Disclaimer

Troubleshooting the H&E Stain

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Topics of Discussion

- History of Stains-Hematoxylin and Eosin
- Chemistry 101
- H&E Criteria
- Steps of H&E stain
- Troubleshooting the H&E stain
- QA/QC

Hematoxylin

Hematoxylin is a natural dye from tree (logwood) Campechi Bay Area

- Discovered when Spanish landed in Mexico in 1500's
- Pirate battles over Hematoxylin shipments-due to high value placed on Hematoxylin
- English parliament banned import of Hematoxylin until discovery of adding metal mordants made fabric colorfast
- Hematoxylin is positively charged dye

Eosin

Eosin is a synthetically produced dye-2 versions

- Eosin Y-Slightly yellowish cast-alcoholic
- Eosin B-Slightly bluish cast-aqueous
- Used in inks and paints
- Negatively charged dye
- May have other additives such as Orange G or Phloxine, Bismark Brown, etc.

In his *Field with Irises near Arles* painting, Van Gogh incorporated red eosin dye into the color of the irises depicted at the bottom of the painting. Due to eosin's tendency to fade, the petals have now attained a blueish hue from their original purple coloration.

H&E Chemistry Law

- **Law #1-Opposites attract-**
  - Hematoxylin dye is positively charged (Basic)

    Nuclei ← Phosphoric Acid (DNA), Carboxylic acid, Sulfonic Acids → Goblet Cells

    These tissues negatively charged
  - Eosin Dye is Negatively charged (Acidic)

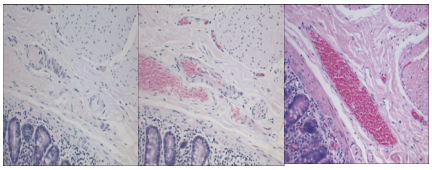
    Amino groups ↓ Proteins

    These tissues positively charged

H&E Chemistry Law

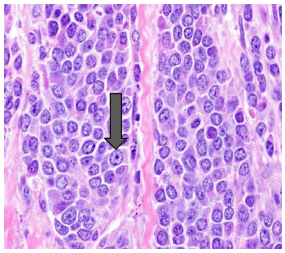
- **Law #2-Solution pH matters**
- Hematoxylin range is 2.2-2.8 (must be under pH 3.0)
  - Above pH 2.8-Background staining of collagen and muscle
  - Below pH 2.0-Solution turns red/yellow
- Eosin range is 4.0-4.5
  - Above pH 4.5-Tissues stain light
  - Below pH 4.0-Eosin will precipitate out of solution

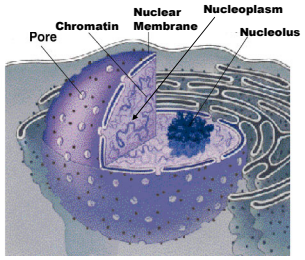
Eosin (left to right)
pH-8.0
pH-6.5
pH-5.0



Good H&E Criteria

Hematoxylin Stain

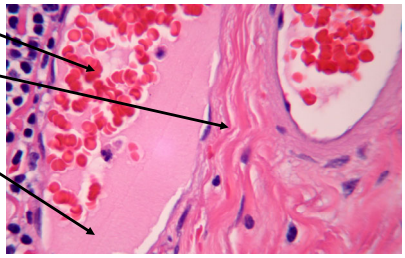




Qualities of Good Eosin-3 Shades (tones)

Eosin 3 tone effect

- Red-orange erythrocytes, keratin
- Pink collagen
- Lighter pink smooth muscle



A good H&E stain is dependent on many factors…

Adequate & prompt fixation | **Proper processing & paraffin infiltration** | **Well cut tissue sections** | **Hematoxylin and Eosin solutions** | **Validated protocol** | **QC/QA Controls**

"The quality of the final sections can be compromised by failures of any of the intermediate steps."

-John Tarpley

H&E Stain Steps

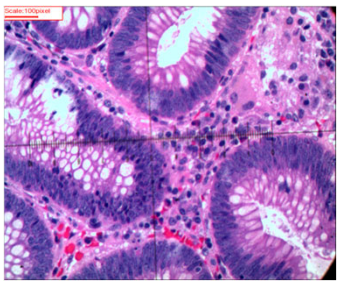
- First part-Deparaffinization and hydration
- Second Part- Hematoxylin, rinses, Eosin, acid rinses(Clarifier), Bluing
- Third Part-Dehydration and Clearing

NSH Guidelines

Deparaffinization

Deparaffinization-remove the paraffin

- Xylene-3 stations for 3 minutes are considered best practice-better than 2 stations for 5 minutes each
- Xylene Substitutes-3 stations usually considered mandatory and may need to consider longer times per station rand keeping solutions fresh
- Xylene can hold up to .5% water and still act as 100% at higher than .5% water droplets fall out

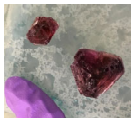


Hydration of slides

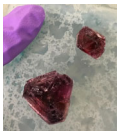
- Purpose-hydrate the sections to water by passing through a series of decreasing concentrations of alcohols
  - Best practice-minimum of 2 x 100% for 1 minute each
    - First alcohol should be for 1 minute
- Reagents-Ethanol, Reagent, isopropyl alcohol
  - Last alcohol bath can be 95% (or lower) which prevents surface tension from causing tissues to wash off of slides
  - If clearant is not dispersed by alcohol, clearant droplets (oil droplets) will be visible in lower alcohols and sometimes in water rinses
  - In extreme cases may interfere with hematoxylin staining
- End of Part 1-Hematoxylin and Eosin staining

Part 2-Hematoxylin solutions not all the same

- Hematoxylin Dye
  - Vary from 1 to 20 grams
- Amount of mordant – Mordants strengthen the positive ionic charge of the hematein.
  - Aids bonding of the hematein to the anionic tissue component most commonly chromatin.
  - Can fall out of solution in cold weather or if supersaturated solution
- Solvent
  - Tap water
  - Mixtures of water, ethanol, methanol, and isopropyl, may include glycerol, ethylene glycol or propylene glycol
- Acid-pH
  - Added or not
  - Type of acid used



Mordants



- Addition of the mordant improves the ability of the hematein (oxidized Hematoxylin) to attach to the anionic (negatively charged) components of the tissues.
- Mordants strengthen the positive ionic charge of the hematein.
  - Aids bonding of the hematein to the anionic tissue component most commonly chromatin.
- The type of mordant influences the final color of the stained components
- Most common mordant used in routine histology is aluminum ammonium sulfate (alum).
- This mordant causes the nuclei to be red in color, which is then changed to the more familiar blue color when the sample is later rinsed with a weakly basic solution.

Alum Hematoxylin Stain Composition

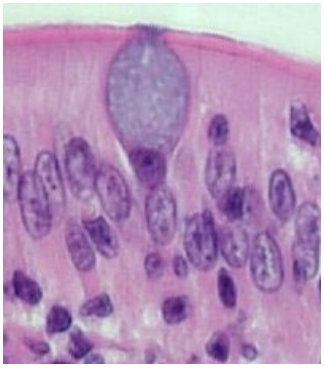
Ingredient	Harris Hematoxylin	Gill Hematoxylin	Proprietary Hematoxylins
Solvent	Deionized Water	Deionized Water	?
Dye	Hematoxylin	Hematoxylin	Hematoxylin
Mordants	Aluminum Ammonium Sulfate	Aluminum Sulfate	??
Oxidizers	Sodium Iodate	Sodium Iodate	??
Stabilizer	Ethyl Alcohol	Ethylene Glycol	??
pH Adjuster	Glacial Acetic Acid	Glacial Acetic Acid	??

- Gill 1, 2, and 3 Hematoxylin – 2.0, 4.0, 6.0 grams of Hematoxylin per liter respectively
- Harris Hematoxylin – 5.0 + grams of Hematoxylin per liter

Mucin Staining?

Negatively charged and will stain with Hematoxylin- depending on formulation and pH of stain

What does your pathologist prefer?



Critical Steps of the H&E

Hematoxylin stain → Water Rinses → Acid rinse/Clarifier → Water rinse → Bluing → Water rinse → Alcohol before Eosin → Eosin stain

Water Rinse #1

- Purpose-removes unbound Hematoxylin
- If inadequate-
  - Uneven staining
  - Blue Glass staining
- Good practice to have 2 changes of water
  - Check water flow so that no Hematoxylin left in container
  - If still blue the slides are not rinsed enough

Hematoxylin–Rinses are Important!

Periodic cleaning of the containers and stainer lines may be necessary for adequate flow

Tap water can change with the seasons due to addition of chemicals

Hematoxylin-Too Dark/non-specific

- Staining time -too long-4-5 minutes is usually sufficient
- Microscope slide staining blue
  - Excessive slide adhesive
  - Glass Slides have charges
    - Tissue has a net negative charge
    - Adhesive-Positively charged slides have a positive charge
    - Glass has a negative charge
  - Filter hematoxylin prior to use if sheen is present
- pH > 3.0
  - Increases with time and carryover of water
  - Collagen and smooth muscle starts to pick up the stain
- Section thickness

Hematoxylin –too light

- Thin sections
- Incomplete deparaffinization
- Staining time too short
- Lost of potency of hematoxylin
  - Water carryover
  - Beyond expiration date
    - Different expiration date once bottle has been opened due

Hematoxylin-Too Light

- Increase staining time
- Decrease time in differentiation steps (acid/alcohol)
- Hematoxylin may be over-oxidized-replace
- Tap water rinses may be decolorizing sections
  - Highly chlorinated water

Differentiation- no longer just for regressive stains

Purpose-To remove non-specifically bound hematoxylin-clean up the background	Tissue Adhesive Glass
Common Acids Used	HCl acid--Harris Glacial Acetic Acid
If using proprietary stains-make sure that the differention solution matches the stains. Manufacturer has optimized the solution for the stains-IF having issues call the company!	

Water Rinse #2-Purpose-remove and stop action of acid rinse!

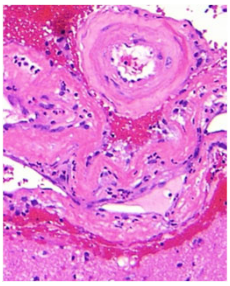
Bluing

- Purpose-Changes cells from Red-blue tint (pH less than 3) to Blue-purple tint (pH greater than 3) by raising the pH of the hematoxylin from acidic range to basic range
- Recommended Proprietary Reagents:
  - Gentle on the tissues
  - pH consistent
  - Color Indicator helpful to prevent confusion with "differentiation rinse"
- Not recommend:
  - Tap water-pH may be unpredictable
  - Ammonia Water-pH may be too high-a cause of section loss

Water Rinse #3-Purpose-remove and stop action of Bluing Solution-important to remove all bluing solution from slides prior to Eosin

Eosin Y- Counterstain-Counterstain

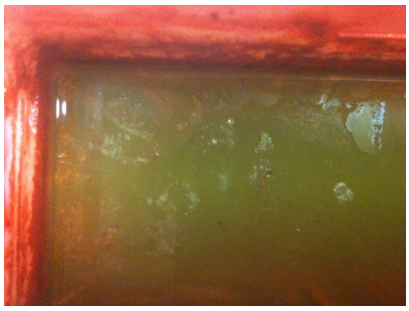
- Purpose-Cytoplasmic Counterstain
- Reagents-Alcoholic and acidified, aqueous
  - Eosin Y
  - Eosin Y with Phloxine B
  - Eosin B
- Recommended pH is 4.0-4.5
- Variables that influence staining
  - Time in the eosin solution
  - Dye(s) and concentration of dye
  - Concentration of alcohol after eosin
  - Time in alcohol rinse
  - Pathologist Personal Preferences



Eosin Counterstain

- Reduced eosin staining intensity or decreased staining capacity is often the result of an increase in the pH of the eosin solution
  - Carry over of alkaline tap water-orange salts accumulate at the top of container
  - Inadequate rinsing to remove bluing agent
  - Contamination with bluing agent
  - Use 95% Alcohol rinse before Eosin Stain

Eosin Precipitation



Eosin-Troubleshooting

- Too Dark
  - Stronger dye concentration due to evaporation-don't top off
  - Thick sections
  - Excessive staining times
  - pH of the eosin-too low

Eosin-Too Light

- pH too high (>4.5)
- Alcohol Rinse
  - Too aqueous or too long
  - Type of alcohol
  - Recycled alcohol
  - Thin sections
  - Inadequate staining time
  - Inadequate rinsing after the buffer

Lack of 3 tones

- Poor Fixation
- Overstained
- Eosin too strong
  - Eosin with Phloxine may be more difficult
- End of Part 2-Critical Steps for H&E Stains

Dehydration-Part 3

- Purpose
  - Obtain correct three tone effect-Eosin
  - Remove all water from the tissue sections so xylene can penetrate
- Good Practices
  - Minimum of 3 changes of 100% alcohol
  - 1 minute each
  - Increasing volumes
  - Do not use recycled alcohol-not 100%

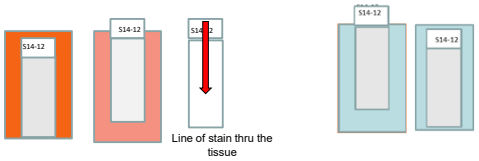
Dehydration Troubleshooting

- Excessive rinses-
  - Weak eosin staining
- Inadequate rinses-
  - Hazy slides-water and mounting media
  - Carryover of water into Xylene-Pink Xylene
  - Rotate alcohols frequently-alcohol is hygroscopic-absorbs water from the air-especially when humidity is high and using a xylene substitute

Bleeding of eosin is not an eosin problem, it is a water carryover/alcohol absorption of water problem

Fluid Levels

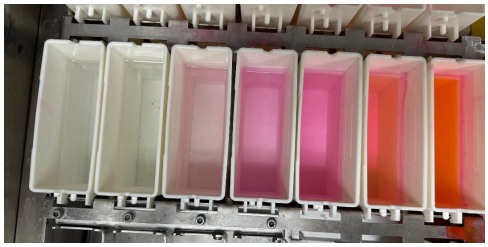
- Decreasing Levels
- Increasing Levels



Clearing

- Purpose
  - Remove alcohol
  - Prepare for coverslipping-mounting media
    - Solvent compatible with mounting media
- Reagents-Xylene
  - Best Practice-3 changes 1 minute each
- When using Xylene Substitute-Last alcohol must be anhydrous alcohol or eosin will bleed out of the sections after coverslipping
- Last alcohol should not have any pink coloration in the solution

First Xylene Pink?



Xylene and Xylene Substitutes

Xylene
- Can tolerate 0.5% water
- Must be compatible with mounting media

Xylene Subss
- Most tolerate no water
- Requires perfect dehydration

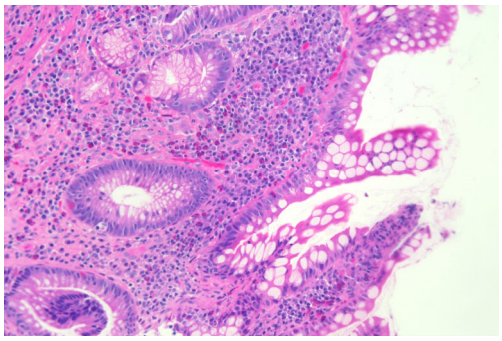
Xylene Substitute-Troubleshooting

- Eosin fading/bleeding
  - Left alcohol or water in section
  - Use limonene xylene substitute without anti-oxidants added
- Eosin fading/bleeding
  - Mounting medium must be compatible with the xylene substitute

H&E Balance of Color

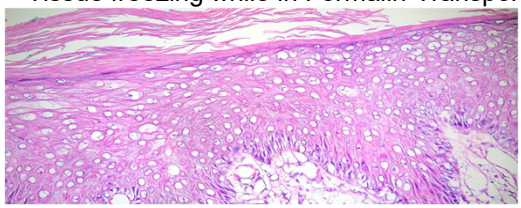
- Overall balance of H&E stained specimen is the result of balance of the intensity of the alum-hematoxylin and eosin
- Hematoxylin can stain cytoplasm
- Eosin can stain nuclear basic proteins

Smudgy Nuclei-Fixation Issue



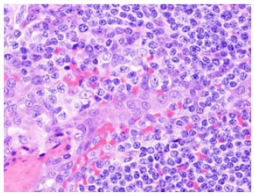
Bubbly Nuclei

- Possible causes:
  - Trapped water under the section-Hot dryer
  - Tissue freezing while in Formalin-Transport



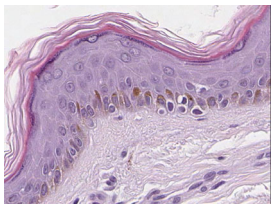
The H&E Stain Balance

- Hematoxylin and Eosin must work in harmony, not overpowering each other but complimenting each other
  - Eosin shouldn't overpower the Hematoxylin
  - Hematoxylin shouldn't overpower the Eosin

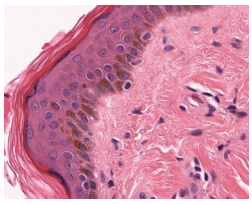


H&E Balance

Skin, High Power. Poor contrast



Skin, High Power, Good Contrast



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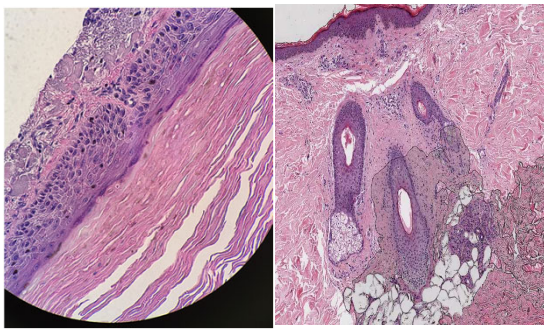
Coverslipping Issues



QA/QC-Rotation/Replacement

- All solutions have a definite useful life dependant on
  - Work volume
  - Type of solutions used
  - Mode of staining
- Wash and rinse each container thoroughly
- Alcohol/clearant rotation
  - Last alcohol is pink rotate alcohol/xylene immediately
    - Indicator that the alcohol has water in it

QA/QC-Record Keeping

- Record Daily
  - Brand and lot number of each stain/solution
  - (pH of the eosin/hematoxylin)
  - Filtering of the hematoxylin
  - Rotation/discarding/replacement of reagents
  - Results of H&E control slide
    - Pathologist review

H&E Control Daily Log Sheet (example)

DATE	NUCLEAR STAIN	CYTOPLASM STAIN	HEM LOT #	EOSIN LOT #	TECH INITIALS	COMMENTS-RESOLUTION
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
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15						
16						
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