⟨1285⟩ PREPARATION OF BIOLOGICAL SPECIMENS FOR HISTOLOGIC AND IMMUNOHISTOCHEMICAL ANALYSIS

INTRODUCTION

Histology and immunohistochemistry (IHC) are commonly used to visualize the cellular and biochemical constituents of tissues. Whether the attributes to be probed are detected based on chemical reactivities (histochemistry) or are detected using antibodies (IHC) or lectins with known specificities, the preparation of the specimen for staining is a critical phase of the analysis. Preparation typically involves (1) tissue fixation, (2) mounting the tissue in an embedding medium to permit sectioning that is sufficiently thin for the tissue to be viewed microscopically, and then (3) removal of any embedding medium before histochemical or IHC evaluation.

The purpose of this chapter is to identify factors that should be controlled to optimize the consistency of tissue staining results. The following sections address tissue fixation used to prevent degradation and to prepare the tissue for sectioning and staining. Since these methods are often used to characterize tissue-based therapeutic products and the adequacy of their process methods, USP sometimes includes visual tools to support those product monographs. USP Authentic Visual References (AVRs) are often histology images that have been prepared as described in this chapter and are used as reference standards associated with product monographs. The purpose of histologic analysis is to generate visual images that can be used either for illustration or as a visual reference. When used as AVRs, the image set should include representative images of both failed and passing samples at different magnifications. AVRs can be used to better clarify specifications and acceptance criteria related to, for example, cell content, collagen structure, or integrity.

BASIC PRINCIPLES

The sample tissue must be properly and adequately treated or fixed to limit changes to the extracellular matrix elements or to specific constituents such as cells or proteins. Tissue fixation typically employs a chemical that can rapidly permeate the tissue to effectively cross-link proteins and limit degradation caused by either chemical or enzymatic action. Standard fixation methods typically use 10% neutral buffered histological-grade formalin and are adequate for most, if not all, histochemical analyses. Formalin fixation provides material suitable for IHC analysis provided that proper attention is paid to postfixation treatments (collectively known as antigen retrieval). Once tissue is properly fixed, it can be embedded.

The tissue is embedded in a medium that is sufficiently stiff to maintain sample geometry and the desired orientation for sectioning but is soft enough to be easily and rapidly cut through without distortion. The most common medium used is paraffin, although it is not suitable for nondecalcified hard tissues and other stiff materials (e.g., hard polymers or metals). Because paraffin is not water soluble, tissue generally is passed through a graded series of increasing concentrations of ethanol and finally into xylene (or a suitable substitute) in which the paraffin wax is soluble. Sufficient time is allowed for the paraffin to permeate the tissue, and then the block is sectioned to the desired thickness. Hard tissues commonly are embedded in hard plastics such as poly(methyl methacrylate).

After sectioning, the samples are floated on warm water with or without gelatin so they can be transferred to microscope slides. If the sections are to be used for IHC, it is recommended that gelatin not be used in the water because it can contribute significantly to background staining. Positively charged slides also are recommended to aid in sample retention throughout the IHC processing regime. The sections are allowed to dry in air, in a slide warmer, or in an oven at no more than 50°. Because most histochemical and IHC procedures are carried out under aqueous conditions, the paraffin within the mounted tissue sections must be removed. This is accomplished by reversing the xylene and alcohol rinses until the samples are rinsed in water immediately before staining.

The mounted sections are now ready for histochemical staining. If an immunological staining method is to be used, in most cases an antigen retrieval step will be necessary to break the protein cross-links formed by formalin fixation. Many procedures are available to accomplish this, and although heat treatment in acid vapor appears to be effective in recovering the reactivity of most targets in tissues, the ultimate choice may depend on the characteristics of the target to be evaluated.

PROCEDURES—POINTS TO CONSIDER

Fixation

The most common fixatives are aldehydes, alcohols, and oxidizing agents. Each has advantages and disadvantages depending on the particular purpose (see *Table 1*).

Table 1. Attributes of Common Tissue Fixatives

Fixative Name	Mechanism	Advantages	Disadvantages	Notes
Formaldehyde (e.g., 10% neutral buffered formalin)	Cross-links protein	Minimal alteration of protein structure and antigenicity; good penetration	Buffered version is preferred to counteract oxidation of formaldehyde to formic acid, which tends to cause a brown artifact.	Unbuffered precipitates can form, and will be acidic (pH 3–4.6).

Table 1. Attributes of Common Tissue Fixatives *(continued)*

Fixative Name	Mechanism	Advantages	Disadvantages	Notes
Glutaraldehyde (e.g., 0.25%–4%)	Cross-links protein	Morphology suitable for electron microscopy	Deforms α-helix structure of protein, so not good for IHC; slow penetration	Use with 2–3 µm thick sections. Opens structures such as blood vessels to improve access to all tissue surfaces and to prevent opposing surfaces from bonding to each other
Alcohols (e.g., methanol or ethanol)	Denature proteins	Best applied to cytologic smears; good penetration	Can cause tissue hardness and brittleness, making sectioning difficult	
Permanganates, dichromates, or osmium tetroxide	Oxidizing agents that cross-link proteins	Fixation of cytoplasm without precipitation; fixation of lipids, especially phospholipids	Causes substantial denaturation; uncommon	
Picrates	Oxidizing agents that cross-link proteins	Good retention of nuclear structure with limited tissue hardening	Explosion hazard; causes cell shrinkage	

Tissue that is not appropriately fixed will not embed and section well, and subsequently will not stain well. Adequate fixation time is of primary importance in quality assurance. Fixatives usually are delivered at a 15:1 to 20:1 ratio of fixative volume to tissue mass. Alternatively, multiple changes of fixative can be used with agitation. It is important to expose tissues to fixatives for a sufficient time, making sure that the sample does not dry before or during this process. Even small tissue pieces may require 12–24 h of exposure time. Tissue handling also is important because areas that are physically gripped may not be fixed as rapidly as areas that are not gripped.

Typical concentrations for some fixatives are shown in *Table 1*. Note that if fixation concentrations are too high or if fixation time is too long, structural artifacts can form. The fixation temperature can be increased somewhat but not to the extent that will cause protein denaturation.

Buffers also are important for high-quality fixation. A pH range of 6–8 usually is best because it will most likely maintain the native tissue structure. Buffering must be sufficient to overcome acidification caused by hypoxia and to prevent black deposits of formalin–heme.

Tissue Processing

DEHYDRATION

Once the tissue is properly fixed, it is ready for dehydration and the embedding process. The most common dehydrants are alcohols, but sometimes acetone is used because it rapidly fixes the tissue (although it is a fire hazard). Tissues may be physically damaged if they are insufficiently dehydrated, often because of water contamination of ethanol wash solutions. After dehydration, the tissue is placed in a clearing agent before embedding. Some common clearing agents are the following: xylene, toluene, chloroform, methyl salicylate, limonene (a volatile oil found in citrus peels), or some commercial xylene substitutes.[1]

EMBEDDING

Depending on the tissue type and subsequent staining protocol, there are several embedding and mounting-media options. Paraffin is most commonly used to embed tissues. Its density is similar to that of most tissues, which facilitates sectioning, and multiple paraffin types with different melting points and hardness can be evaluated if tissue sections do not cut smoothly. If very thin sections are needed or if harder tissues (e.g., bone) will be embedded, plastics are good options (e.g., methacrylate, glycol methacrylate, araldite resin, or epon). Once the mounting medium is selected, the specimen should be carefully aligned and oriented in the medium relative to the desired cutting plane. Tissues that are properly fixed and embedded, if stored appropriately, can stay in this form indefinitely before staining.

Tissues can be embedded manually or with automated systems. Some embedding systems have a chamber for keeping molds and samples hot and ready for embedding. They also may have a separate chamber to melt paraffin, an attached dispenser for pouring paraffin into embedding molds, and a separate cold plate to cool the recently embedded tissues. Both manual and automated station methods are essentially the same and are well accepted as tissue embedding methods. The only difference is that the latter are semi-automated so that the system is operational within an hour or less (i.e., paraffin is melted, heating chambers are warmed, and the cold plate is cooled). Manual methods are somewhat slower. Regardless of the system used, the integrity of the histological section must not be compromised.

SECTIONING

When tissue blocks are ready for staining, sections are cut with a sharp knife or disposable blades. Paraffin-embedded tissue sections typically are 4–8 µm thick, but plastic-embedded tissues usually are 2 µm thick. The tissue sections are placed on slides and can be stored in this state. The slides are cleared again (to dissolve the paraffin) and are rehydrated before staining or IHC. In all cases analysts should be aware of the importance of each step in order to avoid producing artifacts (e.g., fixation-related shrinkage or nicks, folds, or tears in the tissue sections caused by improper microtome work or a knife that is too dull). Once the staining procedure is complete, analysts should apply sufficient mounting medium over the tissue sections in order to avoid entrapment of air bubbles when a cover slip is applied.

[1] For example, Clear Rite, Pro Par Clearant.

Example Protocol

The following is an example of a fixation, paraffin-embedding, and processing method that is commonly used before hematoxylin and eosin (H&E) staining to study the effectiveness of tissue decellularization processes.

Fixation solution: 0.1 M sodium phosphate, pH 7, containing 10% formalin

Paraffin: Melt paraffin at 60° for 3 h before analysis. [NOTE—For proper infiltration and success in later steps, it is best to keep the *Paraffin* fresh (free of clearing agent) with frequent changes of paraffin (at least two changes). The temperature of the *Paraffin* must be NMT 2°–4° above its melting point; otherwise, the tissues exposed to overheated *Paraffin* will overharden.]

Paraffin solution 1: *Paraffin* and xylene (1:3) or a xylene substitute[2]

Paraffin solution 2: *Paraffin* and xylene (1:1) or a xylene substitute

Paraffin solution 3: *Paraffin* and xylene (3:1) or a xylene substitute

Fixation and dehydration, clearing, and paraffin infiltration of the tissue (DCI): Place each tissue sample (NMT 1 cm^3) in *Fixation solution* for a minimum of 6–8 h. The volume of *Fixation solution* should be NLT 15–20 times the volume of the tissue sample. [NOTE—When processing bone samples, thoroughly fix before decalcification and DCI. In this case, after fixation, rinse the sample with Purified Water, and place in a rapid decalcifier solution[3] for the recommended time. After decalcification, rinse the bone samples with Purified Water again, and place them back in the *Fixation solution*.] Place each tissue sample in an embedding cassette, and label the cassette with a pencil or histology pen. Place cassettes in the following series of solutions for 30 min each: 2 changes of 70% ethanol, 2 changes of 95% ethanol, and 3 changes of 100% ethanol. [NOTE—An optional approach is to transfer cassettes directly from 100% ethanol to 100% xylene for 3 changes. Place cassettes in *Paraffin* heated to 2°–4° above its melting point for 1–3 changes for NLT 90 min and NMT 180 min. Exposure to prolonged heat causes shrinkage and hardening of tissues. Avoid overnight treatment.] Next, place cassettes in the following series of solutions for 60 min each at 60°: *Paraffin solution 1, Paraffin solution 2,* and *Paraffin solution 3*.

Tissue embedding: [NOTE—This example is a manual method, but an automated embedding method can be substituted.] Move the container with the cassettes described above from the incubator into a 60° water bath to prevent the infiltrated paraffin from solidifying during the embedding procedure. [NOTE—For easier release later from the embedding molds, molds can be sprayed with a diluted mold-release concentrate.] Place a small amount of *Paraffin* into a room-temperature base mold. Using forceps, remove a tissue-containing cassette from the liquid *Paraffin*, and position the tissue section with the desired orientation in the mold before the *Paraffin* solidifies. [NOTE—If it is not positioned properly, the tissue sample can be placed back into the tissue cassette containing the liquid *Paraffin,* and then a new mold can be prepared and tissue positioning can be attempted again.] Once the tissue is in place, place the labeled portion of the tissue cassette onto the base mold, and add *Paraffin* to a level just below the lip of the cassette. Place the cassette mold assembly onto a frozen cold plate. When the *Paraffin* begins to solidify on top, transfer the assembly to an ice bath until the entire block is solidified (about 20 min). Once the *Paraffin* is solidified, carefully separate the base mold from embedded tissue without using excessive force. Store blocks at 4° until needed for sectioning. Blocks can be sectioned immediately, but it is advisable to wait at least 24 h for best results.

Sectioning paraffin-embedded tissue: Using an appropriate marker, label glass slides with a tissue-sample identifier. Select an embedded tissue block, and trim any excess wax remaining around the edges of the block. Fill a flotation bath with Purified Water, with or without gelatin (histology samples only), and warm to 36°–46°. Carefully place a new microtome blade into the blade holder. Ensure that the blade is locked into place. If available, select the manual or motorized mode of the microtome. Place the tissue block in the microtome cassette holder, and adjust its orientation using the adjusting screws until the block is correctly positioned vertically and horizontally in relation to the blade. Select the *trim* setting. [NOTE—The trim cut usually is set to 15–16 µm, but this can be increased if a large amount of trimming is necessary, or can be decreased if only a small amount of tissue is present in the block. In addition, blocks can be refrigerated or placed on ice before sectioning to keep them cool and firm.] Advance the block forward to the desired position. Once the block is positioned, start trimming or "facing" the block. After the tissue is visible in the sections, set an appropriate sectioning depth and begin collecting tissue sections. [NOTE—Routine H&E sections should be cut at approximately 6 µm, but the section thickness can be adjusted between 4 and 8 µm as necessary if there is difficulty obtaining good-quality sections.] Using forceps, transfer the sections to the tissue flotation bath. Gently place the ribbon of sections onto the water surface while gently pulling to eliminate any wrinkles from the ribbon. [NOTE—If the water in the flotation bath becomes too hot, the tissue sections will fragment. If this occurs, decrease the heat setting on the bath, and allow the water to cool before proceeding.] Next, separate one or two adjacent, intact, full sections from the ribbon with a sharp dissecting probe or forceps. Using a glass slide placed at approximately a 45° angle beneath the section, lift the sections onto the slide. Gently tap off the excess water, and allow the sample to dry. As needed, repeat the sectioning process for additional samples. After all slides have been prepared and before staining, allow the slides to air-dry for NLT 15 min or until no water droplets are visible. Remove the wax blocks from the microtome, and store appropriately.

Deparaffinization and rehydration of histological slides: Before staining, place slides with mounted tissue specimens in either Coplin jars, reagent buckets, or staining racks. Place slides in the following solutions for at least 3 min each: 3 changes of xylene (or a xylene substitute), then 100% ethanol, then 95% ethanol, then 70% ethanol, then 2 changes of Purified Water. Proceed with the desired staining procedure. [NOTE—Slides can be left in the water for several hours but then must be stained.]

[2] For example, CitriSolv, Clear Rite 3, Pro Par Clearant, or equivalent.
[3] For example, Decalcifying Solution, Richard-Allen.

METHOD DEVELOPMENT AND VALIDATION: POINTS TO CONSIDER

Histological methods sometimes are used to demonstrate that cells within a tissue sample are still viable (e.g., when evaluating a cryopreservation method's performance). In other circumstances, the method is used to evaluate the absence of viable cells following a decellularization process. Each case presents specific validation challenges.

H&E staining provides a useful tool for determining whether significant quantities of cells are still viable after preservation. During method validation, immunohistological stains can be used to ensure that specific cell types are still present in the preserved samples. Testing samples for all expected cell types during the validation phase provides support for the less specific H&E staining (that will stain all cells and not just specific cell types) during routine monitoring.

When validating any histological method, analysts should demonstrate that different observers can detect the same quality (or quantity) of an object, tissue type, cell body, nucleus, etc., and that the characterization by different observers can be replicated in several specimens following the same process. Samples for evaluation may consist of multiple types of cells, extracellular matrix proteins, and glycosaminoglycans, or other components. Some of these components may be resistant to decellularization processes or may be difficult to stain. During assessment of method reproducibility, analysts should evaluate multiple samples throughout the sample to ensure that they are representative of the types of materials expected to be found in the matrix. In addition, because inadequate decellularization procedures may not remove all cells throughout the material, it is important to take samples from multiple locations in the tissue so that remaining cells are not underestimated. Intra-operator reproducibility also should be a part of the assessment because the relatively weaker signal from fewer positively stained cells may make visualization more challenging for some technicians. The method validation should use samples that underwent worst-case decellularization processing conditions as determined by statistical modeling to ensure that residual cells can still be identified and quantified in these marginal samples. Finally, the validation of the method should include control samples to confirm the suitability of the staining system (see *Hematoxylin and Eosin Staining of Sectioned Tissue for Microscopic Examination* ⟨1285.1⟩).

The histological assessment of decellularized tissue-based matrix products is one tool to characterize the matrix but cannot be the sole indicator of product quality. On the one hand, processing may generate decellularized matrix products that have no detectable cells but may not possess other critical quality attributes. On the other hand, tissue matrix products undergoing other decellularization processes may result in products with greatly reduced cellular content (although some cells may remain) that retain the necessary performance characteristics for that therapeutic purpose.

Sample Selection for the Analysis of a Decellularization Process

Samples cannot always be taken from a random location within the product. For example, it is not practical to pull a sample from the center of a sheet of decellularized pericardium without creating a defect that would make the end product undesirable. If routine sampling plans require sampling from the edges or waste portion of a given matrix, analysts should conduct an initial validation study that confirms that such a sample is representative of the whole. This is important because the geometry of the matrix may affect the exposure of the matrix to the decellularization processing solutions. For example, if the tissue matrix is tubular, e.g., a nerve segment, and a sample is taken from the end for histological assessment, then this sample will have different exposure to solutions than a sample taken from the center of the nerve segment. Thus, the sampling may not represent a worst-case assessment, and the suitability of the sample should be determined by means of a validation study.

CONCLUSIONS

This chapter outlines common steps to prepare tissue or organ specimens for light microscopic analysis following either staining or treatment with structure-specific probes. Each of these steps can produce specimen artifacts that either interfere with the observation or actually introduce structures that were not present in the original sample (e.g., certain well-known chemical artifacts). Therefore, proper attention and consistent execution of each step are critical factors in generating sections that reflect the character of the tissue and that stain in a reproducible manner.