

The Science and Application of Hematoxylin and Eosin Staining

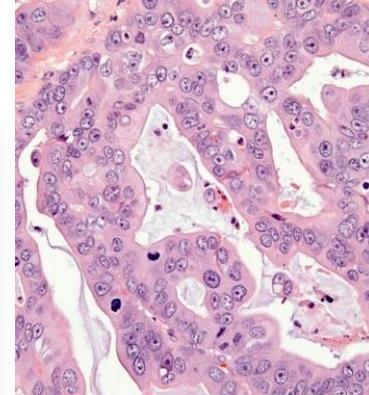
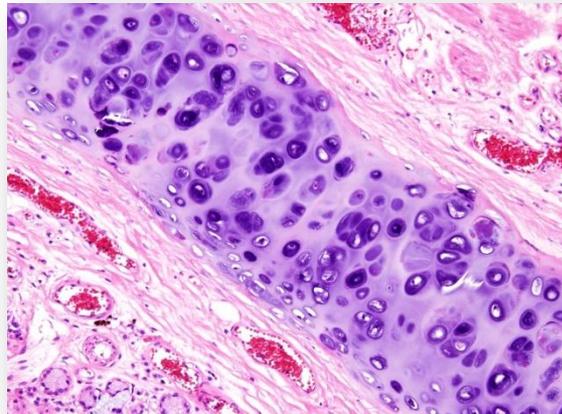
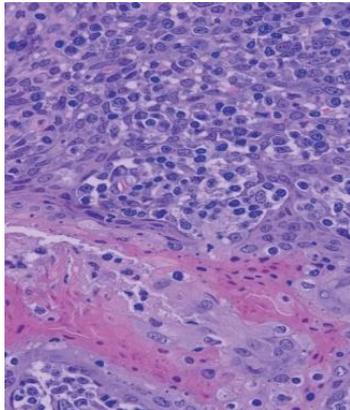
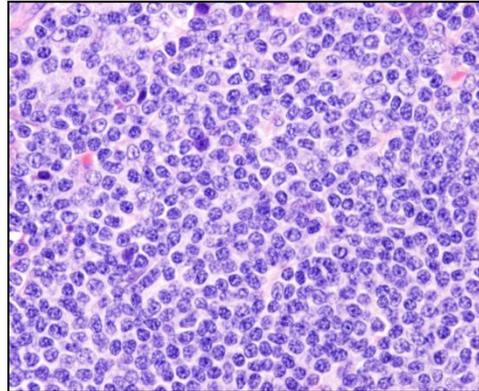
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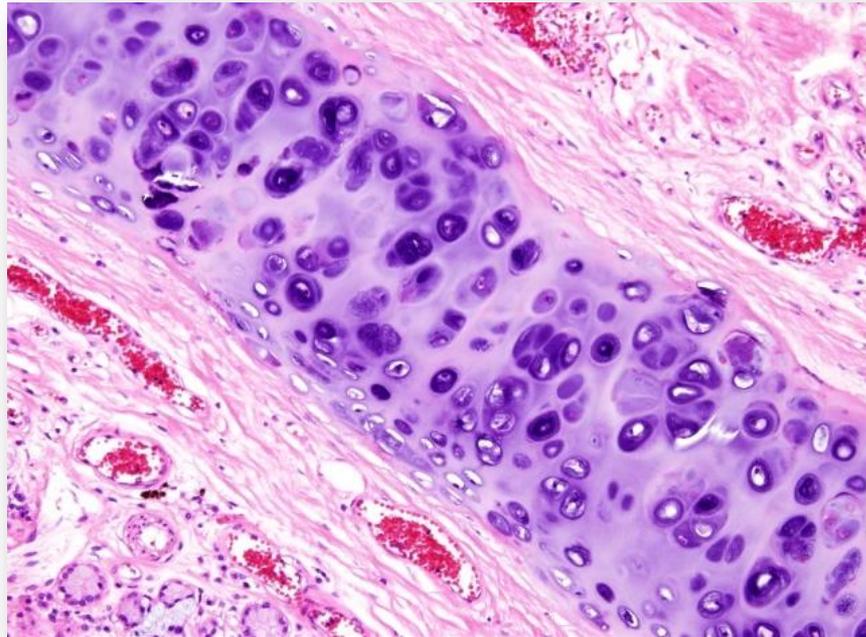
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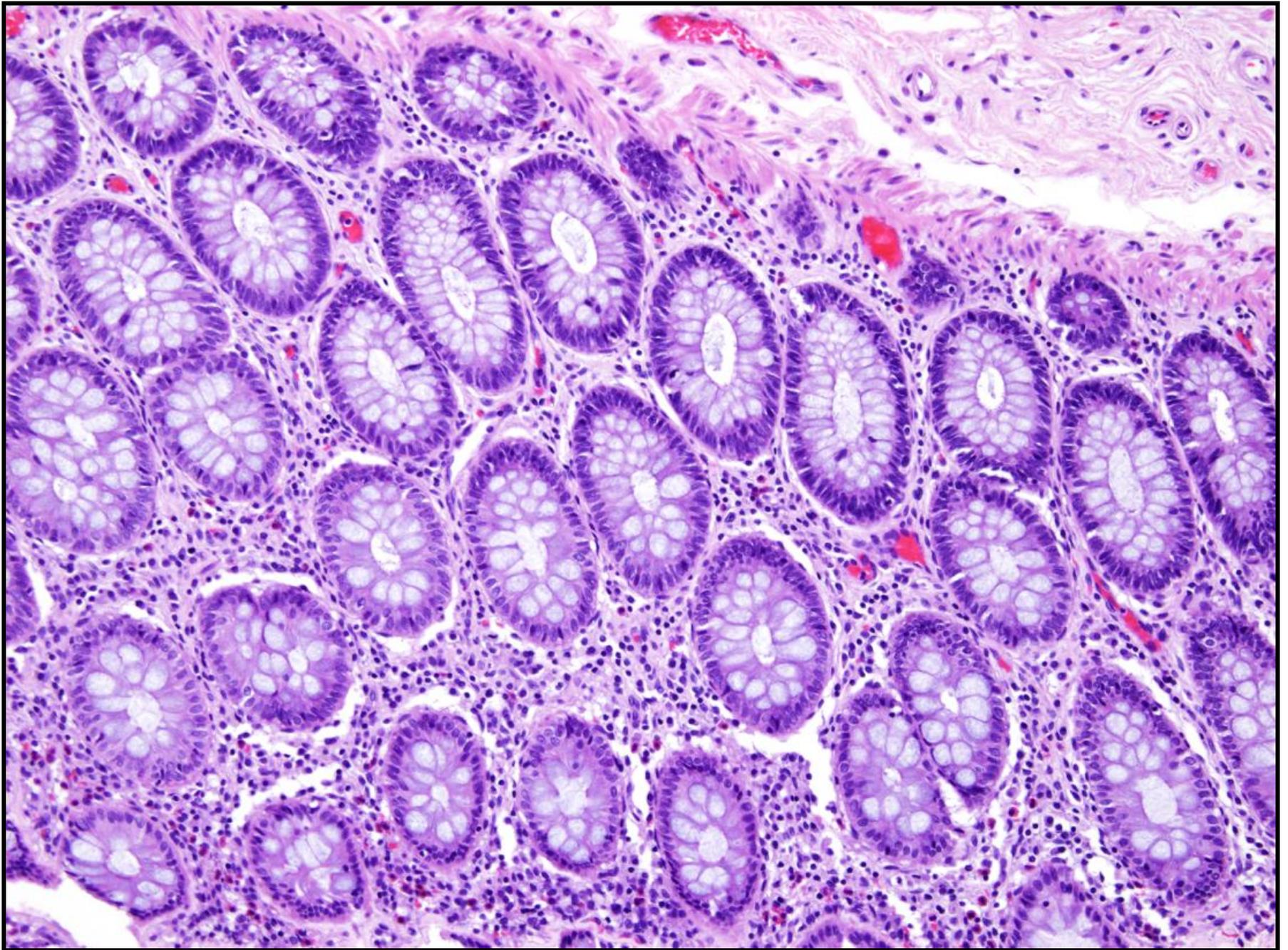
Stains and Staining Systems



Routine Staining (Hematoxylin & Eosin)



Nuclear detail/definition - Hematoxylin
Contrasting counterstain - Eosin



The Hematoxylin and Eosin Stain

- Long history of use
 - Mayer 1904
- Primary diagnostic technique in the histopathology laboratory.
- Primary technique for the evaluation of morphology
- Widely used
 - 2.5 to 3 million slides per day

Introduction

- The Hematoxylin and Eosin combination is the most common staining technique used in histology.
- The diagnosis of most malignancies is based largely on this procedure.
- Customer expectations or preferences are extremely subjective

The Hematoxylin and Eosin Stain

- A better understanding of the basic chemistry of the H and E stain will provide the attendee with:
 - A greater knowledge of how to optimize or modify the technique to achieve the results desired
 - A greater knowledge of how to solve staining issues.

Introduction

- There are a number of commercially available formulations for both hematoxylin and eosins
- Customers preference for a specific formulation is based largely on desired staining characteristics.
- Other factors may include ease of use and throughput.

Some Basics of Dye Chemistry

- Why do dyes stain specific elements of cell and tissues?
- Dyes demonstrate an affinity for molecules within cells and tissues.
- Affinity is the result of attractive forces between the dye molecule and molecules within the tissue.
 - Dyes have a greater affinity for tissue molecules than solvent molecules

Some Basics of Dye Chemistry

- The affinity of dyes for tissue elements is affected by a number of factors
 - The structure of the dye molecule
 - The shape of the dye molecule
 - The charge distribution of the dye
 - Solvent characteristics

Some Basics of Dye Chemistry

- Acidic pH (< 7) *****Acidophilic Stains**
 - higher concentration of H^+
 - Some tissue molecules will bind H^+ and will carry a positive charge
- Basic pH (> 7) *****Basophilic Stains**
 - Lower concentration of H^+
 - some tissue molecules will give up H^+ and will lose a positive charge.

Some Basics of Dye Chemistry

- Charge distribution of the dye determines attractive or repulsive characteristics of the dye.
- Positive charges (+)
 - Cations
 - Attracted to negatively charged molecules
- Negative charges (-)
 - Anions
 - Attracted to positively charged molecules
- Charge is largely determined by pH.

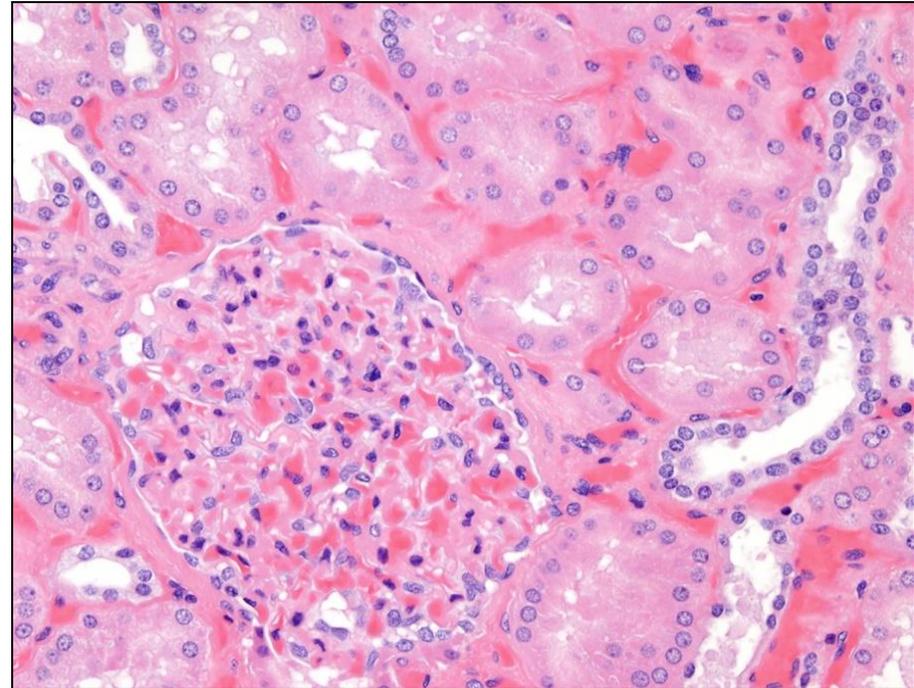
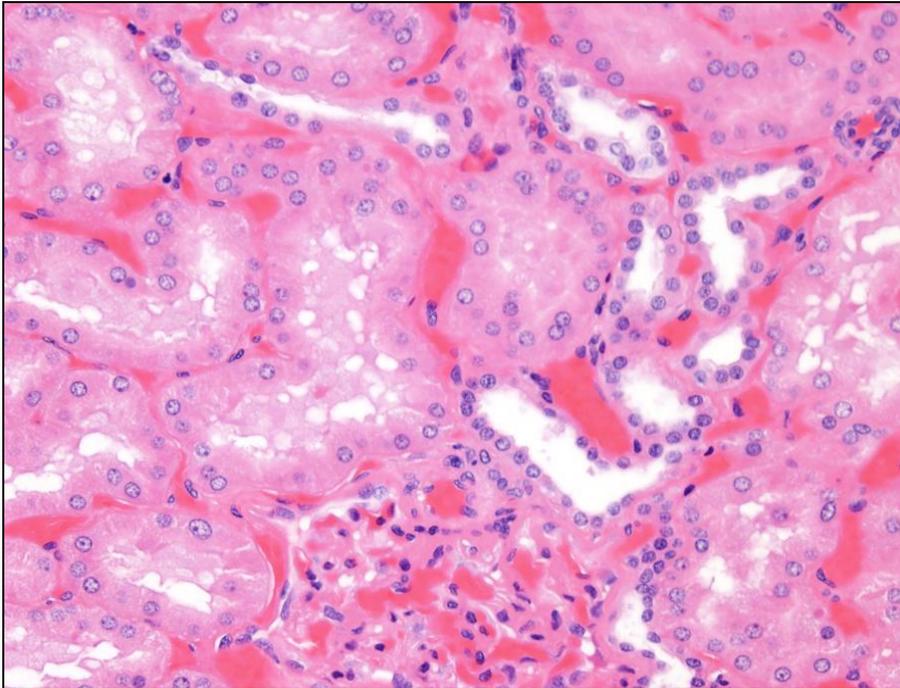
EOSINS

(pH effects)

Eosin Y Staining

pH = 4.3

pH = 5.2



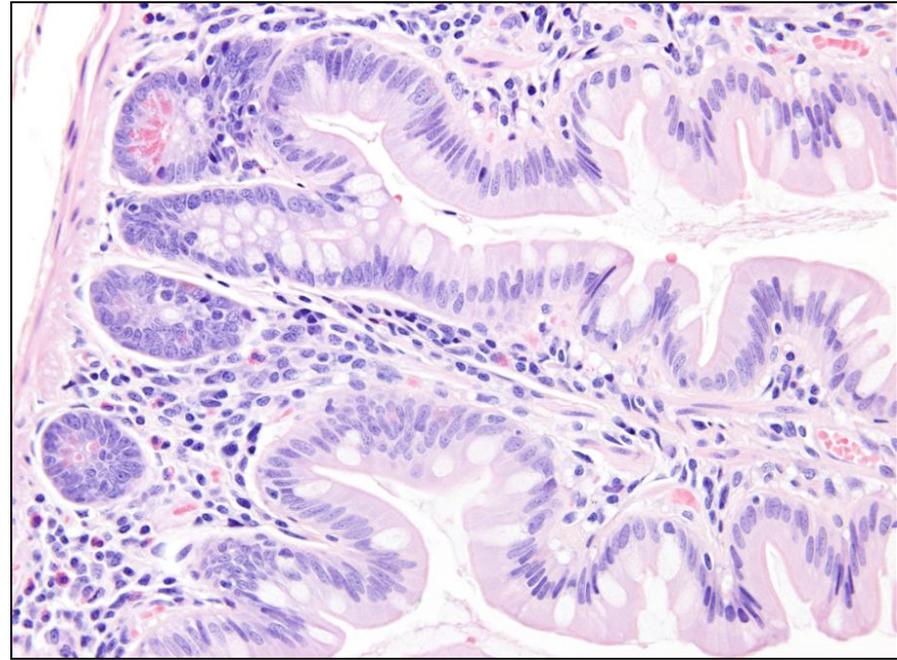
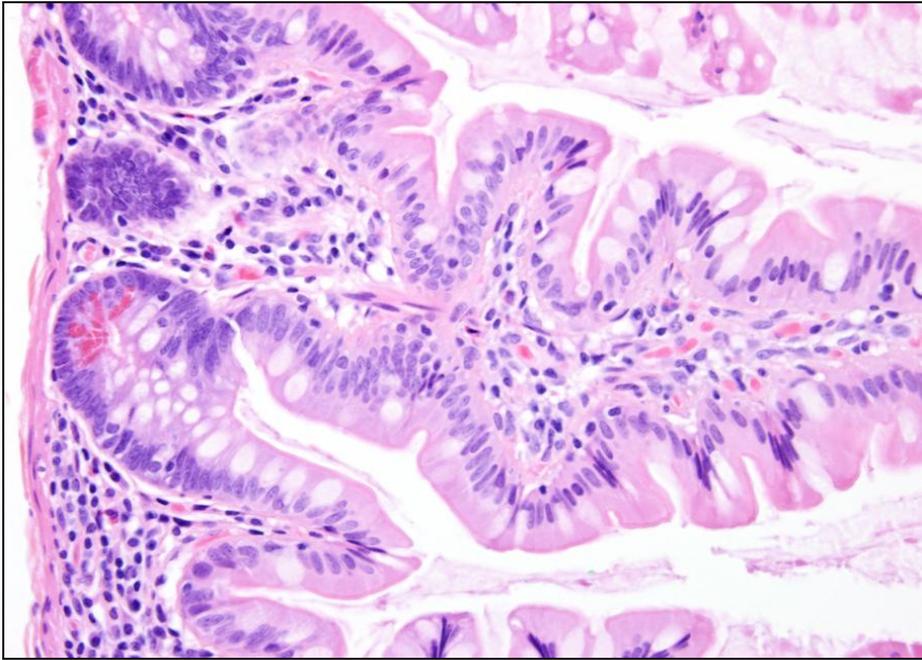
EOSINS

(pH effects)

Eosin Y Staining

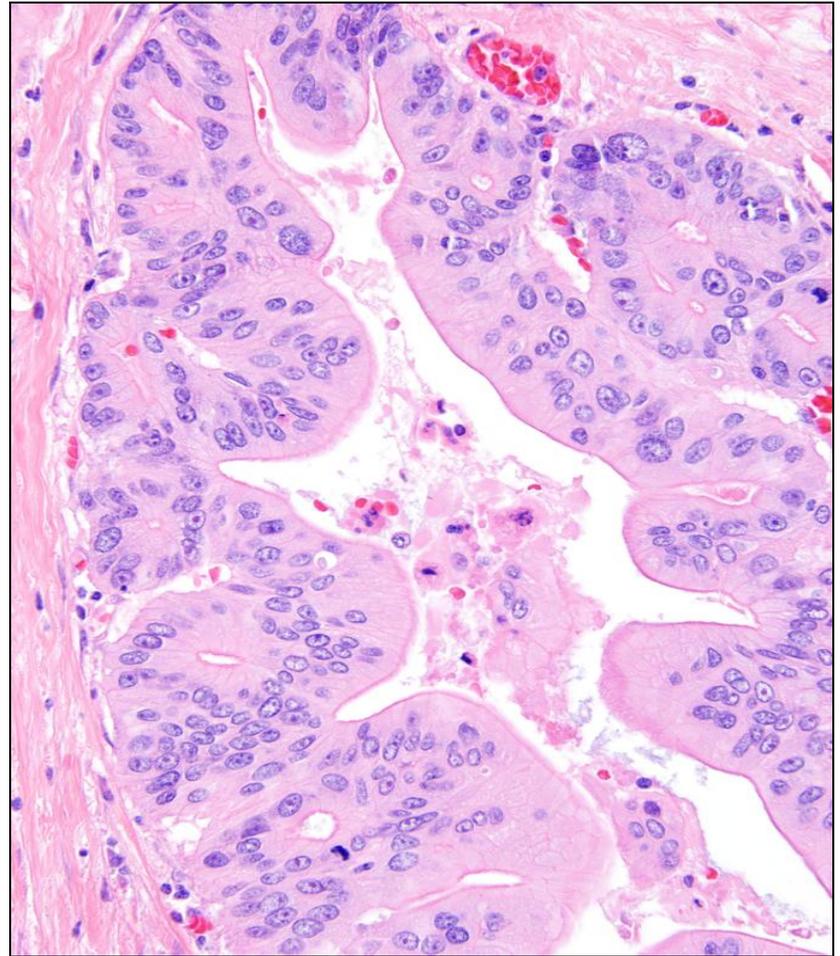
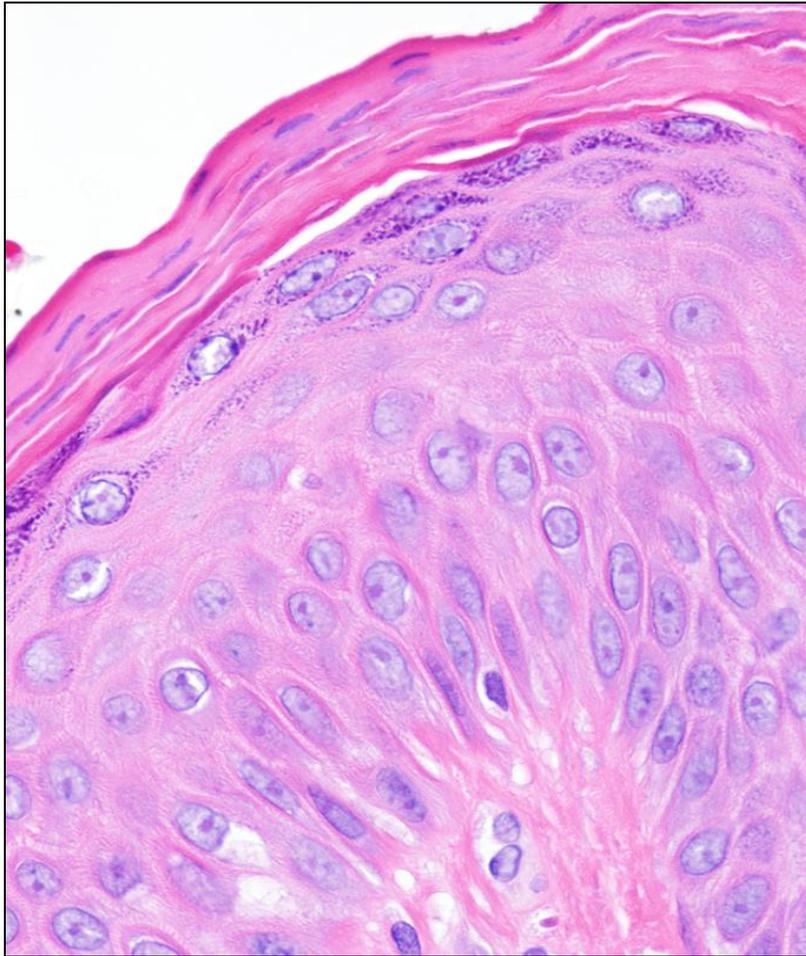
pH = 4.3

pH = 5.2



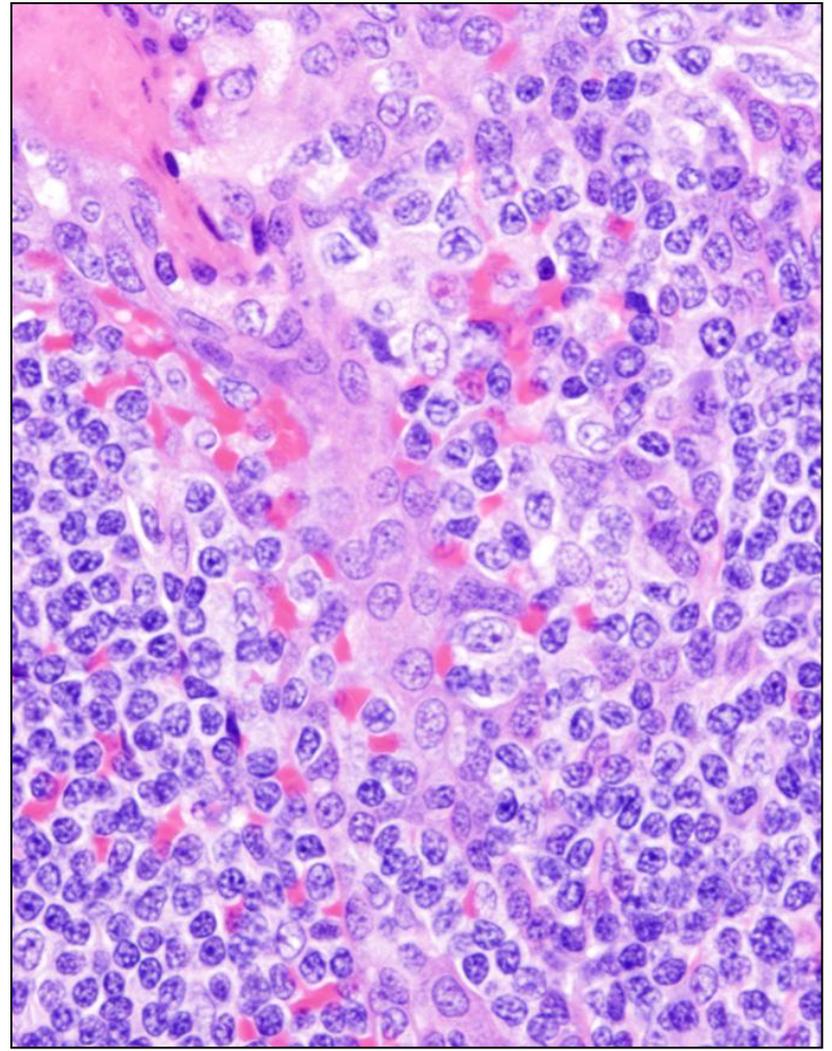
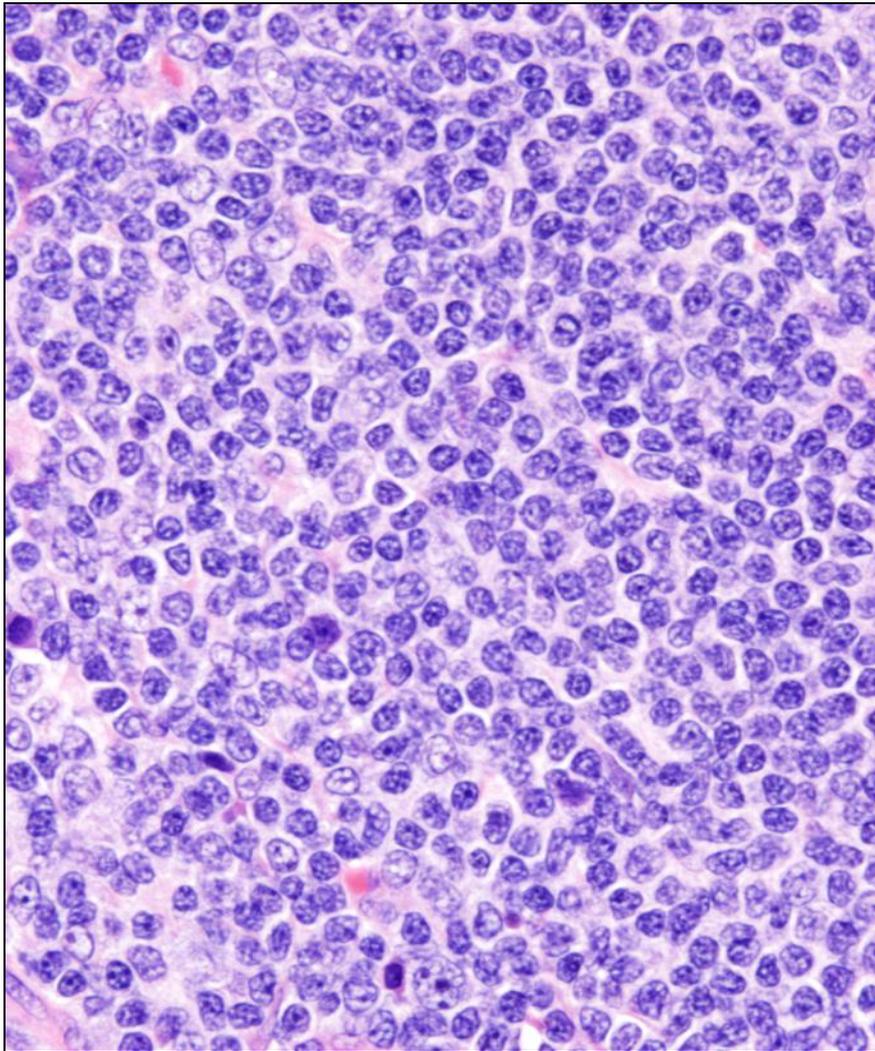
The H and E Stain

(balance of coloration)



The H and E Stain

(balance of coloration)



Staining Systems (Progressive v/s Regressive)

REGRESSIVE

- Hematoxylin stain is applied
- Tissue is overstained with hematoxylin
- Differentiator is used to aggressively remove excess hematoxylin
- Stain procedure continues on with counterstain

Staining Systems (Progressive v/s Regressive)

PROGRESSIVE

- Hematoxylin stain is applied
- Tissue is stained with hematoxylin only to a point
- Traditionally no differentiator is used to remove excess hematoxylin
- Stain procedure continues on with counterstain

Staining Systems

(Progressive v/s Regressive)

Regressive:

Stain \longrightarrow Regress \longrightarrow Continue staining
(Harris hematoxylin)

Progressive:

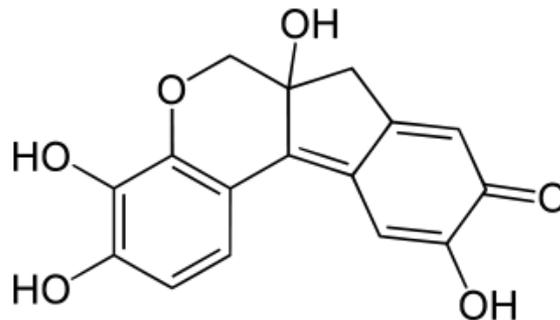
Stain \longrightarrow Rinse \longrightarrow Continue staining
(Mayer's, Gill's I & II)

Basic Chemistry

- Progressive staining:
 - Intensity is controlled through varying the time in hematoxylin. Hematoxylin will only stain to a point.
- Regressive staining:
 - Tissue is purposely over stained with hematoxylin.
 - Background is removed with differentiation.

Hematoxylin

- Natural dye extracted from the wood of the Logwood tree found in Mexico and Central America
- Hematoxylin has little or no staining capacity
- Hematoxylin is oxidized to hematein



Basic Chemistry

- Hematoxylin solution is a mixture:
 - Hematoxylin
 - Hematein
 - Aluminum ions
 - Hematein/aluminum
 - Solvent mixture

Basic Chemistry

- Hematein bound to aluminum has an overall positive charge.
- Hematein/aluminum binds negatively charged molecules in cell and tissues.
- Chromatic material in cell nucleus is strongly negative in it's ionic charge.

Hematoxylin

- Oxidation of hematoxylin to hematein
 - Air/light
 - Mercuric oxide
 - Sodium iodate
- Today almost all hematoxylin are oxidized by sodium iodate
- Oxidation continues after formulation is complete

Hematoxylin

- Staining patterns of hematoxylin are consistent with a cationic hematein- Al^{+3} molecule
 - Acid mucins may stain
 - Proteoglycans may stain
 - Staining profile is similar to other cationic dyes
- Decreased pH results in decreased intensity of stain

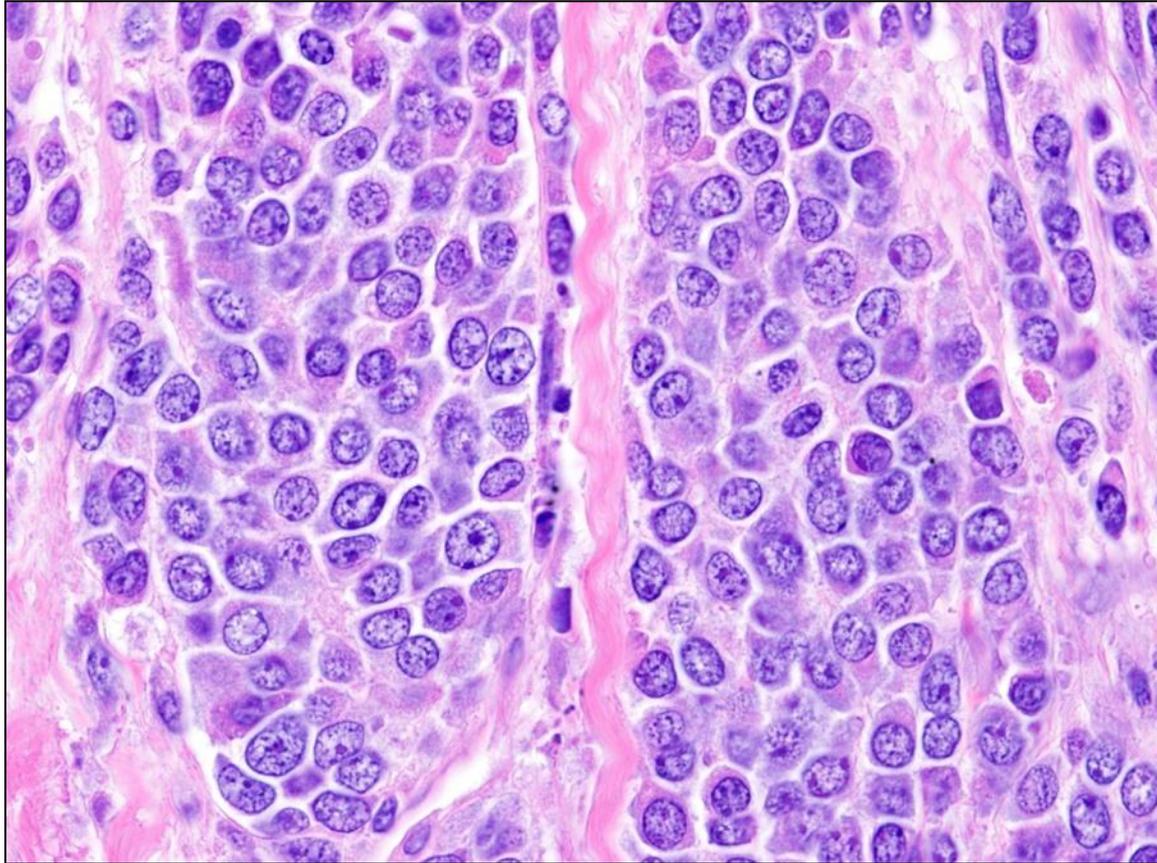
Hematoxylin

- Hematein complexed with Al^{+3} is the most common form of hematoxylin used for nuclear staining.
 - alum hematoxylin
 - Purple/blue coloration
- Alum-hematoxylin stains nuclei various shades of purple/blue –Specific staining
- Non-specific staining may also occur
 - Cytoplasm
 - Mucin

Basic Chemistry

- Specific staining:
 - Nuclei; DNA in chromatin
- Non specific – Background staining:
 - Cytoplasm
 - Goblet cells of G.I. tract; mucin

Hematoxylin



Basic Chemistry

- Background staining is reduced or removed by differentiation.
- Differentiation:
 - Decreases the ability of hematoxylin to bind to tissue sites
- Acidic solutions
 - Acetic acid diluted in water or 70% ethanol
 - HCl diluted in alcohol

Basic Chemistry

- Common progressive hematoxylin;
 - Mayer's, Gill I, Gill II, Gill III(?)
- Common regressive hematoxylin;
 - Harris
- With some hematoxylin the tissue is not purposefully over stained but light differentiation is necessary to optimize staining contrast

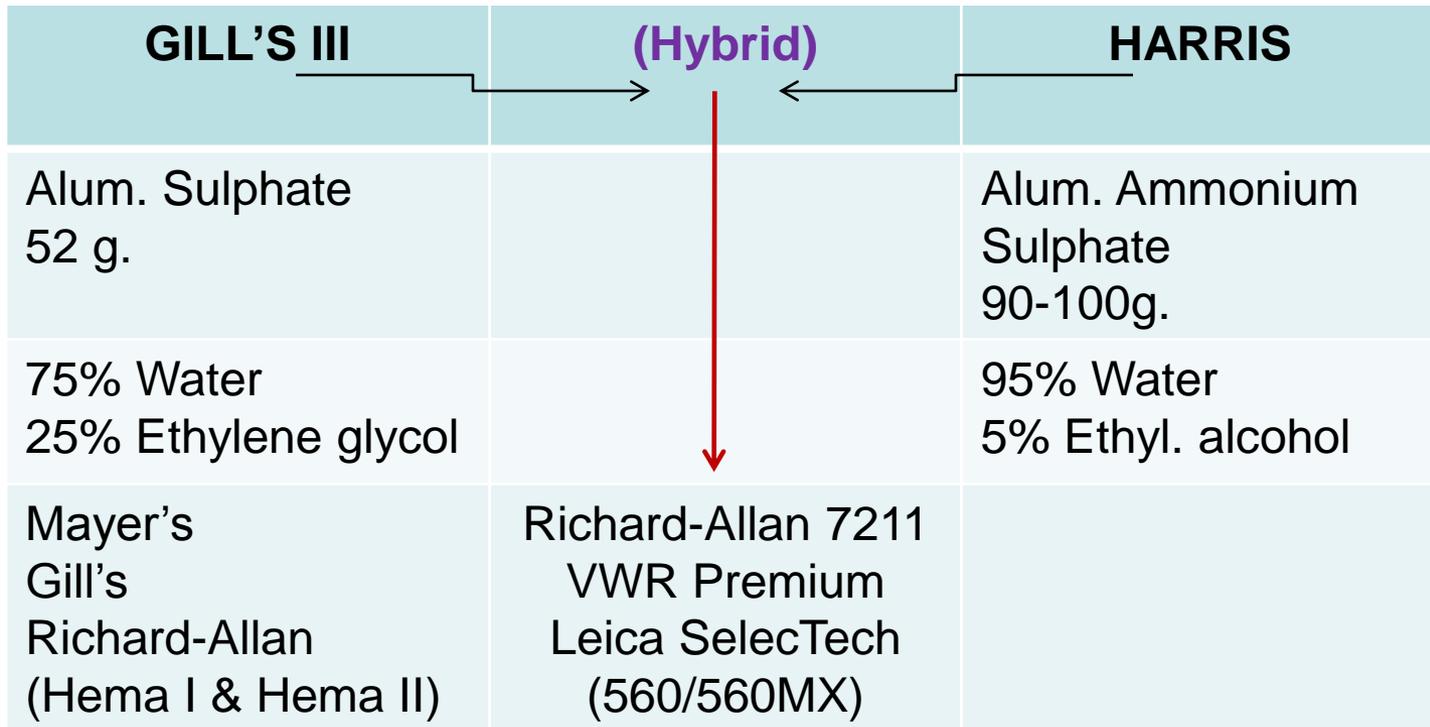
(SelecTech, VWR, some R.A. stains, Gill's I & II)

Introduction

- Commercially available hematoxylin:
 - Harris
 - Mayers
 - Gill I, II, III
 - Proprietary formulas

What makes one hematoxylin different from others

| GILL'S III | (Hybrid) | HARRIS |
|----------------------------------------------------------|---------------------------------------------------------------------|----------------------------------------|
| Alum. Sulphate 52 g. | | Alum. Ammonium Sulphate 90-100g. |
| 75% Water 25% Ethylene glycol | | 95% Water 5% Ethyl. alcohol |
| Mayer's Gill's Richard-Allan (Hema I & Hema II) | Richard-Allan 7211 VWR Premium Leica SelecTech (560/560MX) | |



Harris Hematoxylin

- Harris hematoxylin:
 - Common hematoxylin used in histology
 - Strong, regressive stain
- Harris hematoxylin produces well delineated crisp nuclear staining.
- Harris may require filtering to remove precipitates.

Harris Hematoxylin

- Because of the strength of Harris hematoxylin, most labs use an aggressive differentiating solution:
 - 1.0% or 0.5% HCl in 70% alcohol is commonly used
 - Sections typically are exposed to these strong differentiation solutions for 2-10 seconds

Differentiation

- Differentiation- the use of acidic solutions to remove excess background staining
- Differentiation solutions used with regressive stains are strong acids.
 - 70% alcohol with 0.5-1.0% HCl
 - Alcohol softens the differentiation process or makes it more controllable.
 - Aqueous differentiation solutions are more aggressive.

Differentiation

- Differentiation solutions are acidic
 - Increases the concentration of (H⁺)
- Differentiation breaks
 - The bond between Al⁺³ and the tissue
 - The bond between Al⁺³ and the hematein
- Over differentiation will remove staining from the nuclei and dull the crispness.

Differentiation with Progressive Stain

- Differentiation solutions used with progressive stains are weakly acidic.
 - Acetic acid is weaker than HCl
 - Acetic acid in aqueous or alcoholic solution
 - Buffered solutions provide more control of pH

Differentiation with Progressive Stain

- Why would differentiation be used with a progressive stain?
 - To remove mucin staining.
 - To remove background staining from the slide.
 - To increase the crispness of the stain.

Progressive Stain Differentiation Reagent

- Buffered solution of organic acids:
 - Designed to maintain specific pH
 - Increases the clarity and definition of cytoplasmic stains
 - Removes background staining of mucin and slides.
- Contains no alcohol

BLUING REAGENT

- Buffered solution:
 - Designed to maintain a specific pH (8.0 – 8.2).
 - Produces crisp blue/purple chromatin stain.
 - Gentle to tissue sections.
 - Excellent for use with frozen sections
 - May be used with any hematoxylin.

BLUING

- Bluing is necessary to convert nuclear coloration from reddish purple to a crisp blue/purple
- Bluing agents typically are alkaline with a pH range of 7.5-9.0 optimally
 - Tap water
 - Scott's tap water
 - Ammonia water
 - Lithium Carbonate
 - Buffered solutions
- Bluing agents enhance the contrast of the H&E stain by increasing the crispness of the hematoxylin
- Hematoxylin coloration is changed from purple to blue/purple.

BLUING

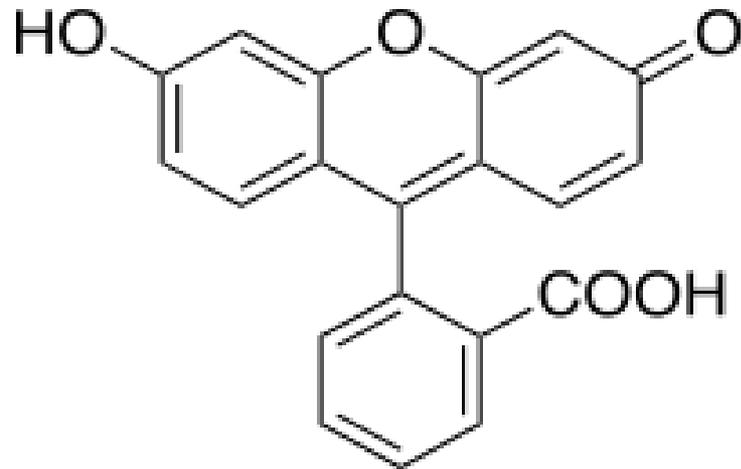
- How does bluing work?
- Elevated pH
 - Fewer H^+ in solution effects the structure of hematoxylin
- Loss of H^+ from ring structure.

BLUING

- Tap water- pH may be unpredictable
- Ammonia water- pH may be too high
 - Section loss may occur
- Scott's tap water or buffered solutions are more effective at maintaining the optimal pH
 - Gentle for tissues

EOSINS

- Unlike hematoxylin, eosin Y is a synthetic dye.
 - Derived from fluorescein
- Eosin Y is a member of the xanthene family of dyes
 - Eosin Y
 - Eosin B
 - Phloxine B
 - Fluorescein



Basic Chemistry

(Eosin)

- Eosin is an orange/pink dye that is a member of the xanthene family of dyes
 - Phloxine B
 - Fluorescein
- Eosin is acidic
 - Negatively charged
- Eosin binds to positively charged proteins in the cytoplasm and in connective tissue

Eosin Y Alcoholic Stain

- Alcoholic eosin Y formulated to produce optimal contrast with hematoxylin.
- Stains erythrocytes bright red/pink and collagen, muscle and cytoplasm varying shades of orange/pink.

EOSINS

(pH effects)

- Eosin Y is an acidic/anionic molecule
 - Negatively charged at a pH of 3.5 or higher
- Attracted to cationic tissue sites.
- **Staining intensity is highly dependent on pH**
- Above a pH of 5 the staining intensity drops significantly.
- Below a pH of 4 the intensity drops and staining becomes murky.

EOSINS

(pH effects)

- Binds cytoplasmic and connective tissue cationic proteins
 - Arginine
 - Lysine
 - Histidine - at lower pH
- Basic (cationic) nuclear proteins also bind eosin
 - Red colored nucleoli

EOSINS

(formulations)

- Commercially available eosins:
 - Alcoholic eosins
 - Aqueous eosins
 - Eosins with phloxine
- Eosins may differ with respect to eosin concentration, solvent properties and pH.

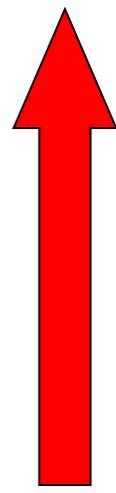
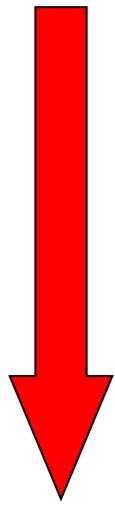
Some Basics of Dye Chemistry

- pH is critical to determining the charge on both the dye and the tissue molecules.
- pH
 - Measurement of acidity
 - Measurement of the concentration of protons (H^+) in the dye solution

Relationship of pH and charge

pH of dye solution

(+) charge on tissue

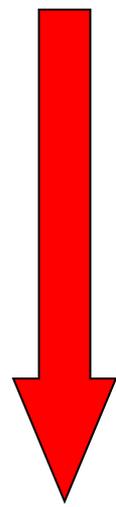
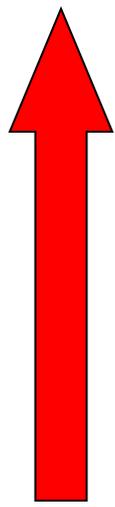


Low pH solutions increase the number of charged groups within the tissue

Relationship of pH and charge

pH of dye solution

(+) charge on tissue



High pH solution decreases the number of charged groups within the tissue

EOSINS

(pH effects)

- 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
 - Inadequate rinsing to remove bluing agent
 - Contamination with bluing agent

EOSINS

(pH effects)

- As the pH is reduced below 3 – 4:
 - The net positive charge of proteins increases
 - Acidic groups of eosin molecules become protonated and eosin becomes neutral
 - Eosin binds nonspecifically by hydrophobic bonds
 - Staining decreases and becomes murky

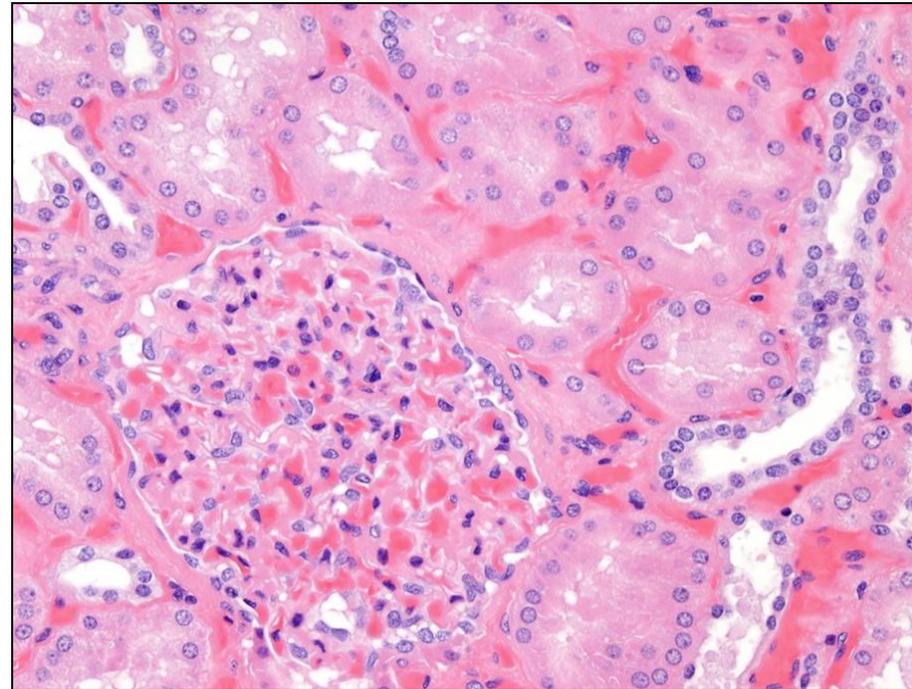
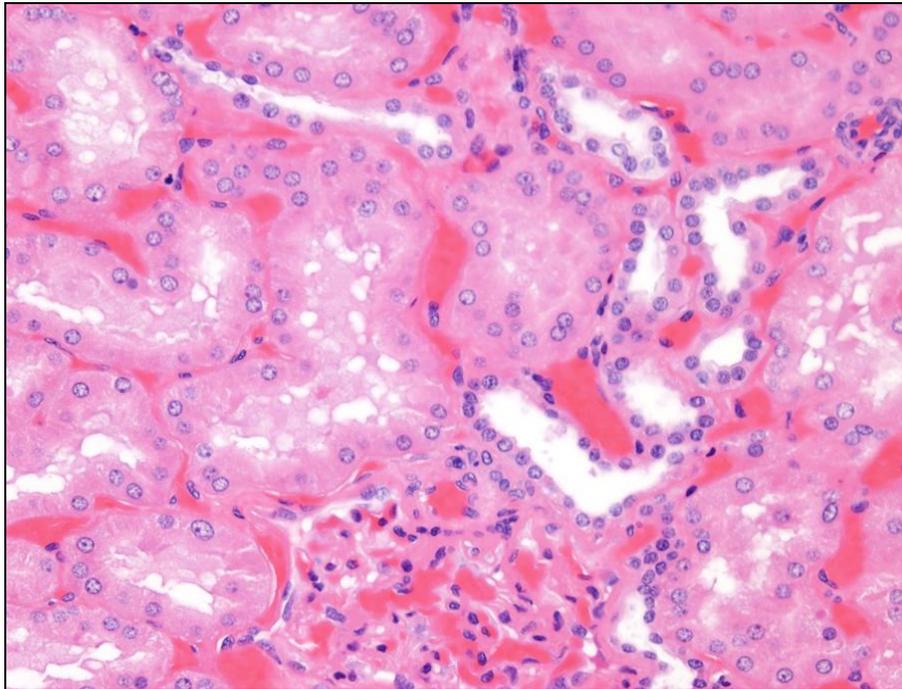
EOSINS

(pH effects)

Eosin Y Staining

pH = 4.3

pH = 5.2



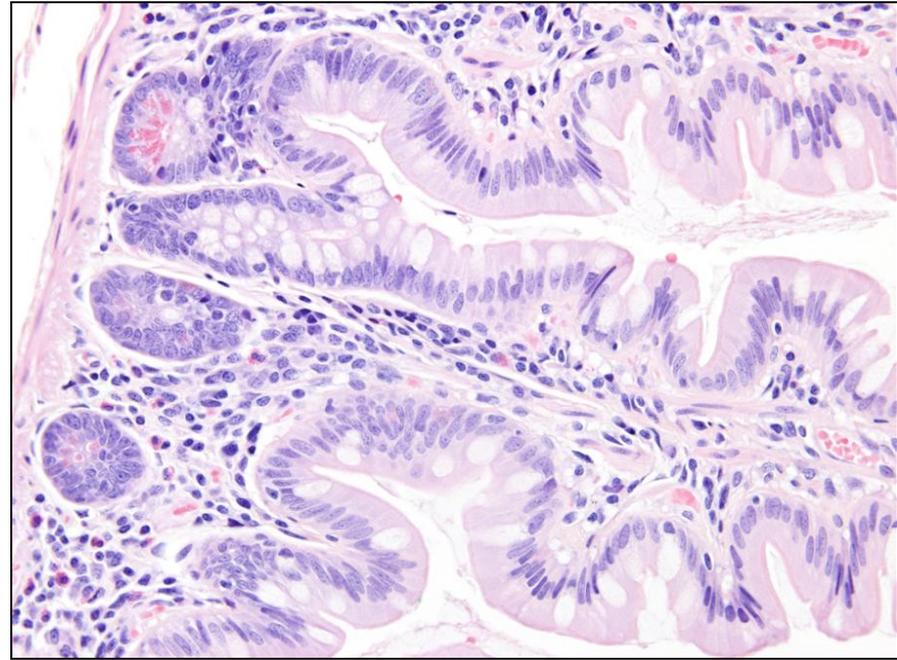
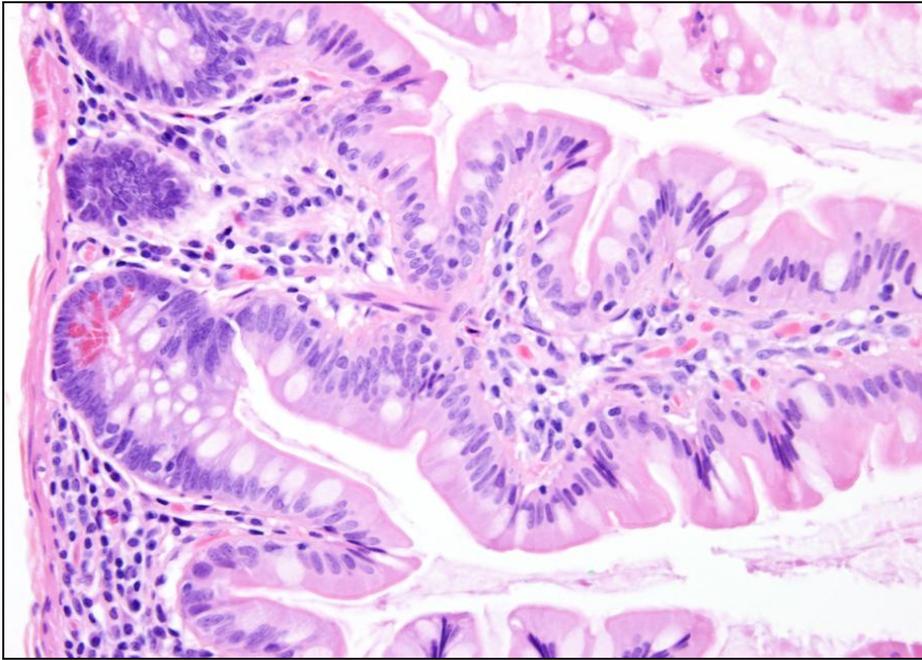
EOSINS

(pH effects)

Eosin Y Staining

pH = 4.3

pH = 5.2



EOSINS

(formulations)

- Addition of water to an alcoholic eosin can cause precipitation.
 - As water is added the pH of the eosin solution decreases
 - Hydrophobic attractions are increased with the addition of water
- When using an alcoholic eosin, an alcohol rinse should be placed prior to the eosin solution

EOSINS

(differentiation)

- The depth or intensity of the eosin stain can be controlled by;
 - The staining time in eosin
 - The composition and length of rinses following staining
- Following eosin staining, slides may be rinsed in water, alcohol or a mixture of the two.

EOSINS

(differentiation)

- Differentiation in a solution containing water must be monitored carefully to prevent the removal of too much eosin.
- Most differentiation solutions are 80-95% ethanol.
- If water is used it must be removed prior to the clearing agent as it will cause leaching of the eosin following cover slipping.

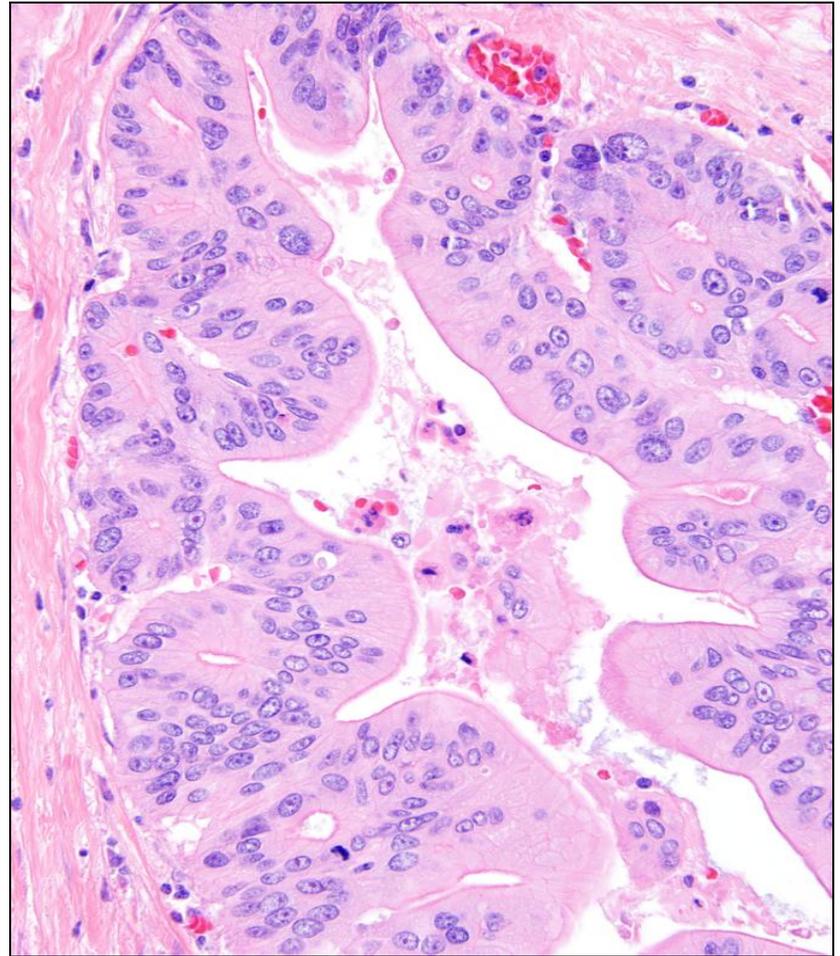
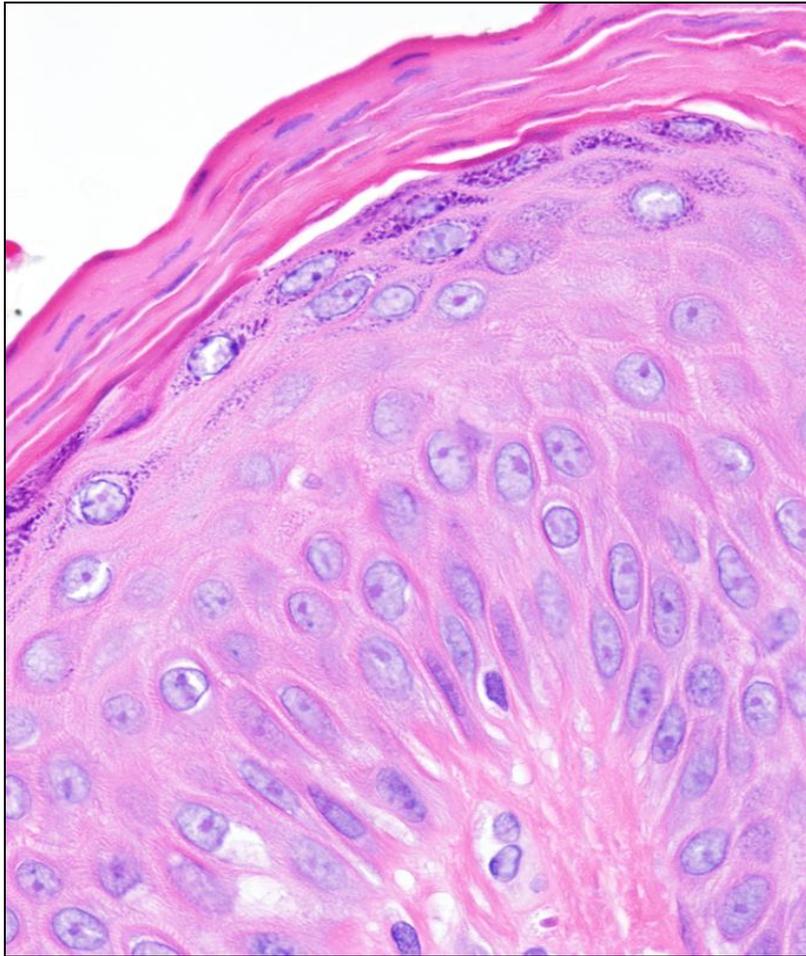
The H and E Stain

(balance of coloration)

- The overall coloration of the stained specimen is the result of the balance of the intensity of the alum-hematoxylin and eosin.
- Remember
 - Alum-hematoxylin can stain cytoplasm
 - Eosin Y can stain nuclear basic protein

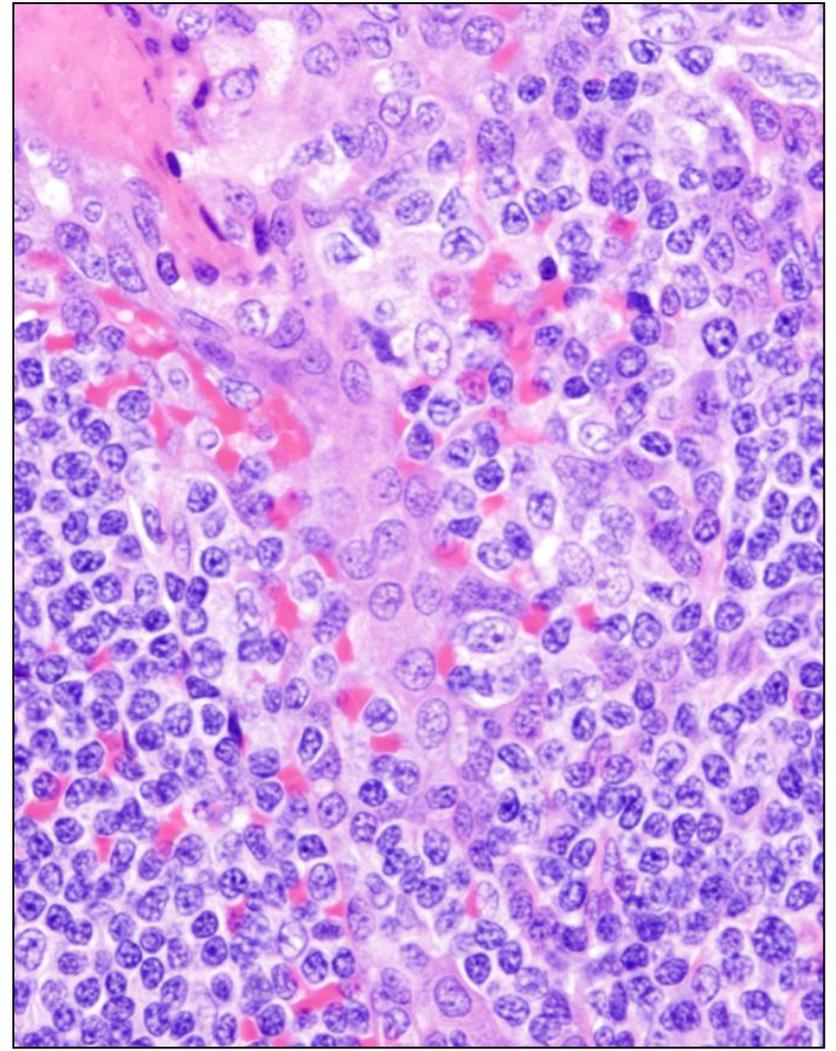
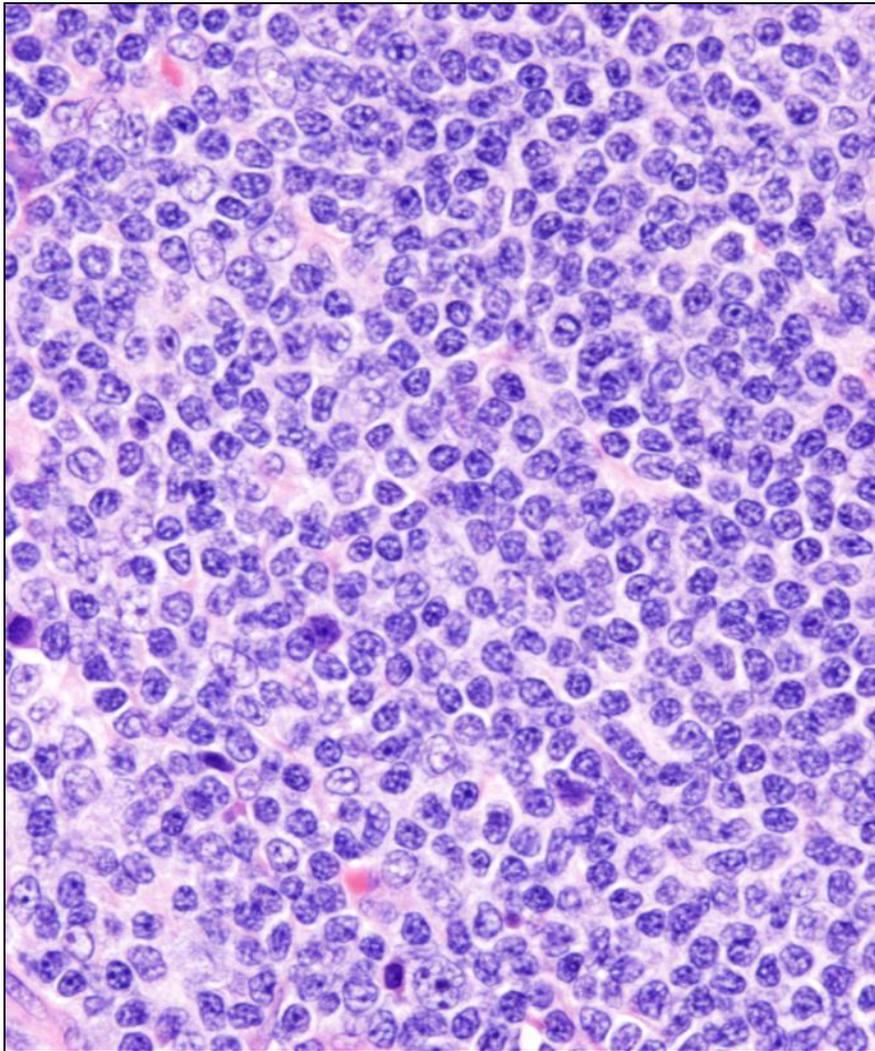
The H and E Stain

(balance of coloration)



The H and E Stain

(balance of coloration)



The H and E Stain

(balance of coloration)

- Over-stained alum-hematoxylin
 - Lack of nuclear detail
 - Poor nuclear/cytoplasmic contrast
 - Cytoplasmic detail is masked
- Light or washed out alum hematoxylin
 - Nuclei are not crisp
 - Poor nuclear/cytoplasmic contrast

The H and E Stain

(balance of coloration)

- Over-stained eosin Y
 - Nuclei may lack crispness-purple
 - Poor nuclear/cytoplasmic contrast
- Light or washed out eosin Y
 - Cytoplasmic detail is lacking
 - Acidophilic nucleoli may not be visible
 - Poor nuclear/cytoplasmic contrast

PREMIUM STAINING SYSTEMS

(Complete Staining System)

- Buffered stains & reagents
- Balanced and consistent pH
- Enhanced Q.A./Q.C.
- Developed to be used as components in an H&E staining system:
 - Hematoxylin
 - Differentiation Reagent
 - Bluing Reagent
 - Eosin
- Essential for high volume throughput

Systematic Changes in Stain

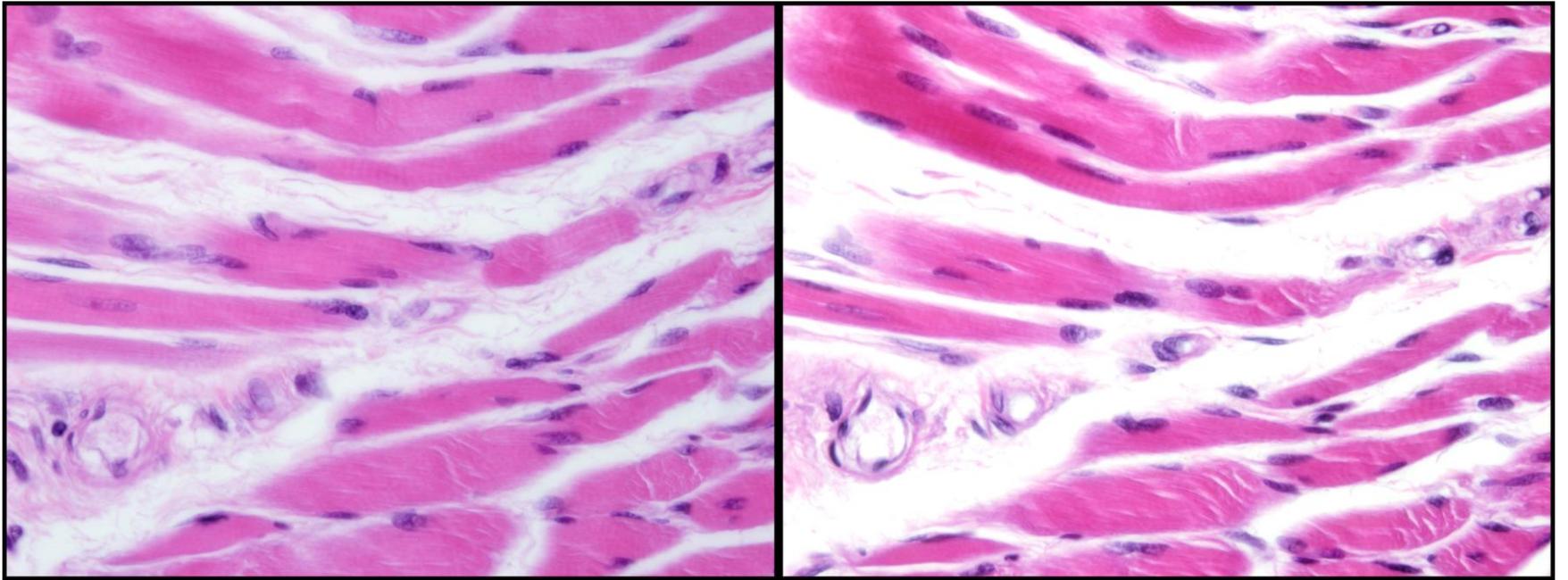
Systematic changes in dye/reagent integrity can present subtle to gross changes in dye/reagent chemistry and performance

Few laboratories monitor their solution pH or stain consistency, resulting in an unmonitored progressive change in stain from the first slide to last.

STAINING 'SYSTEMS'

Premium Hematoxylin Stain

- Produces well delineated crisp nuclear staining.
- Convenience of a Gill-type hematoxylin:
 - No precipitation
 - No filtering required
- High throughput or capacity stain
- Stable pH



Most Common Stain Issues

- Carry over
- Dilution and/or contamination of stain
- Elevation of pH
- Depletion of hematoxylin dye content

Results of Unmonitored Stain Issues

- Loss of stain contrast between nuclear & cytoplasmic stain
- Weak or faded cytoplasmic stain (Eosin)
- Nuclei blurry or smudgy appearance (hematox.)
- Loss of color balance between hematoxylin stain and eosin counterstain
- Repeated change out of stains

OPTIMIZING THE H&E STAIN

- Reduces non-specific staining
- Sharpens nuclear detail
- Optimizes counterstain towards desired preference
- Optimized for pathologists personal preference
- Enhances diagnostic capabilities

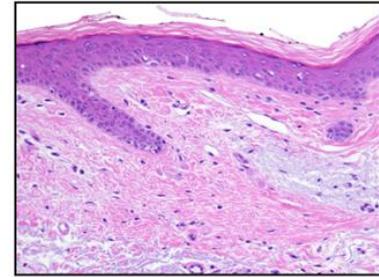
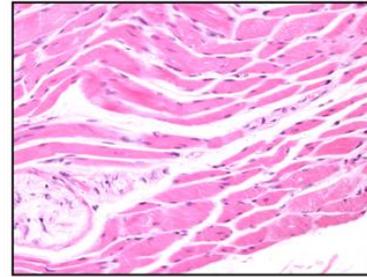
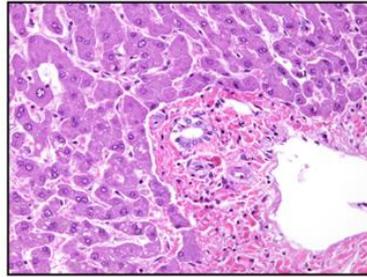
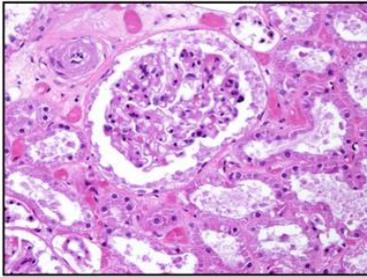
20X

Kidney

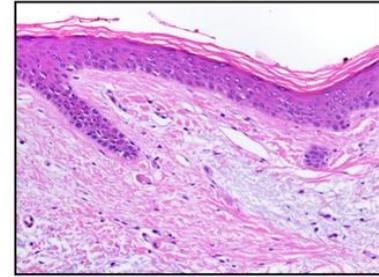
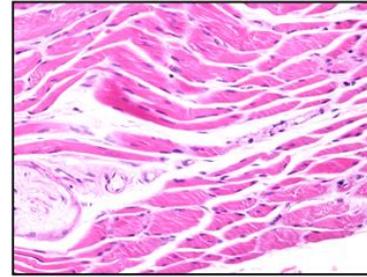
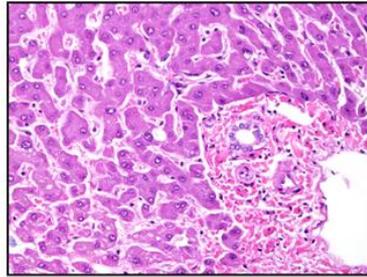
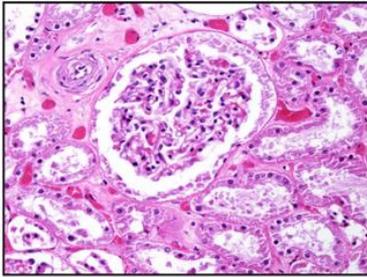
Liver

Muscle

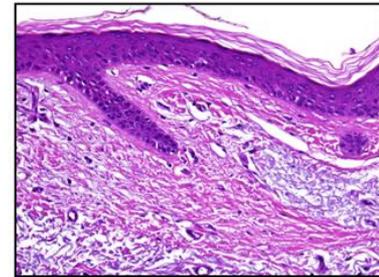
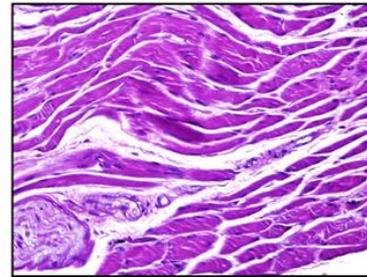
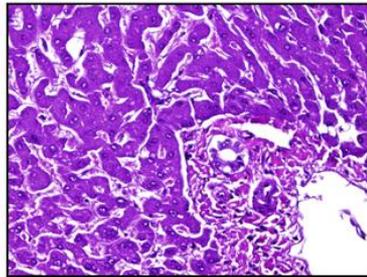
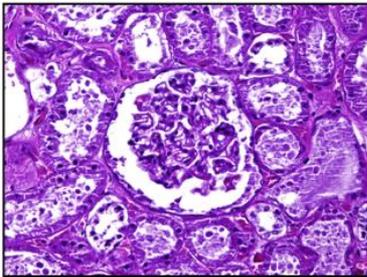
Skin



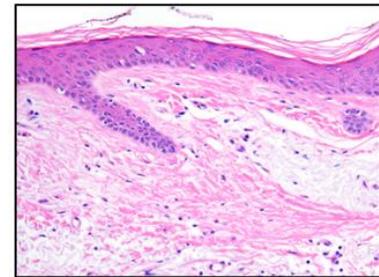
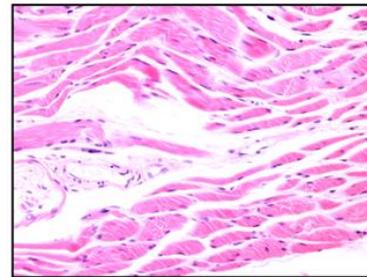
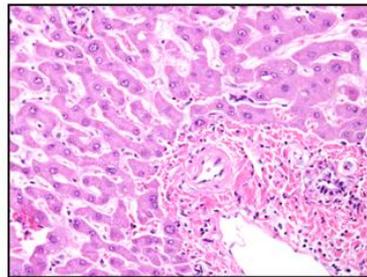
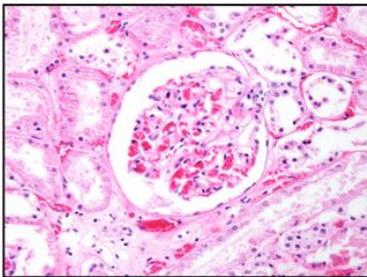
A. Normal Staining



B. Elevated pH



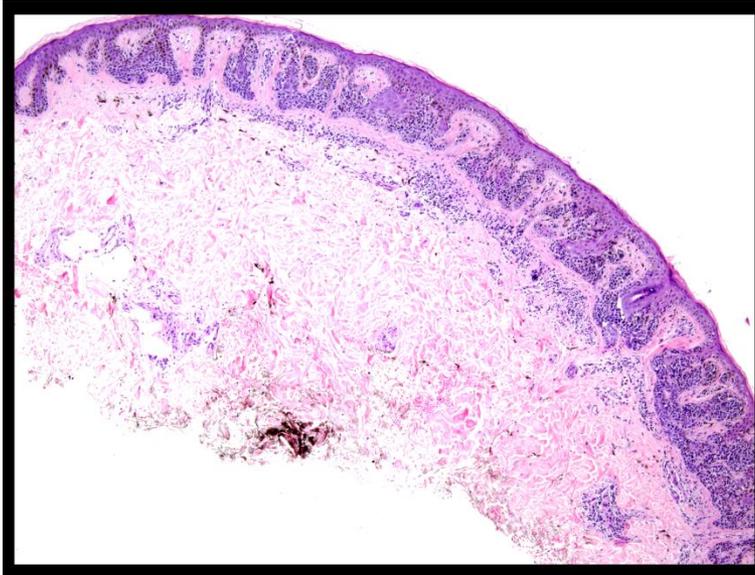
C. No Differentiation (Straight Progressive)



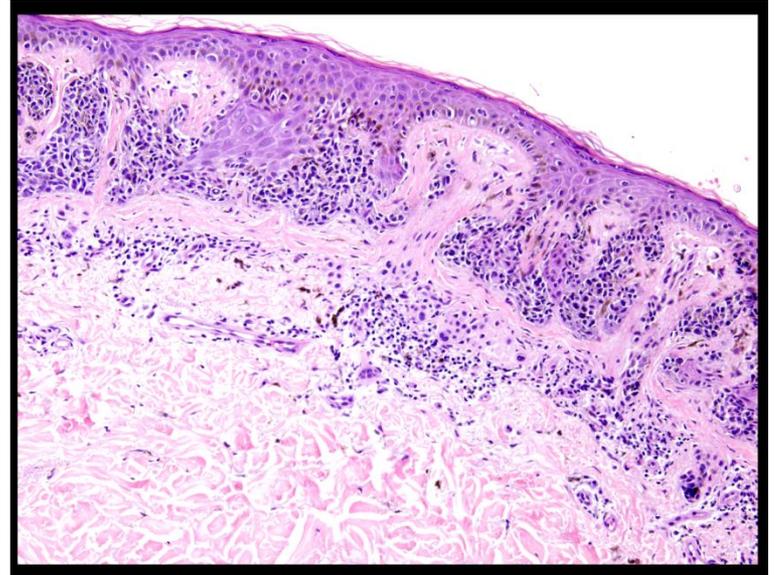
D. Gill's Hematoxylin

| Protocol Station | Standard Protocol (Applications Lab) | ST-1 0 | ST-2 0 | ST-3 0 |
|------------------|-----------------------------------------|-----------|-----------|-----------|
| Xylene | 2 min. | 2 min. | 2 min. | 2 min. |
| Xylene | 2 min. | 2 min. | 2 min. | 2 min. |
| Xylene | 2 min. | 2 min. | 2 min. | 2 min. |
| 100% OH | 2 min. | 2 min. | 2 min. | 2 min. |
| 100% OH | 2 min. | 2 min. | 2 min. | 2 min. |
| 95% OH | 1 min. | 1 min. | 1 min. | 1 min. |
| 80% OH | 1 min. | 1 min. | 1 min. | 1 min. |
| Water | 2:00 min. | 2:00 min. | 2:00 min. | 2:00 min. |
| Hematoxylin | 4:00 min. | min. | min. | min. |
| Water Wash | 3:00 min. | 3:00 min. | 3:00 min. | 3:00 min. |
| Differentiate | 1 min. | | | : |
| Water | 3:00 min. | :30 sec. | :30 sec. | :30 sec. |
| Bluing | 1:00 min. | 1:00 min. | 1:00 min. | 1:00 min. |
| Water | 3:00 min. | 3:00 min. | 3:00 min. | 3:00 min. |
| Eosin | :30 sec. | : sec. | : sec. | : sec. |
| 95% OH | :15 sec. | : sec. | : sec. | : sec. |
| 100% OH | :30 sec. | :30 sec. | :30 sec. | :30 sec. |
| 100% OH | 1:00 min. | 1:00 min. | 1:00 min. | 1:00 min. |
| 100% OH | 2:00 min | 2:00 min | 2:00 min | 2:00 min |
| Xylene | 2:00 min. | 2:00 min. | 2:00 min. | 2:00 min. |
| Xylene | 2:00 min. | 2:00 min. | 2:00 min. | 2:00 min. |

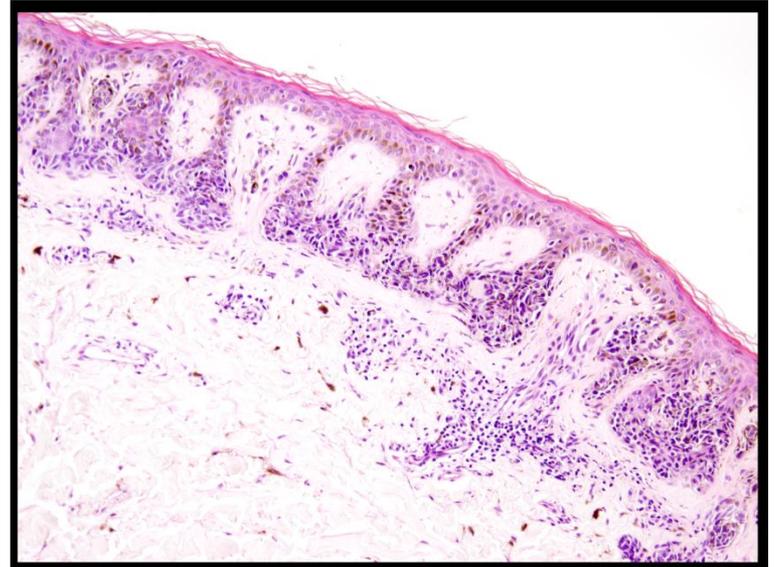
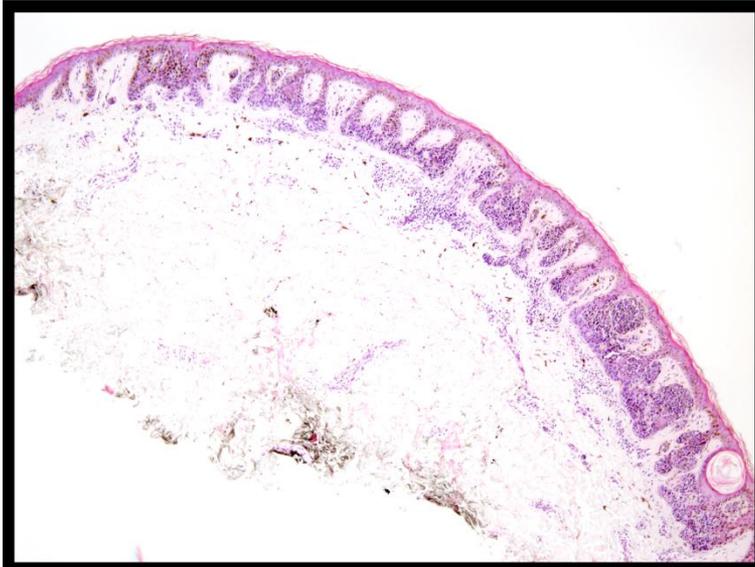
Slide Match Project



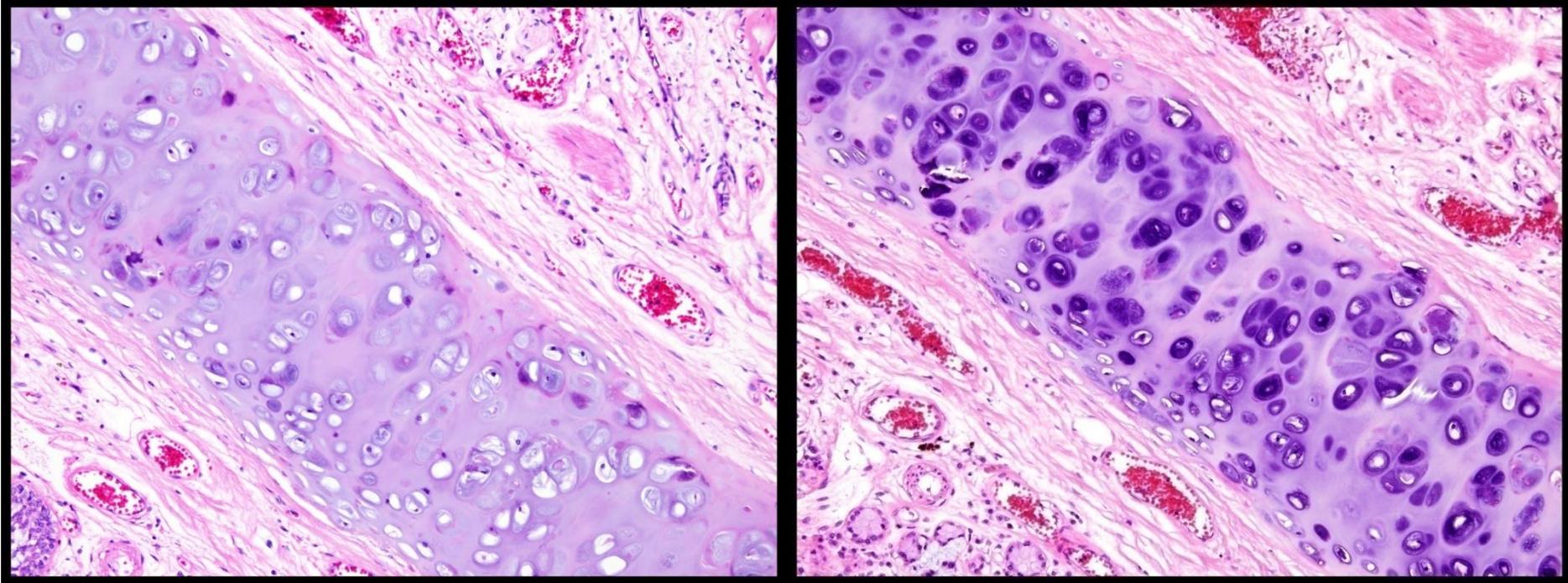
4X



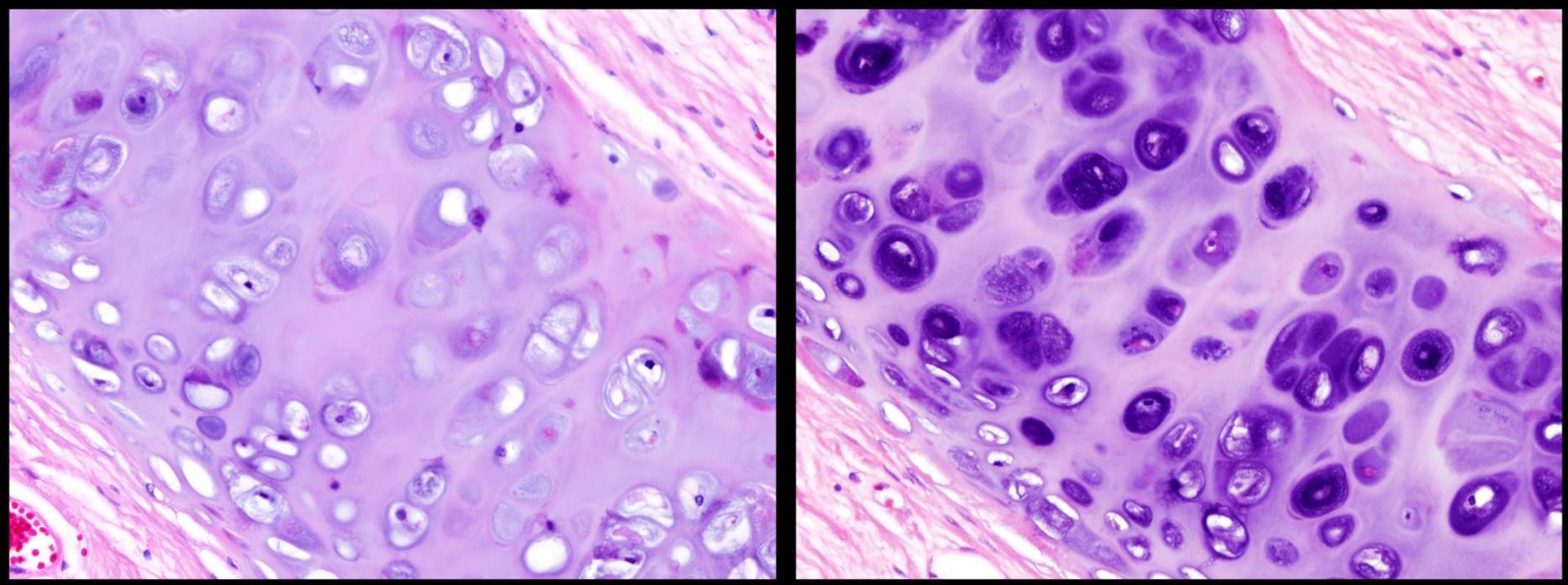
10X

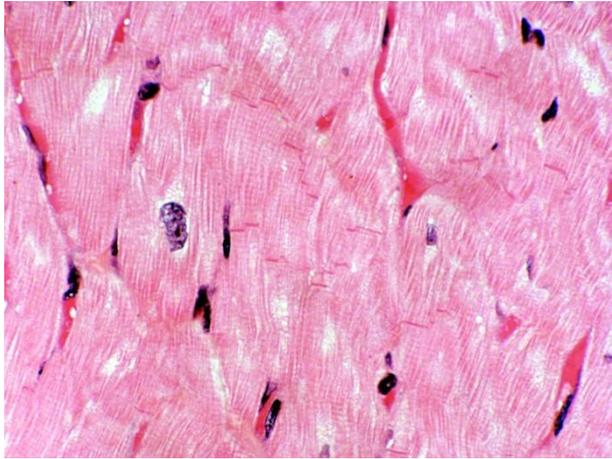


Slide Match Project



Slide Match Project





H&E STAINING TROUBLESHOOTING

Donna J. Emge, HT (ASCP)

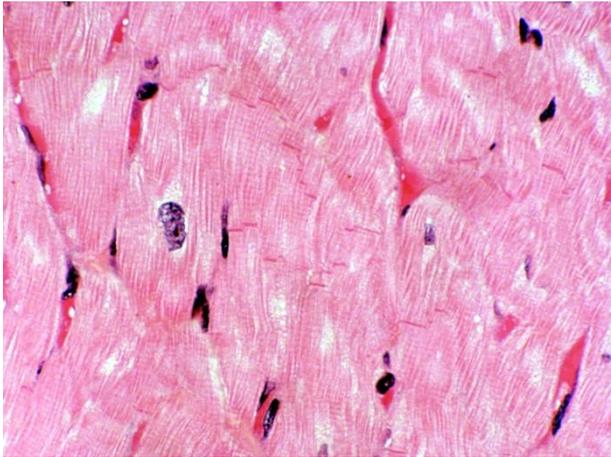
Robert H. Lurie Comprehensive Cancer Center

Northwestern University

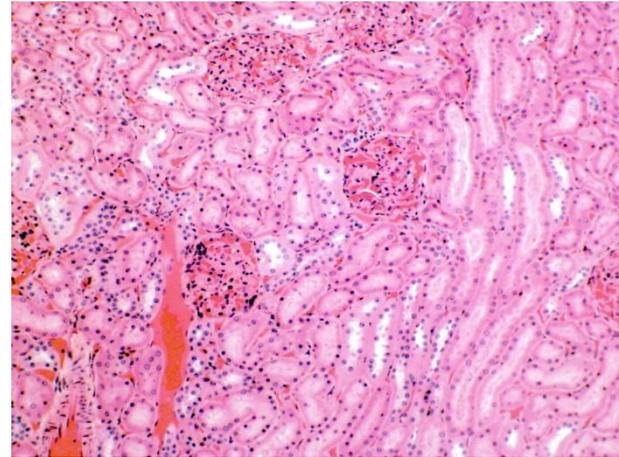
MHPL@northwestern.edu

H&E STAINING TROUBLESHOOTING

- Paraffin sections
- Frozen sections
- Hematoxylin counterstaining IHC
- Troubleshooting



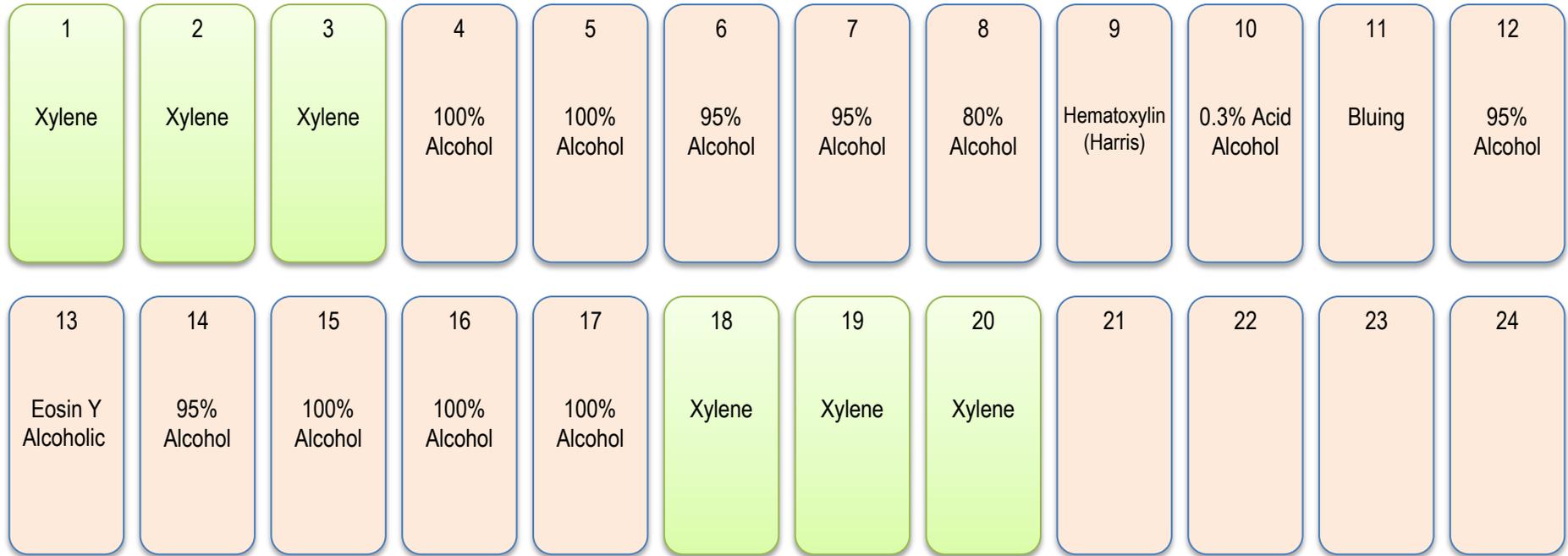
Cardiac muscle, H&E



Kidney, H&E

<http://www.vetmed.vt.edu/education/curriculum/vm8054/labs/lab2/examples/exhe.htm>

H&E REGRESSIVE STAIN SETUP

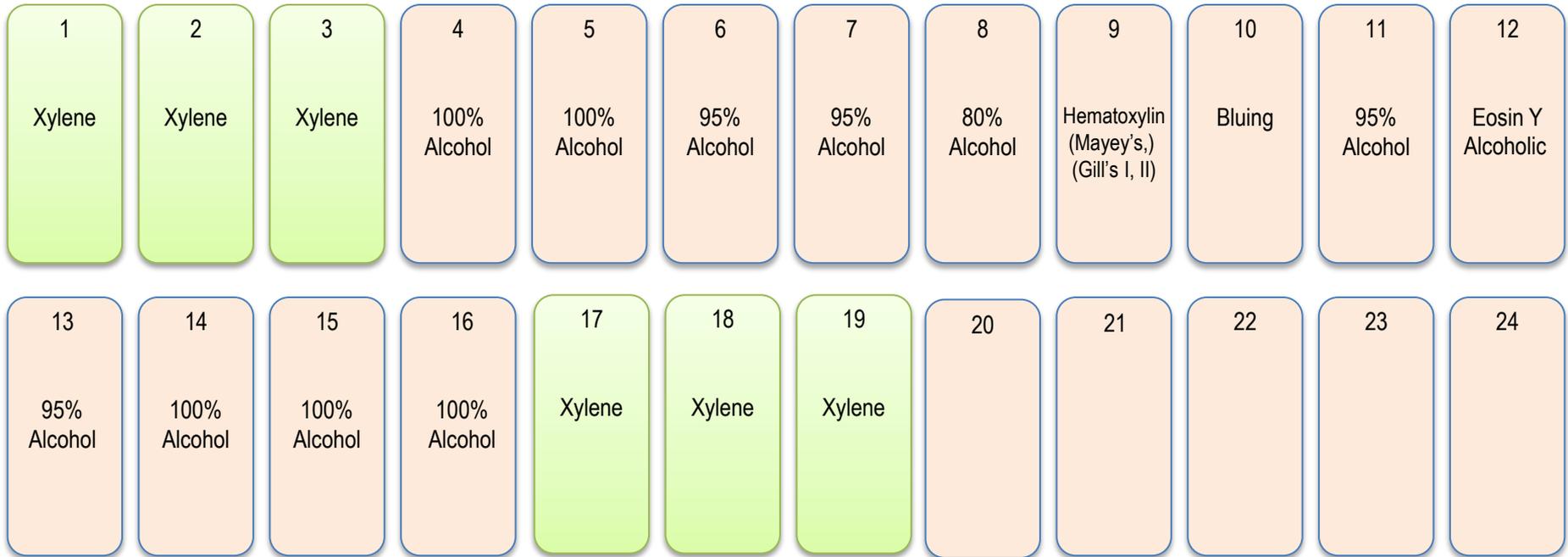


In regressive Hematoxylin stain protocols the nuclear chromatin and other tissue section elements are over-stained with a strong hematoxylin solution and requires the excess stain to be removed by a dilute acid solution to remove the excess stain. This is a common staining method in clinical labs.

Proponents of this method argue, that when differentiated correctly, the nuclear detail stands out brighter and crisper against the Eosin counterstain than other H&E staining methods.

The disadvantage is that over differentiation will cause pale nuclei, or under differentiation will obscure fine detail, prevent correct Eosin counterstaining.

H&E PROGRESSIVE STAIN SETUP



In Progressive Hematoxylin stain protocols the nuclear chromatin and other tissue section elements are stained to the desired endpoint without an acid alcohol differentiating step. NOTE that there is not acid alcohol in the setup.

Proponents of this method argue that the nuclear staining is more consistent and not prone to errors that can be caused by the added differentiation step found in regressive staining.

The disadvantage is that the nuclei may not stain as bright and crisp due to the weaker staining properties of progressive formulated hematoxylin. The progressive hematoxylin cannot be pushed enough to overcome weak nuclear staining caused by certain fixatives (i.e.: Bouin's, Davidson's) and decalcifying agents.

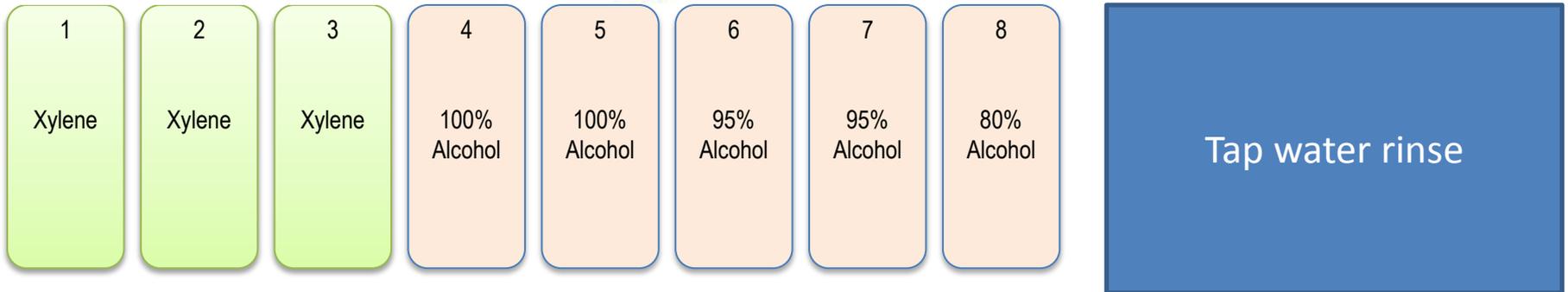
DRYING DEPARAFFINIZATION AND HYDRATION

Quality H&E staining results start from the very first steps of the staining process.



Paraffin sections on slides are given time to drain and then routinely dried in a 60°C oven for 30 minutes to 1 hour – depending upon tissue type.

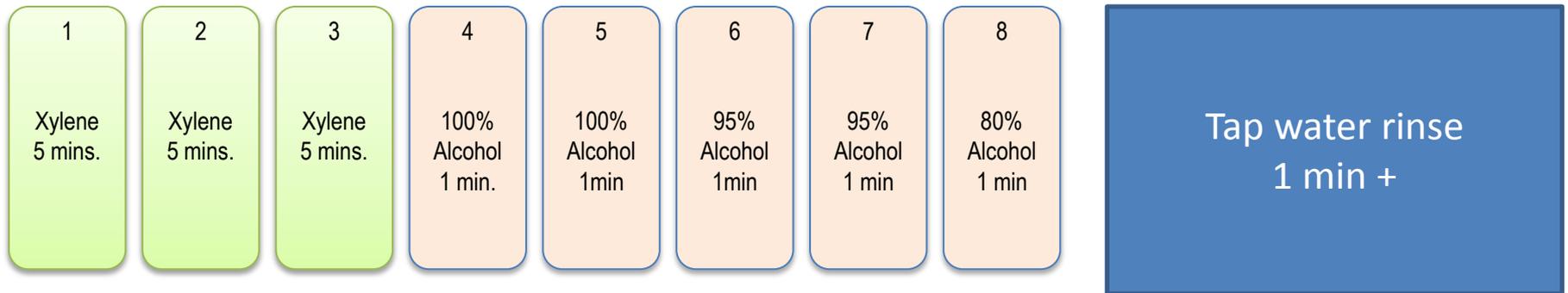
Some tissues benefit from 40°C overnight (i.e. bone and brain sections).



Sections inadequately dried will not deparaffinize sufficiently with xylene and will not pick up the stains consistently. The end result will be poorly H&E stained sections.

DEPARAFFINIZATION AND HYDRATION

Quality H&E staining results start from the very first steps of the staining process.



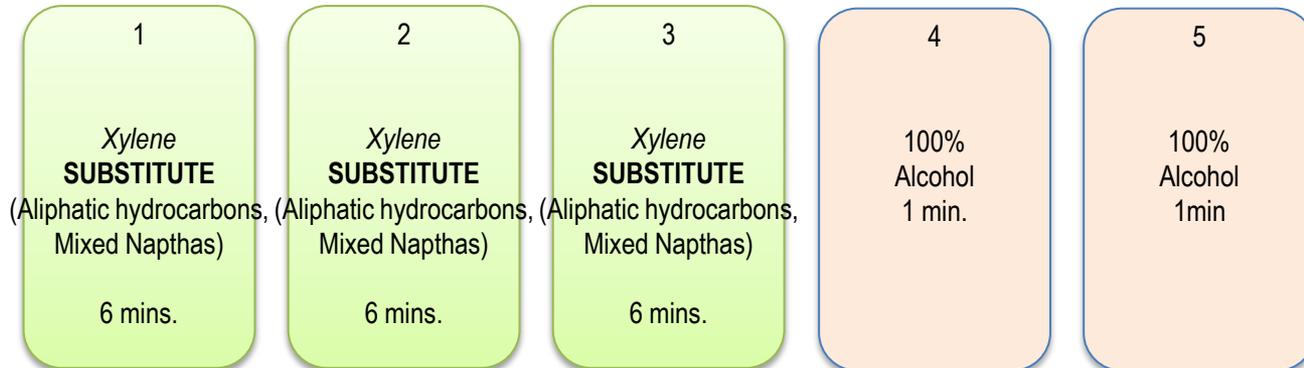
Deparaffinization: The process in which paraffin is removed from the sections. Xylene is not miscible with water so sections must be dried before being placed in xylene. Section drying and paraffin removal are critical to successful H&E staining.

100% Reagent Alcohol / Absolute Ethanol: Is the intermediary between xylene and hydration. Removal of the xylene with 100% alcohol before hydration is critical to a successful H&E stain.

95% Alcohol, 80% alcohol, tap water: Hydration begins the gradual introduction of water to the tissue sections.

DEPARAFFINIZATION WITH XYLENE SUBSTITUTES

Quality H&E staining results start from the very first steps of the staining process.



Xylene Substitutes: There are a variety of xylene substitutes – aliphatic hydrocarbons and mixed naphthas that have been shown to be much safer than xylene and to have less fumes. SlideBrite, Safeclear, Thermo Histosolve, Anatech Pro-Par, Clear-Rite 3, XS from Statlab, CBG Biotech Formula 83, and other brands.

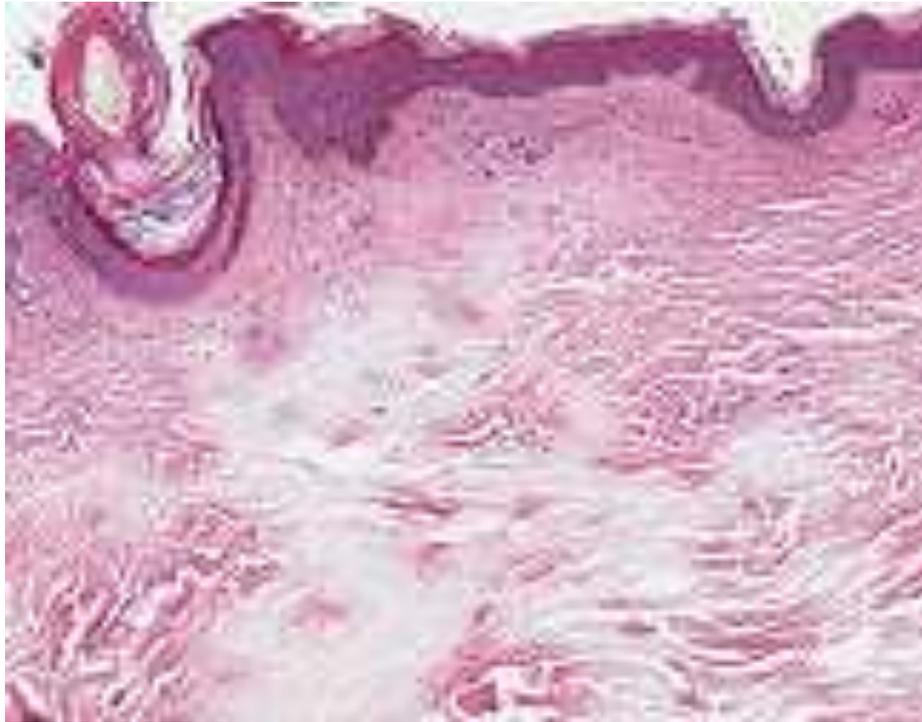
Notes: The safer aliphatic hydrocarbons, or mixed naphthas do a great job:

- Deparaffinizing time must be increased
- They absorb atmospheric moisture so must be rotated at least daily. Solution 1 discarded, solutions 2 and 3 moved ahead. Spot 3 will be filled with fresh.
- The stain setup does not need to be under a hood as required for xylene.
- Not recommended for coverslipping – incompatible with xylene based mountant.

Warning: Xylene substitutes such as the limonenes, citrisolv, Hemo-de, and histosol are just as hazardous or worse than Xylene. Make sure to read the product information and MSDS.

DEPARAFFINIZATION AND HYDRATION

Quality H&E staining results start from the very first steps of the staining process.

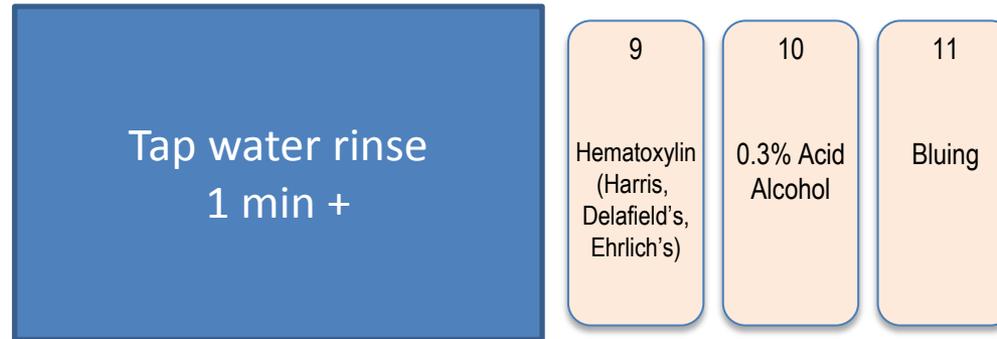


Incomplete wax removal prior to staining

http://www.rcpaqapa.netcore.com.au/cgi-bin/site/wrapper.pl?c1=Notices&c2=Technical_2008_TE08_02

HEMATOXYLIN STAINING BEGINS

Regressive Hematoxylin



Notes: Before use Hematoxylin must be filtered through a large #4 Watman's filter or through a paper towel to remove the metallic sheen that will cause precipitate on stained sections.

If Hematoxylin on the filter paper appears dark bluish purple it may not be fully ripened and will usually stain darker.

If the appearance is maroon red, then the hematoxylin is old and will stain lightly with no crispness. If it appears dark bluish purple with a maroon red top edge then it is perfectly ripened for staining.

Hydration: Hydrate by graded alcohol series to 80% alcohol, 1 min each; Water, 1+ minutes.

Stain: Stain in Harris Hematoxylin or other regressive hematoxylin for 8 to 15 minutes.

Stain Rinse: Remove excess stain by rinsing in running tap water for 1+ minutes.

Destain and Differentiate: This is the critical step that determines the quality of the final preparation. Destain in 0.3% acid alcohol until the cytoplasm has only a faint stain but the sharp nuclear stain still remains. 3 or 4 dips. Nuclei will appear reddish.

Destain Wash: Briefly rinse in tap water to remove excess acid and halt destain.

Hematoxylin Bluing: Dip in 0.3% ammonia water or saturated lithium carbonate water until specimens are blue jean blue (3-6 dips).

Final Rinse: Rinse in running tap water for 1+ minutes.

HEMATOXYLIN STAINING BEGINS

Progressive Hematoxylin



Hydration: Hydrate by graded alcohol series to 80% alcohol, 1 min each; Water, 1+ minutes.

Stain: Stain in Mayers Hematoxylin or other Progressive hematoxylin for 6 to 15 minutes.

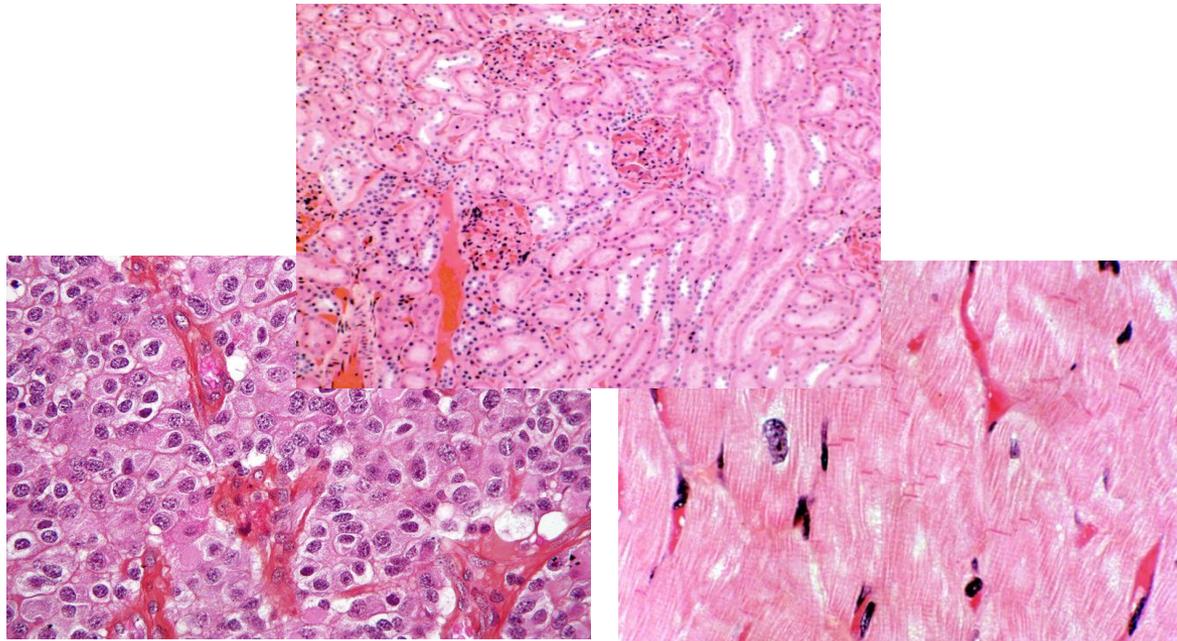
Stain Rinse: Remove excess stain by rinsing in running tap water for 1+ minutes.

Hematoxylin Bluing: Dip in 0.3% ammonia water or saturated lithium carbonate water until specimens are blue jean blue (3-6 dips).

Final Rinse: Rinse in running tap water for 1+ minutes.

EOSIN STAINING BEGINS

Eosin Y, Eosin Y with Phloxine, Eosin B



A quality Eosin stain should show at least 3 color shades from light pink to bright fuschia.

Hematoxylin should appear a sharp purple blue and not be obscured by the Eosin.

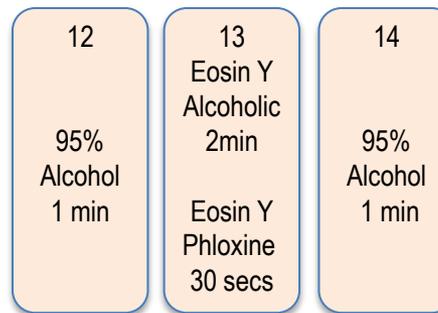
Eosin with Phloxine has deeper bright fuschia areas and stains rapidly.

Eosin Y alcoholic shows color shifts during the 95% differentiation.

Eosin Y hydroous has palest staining with little color shifts.

EOSIN STAINING BEGINS

Eosin Y, Eosin Y with Phloxine, Eosin B



After the bluing rinse of Hematoxylin staining the slides are placed in 95% alcohol before staining in Alcoholic Eosin Y, or Eosin with Phloxine.

Eosin Y staining from 1 to 2 minutes. Eosin Y with Phloxine staining is 30 to 40 seconds.

Differentiation and dehydration takes place at the same time beginning with 95% alcohol.

EOSIN DIFFERENTIATION



Very weak eosin staining, High Power



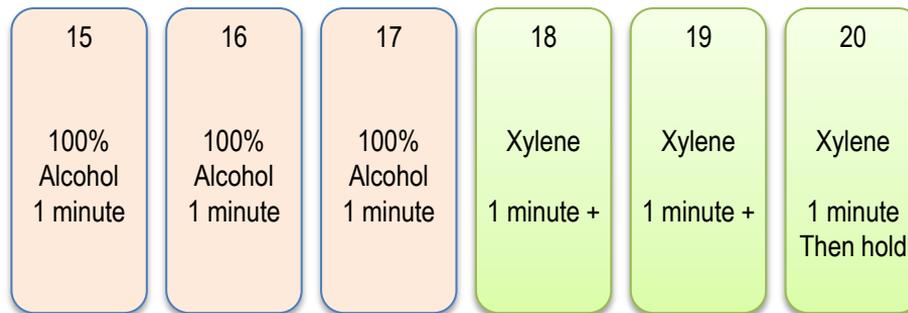
Good color balance of Haematoxylin to Eosin, High Power

http://www.rcpaqapa.netcore.com.au/cgi-bin/site/wrapper.pl?c1=Notices&c2=Technical_2008_TE08_02

If over differentiated bring sections back through the alcohols, stain again in Eosin and differentiate rapidly through the dehydrating alcohols.

If under differentiated leave longer in the 95% alcohol before proceeding through the dehydrating alcohols.

DEHYDRATION AND CLEARING THE FINAL STEPS



Eosin continues to differentiate as the sections are dehydrated through the 3 changes of 100% alcohol.

The sections are cleared through 3 changes of xylene and held in xylene for mounting with a xylene based mounting medium and glass coverslips.

NOTE: If xylene substitute is used for clearing it will not be compatible with xylene or the mounting medium. The xylene substitute must be removed by quickly dipping the slides in 2 changes of 100% alcohol and holding in xylene under the hood for mounting with a xylene based mounting medium and glass coverslips.

TROUBLESHOOTING THE H&E STAIN

| PROBLEM | CAUSE | SOLUTION |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| White spots are seen in the section after deparaffinization step. If they are not recognized at this point, spotty or irregular staining will be seen microscopically on the stained section. | <p>A. The section was not dried properly before beginning deparaffinization.</p> <p>B. the slide did not remain in xylene long enough for complete removal of the paraffin.</p> | <p>A. The slides must be treated with absolute alcohol to remove the water and then retreated with xylene to remove the paraffin. If incomplete drying is severe, the sections may loosen from the slides.</p> <p>B. The slides should be returned to xylene for a longer time.</p> |
| The nuclei are too pale (the hematoxylin is too light). | <p>A. The sections were not stained long enough in hematoxylin.</p> <p>B. The hematoxylin was overoxidized and should not have been used.</p> <p>C. The differentiation step was too long.</p> <p>D. Pale nuclei in bone sections may be the result of over decalcification.</p> | <p>A. The section must be restained. When sections have been placed in an extremely acidic fixative such as Zenker solution, the ability to stain the nucleus may be impaired and the time in the hematoxylin may have to be increased, or a method to increase tissue basophilia may be needed.</p> <p>B. Discard hematoxylin and replace with fresh.</p> <p>C. Run back and restain</p> <p>D. no solution</p> |
| The nuclei are overstained (the hematoxylin is too dark), or diffuse hematoxylin staining of the cytoplasm has occurred. | <p>A. The sections were stained too long in hematoxylin.</p> <p>B. The sections are too thick.</p> <p>C. The differentiation step was too short.</p> | <p>A. Decolorize the section and restain, making appropriate adjustments in the staining time of hematoxylin.</p> <p>B. Recut the section.</p> <p>C. . Decolorize the section and restain, making appropriate adjustments in the differentiation times.</p> |
| Red or red-brown nuclei. | <p>A. The hematoxylin is breaking down.</p> <p>B. The sections were not blued sufficiently.</p> | <p>A. Check the oxidation status of the hematoxylin.</p> <p>B. Allow a longer time for bluing of the sections; it is impossible to over blue the sections.</p> |
| Pale staining with eosin. | <p>A. The pH of the eosin solution may be above 5.0, possibly caused by carryover of the bluing reagent.</p> <p>B. The sections may be too thin.</p> <p>C. Slides may have been left too long in the dehydrating solutions.</p> | <p>A. Check the pH of the eosin solution, and adjust it to a pH of 4.6 to 5.0 with acetic acid if necessary. Be sure the bluing reagent is completely removed before transferring the slides to the eosin.</p> <p>B. Check the thickness of the section.</p> <p>C. Restain with eosin and do not allow the stained slides to stand in the lower concentrations of alcohols.</p> |
| Cytoplasm is overstained, and the differentiation is poor. | <p>A. The eosin solution may be too concentrated, especially if phloxine is present.</p> <p>B. The section may have been stained for too long.</p> <p>C. The sections may have been passed through the dehydrating alcohols too rapidly for good differentiation of the eosin to occur.</p> | <p>A. Dilute the eosin solution.</p> <p>B. Decrease the staining time.</p> <p>C. Allow more time in each of the dehydrating solutions for adequate differentiation of the eosin. (Also, check the section thickness.)</p> |

TROUBLESHOOTING THE H&E STAIN

| PROBLEM | CAUSE | SOLUTION |
|-----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Blue—black precipitate on top of the sections. | The metallic sheen that develops on most hematoxylin solutions has been picked up on the slide. | Filter the hematoxylin solution daily before staining slides. |
| Water bubbles are seen microscopically in the stained sections. | The sections were not completely dehydrated, and water is present in the mounted section. | Remove the cover glass and mounting medium with xylene. Return the slide to fresh absolute alcohol (several changes). After the sections are dehydrated, clear with fresh xylene and mount with synthetic resin. All dehydrating and clearing solutions should be changed before staining any more sections. |
| Difficulty bringing some areas of the tissue in focus with light microscopy. | Mounting medium may be present on top of the cover glass. | Remove the cover glass and remount with a clean cover glass. Review the method used for mounting sections, and modify if needed. |
| The mounting medium has retracted from the edge of the cover glass. | A. The cover glass is warped. B. The mounting medium has been thinned too much with xylene. | A. Remove the cover glass and apply a new cover glass. B. Apply a new cover glass with fresh mounting medium. Keep the mounting medium container tightly capped when not in use. Use a small container for the mounting medium and discard when it becomes too thick. |
| The water and the slides turn milky when the slides are placed in the water following the rehydrating alcohols. | Xylene has not been removed completely by the alcohols. | Change the alcohols, back the slides up to absolute alcohol, and rehydrate the sections. |
| The slides are hazy or milky in the last xylene rinse prior to coverslipping. | Water has not been completely removed from the sections before being placed in the xylene. | Change the alcohol solutions, especially the anhydrous or absolute reagents. Redehydrate the sections and clear in fresh xylene. |
| The mounted stained sections do not show the usual transparency and crispness when viewed by light microscopy. | The mounting medium may be too thick, causing the cover glass to be held too far above the tissue. | Remove the cover glass and mounting medium with xylene. Remount the section with fresh mounting medium. |