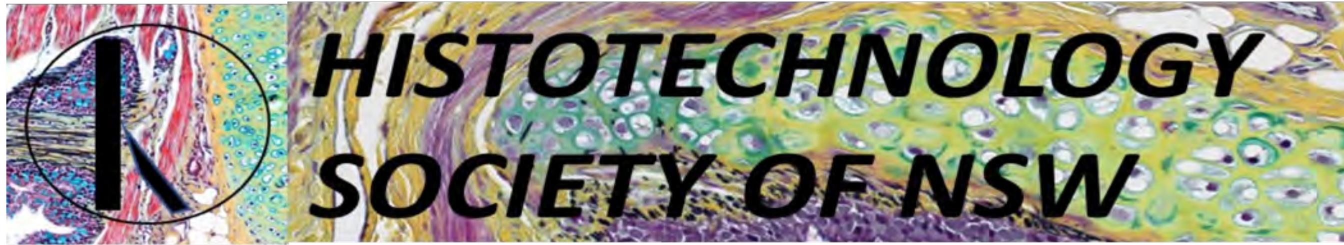

Micro-organism Staining Workshop
Best stain for which Bug

Mrs Dianne Reader and Dr Tamara Sztynda

Saturday 27th July 2019

POCD
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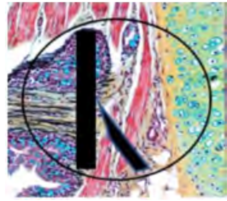

Special Thanks to Our Sponsor for Today

Point of Care Diagnostics (POCD)

Unit B, 19-21 Loyalty Road, North Rocks, NSW 2151

- › Pathology
  - › Decalcifiers
  - › Dyes
  - › Fixatives
  - › Mountants
  - › Reagents
  - › Solvents
  - › Stains

Provided:

- Most of our chemicals
- Morning tea
- Lunch

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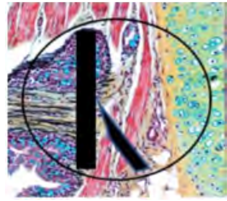
Workshop - Learning Objectives



1. Get an understanding of the dye molecule and its functional groups and their interactions.
3. Be able to explain colour theory, the reasons for dye purity and certification for laboratory use.
4. Understand the principles of dye and tissue chemical interaction to produce same and different colours, by acid, basic, amphoteric and neutral dyes.
5. See what staining methods best highlights specific micro-organisms in tissues by performing the staining techniques and observing the results.

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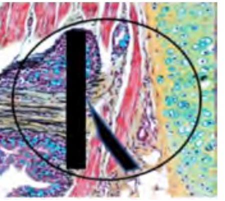
House Keeping Info



- Toilets – Beside the lifts on this floor
  - Out the door through the foyer into a short narrow corridor turn left and 6 steps and you are there
- Safety
  - Phones on SILENT! For phone conversations leave lab please.
  - No food or drink (includes WATER BOTTLES)
  - Need to leave lab, deglove, take off lab coat, wash hands and exit
  - Must wear lab coat, safety specs & gloves when working with any chemical (including Water!), shoes need to be non-porous (no knitted material) without heels and enclose whole foot, long hair must be tied back
  - NO GLOVES when using microscopes.
  - Safety showers and fire extinguishers (point out location)
  - Xylene must only be used in the fume hoods
- Clean-up
  - Use ONLY brown bags in autoclave bags for gloves
  - All paper in bins by the hand wash sinks
  - Notify of any spills immediately

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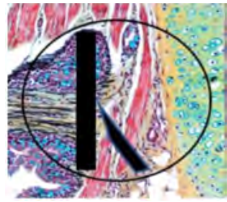

Evacuation
Muster Point in Jones St TAFE complex

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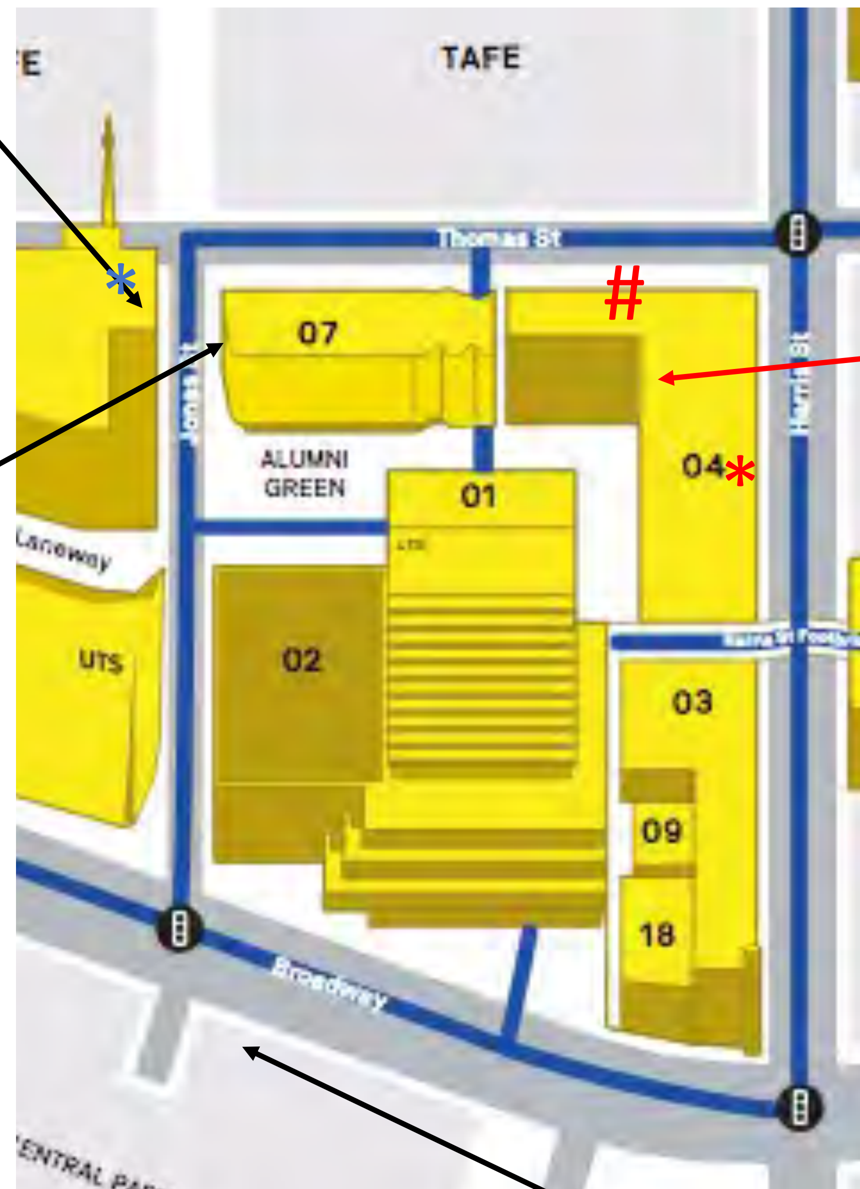
Tea Break
11am to 11:30am
Lunch
1:30pm
Café 10 *

Café 10 *
Building 10 (CB10)
Building 10, Level 2 (CB10.02)

Cornerstone cafe
Building 7 (CB07)
Building 7 level 3 (CB07.03)



Lab here
Level 3 - up the stairs from Harris St entrance *

Or take lift up from level 2 to 3 when entering from Thomas St #

Central Park Woolies and other eateries

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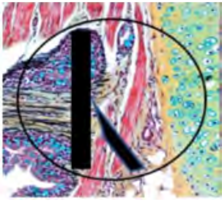

Chromophore - definition

- Atomic groupings are associated with colour
- Are **part** of the dye molecule
  - C=C, C=O, C=S, C=N,
  - N=N (azo), N=O, NO_2

Auxochrome - definition

- A **chemical group** in a dye molecule which **ionize**
- Enhances the action of the chromophore
- It intensifies the colour of that dye
- The type of auxochrome group in a dye determines how the dye will act as
  - Acidic (e.g. the carboxyl group -COOH) or
    - loose H+ act as anions (negatively charged)
  - Basic (e.g. the amino group $-NH_2$)
    - supply H+ act as cations (positively charged)
  - AND what tissue elements it will bind to

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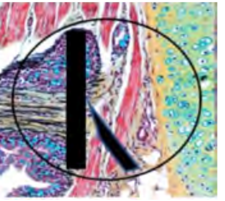
Colour Theory



- The more chromophores that occur in a compound the more pronounced the colour
- Physical chemistry, suggest that colour is caused by the selective absorption of certain wavelengths of light
  - Light which is transmitted or reflected from a substance lacks the absorbed wavelengths of light from the visible spectrum and so appears coloured

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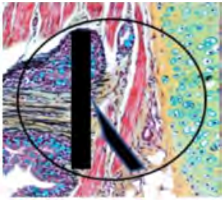
Biological Dyes



- Requirements of such a dye include
  - Revealing detail sought in the tissue
    - Suitable colour intensity
    - Good affinity to bind to the component
  - Chemically reliable
    - Adequate solubility in chosen solvent
    - Stability of the dye solution
    - Purity
    - Consistency of dye for reproducible results
      - consistent within and between batches of the dye
    - Fastness or resistance to changes in colour and intensity from
      - Light
      - Extreme *p*H
      - Changes and leaching by the mountant

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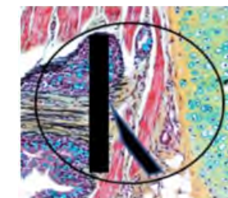
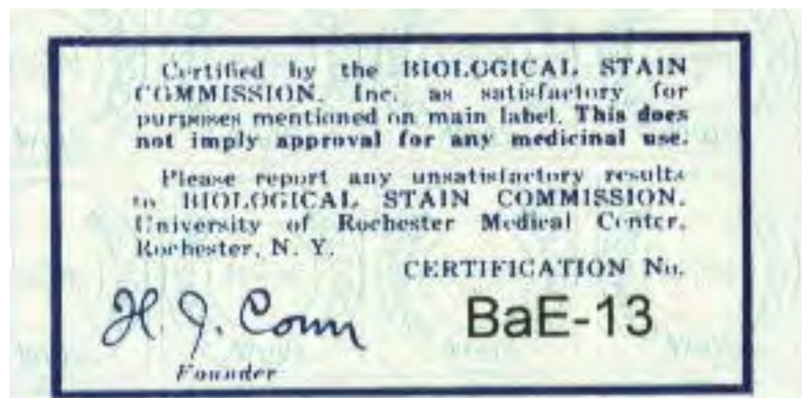


Dye Purity & Certified Dye Labelling

- Determined by **C**olour **I**ndex (**CI**) number
  - Set up by industrial dyers in 1971 and
  - **C**ertification by the US Biological Staining **C**ommission (**CC** after the dye name)
  - Is a confirmation of the dye's composition and it being a formulary preparation
  - Necessary for quality control and consistency in staining with each batch of dye which is produced

Haematoxylin CC
(CI 75290, Natural Black 1)
$C_{16} H_{14} O_6$ mol.wt. 302.286 anhydrous

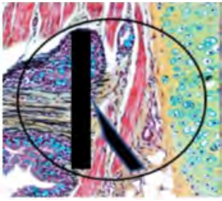

Certified by the BIOLOGICAL STAIN COMMISSION, Inc. as satisfactory for purposes mentioned on main label. This does not imply approval for any medicinal use.

Please report any unsatisfactory results to BIOLOGICAL STAIN COMMISSION, University of Rochester Medical Center, Rochester, N. Y.

CERTIFICATION No.
BaE-13

H. J. Conn
Founder

POCD Scientific



Function of Staining

- Differentiates the cell cytoplasm from the nucleus
- Distinguishes between types of tissue
- Nature of the dye (acid or basic dye, *p*H of solution) gives specificity to particular tissue elements & intensity of staining
  - ***Degree affinity*** of the dye to specific tissue sites rather than other tissue components relates to
    - Intensity of staining - number of binding sites
    - Type and intensity of attractive forces acting

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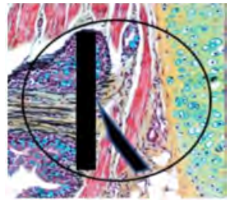

Dye to Tissue Binding

- Chemical and physical forces include
  - Hydrophobic bonding
  - Van der Waal's forces
    - Weak attractive force between atoms or non-polar molecules arising from a brief shift of orbital electrons to one side of one atom or molecule
  - Coulombic attraction
    - Attractive or repulsive electrostatic force
  - Hydrogen bonds
  - Covalent bonds
- Where solvent-solvent, dye-solvent, dye-dye and dye-tissue interactions are all occurring during the staining process.

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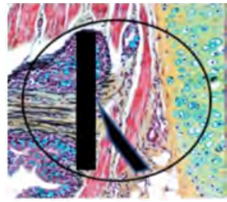
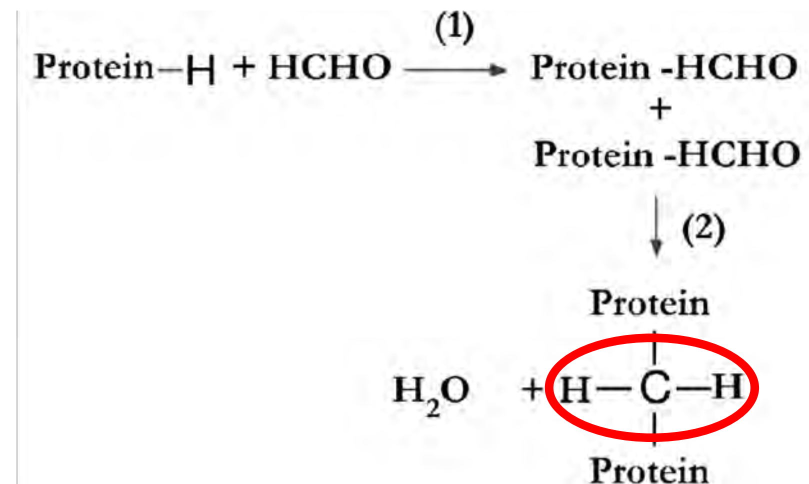


Dye to Tissue Binding: Other Factors

- Staining like other chemical reactions also relies on other factors:
  - Rates of penetration
    - Tissue lattice structure (produced by fixation) and molecular size of dye
  - Rates of uptake/reaction
  - Rates of loss
    - Especially during differentiation



J Oral Maxillofac Pathol. 2012 Sep-Dec; 16(3): 400–405.
doi: 10.4103/0973-029X.102496

Chemical and physical basics of routine formaldehyde fixation

Rooban Thavarajah, Vidya Kazhiyur Mudimbaimannar, Joshua Elizabeth, Umadevi Krishnamohan Rao, and Kannan Ranganathan

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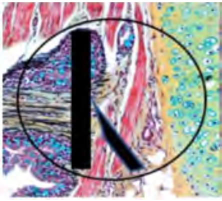
Acid and Basic Dyes



- Most dyes are prepared as salts
- **Auxochromes** on a dye can be classed as either
  - Acidic (anionic [-ive] e.g. the carboxyl group -COOH)
  - Basic (cationic [+ive] e.g. the amino group $-NH_2$)

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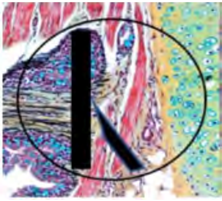

Basic Dyes (Basic Auxochromes)

- **$-NH_2$ auxochrome** supplies $-H^+$ ions
- Dye can act as a cation (basic)
- Binds to form salts with negatively charged tissue elements (e.g. nucleic acids known as ***basophilic*** substances)
- Stains best at basic *p*H's because their ionization is maintained
  Example: Methylene Blue

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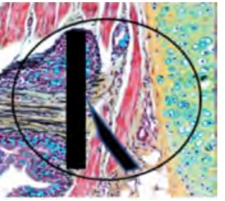
Acid Dyes (Acidic Auxochromes)



- **-COOH auxochrome** can loose $-H^{+}$ ions
- Leaving $-COO^{-}$ ionization of the auxochrome
- The dye can act as a anion (acidic)
- It binds to form salts with positively charged metal ions in or associated with tissue elements (known as *acidophilic* substances)
- Acid dyes stain best at acidic *p*H's because their ionization is maintained

  Examples of acid dyes:
  - Eosin Y
  - Biebrich Scarlet

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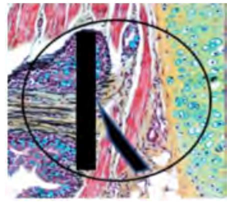
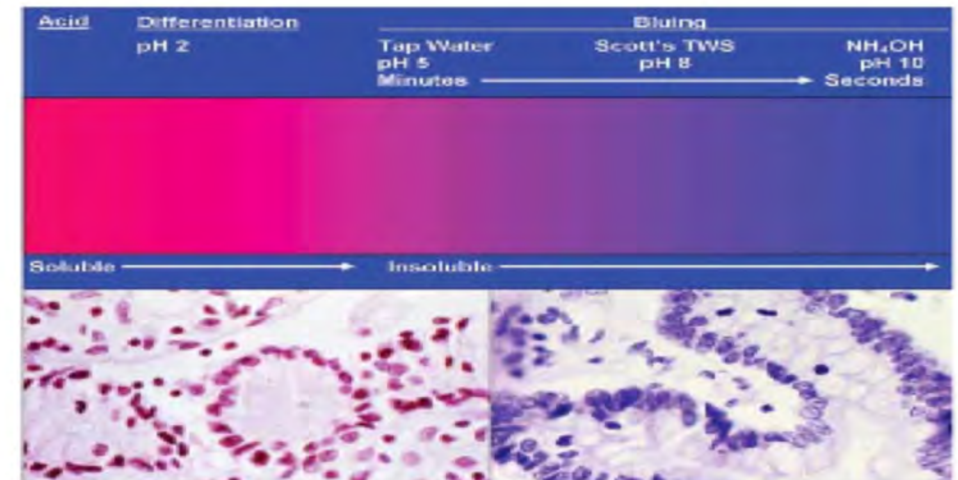
Amphoteric Dyes



- Some dyes can posses **both positively and negatively** charged groups and are known as ***amphoteric***
- These dyes function as acid or basic depending on the *p*H of the solution
- **Tissue proteins are also amphoteric**, their charge will vary with the *p*H of the staining solution so that **proteins can bind either acid or basic dyes**
  - At the **protein's isoelectric** point the molecule is **neutral (**charges are balanced) and it will bind with both acid or basic dyes



http://www.dako.com/08066_guide_to_special_stains.pdf

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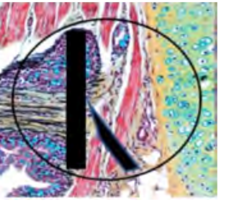
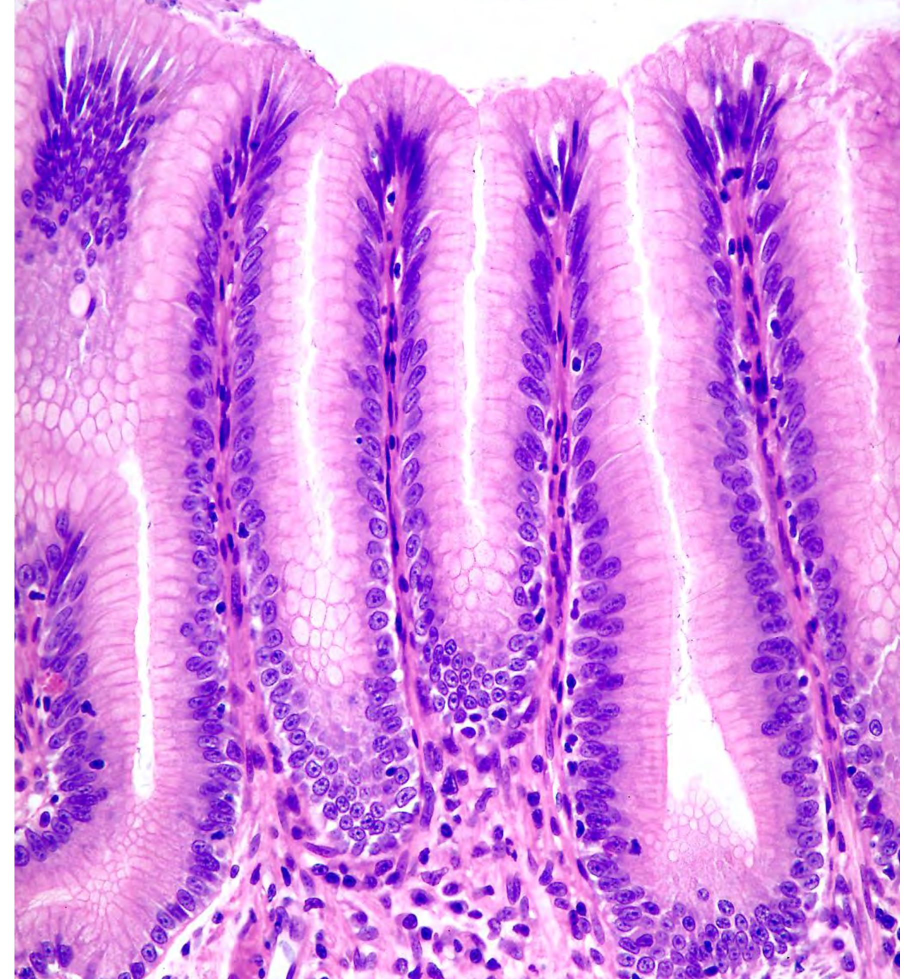


Routine Staining

- Universally is Haematoxylin and Eosin Y (H&E) staining



Kerr, Figure 13.8a

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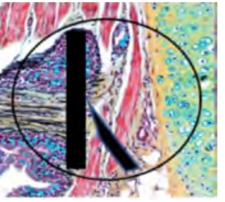

Choice of Counter Stains

- Need contrasting colours
  - Relate to opposite colours on the colour wheel
- For H&E
  - Blue of haematoxylin and
  - Orange/red of Eosin Y

http://img249.imageshack.us/img249/6514/colorwheelou0.jpg

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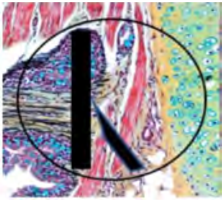
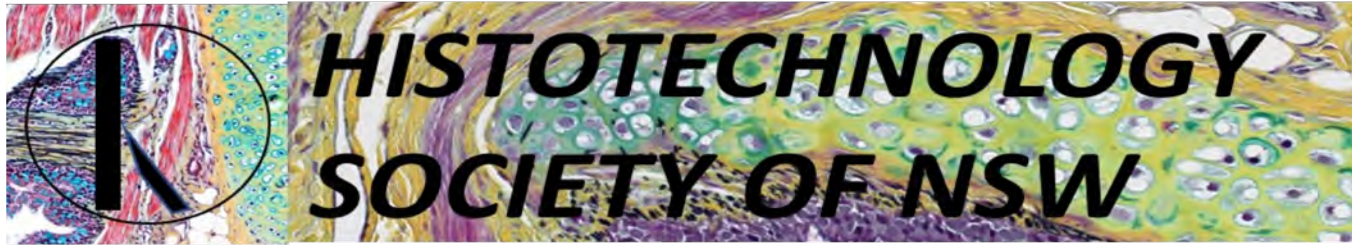


Checking Staining

- Always need ‘WHITE’ light
  - Ensure your microscope has a blue filter in the condenser or on the lamp housing
    - Blue filter not needed with LED light in new microscopes
  - To achieve whiteness of light the **light source needs to be turned up more** than what is used for comfortable observation,
  - The **condenser diaphragm needs to be fully open**
    - This higher light intensity attains correct ‘colour temperature’ giving you the true colour of the stain.



Staining Micro-organisms

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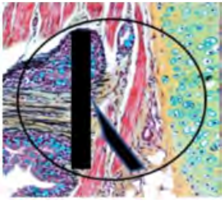
Abbreviations Key:



AB/PAS	Alcian blue/ Periodic acid Schiffs
CAS	Chromic Acid Schiff's
GMS	Grocott’s Methenamine Silver
H&E	Haematoxylin and Eosin
MGP	Methyl Green Pyronin
NBF	Neutral Buffered Formalin
PAS	Periodic Acid Schiffs
RO	Reverse Osmosis
ZN	Ziehl-Neelsen

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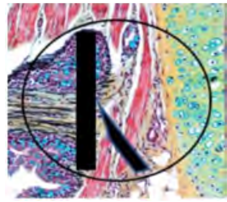
Ziehl Neelsen for Acid Fast Bacilli

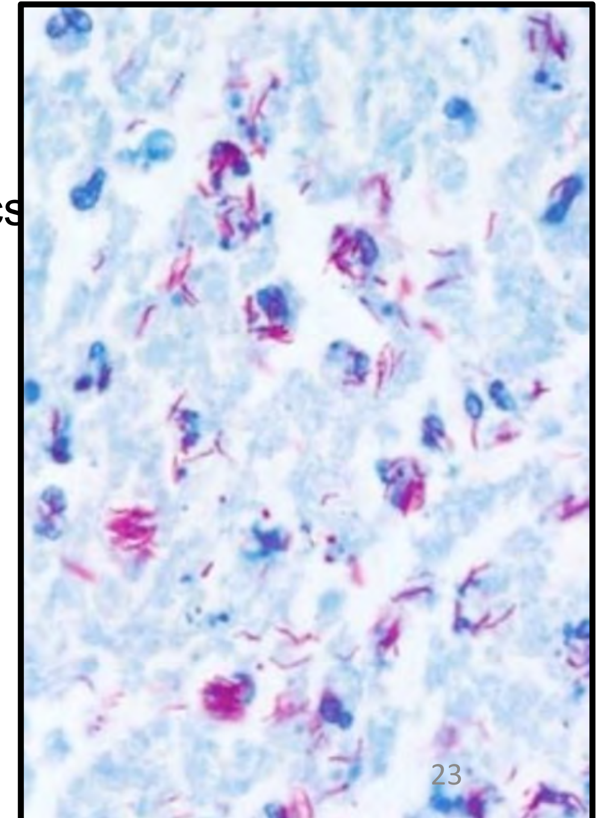


Method

1. Deparaffinize, rehydrate through graded alcohols to RO water.
2. Place slides into a preheated Coplin jar (58-60°C in waterbath) with **carbol fuchsin** – 10 min
3. Remove Coplin jar with slides and gently cool jar running cold tap water – 2 min
4. Remove slides from Coplin jar and gently wash them under running tap water – 1 min
5. Differentiate in acid alcohol until no more colour runs from slide
6. Gently wash slides briefly under running tap water to remove acid alcohol
7. Counterstain with 0.25% **methylene blue** in 1% acetic acid for 15 to 30 secs
8. Wash in tap water.
9. Dehydrate through graded alcohols, clear in xylene and mount

Results

- Acid fast bacilli **Red**
- Nuclei **Blue**
- Other tissue constituents **Blue**



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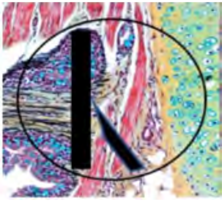
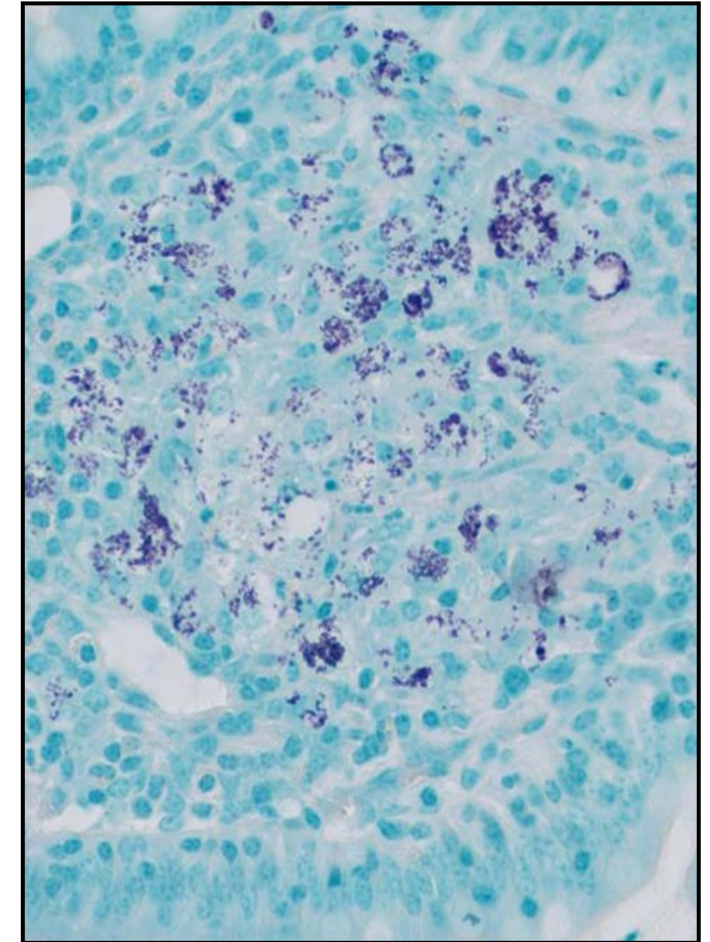
Ziehl-Neelsen (ZN) for Mycobacterium

(Kinyoun 1915)



Method

1. Deparaffinize, rehydrate through graded alcohols to RO water.
2. Place in **carbol fushsin** solution – 30 mins
3. Wash in tap water
4. Differentiate in acid alcohol until solutions are pale pink (2-5dips)
5. Wash in tap water – 8 mins
6. Transfer to RO water wash
7. Counterstain in **methylene blue** (working conc.) until pale blue
8. Rinse in tap water
9. Wash in RO water
10. Dehydrate through graded alcohols, clear in xylene and mount



Results

- Mycobacteria, hair shafts, Russell bodies, and some fungi **red**
- Background **pale blue**

Notes

Blue counterstain patchy if caseation present

Care - avoid over counter-staining as scant organisms easily obscured.

Strong acids (decalcification) destroys acid-fastness; use formic acid

Victoria blue is a substitute for carbol fuchsin with picric acid counterstain (if colour blind)

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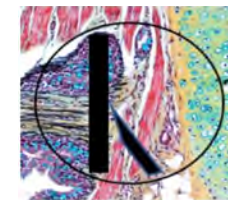


Gram-Twort - Bacteria

Method

1. Deparaffinize and rehydrate through graded alcohols to RO water.
2. Stain in **crystal violet** solution - 3 min
3. Gently rinse in running tap water.
4. Treat with **Gram's iodine** – 3 min
5. Rinse in deionised or RO water
6. Decoluorize individual slides with acetone, until most purple stain removed & tis
7. Rinse briefly in RO water.
8. Stain in **Twort's** for 5 minutes.
9. Wash in RO water, blot dry.
10. Dehydrate through graded acetone, clear in xylene and mount.

Results

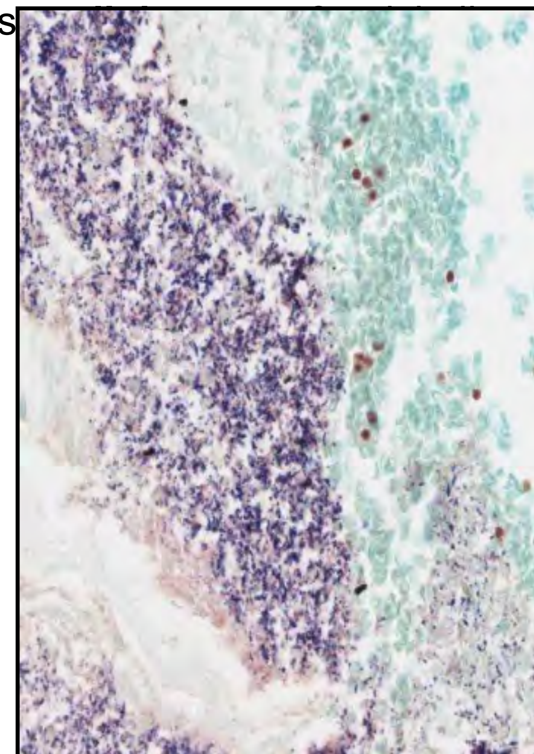
Gram-positive organisms **blue-black**

Gram-negative organisms **pink-red**

Nuclei **red**

Red blood cells and most cytoplasmic structures **green**

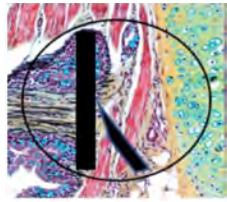
Elastic fibres **black**



POCD Scientific

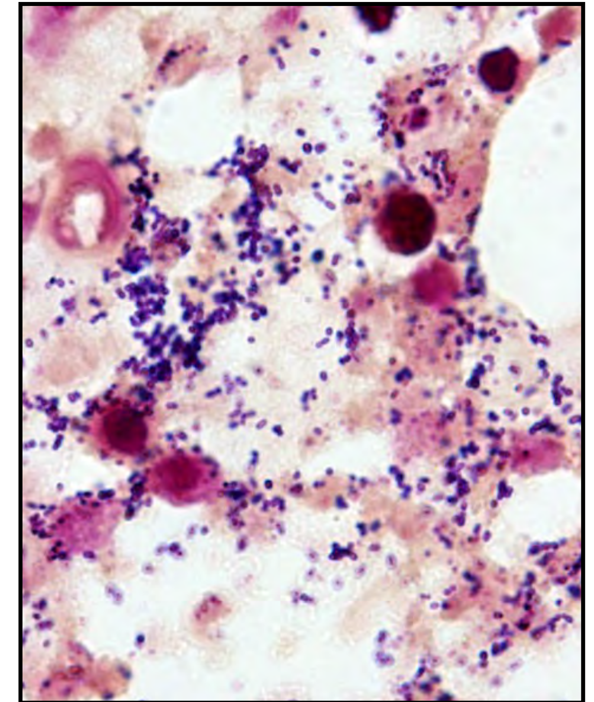
Modified Brown-Hopps Gram - Bacteria

(AFIP modification)



Method

1. Deparaffinize and rehydrate through graded alcohols to RO water.
2. Stain with 1% **crystal violet** solution - 2 min
3. Rinse well in tap water.
4. **Gram's iodine** solution - 2 min
5. Rinse in tap water.
6. Decolorize by dipping in acetone solution until the blue colour stops running. (only 1-2 dips!)
7. Rinse immediately in tap water.
8. Counterstain in working **basic fuchsin** for 5 minute.
9. Rinse in tap water
10. Place in Gallego's differentiating solution for 2 changes - 1 min each
11. Rinse in tap water.
12. Dip in acetone, 1 dip. (30 secs)
13. Dip in picric acid-acetone (2 minutes)
14. Dip several times in acetone-xylene solution.
15. Clear in xylene and mount.



Results

Gram-positive organisms, fibrin, some fungi, Paneth cell granules, keratohyalin, and keratin - **blue**

Gram-negative organisms - **red**

Nucle i- **red**

Other tissue elements - **yellow**

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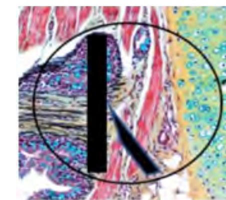
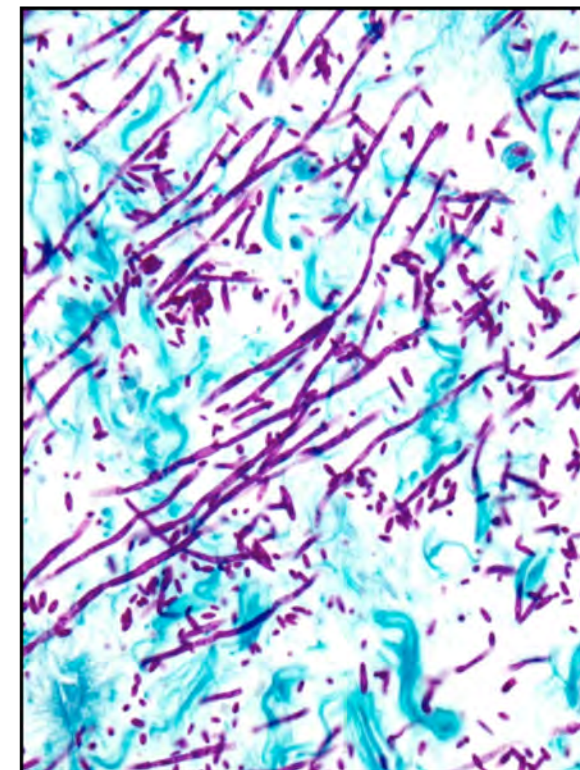


Periodic Acid Schiff's (PAS) - Fungi

Method

1. Deparaffinize, rehydrate through graded alcohols to RO water.
2. Oxidize in **periodic acid** solution - 15 min
3. Rinse in RO water
4. Place in **Schiff's reagent** - 15 min
5. Wash in running tap water - 10 min allows pink colour to develop
6. Counterstain in **light green** solution - 30 secs
7. Dehydrate through graded alcohols, clear in xylene and mount



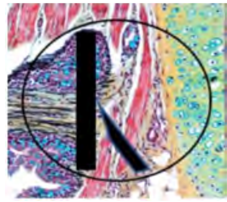
Results

Fungal cell walls and glycogen - **magenta to red**

Background - **pale green**

POCD Scientific

Chromic Acid Schiff's (CAS) - Fungi



Method

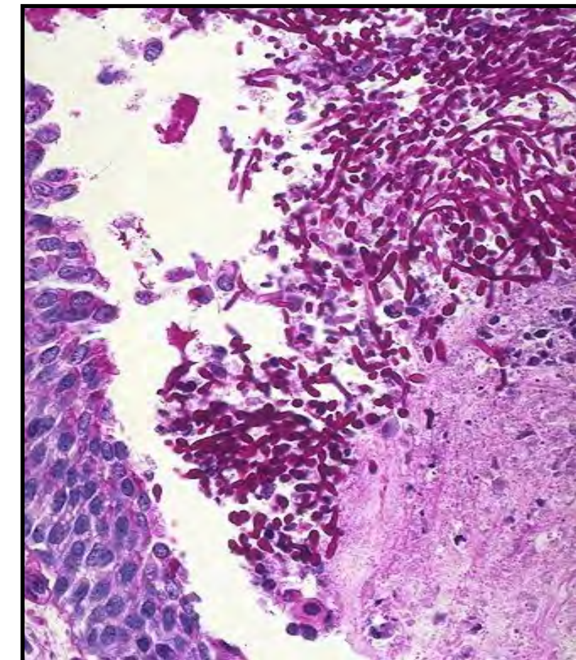
1. Deparaffinize, rehydrate through graded alcohols to RO water.
2. Oxidise in **chromic acid** - 40 mins.
3. Rinse with tap, then RO water.
4. Place into **Schiff's reagent** - 15 min
5. Wash in running tap water - 10 min allow pink colour to develop.
6. Counterstain with Mayer's haematoxylin for 1 minute and blue (neutralize) in running tap water.
7. Dehydrate through graded alcohols, clear in xylene and mount

Results

Glycogen, mucin – **red**

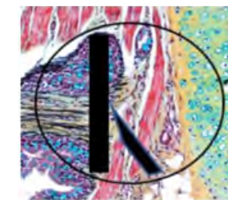
Fungi – **red**

Nuclei – **blue**



POCD Scientific

Mucicarmine - Cryptococci



Method

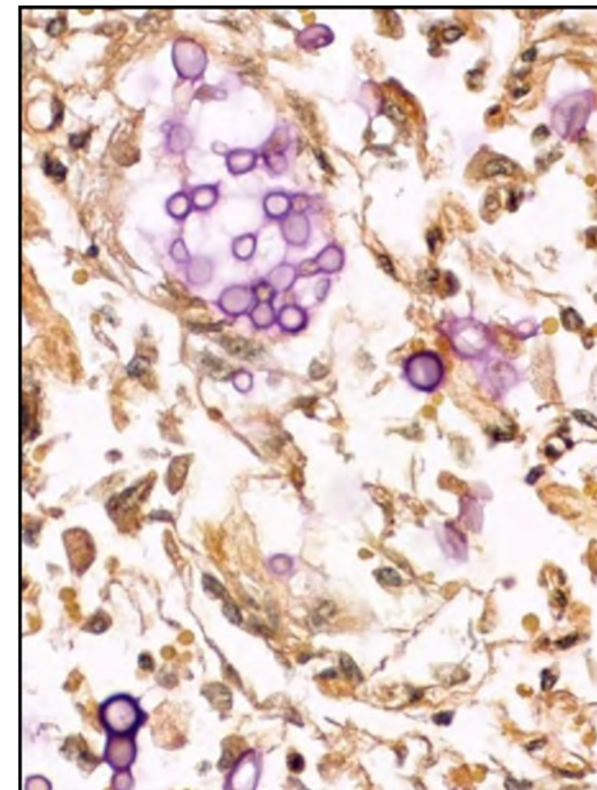
1. Deparaffinize, rehydrate through graded alcohols to RO water.
2. **Mayer's haematoxylin** - 10 min
3. Wash in running tap water - 5 min
4. **Mucicarmine solution**, water-bath at 60°C - 1 hr.
5. Rinse quickly in RO water.
6. **Metanil yellow** - 30 secs to 1 min
7. Dehydrate quickly 3 changes absolute alcohol, clear in xylene and mount.

Results

Mucin - **deep rose**

Nuclei - **black**

Other tissue elements - **yellow**



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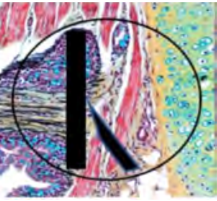
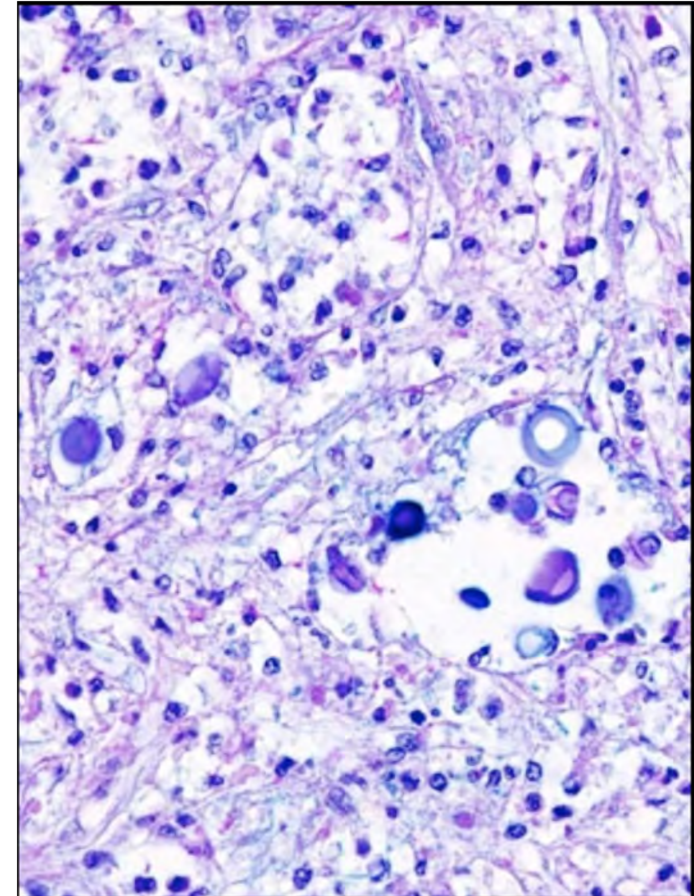
Alcian Blue/PAS (AB/PAS) - Cryptococci



Method

1. Deparaffinize, rehydrate through graded alcohols to RO water.
2. Stain with *pH* 2.5 **Alcian blue** - 30 mins
3. Wash well in running tap water - 2 mins
4. Rinse in RO water
5. Treat with **periodic acid** - 15 mins
6. Wash well in RO water
7. Stain with **Schiff's reagent** - 15 mins
8. Wash well in running tap water - 5 mins
9. Stain nuclei with **Mayer's haematoxylin** - 1 min
10. Wash and blue nuclei in running tap water - 5 mins
11. Dehydrate through graded alcohols, clear in xylene and mount



Results

Cryptococcus - **blue**

Neutral mucins - **magenta**

Mixtures of above - **blue/purple**

Nuclei - **deep blue**

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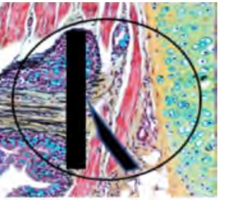
Mystery Slide



- Parasitic Tape worm
- Cross sections of worm found in lumen

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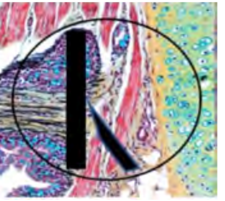

Other Methods in the Booklet

- Tables pages 2,3,& 25
- Warthin Starry (spirochetes & Klebsiella)
- Giemsa (Helicobacter & parasites)
- Gimenez (Helicobacter)
- Solutions list
- Reference List of sources

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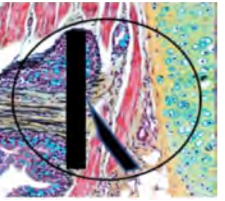

Suggestions for future workshops

Please email Tamara.Sztynda@uts.edu.au

- Basic theory?
- Specialized techniques?
- Equipment use/optimization/ New Tech?
- Histology (System)/Histopathology (disease process)

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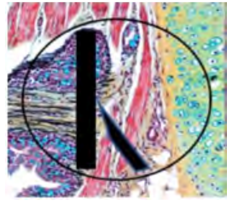

Forthcoming Workshops

Webinar:
Importance THE Glass slide

Monday 26th August

- Properties of glass
- Hope to clean milky glass slides
- Different coatings of glass slides
  - Why?
- Charged glass slides

Microscopy:
Advanced Tissue Identification

Saturday 14th September

- The gastro-intestinal tract
  - Distinguishing features & similarities of segments
    - Oesophagus
    - Stomach
    - Small intestine
      - Duodenum
      - Jejunum
      - Ileum
    - Colon
    - Appendix

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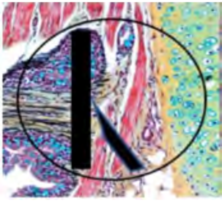
Special Thanks



Mrs Reader (first and foremost!)

- Who sought out the techniques
- Prepared and printed the booklet
- Made lists of reagents and equipment
- Cut the slides

Mr Sinai

- Provided most of the tissue blocks

Rest of the Committee

- Registrations
- Preparing certificates (emailed)
- Sponsors
- Email enquiries
- Advice and help on their days off

Faculty of Science (Dr Bill Booth)

- For use of equipment & glassware and wash-up free of charge
- Technical staff Nilufa Sultana for getting equipment together