Published with Open Access at Journal BiNET
Vol. 02, Issue 01: 57-67
Journal of Fisheries, Livestock and Veterinary Science
Journal Home: https://www.journalbinet.com/jflvs-journal.html

A better and copacetic protocol for histopathological slide preparation using H&E stain: A review

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Article received: 10.11.21; Revised: 21.12.21; First published online: 29 December 2021.

ABSTRACT

The hematoxylin and eosin (H&E) stain is one of the most common stains which is frequently used all over the world for histochemical and histopathological results or observations. Many hematoxylin and eosin (H&E) stain protocols are used in the laboratories, but sometimes after following the protocol, the slides do not produce a better visualization, which affects the results massively. H&E staining faults can result from any problems in the key steps fixation, processing, embedding, sectioning, and staining. Also, inaccurate timing of staining, not considering the quality and stability of the stain, not following the protocol can cause damage to the slide and ultimately the result. A respectable number of papers, articles and books are reviewed and several practical protocols are analyzed; therefore, this study is done at the Department of Anatomy and Histology & Department of Pathology in Bangladesh Agricultural University, Mymensingh to determine and produce a better copacetic protocol for H&E stain for precise and actual visualization of slide when viewing in light microscope; which will help to render a better slide and produce an accurate result every time.

Key Words: *Histopathological slide, H&E, Stain, Histopathology and Tissue visualization protocol*

Cite Article: Hasan, M. I., Das, S. K. and Chowdhury, E. H. (2021). A better and copacetic protocol for histopathological slide preparation using H&E stain: A review. Journal of Fisheries, Livestock and Veterinary Science, 02(01), 57-67.
Crossref: https://doi.org/10.18801/jflvs.020121.07

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I. Introduction

An animal or plant cell or tissue studied under a microscope (electron or light) is called histology, which is done by staining and cutting the cell or tissue. There are many ways to look at tissue characteristics and the microscopic structures of cells. An autopsy, diagnosis and education are all made with histological studies. Besides that, histology is used all over medicine, mainly to study diseased tissues so that doctors can help treat patients (Black, 2012). Histological staining is a series of technical steps used to prepare sample tissues for microscopic study by staining them with histological stains (Anderson, 2011). Staining is a common medical process used to diagnose tumours. A dye colour is put on the back and front edges of the tissue samples to help doctors find the diseased or tumorous cells or any pathological cells (Musumeci, 2014).

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Staining is used to show off the essential parts of the tissue and to make the tissue look more contrasted. A stain named hematoxylin, a basic dye is used in this process. It gives the cell's nucleus a bluish colour. Eosin, another stain dye used in histology, gives the cell's cytoplasm a pinkish colour. However, there are a lot of different staining methods used for different cells and parts (Black, 2012). Histological staining is a process that goes through five main steps. These steps are fixation, processing, embedding, sectioning and staining (Titford, 2009). Differential staining, double staining, or multiple staining methods are some ways that staining can be done (Iyiola and Avwioro, 2011). Staining is used in biological studies to mark cells, flag nucleic acids, proteins, or gel electrophoresis to do microscopic examinations more rapidly (Jackson and Blythe, 2013). The most commonly used histochemical stain in clinical and research laboratories is hematoxylin and eosin (H&E). For over a century, this stain has been used to highlight the structures of cytoplasmic and nuclear components in cells and tissues (Ortiz-Hidalgo, 2019), where hematoxylin stains nuclear structures and eosin stains cytoplasmic structures. Because the H&E section can provide a tremendous amount of information (Chan, 2014), it is routinely used in many applications, resulting in a large volume of sections processed using this stain by nearly all histology laboratories. As a result, the quality and reproducibility of H&E staining are critical considerations in interpreting results (Larson, 2011). The H&E stain is widely used in biology laboratories for gross (histological) evaluation of tissue morphology, cellular structure (cell morphology), as well as the cytochemical examination of isolated cell organelles and intracellular constituents. The H&E stain can be used to identify cellular components such as nuclei, nucleoli, mitochondria, Golgi apparatus, lysosomes, lysosome-associated bodies (LACS) and other vesicular organelles (Andrew, 2014).

As a way to give pathologists and researchers a close-up view of the tissue under examination, histopathology labs routinely employ the H&E staining method. The cytoplasm, nucleus, organelles, and extracellular components are all visible thanks to the dye's ability to do so. Many diseases can be diagnosed based on the organization (or disorganization) of cells, and this information is often enough to show any abnormalities or specific indicators in the cells themselves (such as nuclear changes typically seen in cancer). The H&E stain is still critical to the diagnostic picture even when advanced staining methods are used because it reveals the underlying tissue morphology, allowing the pathologist/researcher to correctly interpret the advanced stain (Anderson, 2011). Traditionally, special stains referred to any staining other than an H&E. It encompasses a wide range of techniques that can be used to visualize specific tissue structures, elements, or even microorganisms that are not identified by H&E staining (Anderson, 2011).

II. Materials and Methodology

The study collected quantitative and qualitative data on histological stains used in previous and recent cases through an extensive exploration and review of historical, recent and current medical research studies and case studies (Silverman, 2011). A database of clinical pathology journals, different histological journals, histopathological and histological books involving past and recent usage of histological stains, specifically H&E stain, was created in this case. These journals, articles, books and case studies were reviewed and analyzed and key trends in applying histological stains were identified. Rich data on stains used in the past and present were gathered through integrative and intensive literature and case study reviews to consider how histological stains should be improved. This triangulation aids in the collection and evaluation of detailed data on past, present and future stain and staining techniques (Silverman, 2011).

III. Literature Review

History of staining techniques in medical and biological Studies

According to the history of staining, the application of histological techniques is a relatively new area of disease diagnosis (Rodrigues et al., 2009). Early pathologists and surgeons borrowed historical staining techniques from a seventeen-year-old scientist named Leeuwenhoek, a pioneer in histology, using substances like madder, indigo and saffron to stain tissues and study them with rudimentary microscopes (Titford, 2009). These early researchers used microanatomy to delineate a normal plant cell structure from that of an animal and draw a relationship between differences in cells (Bancroft and Layton, 2013). Later, newer techniques were developed to aid in the detailed study of cell structure by preserving tissues in their natural state before staining with various laboratory chemicals

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(Titford and Bowman, 2012). When Joseph Von Gerlach successfully stained cerebellum cells with ammoniacal carmine in 1858, he was regarded as the father of microscopical staining (Costa et al., 2010). To prepare tissues for microscopic studies, early histologists used readily available laboratory chemicals such as potassium dichromate, alcohol and mercuric chloride to hard cellular tissues (Iyiola and Avwioro, 2011). These fixatives and staining agents were ingenious and coloured staining agents were developed over time that is still used in modern laboratory staining techniques (Black, 2012). Trichrome, which is used in liver and renal biopsies, as well as silver nitrate, which is used in other organisms, are examples of these ingenious coloured stains that are still in use (Musumeci, 2014). The improved technological development of microscopes and the establishment of the histologic stains (aniline dye) in 1856 in Germany, which manufactured a variety of new histological stains, shaped significant advancement in histologic stains (Shostak, 2013). Simultaneously, research and knowledge of human anatomy and tissues grew and this knowledge was used to develop new histological techniques for the study of diseased tissue (Titford, 2009). Many medical centres hired physicians, pathologists and surgeons to handle surgical issues in the aftermath of the nineteenth century (Titford and Bowman, 2012). This generation of pathologists is responsible for developing intraoperative staining techniques for frozen tissues sections by adapting a histopathology staining technique. The paraffin infiltration staining technique was developed around this time (Shostak, 2013). The non-malignant and malignant tumours were studied due to this achievement and a bacterium was identified as the disease's causal organism in the nineteenth century (Avwioro and Godwin, 2011). In 1875, a Danish inventor named Hans Christian Gram invented the Gram staining method to distinguish bacteria species (Anderson, 2011). Gram developed the staining technique while working at the city morgue with his colleagues to distinguish the type of bacterium infection and make the bacteria visible on selected and stained lung tissues during examination (Black, 2012). Although it was found to be unsuitable for certain bacterium organisms, this technique is still in use today and competes fairly with modern molecular histology techniques (Shostak, 2013).

History of H&E staining

The most used stain in the histological laboratory. It is a constituent of the heartwood of the logwood tree *(Haematoxylon campechianum),* which grows in Campeche, Mexico. (Culling, 1984). These are naturally occurring substances that have been used in histopathology throughout history (Titford, 2009). Wilhelm von Waldeyer developed the stain in 1863 using a log tree he discovered in Central America. Hematoxylin is a weak stain used in combination with other oxidized solutions (Shostak, 2013). The stain is combined with an oxidizer mordant to enhance its capacity for differentiating cell components; these solutions are referred to as Hematoxylin. The stain's versatility has aided in the development of numerous Hematoxylin methods (Titford and Bowman, 2012). Historically, hematoxylin was transformed into a nuclear stain with a faster staining time and resistance to acidic solutions; this made it ideal for histologic staining techniques requiring multiple steps (Anderson, 2011).

Although Hematoxylin stains have been used historically, they have undergone significant laboratory changes; nearly all tissue specimens are now stained with Hematoxylin and Eosin (Bancroft and Layton, 2013). Additionally, numerous Hematoxylin staining methods have been developed, but all involve staining tissue specimens in hematoxylin, alcohol and tap or alkaline water to remove argentaffin agents. The Hematoxylin and Eosin procedures are adequate for studying most histopathological processes (Titford and Bowman, 2012). Similarly, the method is simple to implement, inexpensive and adaptable. However, Hematoxylin and Eosin are inefficient in that they do not capture all of the characteristics of a substance, necessitating the use of special stains (Musumeci, 2014).

Effects of staining on tissue visualization

Although hematoxylin has been used for tissue staining for nearly 150 years, failure to obtain optimal results remains a problem. Hematoxylin is a natural product derived from the Central American logwood tree (*Haematoxylum campechianum*). That dye does not stain until it undergoes two-step oxidation followed by attachment to a mordant. Oxidation can occur naturally due to exposure to air and sunlight, or artificially, as a result of the addition of an oxidizer (mercuric chloride, potassium permanganate, and sodium iodide have all been used) to form hematein. Hematein does not stain until it is attached to a metal mordant, linking the hematein and the tissue. Iron and alum are the two most

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frequently used mordants with hematoxylin. Alum hematoxylin is frequently used in the HE stain, which is more properly referred to as aluminium-haematein-eosin. In European publications, HE is frequently referred to as "hematein and eosin." The mordant chosen is critical because it affects the final colour and maximum density of stained nuclei and the contrast between hematoxylin and eosin in HE stained slides. While iron hematoxylins stain nuclei black and alum hematoxylins stain them blue, the final blue colour of alum hematoxylin-stained nuclei varies according to the mordant formulation, ranging from blue-black to purple with Harris's hematoxylin, to deep blue with Gill's #2, to mid to light blue with Mayer's hematoxylin. Harris's hematoxylin produces the highest grayscale value of the alum hematoxylins in a section with appropriately differentiated nuclei (McGavin, 2014).

The tissue visualization is affected by the faulty staining process can be minimized by maintaining; accurate timing in each step of staining; Control slides should be stained regularly to monitor the staining quality; Agitation, wash and drain times are optimized for all steps during staining; Ensuring complete dewaxing; Solvents and reagents should be regularly replaced based on the number of slides had been stained or number of slide racks had been stained; Slides should be appropriately hydrated prior to the hematoxylin staining; Avoid the uneven eosin staining; P^H of eosin stain should be maintained at 5.0; Slides should be thoroughly dehydrated before putting in xylene solution; Avoid drying and crystal formation in the cover slipping process (Cindy and Geoffrey, 2021).

IV. Histopathological slide preparation

Sample collection

The tissues are collected from the animal's body, usually in small pieces (not more than a size of few millimeters thick in order to permit adequate penetration of fixative solution), with the help of a sharp scalpel since any pinching, squeezing and pressure can change and distort the appearance of the normal tissue. The sample's length, width and breath shall not exceed 5mm. Specimens are usually received in fixative (preservative) but sometimes arrive fresh and must be immediately fixed (Rolls, 2021).

Tagging

The sample marking was carried out by thread to identify the specific sample in the following steps. It is crucial because if the samples cannot be identified at the later stages, the results of the study will permanently affect and cause difficulties in producing results. Correct labelling and specimen identification are the first requirements of any processing technique because a mistake in labelling may result in a major operation on the wrong patient or an incorrect diagnosis, resulting in the patient's death (Culling, 1984). Before a laboratory accepts a specimen, the identification (labelling) and accompanying documentation will be thoroughly checked, all details will be recorded and "specimen tracking" will begin (Rolls, 2021).

Fixation

We used a 10% formalin solution to fix the samples after tagging and the fixation process took anywhere from three to seven days to complete here. Using chemicals to preserve the natural tissue structure and protect the cell structure from degradation is known as fixation in histology. Neutral buffered formalin is the most common choice when using a light microscope. Fixatives improve tissue and cell preservation by cross-linking proteins in an irreversible manner (Table 01). Even though this procedure preserves the cell's structure for histological studies, it has been discovered to destroy and denature proteins, rendering them ineffective (Young et al. 2010). The DNA, mRNA and mRNA tissues are denatured by formalin fixation, making it risky to extract them for histology (Anderson, 2011).

Hardening the cells or tissues for sectioning and delaying degradation are some of the effects of the fixation phase (Titford, 2009). Fixatives also alter tissue penetration and affect antigen exposures, either beneficial or harmful depending on the situation (Iyiola and Avwioro, 2011). Fixatives can be injected into the prepared tissue in two ways: either through perfusion or immersion. Diffusion is used to get the fixatives into the animals. Perfusion is a more time-consuming procedure that uses a single fixative at a time (Shostak, 2013). For the most part, formaldehyde-based fixatives are used, but others exist (Black, 2012). For delicate and soft tissues like embryos and brains, the Bouin fixative has proven effective (Musumeci, 2014). The nuclei and glycogen are well preserved with Bouin fixative, but its

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penetration is slow and it distorts mitochondria and kidney tissues as a result (Weiss et al., 2010). Tissue preservation (fixation) is the first and most important step in specimen handling. As far as anatomical pathology is concerned, formaldehyde is the fixative of choice (Christina, 2014).

Table 01. Common Fixatives with their recommended use and limitations (Christina, 2014).

Fixative	Primary/Recommended use	Limitations
Alcohol	Routine cytology specimens. Cases where gout is suspected. Fixation for frozen sections, smears, and touch preps.	Methanol and ethanol cause cell shrinkage and make tissue brittle if over fixed.
Alcoholic formalin	Completion fixation with incompletely fixed tissue; primary fixative for fatty specimens (allows for easier detection of lymph).	Acidic pH can allow for formation of formalin pigment precipitates.
B-5 fixative	Hematopoietic and lymphoid tissue.	Sections require removal of mercury pigment prior to staining; tissue cannot be stored in this; low molecular weight or no extractable nucleic acid.
Bouin's Fixative	Gastrointestinal and genitourinary tissue.	Slowly removes small calcium and iron deposits; lysis of erythrocytes; low molecular weight or no extractable nucleic acid.
Decalcifying solution (acid based)	Large bone sections where future molecular testing is not required.	Poor staining with low molecular weight or no extractable nucleic acid. Prolonged immersion can completely dissolve specimen.
Decalcifying solution (EDTA based)	Bone biopsies for primary diagnosis or secondary diagnosis (metastasis), bone aspirates.	
Formalin	Routine processing	Dissolves uric acid crystals; can dissolve breast microcalcifi cations if fixed >24 h prior to processing; reduced high molecular weight nucleic acids with time. Unbuffered formalin can allow for formation of formalin pigment precipitates
Glutaraldehyde microscopy	Electron microscopy	Can cause false positive PAS staining; for light tissue is fixed for 2–4 h then transferred to buffer solution until processing; low molecular weight or no extractable nucleic acid.
Hollande's	Gastrointestinal and endocrine tissues, small decals, and bones.	Picric acid component limits use for molecular testing; low molecular weight or no extractable nucleic acid.
Michel transport medium	Renal biopsy transport. Cases requiring immunofluorescence.	Requires tissue to be washed with PBS prior to processing.
Zenker's solution	Bone marrow biopsies.	Poor antigen preservation for IHC; slow penetration; contains mercury; lyses red blood cells; can dissolve iron; silver stains poor after fixation; low molecular weight or no extractable nucleic acid.

The most popular fixing agent is formaldehyde, usually in the form of a phosphate-buffered solution (often referred to as "formalin"). Ideally, specimens should be fixed by immersion in formalin for six to twelve hours before processing them (Rolls, 2021).

After that washing the samples in running water overnight to remove excess formalin from the sample and cutting the tissue samples in 3-6mm in thickness (less thickness results in better slide preparation and allows for staining easily). Then the samples are placed in 50% Alcohol for 1 hour; 70% Alcohol

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for 1 hour; 80% Alcohol for 1 hour; 95% Alcohol for 1 hour; 100% alcohol 3 times for 1 hour each to remove the excess water from the tissue samples to solidify and give advantages in cutting them for the slide section. Aiming to remove water from the selected tissues in order to solidify them and facilitate the cutting of thin sections of slides for use in light microscopes and thick sections for electron microscopes is the goal of the dehydration step. The dehydration method uses ethanol to remove water from the tissues (Shostak, 2013). The routine dehydration method is done by using a series of ascending grades of alcohol (70%, 80%, 95% and 100%) solutions at room temperature for 2 hours each (Hegazy, 2015). Dehydration displaces the residual aqueous fixative as well as cellular water. Water is found in the tissue in two forms: free water and bound water (Charley, 1982).

Clearing and infiltration

After the dehydration step, the samples are placed in chloroform or xylene solution 3 times for 1 hour each to make it clearer and more penetration can be done. Chloroform, benzene, xylene, toluene, carbon tetrachloride, and cedar-wood oil are the most commonly used degreasers (Culling, 1984). Tissue processing facilities around the world use xylene as their primary clearing agent. It's an excellent lipid solvent, but it has the unfortunate side effect of drying tissue samples. For fat tissue, xylene can remove water from the tissue by its "practically insoluble" nature in water (Christina, 2014). The routine method of the clearing and infiltration is done by using two xylene (xylene-1 and xylene-2) solutions at room temperature for 2 hours each (Hegazy, 2015).

Embedding

For embedding step, paraffin wax is the best embedding material for any samples used in permanent slides using H&E stain. The samples are then placed in 56°C-58°C liquid paraffin and 58°C-60°C liquid paraffin one time each for 1.30 hours at an oven. This embedding will help to extract the cellular structures of the samples. In staining, paraffin wax is used in the embedding process to facilitate the extraction of cell structures. Plastic resin or wax, or a combination of fixatives, produces good morphology in complex cellular tissues (Musumeci, 2014). In an oven heated to 54-60°C, paraffin wax impregnation occurs. The oven temperature is determined by the melting point of the wax used (Culling, 1984).

After clearing, sections of tissue are infiltrated (impregnated) with paraffin wax to be cut into thin sections. For the wax to harden properly, the infiltration must be sufficient to remove the clearent from the tissues. The length of time the section is exposed to the molten paraffin is critical to its quality. Insufficient time for tissue infiltration results in soft, crumbly tissues that are difficult to section. Excessive shrinkage and dry, brittle tissues can result from prolonged exposure to high-temperature wax. Encasing paraffin should be 2° to 4° above the wax's melting point. There should be no more than 2 hrs of total paraffin infiltration time (Christina, 2014). The embedding phase done routinely with paraffin wax and done three times at the temperature of 48◦-62◦C for 2 hours each (Hegazy, 2015).

Blocking

The samples were then thoroughly infiltrated with liquid paraffin 58°C-60°C. Then the samples are placed into a mold; pouring 60°C-62°C liquid paraffin on that mold and turn the samples into block. This mold is kept overnight for cooling at room temperature so that the liquid paraffin wax becomes harder and the block itself along with the sample. Tissue is blocked by transferring it from the final wax bath to a mold filled with molten wax, inverting the tissue to free the surface to be cut from air bubbles and orientated so that this surface rests on the base of the mold. The block is then quickly cooled (Culling, 1984).

Sectioning

Sectioning is the most important factor over the permanent slide preparation as it plays a crucial role as the tissue sample for the slide is being cut at this stage. Sectioning should be done by cutting the block with the help of Microtome in the thickness of 4-6µm so that the tissue sections can be stained accurately and easily. If the tissue sections are less than 4µm will give a thinner section which can be stained very largely and concludes it to a false result; whereas more than 6µm will result a thicker section which will result in more time consuming, less stained section and also concludes it to inaccurate result.

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'Ribbon-like' microtomes of tissue are prepared in histology for mounting on a microscope slide for examination (Cai et al., 2014). In order to perform microtomy correctly, the user's manual should be consulted. Section thickness is determined by the pathologist, researcher or project. Sharpness and cutting angles, i.e., blade facet and alignment between the paraffin block face and blade edge, are critical to section quality (Christina, 2014). For routine work, 6 microns will give moderately thin sections with ease of cutting. It should be remembered that dense tissue, such as spleen, should be cut thinner than open tissue, such as lung (Culling, 1984). To stain with Harris hematoxylin and eosin (H&E), as well as other techniques such as periodic acid-Schiff (PAS), Masson's trichrome (MT), and Papanicolaou's stain (PAP), serial sections of 2-5 um were cut, deparafinized, hydrated, and stained (Hegazy, 2015). Sections are cut on a "microtome" using extremely fine steel blades on a precision instrument. The thickness of paraffin sections is typically between 3 and 5 micrometres (Rolls, 2021).

Floating of sections in water bath and placing over slide

After the sectioning step, the sectioned tissue samples are floated in a floating water bath containing 5gm gelatin/liter water at 37°C -42°C so that the wrinkled section of tissues can be flattened and also can easily be placed over the glass slide. Then the slides with the tissue section are kept in an incubator at 37°C or can be kept at room temperature overnight or 12 hours for drying. In order to remove wrinkles, the tissue section should be floated in a warm water bath for a few minutes. When working with wax, the water bath temperature should be between 5° and 10° lower than the melting point (Christina, 2014). During the cutting process, crinkled and compressed sections of paraffin-wax embedded sections are formed. These creases must be removed and the section flattened before being attached to slides, and this is now possible with the advent of thermostatically controlled baths with black interiors. Baths are kept at a temperature 5-6 degrees lower than paraffin wax's melting point in order to keep the temperature stable (Culling, 1984). After being flattened in a flotation bath, sections are now "floated out" and picked up onto microscope slides. They are now ready for staining after a thorough drying period (Rolls, 2021).

Hematoxylin and eosin staining

The slides kept overnight are placed in slide rack and place in xylene solution 3 times each for 5 minutes for clearing paraffin from the sample; And then hydrate the samples by placing the slide rack through 100% alcohol 2 times for 5 minutes, 95% alcohol for 5 minutes, 80% alcohol for 5 minutes, 70% alcohol for 5 minutes and rinsing in running tap water for 10 minutes at normal room temperature; After that dipping in distilled water for 2-4 times so that if there is any minerals deposited over the sample from the tap water could be removed. Then placing the rack in Hematoxyline stain for 5-15 minutes according to the quality of stain. To remove excess stain from the samples placing the rack in running tap water for 10 seconds, dipping in Acid Alcohol for 2-4 times (To decolorize), running tap water for 10 seconds, dipping in Ammonia water for 2-4 times (To decolorize) and ultimately running tap water for 10 minutes; Placing the slide rack in Eosin stain for 2-4 minutes; After that to dehydrate the tissue samples the slide rack is place in 95% Alcohol 2 times for 5 minutes each, 100% Alcohol 3 times for 5 minutes each; To clear the samples the rack is placed in xylene solution 3 times for 5 min each and then placed in a dry place for sometimes so that excess xylene solution is removed from the slides.

In staining racks, place the glass slides containing the paraffin sections. Remove the paraffin from the samples with three changes of xylene for two minutes each. Hydrate the samples in the following manner: Transfer the slides through three changes of 100 percent ethanol for two minutes each, 95 percent ethanol for two minutes, and 70 percent ethanol for two minutes. Rinse the slides for at least 2 minutes in running tap water at room temperature. 3 minutes staining in hematoxylin solution Place the slides in a tub of running water at room temperature for at least 5 minutes. Stain the samples for 2 minutes in a working eosin Y solution; Dehydrate the samples in the following manner: Dip the slides about 20 times in 95 percent ethanol, then transfer to 95 percent ethanol for 2 minutes, then through two changes of 100 percent ethanol for 2 minutes each. Clear the samples in three xylene changes for two minutes each (Robert et al., 2014). The hematoxylin and Eosin staining schedule can be done by clearing the paraffin from the sample by using clearant three consecutive times each for 3 min; 100% alcohol 2 times for 1 min each; 95% alcohol for 1 min; 70% alcohol for 1 min; distilled water for 1 min; hematoxylin stain for 2-4 min depending on the desired intensity; distilled water rinse for 1 min; 10% acetic acid in 95% alcohol for 1 min; distilled water for 1 min; Scott's tap water for 1 min; distilled

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water rinse for 1 min; 70% alcohol for 1 min used as prerinse; Eosin stain for 0.5-3.0 min depends on desired intensity; 95% alcohol for 1 min; 100% alcohol 3 times each for 1 min; Clearent for 3 times each for 1 min (Christina, 2014).

Slides should be staining in glass staining racks. The solutions are poured into square glass staining jars. For a quick dip Slide Staining System, each jar contains 80 mL of reagent and 12 slides in a staining rack; Deparaffinize the sections in two 10-minute xylene baths. Hydrate the lung sections by passing them through a series of decreasing-concentration alcohol baths: 2 absolute alcohol changes of 5 minutes each, 95 percent alcohol for 2 minutes and 70 percent alcohol for 2 minutes; Wash your hands briefly in distilled water; 8 minutes staining in Mayer's hematoxylin solution; 10 minutes in warm running tap water; Rinse thoroughly with distilled water. Counterstain for 30 seconds to 1 minute in a 1 percent eosin Y solution; Dehydrate in 95 percent and absolute alcohol for 2 minutes each, or until excess eosin is removed. Examine under a microscope; 2 xylene changes of 5 minutes each; Resinous mounting medium for coverslip slides. Mount an H&E-stained slide on the stage of an optical microscope to observe (and photograph if necessary) (Zhou and Moore, 2017). Place the slide in xylene solution 2 times for 2 min each; 100% alcohol 2 times for 2 min each; 95 % alcohol for 2 min; water wash for 2 min; Hematoxylin stain for 3 min; use of differentiator (mild acid) for 1 min; water wash for 1 min; bluing for 1 min; water wash fir 1 min; 95% alcohol for 1 min; Eosin stain for 45 seconds; 95% alcohol for 1 min; 100% alcohol2 times for 1 min each; xylene solution for 2 times for 2 min each (Cindy and Geoffrey, 2021).

Mounting

After staining and drying the slides, the slides are removed from the slide rack and on each slide adding 1-2 drops of DPX of Canada Balsam or any mounting agent. Then adding coverslip and observed under a microscope. One or two drops of Canada balsam (depending on the size of the coverslip) are placed on the section; being laid along the middle of the section to reduce the likelihood of trapping air bubbles (Culling, 1984). After the eosin stain, the slide is rinsed in several baths of xylene to "clear" the tissue and make it completely transparent before being passed through several changes of alcohol to remove any remaining water. Glass coverslips and a thin layer of polystyrene are then applied (Cindy and Geoffrey, 2021).

The copacetic protocol

1.	Collection of samples	Experimental samples are collected not more than a few mm thick. Tagging was done to identify samples
2.	Fixation of sample	Immediately after tagging, placed in 10% formalin solution for 3-5 days Washing the samples in running water overnight to remove excess formalin solution Cutting the tissue samples in 4-6mm in thickness.
3.	Dehydration of sample	Placed in 70% Alcohol solution for 1 hour. 80% Alcohol solution for 1 hour. 95% Alcohol solution for 1 hour. 100% Alcohol solution for 1 hour. 100% Alcohol solution for 1 hour. 100% Alcohol solution for 1 hour.
4.	Clearing of sample	Placed in chloroform for 1 hour. Chloroform for 1 hour. Chloroform for 1 hour.
5.	Impregnation of sample	Placed in 56°C- 58°C liquid paraffin wax for 1.30 hour. Placed in 58°C- 60°C liquid paraffin wax for 1.30 hour.
6.	Embedding of sample	Infiltration of the samples is done thoroughly by placing it in a mold and pouring 60°C-62°C liquid paraffin wax over it to form a block. The mold with block is placed for cooling overnight.
7.	Sectioning of sample	Cutting the blocks with the help of microtome in the thickness of 4-6µm. Cut sections are floated at 37°C-42°C water bath containing gelatin 5gm/liter water. The gelatin will act as adhesive when the sections are placed over the slides. The sections were then placed over a glass slide.

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	Slides with the sample are dried overnight.
8. H&E staining	Slides with sample placed in the slide rack and placed in Xylene solution for 5 minutes. Xylene solution for 5 minutes. Xylene solution for 5 minutes. 100% Alcohol solution for 5 minutes. 100% Alcohol solution for 5 minutes. 100% Alcohol solution for 5 minutes. 95% Alcohol solution for 5 minutes. 80% Alcohol solution for 5 minutes. 70% Alcohol solution for 5 minutes. Running tap water for 10 minutes. Dipping in distilled water for 2-4 times. Hematoxylin stain for 5-15 minutes. Running tap water for 10 seconds. Dipping in Acid Alcohol solution for 2-4 times (to decolorize). Running tap water for 10 seconds. Dipping in Ammonia water solution for 2-4 times (to decolorize). Running tap water for 10 minutes. Eosin stain for 2-4 minutes. 95% Alcohol solution for 5 minutes. 95% Alcohol solution for 5 minutes. 100% Alcohol solution for 5 minutes. 100% Alcohol solution for 5 minutes. 100% Alcohol solution for 5 minutes. Xylene solution for 5 minutes Xylene solution for 5 minutes Xylene solution for 5 minutes
9. Mounting of slide	1-2 drops of mounting agent (e.g. DPX, Canada Balsam etc). Adding coverslip over the sample. Wait to fix the coverslip over the slide.
10. Observation	Observation under microscope.

V. Conclusion

The authors of this paper tried to produce a copacetic protocol for having a better and clear visualization, which will be time and money-saving. The final goal of this study to abate errors in producing permanent histological slides and disseminate this protocol to others. Accurate timing and step by step following should be done and changing the stain reagents after the maximum number of staining slides will reduce the effects of staining on the tissue visualization. The future plan of the authors are to analyze more about the different histological techniques using different staining agents and produce a better protocol for each of them. The authors think that this research will be helpful for the histologists, pathologists, cell biologists, and autonomous learners in this realm to acquire knowledge about the permanent slide preparation and help prepare better histopathological slides by H&E stain using their protocol.

VI. References

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HOW TO CITE THIS ARTICLE?

MLA
Hasan, M. I. "A better and copacetic protocol for histopathological slide preparation using H&E stain: A review". Journal of Fisheries, Livestock and Veterinary Science 02(01) (2021): 57-67.

APA
Hasan, M. I., Das, S. K. and Chowdhury, E. H. (2021). A better and copacetic protocol for histopathological slide preparation using H&E stain: A review. *Journal of Fisheries, Livestock and Veterinary Science*, 02(01), 57-67.

Chicago
Hasan, M. I., Das, S. K. and Chowdhury, E. H. A better and copacetic protocol for histopathological slide preparation using H&E stain: A review". Journal of Fisheries, Livestock and Veterinary Science. 02(01) (2021): 57-67.

Harvard
Hasan, M. I., Das, S. K. and Chowdhury, E. H. 2021. A better and copacetic protocol for histopathological slide preparation using H&E stain: A review. Journal of Fisheries, Livestock and Veterinary Science, 02(01), pp. 57-67.

Vancouver
Hasan MI, Das, SK and Chowdhury, EH. A better and copacetic protocol for histopathological slide preparation using H&E stain: A review. Journal of Fisheries, Livestock and Veterinary Science. 2021 December 02(01):57-67.

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