

# A Study on Tissue Processing

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## ABSTRACT

Histology, the study of tissue had been done for century in order to detect and observe normal structure of tissue. However, there are several reasons that might alter tissue from its original structure. Several processes, such as fixation, processing, embedding, cutting and staining had been done to reduce the limitation of tissue observation. In this study, ileum, skin, and appendix were experimental by using neutral buffered formaldehyde as fixative and stained with Van Gieson stain, Haematoxylin and eosin, Masson's Trichrome stain and Periodic Acid Schiff.

**Keywords:** Haematoxylin and Eosin, Van Gieson, Masson's Trichrome and Periodic Acid Schiff

## INTRODUCTION

'Histology' term is derived from Greek: *Histos* means tissue while *Logia* means study of or knowledge. By refer to these terms, it actual refer to study of tissues for living organism. Nowadays, histology is used loosely to include all subdivision of microscopic anatomy. The correlation between structure and function provide the evidence that show the histology is an intriguing and readily understandable subject. In the study of histology, preparation of tissue for microscopic viewing is an important consideration. This is because cell and tissue cannot be studied to advantage unless they are well-prepared for microscopic examination. There are two subdivisions in whole method of tissue preparation: method involving direct viewing of living cell and method employed with dead cell (fixed and stained). In this practical session, fixed and stained preparation was applied. Ileum, thick skin, and appendix were selected. Tissues, collection of cells which carry out same, specific functions in the body, play an important role in daily life. However, different tissues that stacked together are hard to be recognized and differentiated, as most of them appear as colourless compound. This study provided with a full processes which allow the observation of tissue become easier.

## METHODS

### Fixation

Fixation was done as the first step to preserve the tissue substrate and render protein structure and other tissue components insoluble in all reagents exposed later in either processing or staining process. This step must be done as soon as possible as most cells contain lysosome which will carry out cell autolysis and release digestive enzymes to break down cell components after the cells have died (Bancroft & Gamble, 2002). Besides that, extracellular microorganism, mostly bacteria, will take opportunities and break down the dead cell through putrefaction for nutrient absorption (Bancroft & Gamble, 2002). By fixation, autolysis and putrefaction can be halted and tissue substrates can be preserved. In fixation, ideal fixatives are listed below (Bancroft & Gamble 2002, pp 25-26)

- Do not shrink, swell or harden tissue substrates
- Do not dissolve tissue components
- Kill microorganisms
- Retain the original form of tissue constituents throughout subsequent processing of tissue
- Compatible with subsequent staining method
- Adequate penetration rate

In the experiment, 10% neutral buffered formalin, which is the chemical fixative, was used due to:

- High penetration rate
- Inexpensive
- Reaction slow and reversible for first 24 hour
- Preserve tissue for quite long period if buffered
- Non coagulative gel: better preservation for cellular organelle

Formalin is inflammable and a strong mucous membrane irritant (Bancroft & Gamble 2002), thus it must be handle carefully.

## Processing

After fixation, tissues were insufficiently firm and cohesive to allow thin section to be cut (Gormley, 2012). This is why processing must be carried out. However, before the processing, about 3-5mm thick of the tissue were obtained and put into tissue dek cassettes (Gormley, 2012). The cassettes were then labeled for their laboratory accession number with chemical resistant lead pencils (Gormley, 2012). Dehydration was then carried out to remove all water in tissue substrate so that water droplets will not trap and affect the specimen from being processed (Gormley, 2012). Ethanol was used in dehydration process as it has least side-effects on tissue substrates compared to other dehydration agents. Water molecules were removed in ascending grades of alcohols to absolute alcohol.

Wax is insoluble in ethanol. Clearing agent that was miscible in both ethanol and wax are required. As xylene has least effect on tissue substrate (Bancroft & Steven, 1990), it was used in the experiment. However, xylene can harden the tissue, thus the immersion time cannot be prolonged (Bancroft & Steven 1990).

After the clearing process, tissues were then infiltrated with wax. In this process, paraffin wax which had a melting point about 54<sup>0</sup>C to 58<sup>0</sup>C was used (Gormley, 2012). Additives such as bee's wax or synthetic resin were added to increase the hardness and stickiness of wax in order to give better cutting properties on tissue by altering the size of wax crystals (Bancroft & Gamble, 2002). Temperature of the wax was kept 2 to 3<sup>0</sup>C above the melting point of wax (~60<sup>0</sup>C) so that the wax will remain as liquid form throughout tissue infiltration process (Bancroft & Gamble, 2002). However, it is important to make sure that the wax did not overheated as it will destroy plastic polymers (Bancroft & Steven, 1990) and cause the cutting process to become more difficult.

In the overall view, the tissue substrates were immersed in the chemical as shown:

- 1) 70% ethanol for 1 hour

- 2) 70% ethanol for 1 hour
- 3) 95% ethanol for 1 hour
- 4) Absolute ethanol for 1 hour
- 5) Step 4 repeated for other 4 times
- 6) Xylene for 1 hour
- 7) Xylene for 1 hour
- 8) Paraffin wax for 2 hour
- 9) Paraffin wax for 3 hour

Whole process (dehydration, clearing and infiltration of wax) was done in Shandon Duplex Automatic Tissue Processor.

### **Embedding**

Before the cutting, processed tissue needs to be embedded in wax as a 'block' form to enable the section to be cut. Tissue cassettes molds method was applied as it did not required further trimming of wax around the tissue. Different tissues have different embedding section. For instance, appendix was embedded with the cross section across lumen (Bancroft & Steven, 1990). Skin was embedded with the plane section across all tissue layers with the epithelial on top which was cut last to minimize pressure distortion of the epidermis (Bancroft & Steven, 1990). The embedding process (Gormley, 2012) was done as shown:

- 1) Little wax was poured into the tissue molds and allowed to form at the bottom layer of the molds.
- 2) Tissues were then picked with warm forceps and put onto the centre of the molds.
- 3) Plastic tissue cassettes were placed over the mold. More wax was added into the molds if necessary.
- 4) The molds were then moved to cold plate for rapid cooling and give fine crystalline structure to wax for better cutting properties.

### **Cutting**

In this experiment, rotary microtome was used for cutting sections. This is because rotary microtome is good in cutting semi-thin section for light microscopy (Bancroft & Gamble, 2002). Besides that, it can be motorized to facilitate the cutting of plastic embedded tissue. The full cutting method is shown in appendix 1, while the faults and remedies are shown in Appendix 2. There are several precautions (Bancroft & Gamble, 2002) that needed to be considered during cutting section, such as:

- Microtome knife MUST be set at clearance angle about  $5^{\circ}$  to prevent compression and chatter in the section
- Water bath must be set at temperature about  $45^{\circ}\text{C}$  to  $50^{\circ}\text{C}$ . Small amount of alcohol or detergent should be added into water bath to reduce surface tension and allow the section to flatten out easily.
- Floating of tissue section should be done more carefully to prevent water bubbles from being trapped under section. Fold in section can be removed by simply teasing with forceps. Section

should be allowed to float for about 30s as prolonged floating will cause excessive expansion and distorting of tissue.

- Debris and tissue fragment MUST be cleaned after each block was cut to avoid any overlap of other debris and fragment on tissue section. This can be done by dragging tissue paper across water surface.

## Staining

The constituent part of cell and intercellular material are usually transparent after fixation and processing (Gormley, 2012). Very little details can be viewed microscopically (Gormley, 2012). Thus it needs to be stained with coloured agents, such as dyes. Haemotoxylin and Eosin (H&E) stain, which is general stain, was used on ileum. Special stains were also used in the experiment to highlight either mature collagen or mucous substrates. For example, Periodic acid Schiff (PAS) was used in ileum to highlight the goblet; Van Gieson stain was used to stain skin; Masson's Trichrome stain was used to stain appendix. The staining processes for each stain were shown as below:

- Haemotoxylin and eosin (H&E) stain - Appendix 3
- Masson's Trichrome stain - Appendix 4
- Periodic Acid Schiff (PAS) stain - Appendix 5
- Van Gieson stain - Appendix 6

In the staining process, artifacts formed on the section. This might due to several reasons as shown below:

- Residual wax prevented the staining of the sections (Bancroft & Steven, 1990)
- Incomplete staining of tissue section, which usually due to insufficient stain in the staining dish (Bancroft & Steven, 1990).
- Excess stain deposit on the section (Bancroft & Steven, 1990).
- Contaminated staining solution by microorganism (Bancroft & Steven, 1990).
- Air bubbles under cover glass (Bancroft & Steven, 1990)
- Contamination by dirt or fibres on tissue section (Bancroft & Steven, 1990)
- Water molecule in tissue section (Bancroft & Steven, 1990)
- Cracking of synthetic mounting media with age (Bancroft & Steven, 1990)

## RESULTS

Ileum which stained with H&E stain was observed under x10 magnification, which is shown in appendix 7. There are numerous finger-like projectile villi on top of visceral epithelial lining. Simple columnar epitheliums cover villi. Lamina propria and lacteal are present within each villi. Besides that, aggregation of lymphatic nodules, which called Peyer's Patches (Holmes & Hourine, 1991), are found located in wall of ileum opposite the mesenteric attachment. Lymphatic nodules originate in the diffused lymphatic tissue of lamina propria. Some of the lymphatic nodules extend into the submucosa, disrupt muscularis mucosa and spread out in the loose connective tissue of submucosa (Eroschenko, 2008). Commonly, villi are absent in the area of the intestinal lumen where nodules reach the surface of mucosa (Davenport

1997). Most lymphatic nodules exhibit germinal centers and coalesce with each other (Rubin, 1971). This causes the boundaries between them become indistinct (Neutra, 1998). Below the lamina propria, there is a layer of muscularis externa, which consist of internal circular layer and outer longitudinal layer. Under serosal mesothelial lining, there are numerous of connective tissue and blood vessels.

In the ileum section that stained with PAS stain, shown in appendix 7, several villi are illustrated under x40 magnification. Simple columnar surface epithelium covers villi. Clear magenta coloured brush border (microvilli) is highlighted on the surface epithelium. Thin basement membrane is partially visible between epithelium and lamina propria. Numerous mucus-secreting goblet cells which stained magenta are found within surface epithelium. Connective tissue, blood capillaries and lacteal, lymphatic vessel are present within the lamina propria (Davenport, 1997).

For the skin section which stained with Van Gieson, illustrated in appendix 8, sub –epidermal papillary dermis layer stained yellow. Less red stained collagen are found in the surface layer compare to deeper layer. In reticular layer, red stained collagen is present as interlacing band form (Eroscehnko 2008). There are also some hair follicles, blood capillaries, sweat gland, sebaceous gland and arrector pili muscle found within the dermis layer. Epidermis layer are composed of stratified squamous non-keratinised cell, which consist of visible stratum corneum, stratum granulosum and stratum spinosum (Parakkal, 1994). Besides that, thin keratin layer also illustrated on the epidermis layer. Meanwhile, hypodermis layer, which located on the bottom layer of skin, is composed of yellow stained adipose cells and red stained collagen fiber (Montagna, 1995). Based on the illustration, it is thin skin.

Appendix section is stained with Masson's Trichrome, illustrated under x40 magnification in appendix 8. There are four layers in appendix. Serosa layer is composed of adipose cell and connective tissue (Kessel, 1998). Muscularis externa layer is composed of smooth muscle tissue (Leeson, 1976). There are inner circular muscularis layer and outer longitudinal muscularis layer. Some barely visible parasympathetic ganglia of myenteric nerve plexus locate between the inner and outer muscularis layer (Wheater et al.2006). Submucosa layer composed of mainly connective tissue and fiber (Ross & Reith, 1985). Numerous blood capillaries and adipose cell are present within the submucosa layer. In some area, there are also present of muscularis layer (Steven & Lowe, 2005). Mucosa layer is mainly composed of connective tissue (lamina propria). It contains numerous lymphatic nodules with germinal centre and diffused lymphatic tissue (Thorek, 1985). Some intestinal glands are present within mucosa layer. Tall, simple columnar lining epithelial layer are found as the visceral layer of the appendix.

## DISCUSSION

H&E stain contain haemotoxylin and eosin. Haemotoxylin is basic stain. It will stain basophilic component, such as nuclei as dark blue (Kiernan, 2008). Meanwhile, eosin, which is acidic stain, will stain acidophilic substrate as pinky red in different degree (Kiernan, 2008). For instance, cytoplasm which fulfilled with granular mass is stained pink (Kiernan, 2008); collagen and muscle is stained pink (Kiernan, 2008) and erythrocyte (red blood cell) is stained intensely red (Kiernan, 2008).

PAS stain contains periodic acid and Schiff reagent. Periodic acid can oxidize the vicinal diol (glycol part) in the glycol-sugar and produce two aldehyde groups at two free tips of each monosaccharide ring while splitting the bond between the two carbons (Kiernan, 2008). On the other hand, Schiff's reagent consists of para-saniline solution (Basic fuchsin), which was decolourised by the sulphurous acid by adding extra sulphurous group to the central carbon of dye (Kiernan 2008). For the reaction with aldehydes in the tissue, the alkyl sulphonic derivative of the dye is formed and restores the quinoid chromophobic group

to give a magenta colour on the tissue (Kiernan, 2008). At the end of reaction, basement membrane and brush border (microvilli) of ileum stain pink and the goblet cell stain magenta red.

The staining mechanism for Masson Trichrome stain is mainly based on molecular size theory. For instance, erythrocyte is stained red by the smallest molecular size dye, Ponceau 2R (MW=480); Acid fuchsin, which has intermediate molecular size (MW=586), stains muscle and cytoplasm pink; Collagen, which is more permeable, stained green by the light green, which has large molecular size (MW=793) (Kiernan, 2008). Meanwhile, phosphotungstic acid is used as mordant to remove red staining from tissue other than in less permeable components (muscle and erythrocyte) and allows easier access for light green to stain collagen (Kiernan, 2008). Besides that, it indirectly affects the diffusion of acid fuchsin dye at different rates based on the tissue attached (Kiernan, 2008). This result in different degree of redness appeared on different acidophilic tissue (Kiernan, 2008). For example, smooth muscle stained red while erythrocyte stained vivid red. As the overall result, nuclei stained black, smooth muscle stained red, collagen stained green and cytoplasm of most cells stained pink.

For Van Gieson stain, the staining mechanism is quite same with the Masson's Trichrome stain, which also depends on the molecular size of the tissue substrate and dye. For instance, small molecules of picric acid (MW= 229) penetrate all of the tissue rapidly, but are only firmly retained in the closed textured tissue substrate, such as erythrocyte and muscle tissue (Kiernan, 2008). Least porous tissue are coloured by the smallest dye molecule (Kiernan, 2008), which is picric acid in this section. Meanwhile, the larger molecule of dye, acid fuchsin (MW= 586) displaces picric acid molecules from collagen fibers, which has larger pores, and allow larger dye molecules to enter (Kiernan, 2008). In overall result, collagen is stained red, nuclei stained dark blue, while other tissue such as erythrocyte and muscle stained yellow.

## CONCLUSION

In conclusion, the fixation, processing, embedding and staining process make the tissue more suitable to be observed microscopically. Fixation keeps the cell in their original form while halted the effect of autolysis and putrefaction. Processing and embedding process alter the hardness of tissue, give sufficient support on tissue and allow the smooth cutting on tissue substrate. Staining process attach coloured compound on specific tissue substrates, rise different colours on the different parts of tissue based on their tissue affinity, so that viewing and distinguish of tissue substrates become clearer. . Suitable fixative agent and staining solution should be choose wisely as it will caused irreversible effect on tissue substrate. Different fixative can cause different effects on tissue. On the other hand, different stain can highlight different tissue compound based on their tissue affinity, the molecular size and the type of counter stain. Fixative can also have effect on stain. It may either remove the tissue compound, or cause the stain loss their tissue affinity or coloured chromogen. In the end of experiment, it can be conclude that, ileum contain many villi projection that use for absorption of nutrient. Each villi consist of numerous goblet cells and microvilli. Appendix contain numerous of lymphatic nodules on the mucosa layer with simple columnar epithelium as visceral layer. Skin contains stratified squamous epithelium with layer of keratin on top. Dermis contains the lamina propria, which is areolar connective tissue, and composed of sweat gland, sebaceous gland, blood capillaries and hair follicles. Hypodermis mainly composed of adipose tissue.

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