm
  • The cytoplasm is over-stained and masks the nuclei
  • The nuclear stain is too dark for the cytoplasmic stain
  • The cytoplasmic stain is too pale for the nuclear stain
Nucleic Acid Stains

**Feulgen Reaction**
- For the demonstration of DNA
- Reagents
  - Hydrochloric Acid
  - Schiff Reagent
  - Sulfurous Acid
- Results:
  - DNA: Reddish purple
  - Cytoplasm: Light green

**Methyl Green-Pyronin Y**
- To differentiate between DNA and RNA
- Reagents
  - Glacial acetic acid
  - Sodium Acetate
  - Methyl green dye
- Results:
  - DNA: green to blue-green
  - RNA: Red to rose
  - Goblet cells: mint green
  - Background: pale pink to colorless
  - Immunoblast and plasma cell cytoplasm: intense red
  - Nuclei: green to blue-green
CPT Codes

• Services 88300 through 88309 include accession, examination, and reporting.
THE END

Lung tissue

artery

GI tissue