

Cells, Tissues & Organs: Methods of Study

1

LEARNING OBJECTIVES OUTLINE

I. HISTOLOGY [Gr. *histos* =web, tissue + *logos* =study of].

- A. **Definition:** branch of science concerned with the microscopic structure of cells, tissues, and organs in relation to their function.
- B. **Synonyms:** microanatomy, microscopic anatomy; cell & tissue biology

II. TISSUE [Fr. *tissu* =fabric, texture, weave]

- A. **Definition:** *societies of cells* and their associated ***extracellular substances (ECM)***, which are specialized to carry out specific functions.
- B. **Four Primary Tissues of the Body**
 - 1. Epithelium
 - 2. Connective Tissue
 - 3. Muscle
 - 4. Nervous Tissue

III. TISSUE PREPARATION / PROCESSING

- A. **Fixation**
 - 1. Immersion
 - 2. Perfusion
- B. **Dehydration***
- C. **"Clearing"***
- D. **Infiltrating & Embedding***
 - 1. Paraffin
 - 2. Plastic
 - 3. Others
- E. **Sectioning**
 - 1. Microtomy (microtome)
 - 2. Cryomicrotomy (cryostat)
- F. **Mounting***
- G. **Staining & Histochemistry**
- H. **Coverslipping***
- I. **Special procedures**
 - 1. Whole mounts and smears
 - 2. Mineralized tissues (bones, teeth)
 - a. Ground sections
 - b. Decalcification
- J. **Artifacts**
 - 1. Definition: artificial structures often introduced during preparation

- 2. Examples
 - a. Folds
 - b. Debris
 - c. Cracks
 - d. Knife marks
 - e. Shrinkage

IV. STAINING & HISTOCHEMISTRY

A. **Dye-cellular interactions**

- 1. Basophilia and acidophilia
- 2. Basic dyes and acid dyes

B. **Important stains and reactions**

- 1. Cationic ("basic") Dyes: *tissue components stained with these dyes are basophilic.*
 - a. Examples of basic dyes
 - i. Hematoxylin (behave as cationic dyes)
 - ii. Azures
 - iii. Methylene blue
 - iv. Toluidine blue
 - b. Examples of basophilic tissue components
 - i. Nuclei and nucleoli
 - ii. Cytoplasmic RNA (e.g., ergastoplasm, Nissl)
- 2. Anionic ("acid") Dyes: *tissue components stained with these dyes are acidophilic.*
 - a. Examples of acid dyes
 - i. Aniline blue: blue
 - ii. Eosin: pink-red
 - iii. Fast green: green
 - iv. Orange G: orange
 - v. Picric acid: yellow
 - b. Examples of acidophilic tissue components
 - i. Most cytoplasm
 - ii. Hemoglobin
 - iii. Keratin
 - iv. Collagens
- 3. Special Stains and Reactions
 - a. Alcian blue (=basic dye) for polyanions and acidic glycoproteins (***alcianophilia***)

- b. Elastic stains (e.g., orcein, resorcin fuchsin, Verhoeff)
- c. Feulgen reaction for DNA
- d. Lipid colorants/stains (e.g., Sudan black, oil red O, osmium tetroxide)
- e. Masson trichrome for differential staining of several tissues
- f. Metachromasia (e.g., toluidine blue for polyanions)
- g. PAS reaction for *vic*-glycols
- h. Silver stains for Golgi, basement membranes, reticular fibers, neurofibrils (***argyrophilia***)
- i. Vital stains (e.g., trypan blue, India ink)
- j. Oxidation-reduction reactions and staining
- k. Romanowsky dyes: mixtures of acid and basic dyes for staining blood smears
 - i. Mechanism
 - Orthochromasia
 - Polychromasia
 - Metachromasia
 - ii. Examples
 - Wright stain
 - Giemsa stain

C. **Labeling using special probes**

1. Immunohisto(cyto)chemistry
2. Radioautography
3. BrdU (Bromodeoxyuridine) Detection
4. *In situ* hybridization
 - a. Isotopic
 - b. Nonisotopic
 - i. FISH
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OVERVIEW

Histology [Gr. *histos* =web, tissue + *logos* =study of] is the study of the structure and function of cells, tissues, and organs of the body at the microscopic level. Hence, it is often referred to as **Cell, Tissue & Organ Biology**. In the past, it has also been called microscopic anatomy.

Tissues [Fr. *tissu* =fabric, texture, weave; from L. *texo* =to weave] are best defined as **societies of cells** and their associated **extracellular substances/matrix (ECM)**, which are specialized to carry out particular functions. Four primary tissues or cellular societies are defined: epithelium, connective tissue, muscle, and nervous tissue. Organs are composites of these primary tissues and

based upon their composite functions are arranged into systems: nervous, integument, cardiovascular, lymphoid, alimentary, respiratory, urinary, endocrine glands, and genital systems.

Microscopic examination. In order to visualize these structures, representative samples are specially prepared, which typically involves preservation and subsequent coloration (staining) prior to microscopic examination (see Fig 1-1 and Table 1-1). Stains, which in a sense can be considered to be a category of histologic **probes***, are commonly used in histology to make the structure of tissues more easily visualized than in unstained preparations, *and* to reveal chemical and physical differences in the various cellular and tissue components. The majority of biological stains are synthetic organic dyes, often borrowed or modified from the textile industry. An older group of stains consists of dyes obtained from natural sources (e.g., **hematoxylin**, from the bark of Logwood trees; **carmine**, from the bodies of certain insects). Natural dyes were used almost exclusively until the synthetic dyes were developed in the mid-19th century. In addition, various metals are used alone or in combination with certain dyes to enhance their action. For example, aluminum, iron, or tungsten are used in combination with oxidation products of hematoxylin to increase the effectiveness of staining specific cellular structures. On the other hand, **silver** and **osmium tetroxide**, which have an innate affinity for certain cell and tissue components, can be used as stains in their own right. Although several thousand stains and other procedures for coloring cells and tissues are in usage, some of the most common are summarized in Table 1-1.

*In medicine, a **probe** [L. *probo* =to test] is an instrument used to explore structures of the body (e.g., cavities, sinuses, wounds). In cell and molecular biology, probes are usually defined as sequence fragments of DNA or RNA, which have been labeled (radioactively or chemically) in order to hybridize with, and therefore locate, complementary nucleic acid sequences of interest. In histology and histochemistry, the term *probe* is also used to define an agent/reagent that is employed to explore, detect, or test for the presence of particular substances located within cells and tissues. Visualization at microscopic levels (LM or EM) results from coupling the probes to colored or electron-opaque reaction products. In addition to *in situ* hybridization (see below), where unique nucleotide sequences are used to probe tissue sections for complementary fragments of DNA or RNA, other examples include immunohistochemistry (see below), where polyclonal or monoclonal antibodies are used to probe the location of specific antigens. Although generally much less specific, histologic stains can also be viewed in a general sense to probe the location of certain cellular structures based upon their molecular chemistry.

TISSUE PREPARATION & PROCESSING

A large number of technical steps must be carried out in order to achieve optimally prepared specimens for microscopic examination. Unfortunately, at each of these steps the introduction of artificial structures, which are commonly known as histologic **artifacts**, can be introduced by the processing or processor. These detractors from normal tissue architecture can be many and include tissue folds; accumulation of external dirt or other debris; cracks in cells and tissues; marks, chatter or gouges in tissues that are introduced by cutting implements; and even such subtle changes as tissue shrinkage. Learning to recognize these artifacts is usually easy and straightforward, but some may present a challenge.

Due to space and time limitations, only a brief overview of tissue preparation and processing will be presented here. For those who are interested in more detailed descriptions, the excellent resources cited in the Staining & Histochemistry section of the General References should be consulted.

Fixation

1. Definition and mechanism of action.

Fixation is defined as the rapid preservation of tissue components in order to arrest cellular processes and to maintain, as close as possible, a resemblance to the living condition. In histology, a **fixative** is typically a chemical agent, which may consist of a simple solution (e.g., 10% formalin: formaldehyde dissolved in an aqueous or buffered medium) or more complex solutions (e.g., Bouin fixative: picric acid, acetic acid, formalin). The end result is a cross-linking and denaturation of tissue components, particularly proteins. Equally important, fixation also resists tissue degradation by endogenous (enzymatic autolysis) or exogenous (bacterial action) mechanisms. While many fixatives are aqueous, some are also alcohol-based.

2. Fixation procedures are generally of two different types: immersion and perfusion.

a. Immersion fixation, which is the most routinely used method, is performed by placing small pieces of tissue into a relatively large volume of fixative. This ratio is chosen to facilitate an interaction between the two that may result is a rapid and complete fixation as this method will allow. The rate of diffusion, which is dependent on the properties of both the fixative and tissue type, is obviously a limiting factor for optimal preservation.

b. Perfusion fixation, which is much more complex and often difficult to perform, depends on the introduction of fixative into tissues via the vasculature. Nevertheless, this method has the greatest potential for rapid and fine preservation of histologic architecture.

Dehydration and clearing. Since histologic techniques are either performed in an aqueous or organic medium, both dehydration and rehydration sequences must be used to move tissue specimens between phases. If a fixative is aqueous and the embedment is water insoluble (e.g., paraffin wax), then a dehydration sequence is employed to gradually introduce an organic phase that will be compatible with the embedding medium. Typically, this sequence is a graded series of ethanols (e.g., 50, 70, 95, 100%). Once absolute ethanol is achieved, the tissue is placed in an intermediate organic solvent that is miscible with both the alcohol and the wax (e.g., xylene, toluene, benzene, or other less toxic synthetics). Since opaque tissue specimens became clear when placed in some of these agents, they were collectively referred to as “clearing agents” or “clearers”, even though not all may result in tissue “clearing”.

Infiltration and embedding. In order for the paraffin wax to mix with and replace the clearing agent within the tissue specimen, the wax is heated to its melting temperature. The specimen is then placed in several changes of molten wax, often under vacuum, in order to replace the clearing agent and completely infiltrate the tissue with the wax embedding medium. Since the paraffin melting temperature is significantly elevated over room and body temperatures, various artifacts (e.g., shrinkage) are introduced by this method. When infiltration is complete, the tissue is embedded in fresh wax, which is then allowed to harden at reduced temperatures within a special embedding mold.

Sectioning and mounting. Embedded tissue is removed from its mold, trimmed, mounted and secured on a cutting instrument called the **microtome**. This device typically advances the tissue in micrometer increments across a cutting knife so that thin slices of tissue (typically 2-10 μm in thickness) are obtained. These **tissue sections** are then mounted on glass slides for subsequent coloration or other processing.

Staining and histochemistry are procedures for visualizing tissue components by means of various coloring techniques. These procedures take advantage of the intra- and extracellular chemistry

of the tissues. Details of some important methods are presented in the section on Dye-Cellular Interactions below.

Coverslipping. Following staining procedures, tissue sections affixed to slides are typically covered by a thin glass disk or plate (square or rectangle) called a **coverslip** or **cover glass**. These coverings not only protect tissue specimens from the environment, but they also facilitate microscopic examination, micrography, and other manipulations.

DYE-CELLULAR INTERACTIONS

Basophilia and acidophilia.

Proteins are the substances that contribute in large measure to the stainability of tissue sections. Since proteins are **amphoteric**, they possess both acidic and basic groups. At the **isoelectric point (pI;** see Fig. 1), the net charge is zero and minimal dye binding occurs. If staining takes place above the isoelectric point of a given protein, its acidic groups (phosphate, carboxyl, or sulfate) will be negatively charged (Fig. 1) and can combine with a positively charged **cationic dye** such as methylene blue.

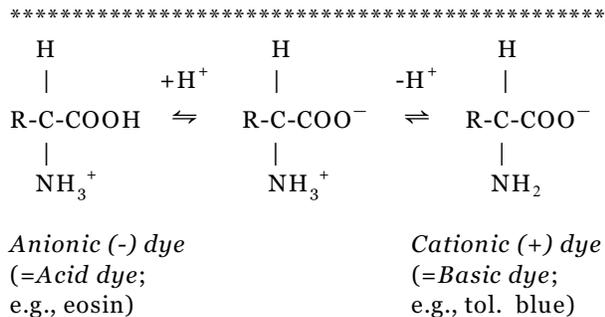


Fig. 1. Schematic representation of dye-protein interactions. At **pI** of protein (center), negligible binding of charged dyes occurs. Below **pI** (left), protein binds acid (=anionic) dyes; above **pI** (right), protein binds basic (=cationic) dyes. At physiologic pH, specific proteins may exhibit net (+) or net (-) charges and are therefore characterized as acidophilic (=affinity for acid dyes) or basophilic (=affinity for basic dyes), respectively.

Since the dye is forming a salt linkage with the residue of an acidic group, it is termed a **basic dye** and the substance stained is termed **basophilic** (base-loving). Conversely, basic groups (primarily amino) are positively charged (Fig. 1) so that at a pH below their isoelectric point they will bind **anionic dyes** such as eosin. Such dyes are therefore called **acid dyes** and the substance stained is termed

acidophilic (acid-loving), or **eosinophilic** in the case of cell components that stain with eosin.

Basophilic substances are of considerable biological importance. The phosphate grouping is abundant in nucleic acids (in both ribose and deoxyribose forms). Thus, nuclei, nucleoli, and ribosomes (free and ER-bound) are basophilic. Negatively charged carboxyl and sulfate groups are found abundantly in *glycosaminoglycans* (GAG's), which are linear sugar polymers that are associated with core and linkage proteins to form complex branched *proteoglycans*. The weakest binding (weakest basophilia) is by phosphates; the strongest is by sulfates. Likewise, a number of acidophilic substances (positively charged substances) are also important: examples include collagen, keratin, hemoglobin, and muscle proteins.

Basic dyes and acid dyes

1. Synthetic dyes. The commonly used aniline dyes are neutral salts. Thus, the colored portion, upon dissociation, may be positively or negatively charged, depending upon the dye. **Methylene blue** and **toluidine blue** exist as chlorides that dissociate in solution to yield a positive charge on the color bearing portion. Thus, these dyes are cationic (basic) dyes. **Eosin** occurs as the sodium salt, and the colored moiety is negatively charged upon dissociation. Thus, eosin is an anionic (acid) dye. Other commonly used anionic dyes are **aniline blue**, **orange G**, **picric acid**, and **acid fuchsin**. (See Table 1-1 for summary of important basic and acid dyes).

2. Natural dyes. Certain stains are obtained from natural sources and differ from the synthetic dyes in their general properties. Their chemistry is not as well understood as that of the synthetics. Perhaps the most important natural dye is **hematoxylin**. Hematoxylin is used as a basic dye but actually is not a dye as such, and must be oxidized ("ripened") to hematein to be a stain. Furthermore, the hematein component of this complex mixture is not a basic dye at all. Rather a mordant or "go-between" of a metallic nature must be used with hematoxylin in order for this mixture to behave as a basic dye. [N.B.: Remember that hematoxylin per se is not a "basic" dye and only stains (basophilic) tissues due to the affinity of its hematein component for metals.] Nevertheless, despite this caveat, hematoxylin is commonly referred to as a basic dye because the cell and tissue structures that are staining are basophilic. The familiar **H&E** preparation employs eosin as a

counter stain. Eosin stains those structures that are positively charged and have no affinity for a metal mordant; recall that such structures are said to be "eosinophilic" or "acidophilic".

Metachromasia. An interesting property exhibited by certain dyes (usually basic varieties; e.g., toluidine blue) is called **metachromasia**. This phenomenon is described as the ability of a dye to change from the **orthochromatic** or usual color (blue in the case of toluidine blue) to a **metachromatic** color (purple in this case) in the presence of highly acidic (polyanionic) substances. This chromatic shift is explained as an effect on dye color due to interactions between the closely adjacent, abundant dye molecules bound by the strongly acidic molecules. In dilute solution, toluidine blue is monomeric and blue in color. In concentrated solution, dye molecules polymerize and thereby exhibit the metachromatic color shift. Thus, the effect of polyanions, due to their high negative charge density, is to functionally polymerize the toluidine blue molecules in tissues. As a result, the metachromatic staining reaction is a great predictor of tissue polyanions. Important examples of cellular structures and tissues that exhibit this property are mast cell granules containing heparin and proteoglycan, and cartilage ECM containing chondroitin sulfate proteoglycans. Other basic dyes that exhibit metachromasia are methylene blue, thionin, and the azures. Metachromatic staining with the acid dye Biebrich scarlet has also been reported (Spicer, *SS Exp Cell Res* 28:480, 1962).

Romanowsky dyes.

1. Background. The Russian physician Dimitri Romanowsky (1861-1921) was apparently the first investigator to combine eosin and methylene blue and use this mixture for staining blood in his studies on malarial parasites (Romanowsky, *DL Imp Med Mil Acad, Dissert No 38, St Petersburg*, 1891). Thus, present day mixtures of acid (eosin) and basic (methylene blue, azures, etc) dyes that are applied simultaneously are referred to as modified **Romanowsky stains**. In these procedures, the pH must be carefully controlled at or near neutrality.

2. Usage and examples. These mixtures are most commonly used for blood or bone marrow smears and produce elegant differential staining of the leukocytes (white blood cells). These stains are also exquisite indicators of cytoplasmic basophilia. **Wright stain** and **Giemsa stain** are common examples of Romanowsky dye mixtures.

3. Properties. Chemically, these mixtures have been characterized as **eosinates of methylene blue** and/or **azure** derivatives of methylene blue. Although Romanowsky appreciated that the combined stain had properties different from the stains used alone, the true nature of the mixture and its mechanism of action would not be fully understood until almost one hundred years later.

4. Mechanism. The staining mechanism has been elucidated to include 3 fundamental processes (see Horobin, RW & Walter KJ *Histochemistry* 86:331-336, 1987): **1) orthochromasia** (cellular components stained either pink/red due to eosin or blue due to basic dye component), **2) polychromasia** (layered staining with both dyes), and **3) metachromasia** (color shift of basic dye from blue to violet-purple due to high concentrations of polyanions). See Table 1-1 for examples of blood smear components that are orthochromatic (O), polychromatic (P), and metachromatic (M). Additional information is presented in Chapter 4 on Blood.

Staining due to physical properties of dyes

1. Vital staining. Vital stains are employed to color certain components within living organisms without doing harm to the vital tissues. For example, **trypan blue** and **India ink** form colloidal suspensions in water. Since these large particulates will not diffuse into cells, they will be cleared from the body by the *phagocytic activity* of macrophages, and are thus excellent markers for members of the **mononuclear phagocyte system** (MPS). **Supravital staining** is a variant of this procedure in which these dyes are applied to living tissues that have first been removed from the organism by, for example, biopsy.

2. Lipid staining. Lipid colorants such as **Sudan black** and **Oil Red O** are soluble in lipid and are used to demonstrate fat in tissues if the lipid has not been extracted; usually these procedures are performed on frozen sections.

Schiff reaction. Some dyes possess other chemical characteristics that are useful in revealing certain cell and tissue constituents. One of the more useful dyes in this regard is **basic fuchsin**, a red dye in solution that becomes colorless when reduced by the addition of acid and an excess of SO₂. The German chemist Hugo Schiff (1834-1915) was the first to recognize that this colorless solution could be recolored to bright magenta by the addition of aldehydes (Schiff, H *Justus Liebigs Ann Chem*

140:92-137, 1866). Thus this solution, which is now called the **Schiff reagent**, is widely used in organic chemistry and histochemistry to detect aldehydes by forming the stable red-magenta reaction product. In histology and pathology, this reaction has not only been utilized in a number of histochemical techniques, but has been of particular significance in studies involving the localization of DNA and complex carbohydrates.

1. Feulgen reaction. An important example of the use of the Schiff reagent is in the **Feulgen** technique for demonstration of deoxyribonucleic acid (DNA). Also known as the *nucleal reaction*, this technique was developed by the German biochemist Robert Feulgen (Feulgen, *R Z Physiol Chem* 92:154-158, 1914; Feulgen, R & Rossenbeck, *H Z Physiol Chem* 135:203-248, 1924). In this two-step procedure, DNA is first hydrolyzed in weak HCl to yield aldehydes, and then the Schiff reagent is used to detect the resulting aldehyde groups in the nucleus. Since this technique is specific for DNA and not RNA, it is an important procedure for discriminating between the two at the cell and tissue level. The specificity of this reaction results from the first step. Since this reaction "is both specific and stoichiometric for DNA, it has become the most important means of staining nuclear DNA for densitometric quantification" (Hardie, DC et al *J Histochem Cytochem* 50:735-749, 2002).

2. Periodic acid-Schiff (PAS). This technique, which is based on the independent work of several investigators (Bauer, *H Z Mikrosk Anat Forsch* 33:143-160, 1933; Lillie, *RD J Lab Clin Med* 32:910-912, 1947; McManus, *JFA Nature* 158:202, 1946; Hotchkiss, *RD Archiv Biochem* 16:131-141, 1948), is widely used for the demonstration of *vicinal*-glycol moieties in carbohydrates. In this procedure, the tissue is first treated with periodic acid. The 1-2 glycol linkages, if present, are then converted (oxidized) to aldehyde groups, which subsequently combine with the clear Schiff reagent to form the colored (magenta) reaction product. Thus, this reaction is also a two-step procedure. *The specificity results from the periodic acid step.*

Metals. Several metals are used in histologic techniques.

1. Silver impregnation methods

are employed to demonstrate the Golgi apparatus, reticular fibers, neurofibrils and some other structures. The results of these "stains" depend on the method used.

2. Osmic acid (osmium tetroxide) is useful for the demonstration of certain lipids because

unsaturated fatty acids reduce it to a black compound.

Osmic acid is often used to demonstrate the Golgi apparatus. The reaction depends on a lengthy impregnation procedure. Osmium is also used as a fixative/stain for electron microscopy due to its ability to preserve membrane lipids and also to give them some contrast.

3. Gold chloride is used in neurology to demonstrate nerve endings and astrocytes; it has also been used to demonstrate Langerhans cells in the skin (Ferreira-Marques, *J Arch Dermatol Syphilol* 193:191-250, 1951; Breathnach, *AS Int Rev Cytol* 18:1-27, 1965).

Special stains.

Whereas the most commonly used staining combination is undoubtedly **H&E** (it is perhaps used more than all other staining combinations put together), many other staining combinations have been employed to emphasize specific tissue characteristics.

1. Connective tissue stains. A wide variety of special stains to enhance connective tissue elements have been developed and consist of a battery of several different colored/shaded acid and basic dyes; e.g., Masson trichrome (Masson, *P Am J Pathol* 4:181-211, 1928), Mallory triple (Mallory, *FB Pathological Technique* WB Saunders, Philadelphia, 1938), Movat pentachrome (Movat, *HZ Archiv Pathol* 60:289-295, 1955). Several images in the database collection exhibit examples of these "polychromes". Although the chemistry of dye binding in many of these techniques is obscure (in some, for example, there are three dyes that are all acid dyes), they are excellent in distinguishing important elements. For example, it is sometimes difficult to distinguish cytoplasm of cells amidst extracellular collagen after H&E and, conversely, to distinguish collagen from cellular material such as in nerves and tendons. The connective tissue stains distinguish these differences clearly, no matter which one of several stains is used. A common stain of this group is the **Masson** procedure. The identifying feature when seeing such a stain is that collagen fibers of connective tissue are stained either blue (aniline blue) or green (fast green). Cytoplasm, in contrast, will be red-orange to red-lilac.

2. Eosin-methylene blue (eosin-azure). Although not as common, several examples of these mixtures are presented in the database collections. Dilute solutions of a basic stain (methylene blue or azure) and an acid stain (eosin) are used in a buffer, so that cytoplasmic basophilia is much more evident than with H&E. This combination yields similar

results to those of the stains for blood smears (Wright or Giemsa). However, here the techniques are adapted to sectioned material rather than for smears.

Special probes.

Labeling components of cells and tissues using special probes for enhanced specificity are valuable tools with important applications for biomedical research and clinical medicine.

1. Immunocyto(histo)chemistry. Histochemistry (cytochemistry) encompasses a very large family of valuable techniques that yield much greater detail than normal staining methods when applied to the localization and distribution of the component parts of cells and tissues. Immunocytochemistry (ICC; IHC), which uses the principles of immunology to locate and identify specific antigens with labeled antibodies, was recognized as having even greater power than traditional histochemical reactions. The first studies used a fluorescent dye as the label or **chromogen** [Gr. *chroma* =color; *gen* =suffix denoting "precursor of"], which was coupled to antibodies in order to identify a certain antigen in tissue sections (Coons, AH et al. *Proc Soc Exp Biol Med* 47:200-202, 1941).

a. Methods. Although there are now many variants on IHC (direct vs indirect; labeled vs unlabeled antibody; enzyme-conjugated vs fluorescent; etc), the basic theme involves the following steps: 1) producing antibodies to specific antigens; 2) using these antibodies to localize antigens of interest in cells and tissues; 3) coupling the antibody-antigen complexes with chromogens in order to form conjugates in tissue sections; 4) developing and/or detecting the chromogens in order to visualize the existence and location of the desired antigen. To amplify chromogen signals (increase sensitivity) in these procedures, it is now common to use a primary antibody for the antigen of interest and either secondary or tertiary antibody-chromogen conjugates, which bind to the primary antibody-antigen complex or a secondary bridge (link) antibody, respectively. Other high affinity bridge techniques (e.g., avidin-biotin) are also used.

b. Applications. In addition to the great advances in detection, localization, and life histories of specific markers either in or on cells in health and disease, virtually every aspect of biomedical research and diagnostic medicine has benefitted from these techniques. For example, in 2000 it was estimated that almost every diagnostic pathology laboratory routinely uses more than 100 antibodies for the

detection of specific antigens by these methods (Coleman R *Acta Histochem* 102:5-14, 2000).

2. Radioautography (autoradiography*) has been an important technical method for localizing radiolabeled substances and studying dynamic events (e.g., secretion pathways, cell turnover) in tissue sections. It was developed more than 50 years ago for light microscopy (Belanger LF & Leblond CP *Endocrinology* 39:386-400, 1946) and subsequently adapted for electron microscopy (Saltpeter M *J Cell Biol* 32:379-389, 1967). Although these applications have made significant contributions to understanding fundamental biologic processes, their use has declined somewhat in recent years partly due to concerns and regulations about radioactive isotopes, and also due to the development of some alternative methods (e.g., BrdU labeling; see below).

a. Methods. "Precursor" molecules (e.g., nucleotides, amino acids, sugars) for larger "products" (nucleic acids, proteins, complex carbohydrates) or certain finished "products" (e.g., hormones) are labeled with a radioactive isotope (¹⁴C, ³H, ³⁵S, ¹²⁵I). Radiolabeled molecules [e.g., tritiated thymidine for labeling DNA (Taylor JH et al *Proc Natl Acad Sci* 43:122-128, 1957)] are injected into the body and tissue samples are subsequently removed at various time intervals (in order to observe dynamic activity over time). Tissue sections, which have incorporated the labeled molecules, can be prepared for either light or electron microscopy. They are then covered with a photographic (silver halide) emulsion and placed in light-proof containers. The emulsion, bombarded by the radioactive emissions, becomes "exposed" while stored in the dark. Development of the emulsion (as in photography) results in the appearance of black silver grains, which are now localized over the sites of incorporated label. Whereas film exposure in photography is a function of shutter speed and aperture settings, optimal "exposure" in radioautography is achieved by keeping a series of specimens in the dark for various time intervals before development.

*Charles Leblond has written "The procedure used for the detection of radioactive elements in histological sections is designated by the term 'radioautography' rather than 'autoradiography'. The latter is a compound word including the term 'radiography' which denotes procedures in which the object under study is located between the source of radiation and the photographic emulsion. Thus, in the clinical use of radiography, X-rays are directed through human tissues before reaching the emulsion. Hence, the structure under study stops the rays; its image is then a photographic *negative*. (Fifty years ago, it was routine in hospitals to print positives from clinical radiographs;

and, as a result, the structures under study, bones for instance, appeared black. Today, physicians have been trained to work with negatives, in which the image of bones and other structures appears white).

When radioactive elements are detected in histological sections the object under investigation is itself the source of the radiation which influences the emulsion. A black image is then produced, which is a photographic *positive*. Such procedure may be referred to as "autography", that is, according to the Oxford English dictionary (1975), "the reproduction of form or outline of anything by an impression from the thing itself". In the initial work of our group with radioiodine and radiophosphorus 34 years ago, the procedure was called "radioactive autography", two words which were later condensed into "radioautography". Even though the term "autoradiography" is the more popular, the term "radioautography" is the more correct of the two" (Leblond CP *Am J Anat* 160:113-158, 1981).

b. Applications. Radioautography has played important roles in contributing to our understanding of many fundamental and dynamic processes of cells and tissues including DNA synthesis and cell division, protein synthesis and secretion, function of the Golgi apparatus, mechanisms of exocytosis and endocytosis, and hormone binding to receptors and target cells.

3. Bromodeoxyuridine detection. Cell proliferation has been studied in many tissues by a variety of techniques such as counting mitotic figures and radioautography following [³H]thymidine incorporation into DNA as mentioned above. In 1982, Gratzner et al. described a sensitive, nonisotopic, monoclonal antibody method for detecting DNA replication in single cells (Gratzner HG *Science* 218:474-475, 1982).

a. Methods. In this immunohistochemical procedure, the thymidine analog 5-bromodeoxyuridine (BrdU) - which is incorporated into nuclear DNA during S-phase before mitosis - is localized in tissue sections and reveals cells that have recently undergone cell division. Although first described using an immunofluorescent chromogen, BrdU IHC like other IHC procedures can use a variety of chromogens.

b. Applications. This technique is a sensitive method that is widely used for detecting DNA replication in vitro and in situ.

4. In situ hybridization. It has long been recognized that double-stranded DNA in solution can be denatured ("melted") into single strands by heat or elevation of pH. Reversal of these manipulations allows the single strands to be recombined ("annealed"). This process of molecular **hybridization** is highly specific since only

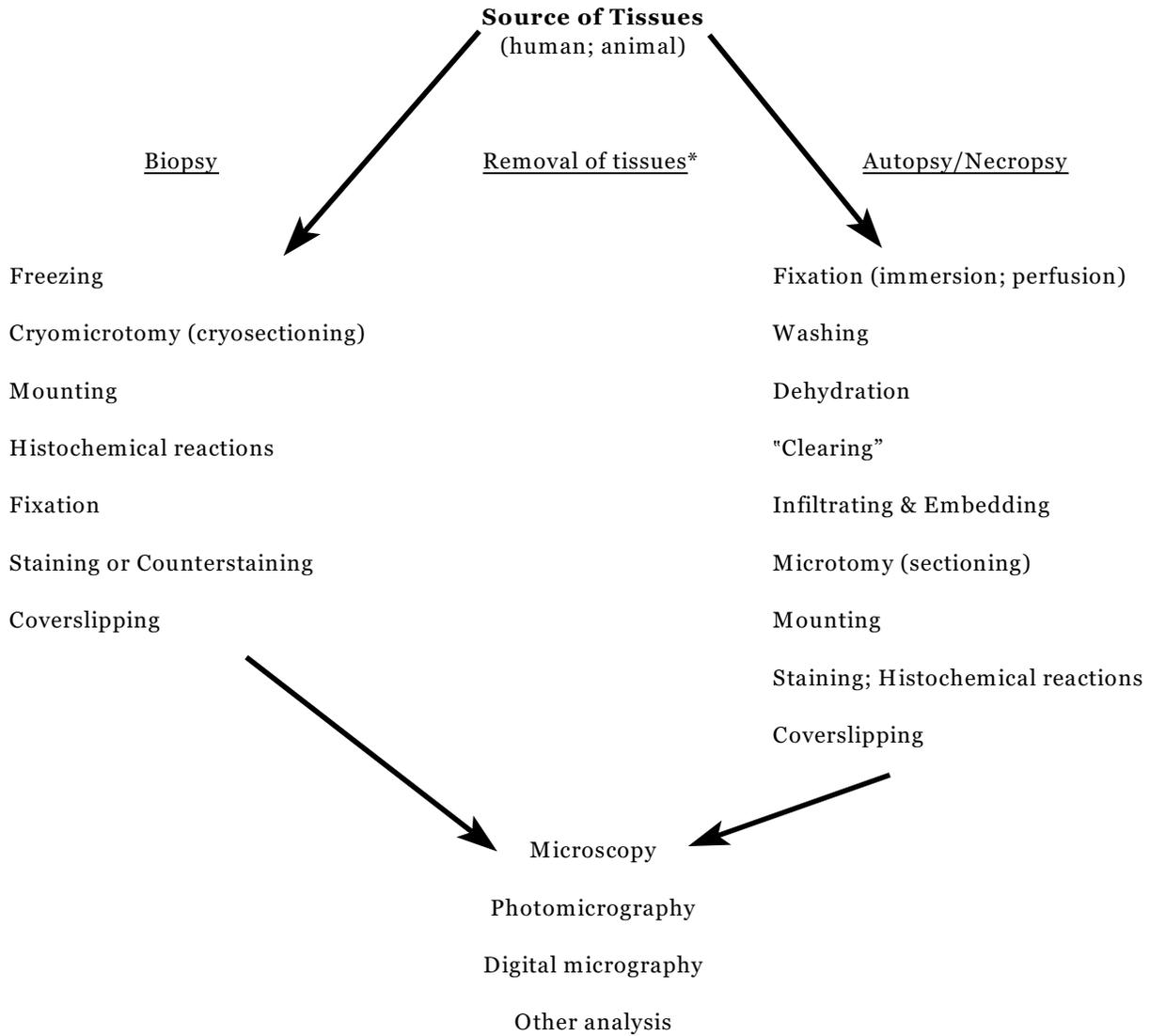
complementary strands will combine. Thus, hybrids between DNA-DNA, DNA-RNA, and RNA-RNA are possible.

a. Methods. These hybridization techniques were then applied *in situ* to cytological preparations on microscope slides to permit detection and localization of RNA-DNA hybrids (Gall JG & Pardue ML. *Proc Natl Acad Sci USA* 63:378-383, 1969) and DNA-DNA hybrids (Pardue ML & Gall JG. *Proc Natl Acad Sci USA* 64:600-604, 1969) at the cell and tissue levels. These studies used radioactive (tritium-labeled) probes, which were detected in the complementary hybrids by radioautography. Subsequently, non-isotopic techniques were employed using enzyme-labeled (e.g., peroxidase-antiperoxidase) or fluorescent-labeled probes, the latter of which is referred to as **FISH** (=Fluorescence In Situ Hybridization). EM techniques have also been developed.

b. Applications. These techniques have had great utility in detecting nucleic acids in their cellular environment. Extremely useful in both research and clinical diagnosis, important applications include time course and location (differential gene expression) of mRNA transcripts, location of genes to specific chromosomes, recognizing chromosome abnormalities and pathologies, and diagnosis of genetic diseases.

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Figure 1-1. Flowchart for the Preparation of Tissues for Microscopic Analysis



 *Although the path at the left for biopsy material may be more typical and vice versa, either paths can be used for biopsy and autopsy/necropsy specimens.

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Table 1-1. Important Stains and Histochemical Reactions (HR*).

Reagent	Type	Color	Tissue components visualized
Hematoxylin	"basic" dye	blue to bl-black	nuclei, cytoplasmic and extracellular anionic substances (e.g., RER, proteoglycans)
Iron hematoxylin	"basic" dye	bl-black to black	nuclei, mitochondria, muscle striations, RBCs, meiotic chromosomes
PTAH	>"basic" dye	bl-black	terminal bars, intercalated disks
Toluidine blue Methylene blue Azures	basic dyes	blue	cellular & EC anions; metachromatic (purple) with high density polyanions (e.g., mast cell granules, basophil granules, cartilage ECM)
Eosin Orange G Picric acid Fast green Aniline blue	acid dyes	pink-red orange yellow green blue	cellular and EC cationic substances (e.g., Hb, collagen, muscle proteins, keratin tonofibrils)
Alcian blue	basic dye	bl-green	high density polyanions (e.g., GAG's)
PAS	HR*	magenta	vic-glycols (e.g., glycogen, mucins, GAG's)
AB-PAS	dye/HR*	bl-green magenta royal blue	anions (e.g., acidic mucins w/out vic-glycols) vic-glycols (e.g., mucins w/out acidic groups) anions and vic-glycols (e.g., mucins w/ both)
Feulgen reaction	HR*	magenta	nuclear DNA (mitochondrial DNA in too low concentration to be detected)
Silver	metal	black	Golgi apparatus, reticular fibers, neurofibrils (argyrophilia: affinity for silver)
Romanowsky dyes Wright Giemsa	mixture of acid and basic dyes	dk purple blue lt purple pink-red	leukocyte nuclei (P), basophil granules (M), platelet granulomere (P) cytoplasm of agranulocytes, esp. lymphs (O) azurophilic granules (P) RBC cytoplasm, eosinophil granules (O)
Vital stains Trypan blue India ink	nontoxic colloidal suspensions	blue black	phagocytic vacuoles in cells of mononuclear phagocyte system, e.g., CT macrophages
Lipid stains Sudan black Oil red O Osmium tetroxide	lipid-soluble particles metal oxide	black red black	in situ lipid droplets containing triglycerides, sterols, etc unsaturated lipids, phospholipids
Elastin stains Orcein Resorcin fuchsin Verhoeff		red purple black	elastin, fibers and sheets
Masson trichrome	polychrome (CT stain)	bl-black red green;blue	nuclei cytoplasm, muscle collagen, mucus

*HR =histochemical reaction

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