Troubleshooting H & E staining

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Troubleshoot H & E staining

Hematoxylin & Eosin staining is the most frequent routine stain in the Mohs Micrographic Surgery tissue preparation.

It has stood the test of time as the standard stain for histologic examination of human tissues since it was independently introduced in 1865 and 1875, by Böhmer and Fischer respectively.

Common problems, pitfalls and troubleshooting tips.

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Why H & E?

- H & E is the primary diagnostic technique for evaluation of morphology in the histopathology labs.
- One of the best nuclear stains.
- H & E provides easier identification of histological features than T-blue.
- It is easy and simple to use.
- Stains are inexpensive, yet reliable and informative.
- It is stable and durable stain, lasting years without fading

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Hematoxylin

Hematoxylin is a natural dye extracted from the heartwood of logwood trees which is indigenous in Central America, Caribbean and other tropical countries.

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Hematoxylin

- It is misleading to call hematoxylin stain as it alone does not stain.
- It has to convert to hematein. Hematein is what we call hematoxylin.
- It is a basic dye and carries a (+) charge.
- Affinity for basic dye is called basophilic.

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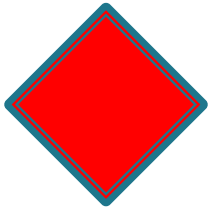
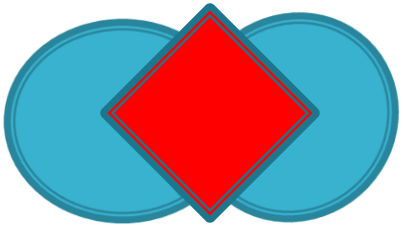
How Hematoxylin Works

Hematein
(+) charge

Chromatin
(–) charge



Mordant
(Al+3,Fe+3,Chr+3)



This complex is held by covalent bonds

Hematein–mordant–chromatin complex

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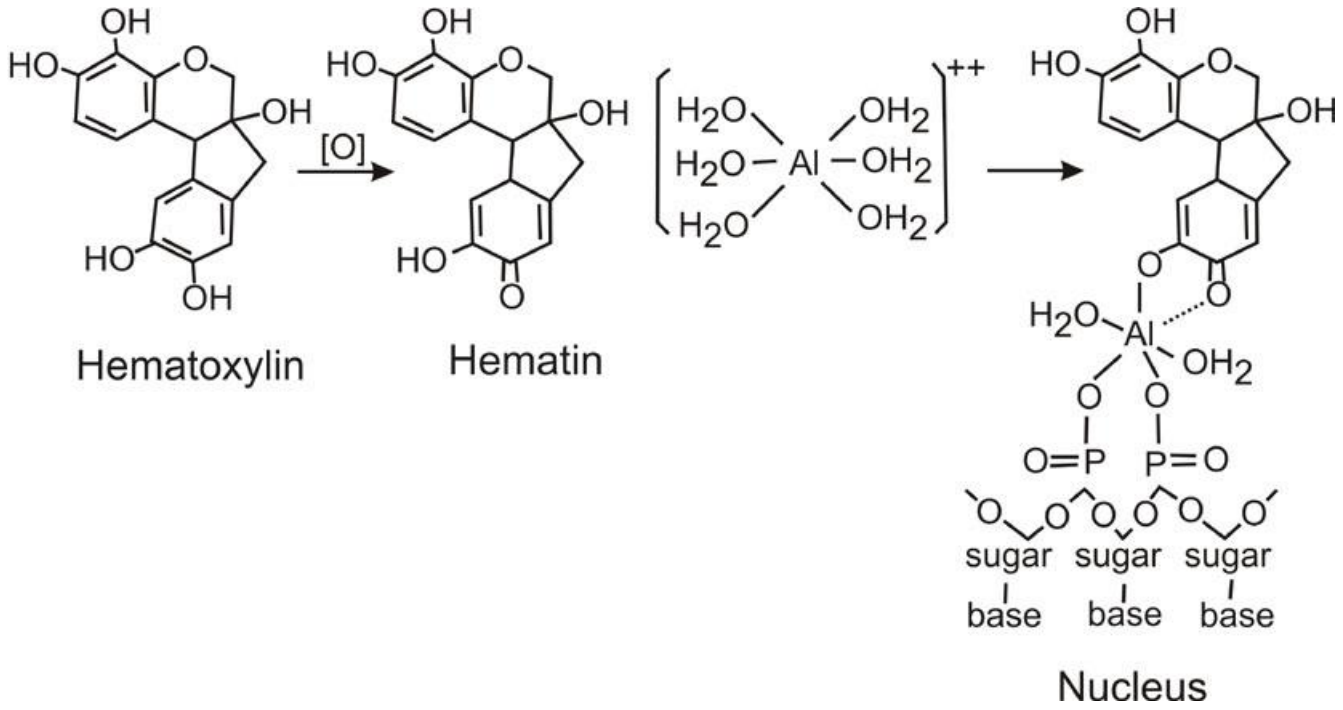
Courtesy of Biotek

Chemical structures



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Mechanisms of Hematoxylin staining

- Progressive vs regressive
- Progressive stains are: Gill's (I-III), Mayer's
- Regressive stains are Harris's, Delafield's, Ehrlich's
- Progressive method : tissue is stained and stopped
- Regressive method: tissue is overstained

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Eosin

- Eosin is a synthetic stain
- It is the counterstain and acts as an acid dye. It is negatively charged (–) and can react with positively charged (+) components in the tissue, such as connective tissues and the cytoplasm.
- This affinity is called acidophilic.
- Tissues stain pink as a result.

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Basics of dye chemistry

- Charge distribution of the dye determines attractive or repulsive characteristics of the dye.
- Positive charges (+) or cation attract to negatively charged molecules.
- Negative charges(–) or anions are attracted to positively charged molecules.
- Charge is determined by pH solution. Changes of pH will change the consistency of the dye, esp. eosin

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Challenges

- One of the challenges in the histology labs is to produce high quality H & E stain day to day.
- All sections of the tissue should look the same either on the same slide or on separate slides.
- The first slide of the day and the last slide of the day should look the same.

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Troubleshoot

In order to troubleshoot H & E, you have to understand the function of each step in the staining.

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Steps	Function	Solution	Time
1	Fixation	Reagent alcohol	30 sec - 1 min
2	Rinse	Water	15 sec - 20 sec
3	Stain	Gills III Hematoxylin	30 sec - 1 min
4	Stain	Gills III Hematoxylin	30 sec - 1 min
5	Rinse	Water	15 sec - 20 sec
6	Differentiate/decolorize	0.1% Acid Alcohol	10 sec - 15 sec
7	Rinse	Water	20 sec
8	Bluing	Scotts Tap Water	10 sec - 15 sec
9	Rinse	Water	10 sec - 15 sec
10	Dehydrate	Reagent alcohol	20 sec
11	Stain	Alcoholic Eosin	10 sec - 30 sec
12	Dehydrate	Reagent alcohol	20 sec
13	Dehydrate	Reagent alcohol	20 sec
14	Dehydrate	Reagent alcohol	20 sec
15	Clear	Clearing agent	Min. 30 sec

Alcohols

- Use in fixation of tissue sections.
- All tissue sections must be fixed and hydrated before H & E staining.

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Water

- It is used in rinsing before and after hema. after acid alcohol and bluing reagent.
- Tap water can destain hematoxylin. Chlorine can acts as a bleach, iron as a mordant and sulfur to acidify water.
- pH of water can fluctuate daily. It can act as a bluing reagent if pH $\geq$ 8.
- Check pH of water. Best use deionized/distilled water.

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15	Clear	Clearing agent	Min. 30 sec

Hematoxylin

- Always run a control before staining with specimen tissue.
- Validate when you start with new batch or new supplier of hematoxylin.
- Every batch or every supplier/vendor is different.

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Weak staining of hematoxylin

- Inadequate staining time
- Too strong or excessive time in destaining due to incorrect formulation of acid alcohol
- Weak hematoxylin due to age or carryover from water
- Decrease in pH of the hematoxylin
- pH of water is off. Too much chlorine in water

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Strong staining of hematoxylin

- Strong potency of hematoxylin
- Excessive staining times
- Too weak or inadequate time in destaining
- Thick section

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Hematoxylin differentiators

- Mild glacial acetic acid.
- Acid rinse is used to remove non-specific background staining and make nuclei crisp.
- Use 0.1% , a weak alcohol-based conc. of acid for automatic staining.
- Two main problems are: Conc. of acid is too high or left in acid alcohol too long.

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Bluing reagents

- Ammonia soln., tap water, Scotts tap water, lithium carbonate
- It turns reddish-purple hematoxylin to crisp blue or purple-blue color
- Usually alkaline with a pH about 7.5-9
- It is important to remove all bluing reagent
- No more than 1 minute, if left too long, sections may fall off

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15	Clear	Clearing agent	Min. 30 sec

Water

- This step rinses the bluing reagent will allow eosin to retain its acidic pH.
- Without this water rinse, the environment will be too alkaline and tissue will stain very pale and uneven with eosin.

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15	Clear	Clearing agent	Min. 30 sec

Strong eosin staining

- Strong eosin solution due to excessive evaporation
- Too long in eosin
- pH of eosin is low (<5)
- No differentiate in 95% alcohol after eosin step

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Weak eosin stain

- Carryover of alkaline tap water
- pH of eosin has increased
- Inadequate rinsing to remove bluing reagent
- Inadequate staining time in eosin
- Overdifferentiation in subsequent 95% alcohol
- Eosin has deteriorated due to excessive carryover

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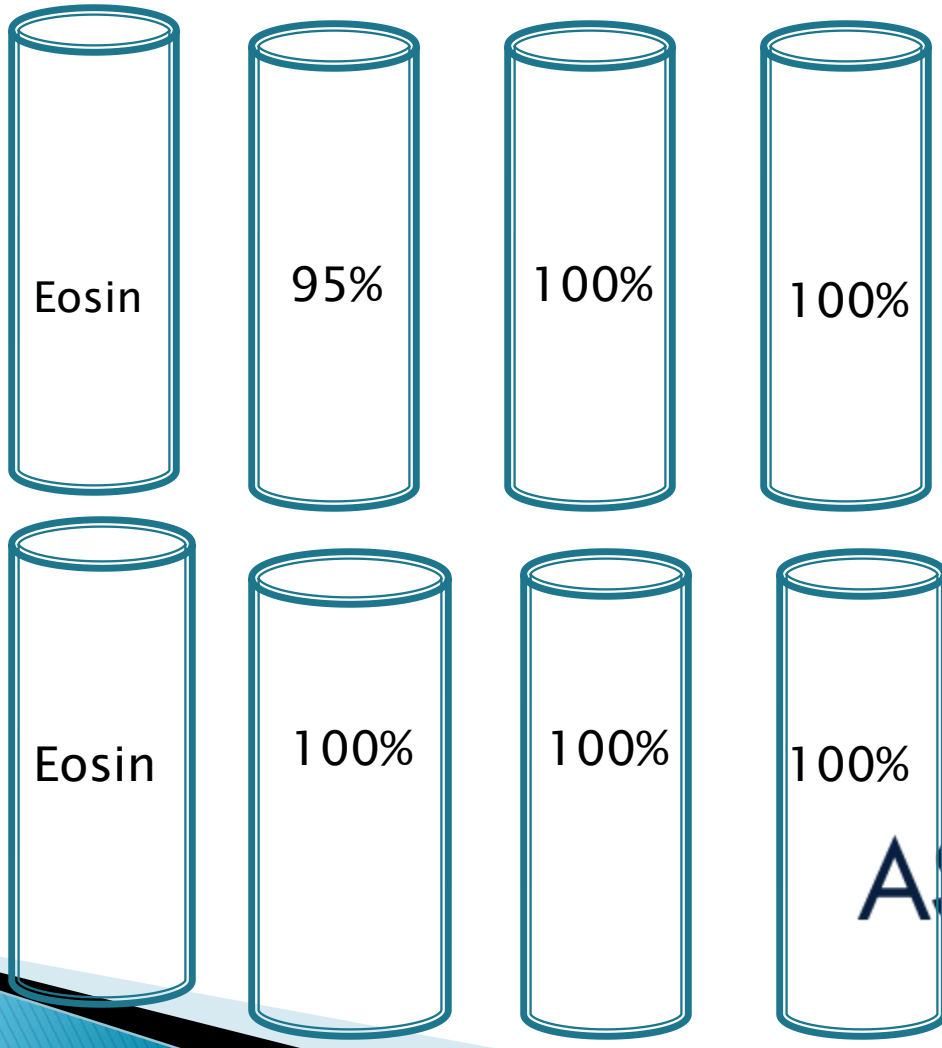
Eosin tones

Eosin is more soluble in water than alcohols



Lighter eosin staining

Darker eosin staining

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15	Clear	Clearing agent	Min. 30 sec

Reagent alcohol

- This step is crucial as an eosin differentiator.
- Different shades of pink will become more prominent.

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100% alcohol

- Dehydration process starts
- Must go through serial 100% alcohol steps
- Incomplete dehydration can result in formation of water beads under the coverslip

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15	Clear	Clearing agent	Min. 30 sec

Clearing agents

- Xylene substitutes
- Inadequate time in clearant will allow alcohol to remain in the sections. Bubbles can be seen under the coverslips
- Clearant should be miscible with mounting media—haziness/milkiness

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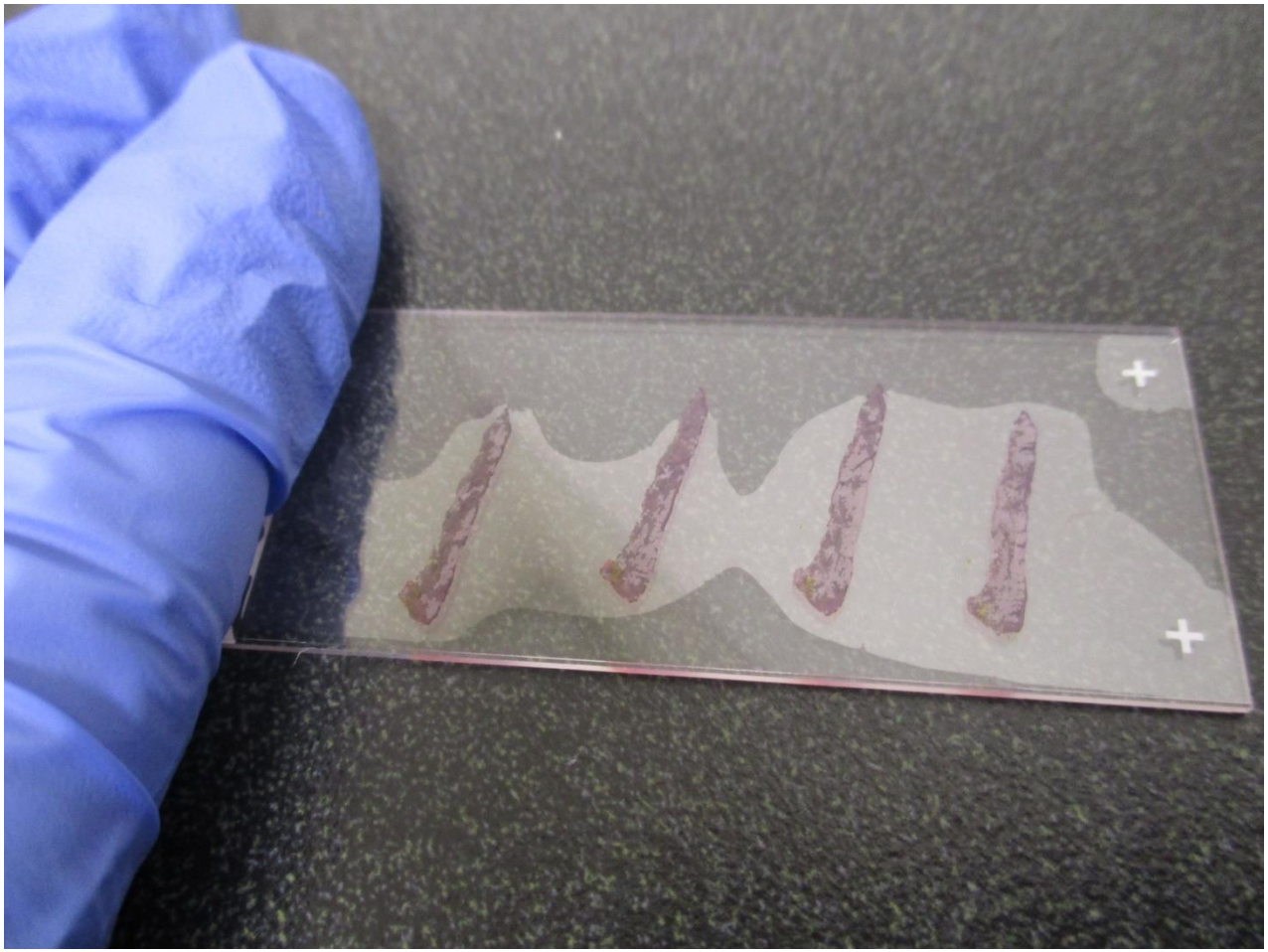
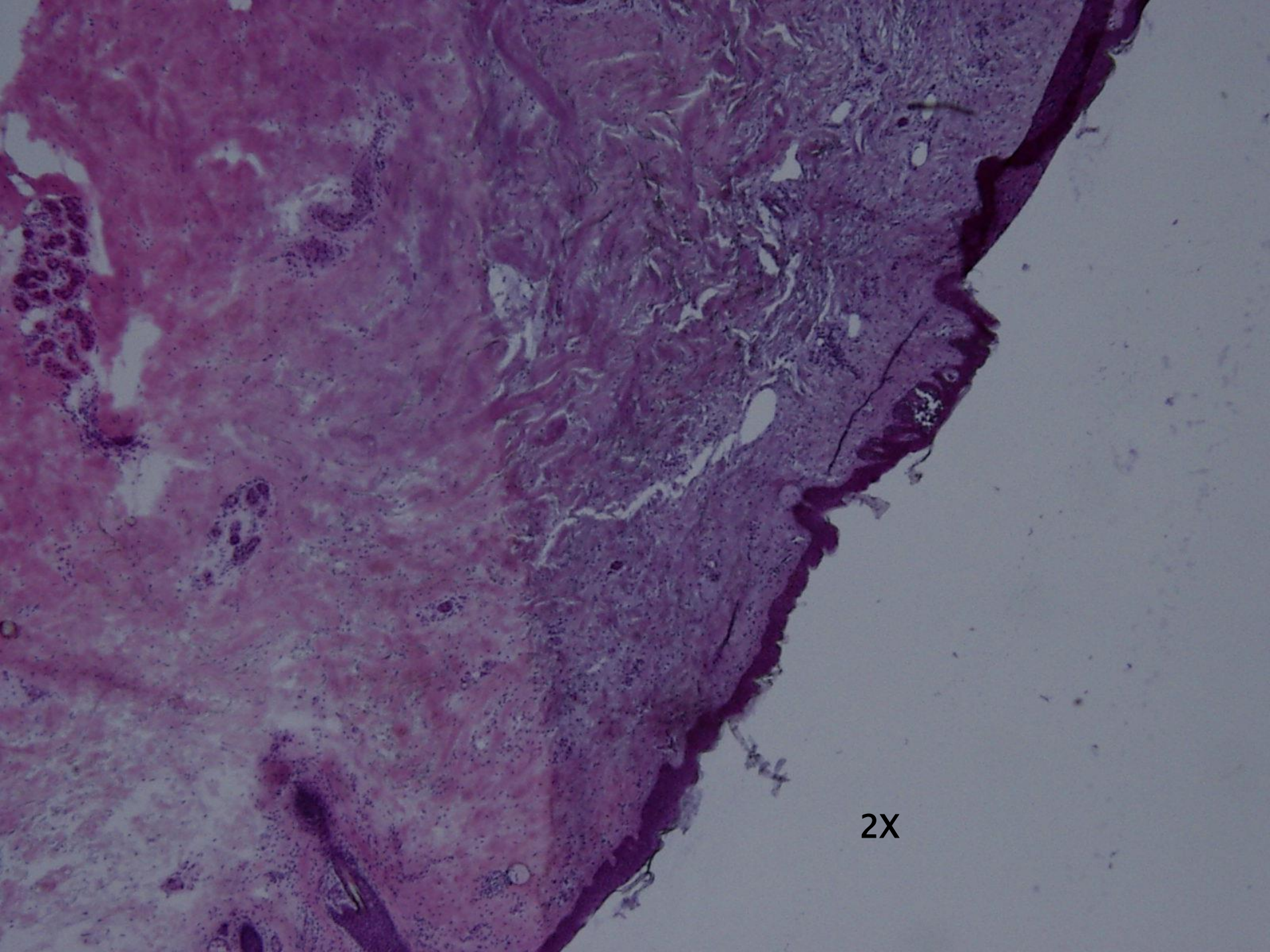
Poor dehydration

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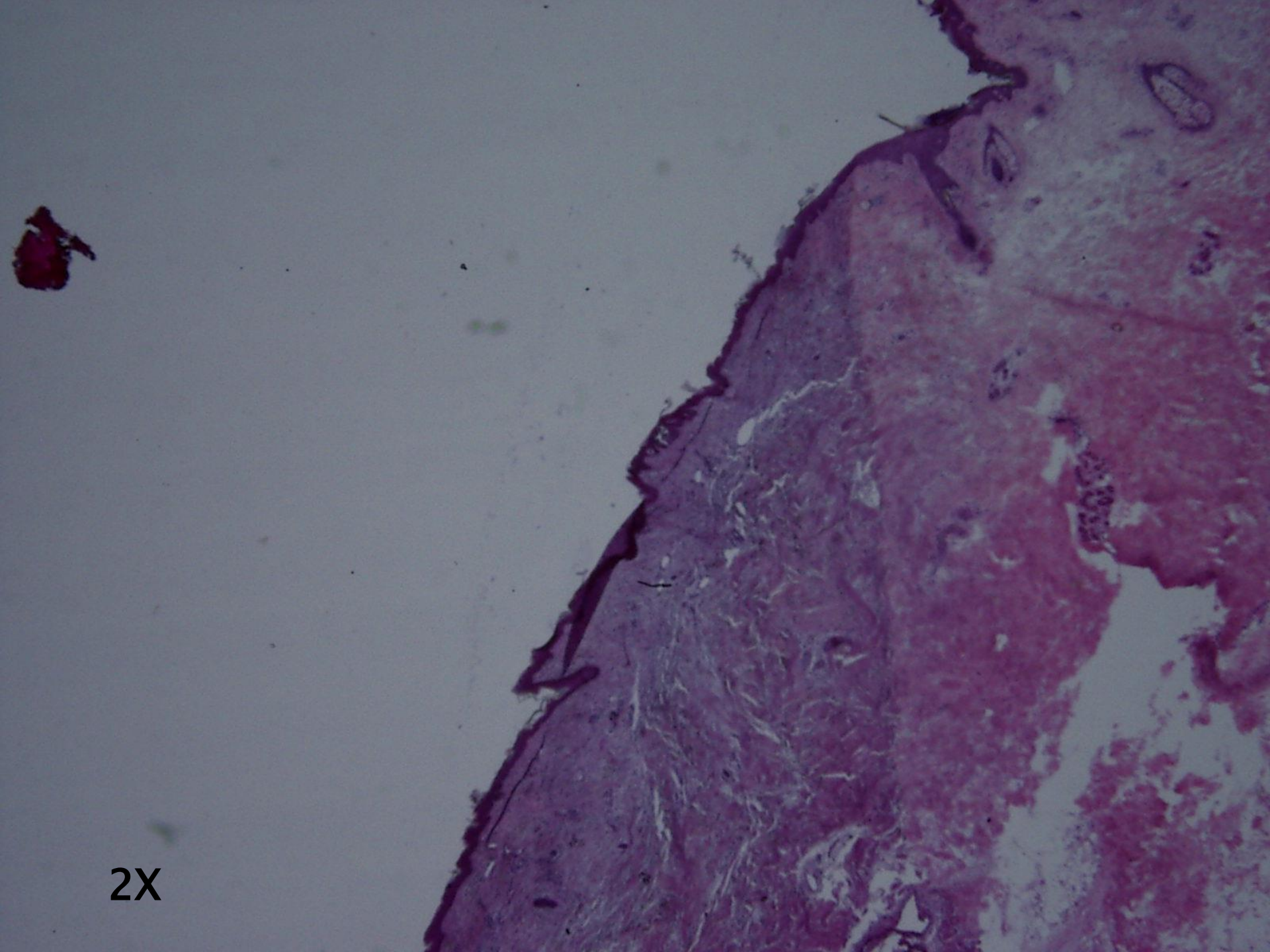
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Sections overlap

Problem	Cause	Solution
• Section partially overlapped another section	• Sections are large	• Trim the embedded block if it too big. • Careful placing sections on slides so that there is no overlap of sections

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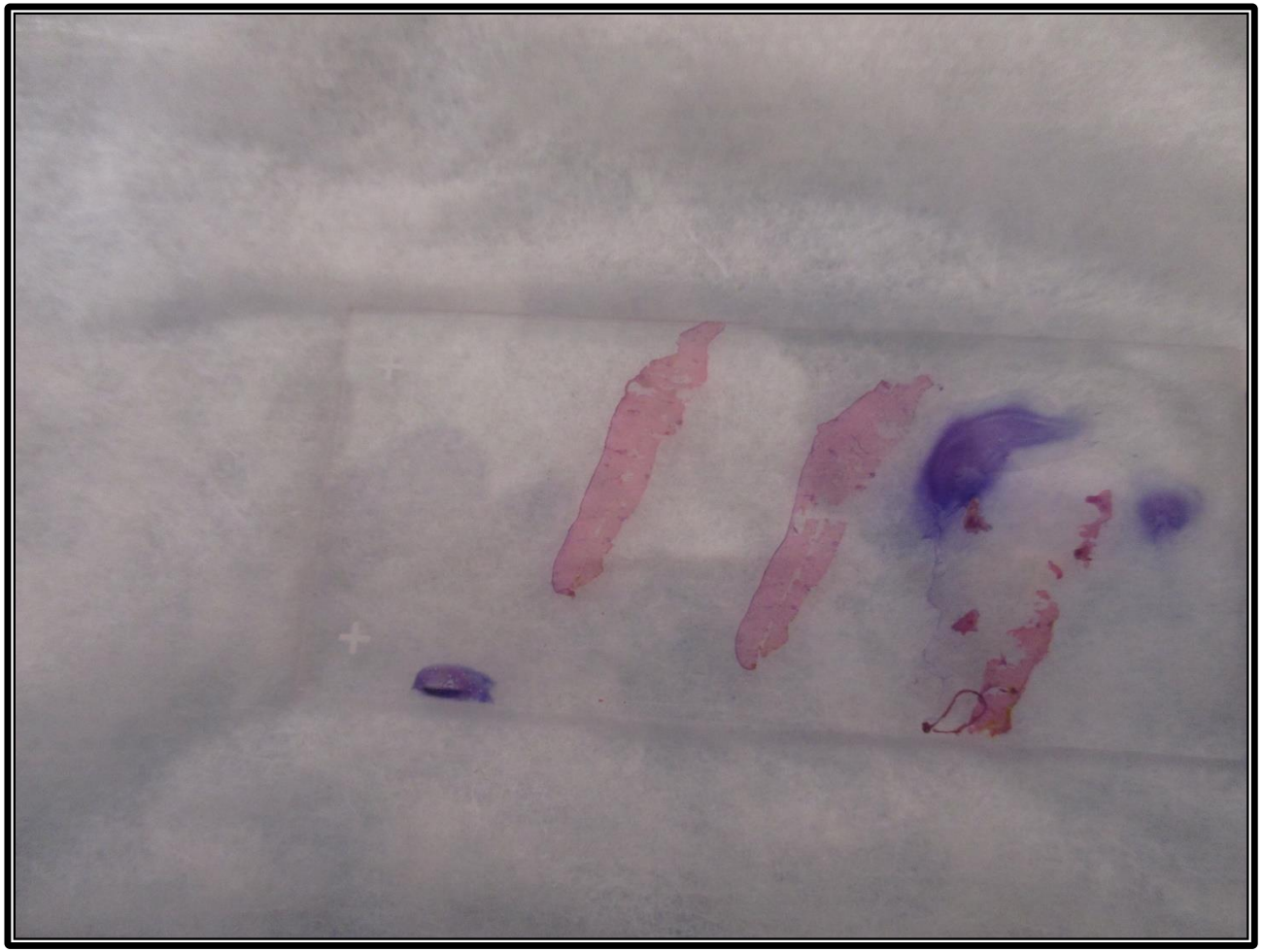

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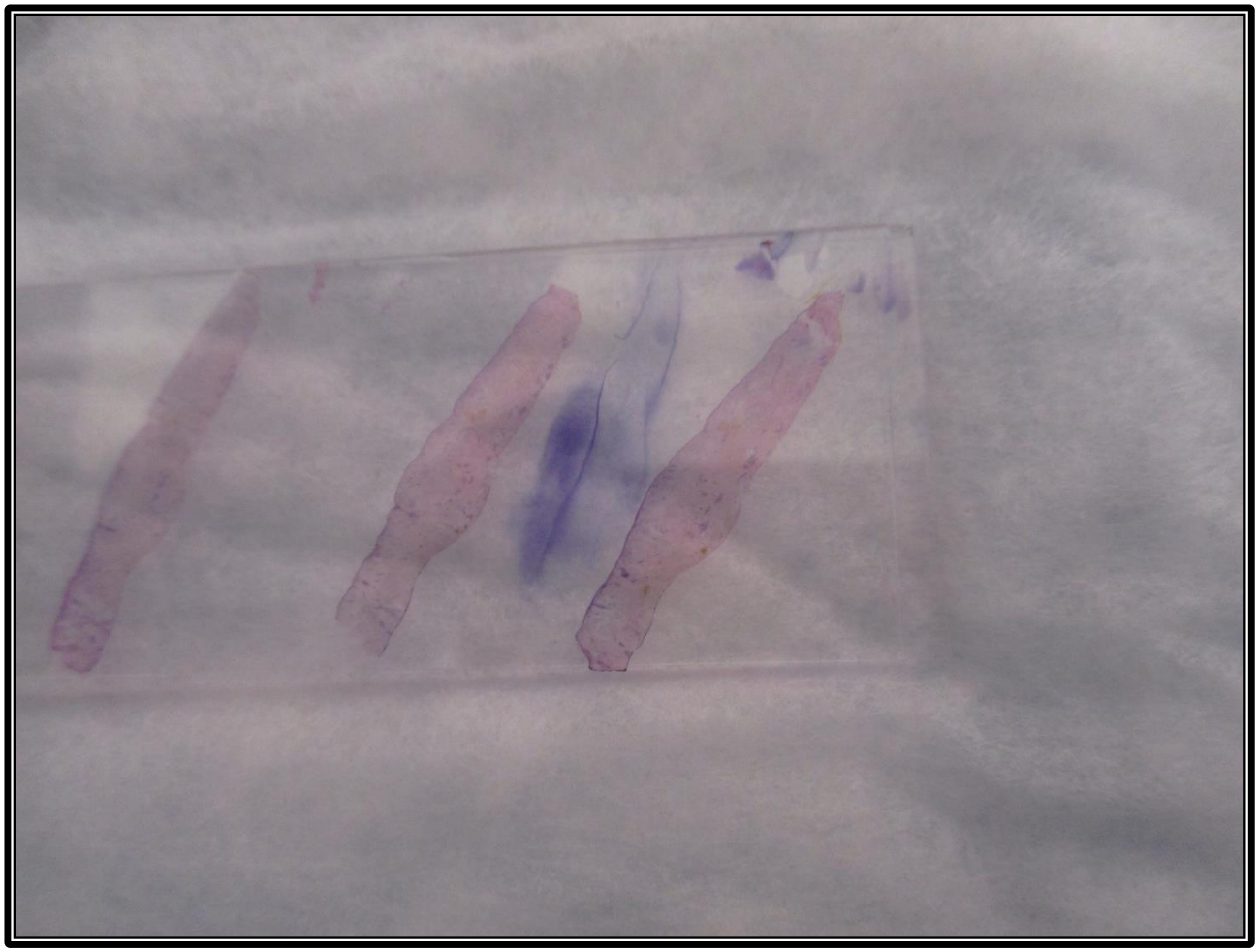

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Background stain

Problem	Cause	Solution
• Background stain	• Precipitation from oxidation of hematoxylin	• Filter hematoxylin • Decolorize sections in dilute acid alcohol after hematoxylin

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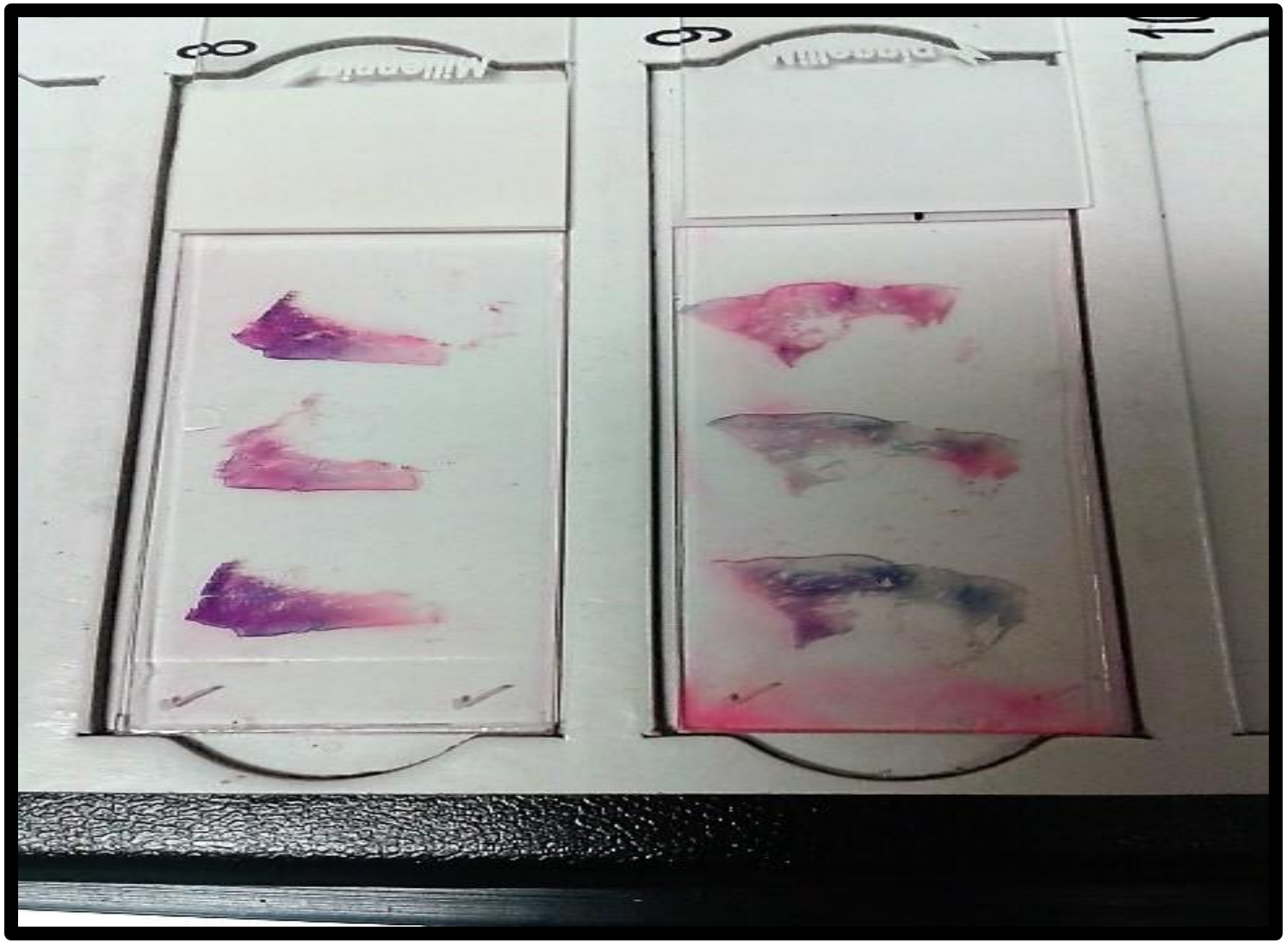

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Eosin bled out

Problem	Cause	Solution
• Eosin leeches out	• Water is left on the slide and draws the eosin out • Water contamination in the alcohols and clearing agent. Most of the citrus smelling clearing agents absorb humidity quite readily	• Change all alcohols/clearing agent after eosin • Make sure dehydrated alcohols are filled to cover slides entirely

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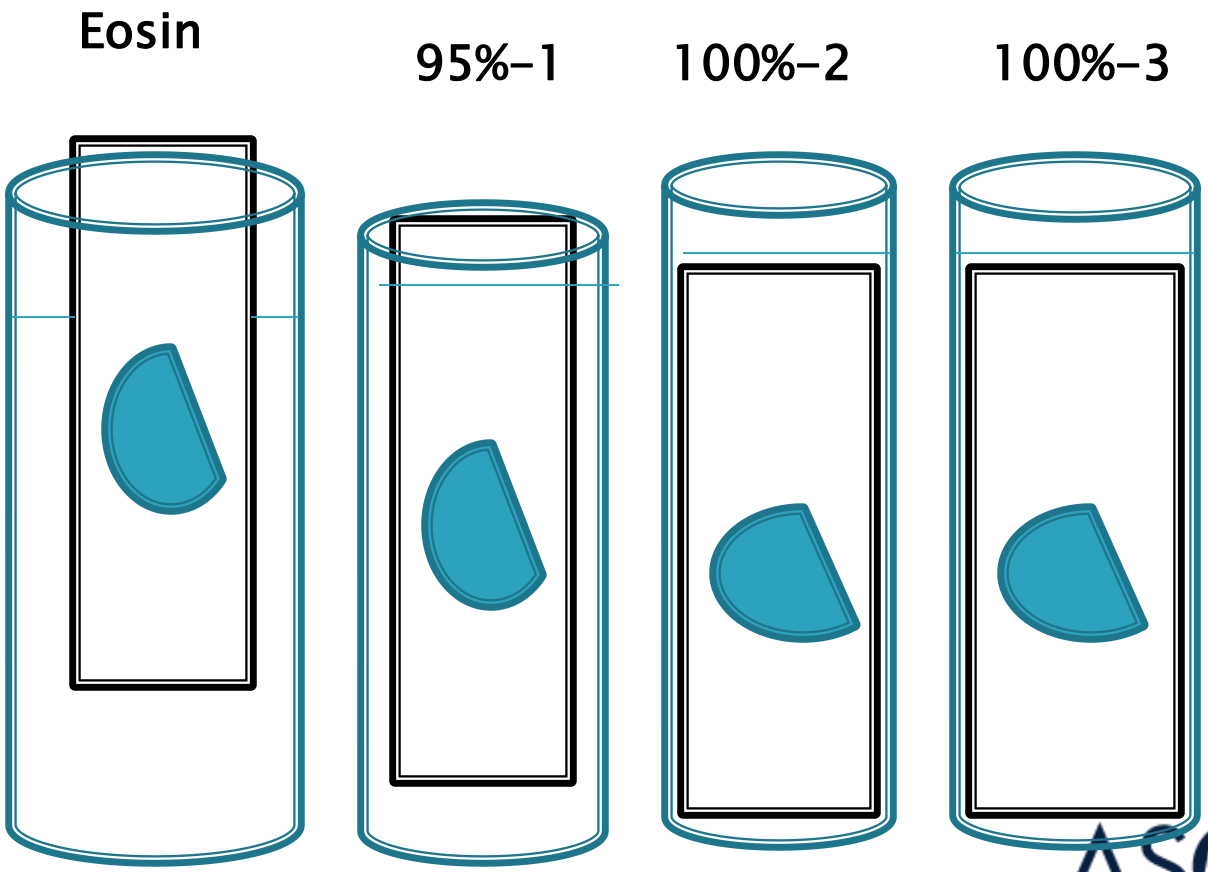
Correct fluid levels



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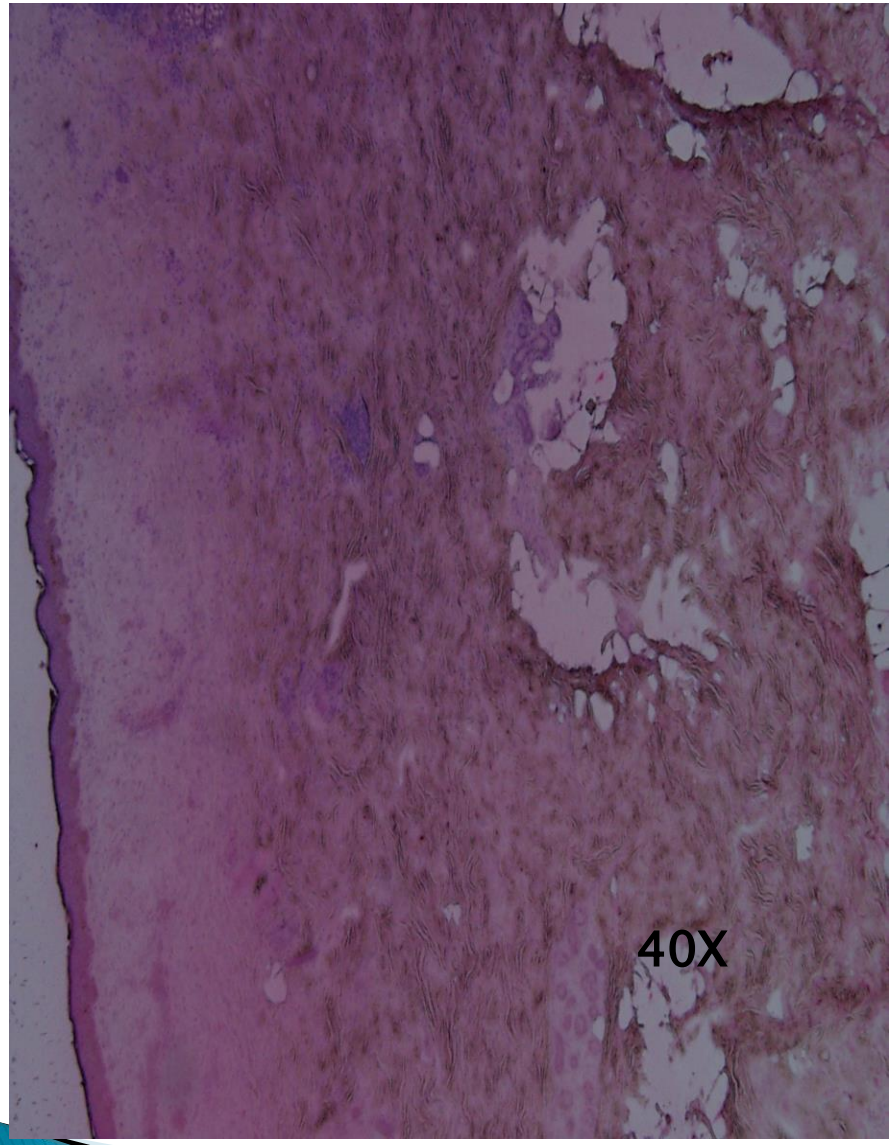
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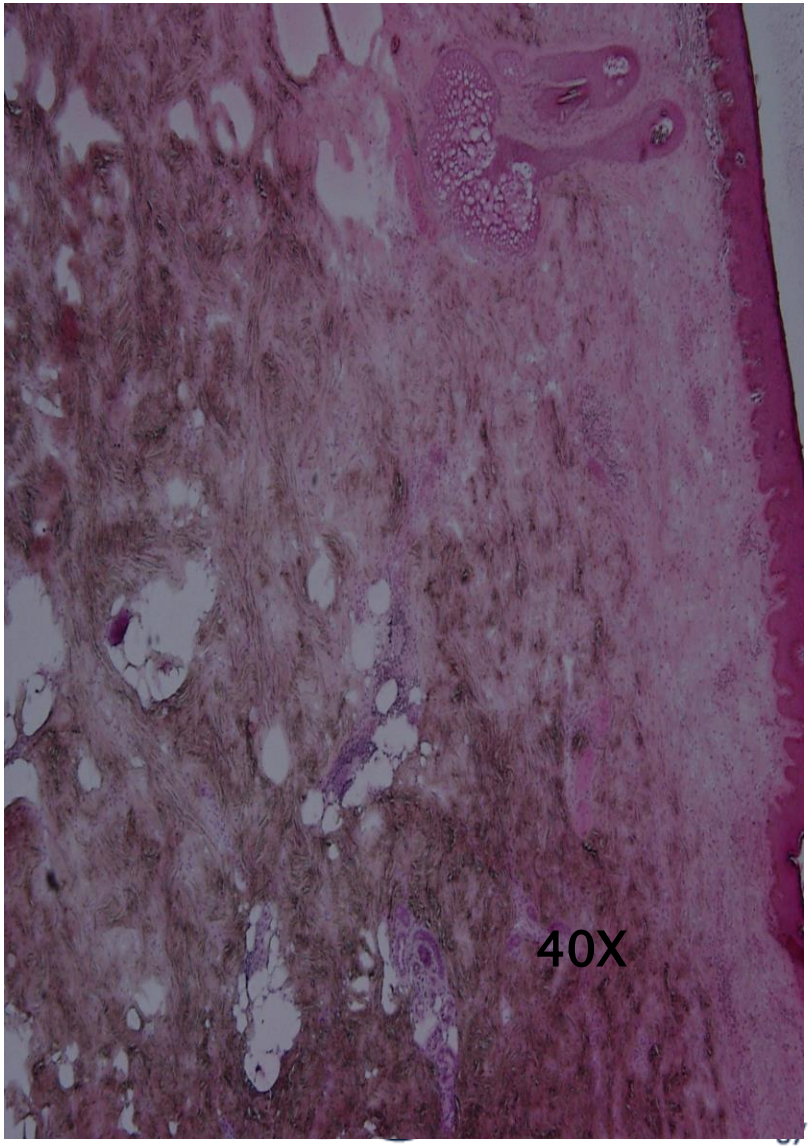
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Murky looking slides

Problem	Cause	Solution
• Murky looking • Nuclei appear smudgy or blurry	• Bluing agent carry over to eosin. • pH is elevated.	• Change eosin. • Put another change of water after bluing agent. • Make sure all punch out holes in automatic stainers are not clogged to allow water to flow freely.

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Manual staining

- Correct the problem as you see it.
- Speed it up if you see the machine is too slow
- Cost saving on capital equipment
- No equipment maintenance/no cost in servicing equipment yearly

Pros

- Inconsistent staining
- Lack of manpower
- Need more time

Cons

Automatic Stainers

Automatic staining

- Free up your time to do other functions
- Consistent staining

Pros

- Reagents carry over
- Murky looking slides
- Maintain equipment

Cons

Resolutions

- Implement a Quality Assurance/Quality Control plan on reagent management
- Use pre-made, buffered, and vendor validated stains/reagents which take the guess work out of reagent mgmt
- Use regimented SOPs for reagent handling and enforce your SOPs
- Rotate alcohols/clearing agent every XX slides.
- Change acid alcohol/bluing reagent after every XX slides
- Replace Hematoxylin/eosin after certain # of slides per manufacturer's recommendation

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Conclusion

It is especially important to have high quality H & E staining in the MMS so accurate diagnoses can be made and good TAT and quality patient care are achieved.

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Conclusion cont'd

I hope you have a better understanding of the mechanism of H & E staining and learn how to troubleshoot the common staining issues.

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References

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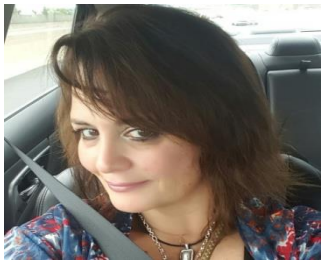
Acknowledgement

- Camille McKay



- Dr. Steven Wang

- Robert Tagliaferro

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Questions?

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