Sample Preparation for Light and Electron Microscopy

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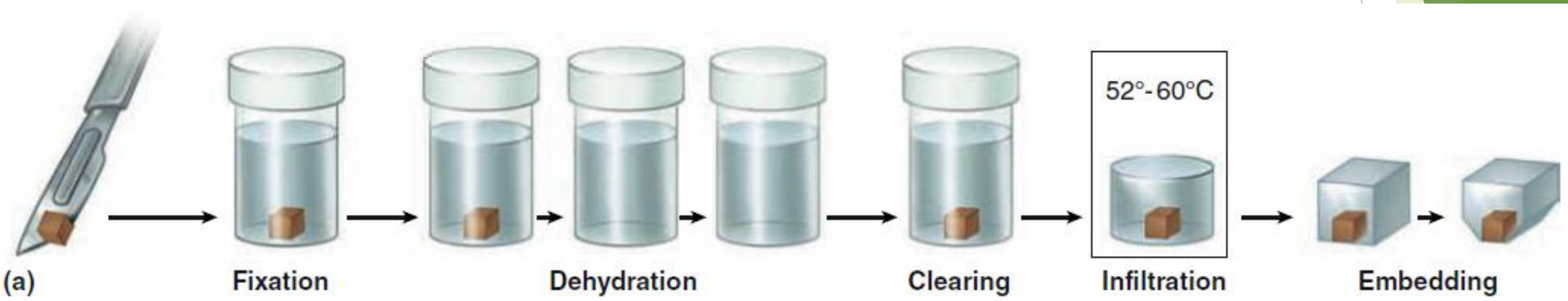
Preparation of tissues for study

- The most common procedure used in histologic research is the preparation of tissue slices or “sections” that can be examined visually with transmitted light.
- Because most tissues and organs are too thick for light to pass through, thin translucent sections are cut from them and placed on glass slides for microscopic examination of the internal structures.
- The ideal microscopic preparation is preserved so that the tissue on the slide has the same structural features it had in the body.


52°-60°C
(a)
Fixation
Dehydration
Clearing
Infiltration
Embedding

Tissue preparation for study

- **Fixation:** Small pieces of tissue are placed in solutions of chemicals that cross-link proteins and inactivate degradative enzymes, which preserves cell and tissue structure (e.g. Formaldehyde for LM, Gluteraldehyde for EM).
- **Dehydration:** The tissue is transferred through a series of increasingly concentrated alcohol solutions, ending in 100%, which removes all water.
- **Clearing:** Replacing the dehydrating fluid with a fluid that is totally miscible with both the dehydrating fluid and the embedding medium (e.g. Xylene for LM & propylene oxide for EM)
- **Infiltration:** Replacing the clearing agent (inside the cell) with a material that can harden to support biological tissue (e.g. paraffin wax for LM & resin for EM)

Together called tissue processing

Tissue preparation for study-cont'd

- **Embedding:** The infiltrated tissue is placed in a small mold and allowed to harden.
- **Trimming:** The resulting block is trimmed to expose the tissue for sectioning (slicing) on a microtome.
- **Sectioning**: slicing using microtome (for LM, and ultramicrotome for EM).
- **Mounting**: The process to place (mount) the tissue sections on the adhesive coated glass slides (for LM, and grids for EM)
- **Staining**: The stain is a chemical substance which reacts with certain tissue components producing a color

Fixation

- To preserve tissue structure and prevent degradation by enzymes released from the cells or microorganisms, pieces of organs are placed as soon as possible after removal from the body in solutions of stabilizing or cross-linking compounds called **fixatives**.
- Because a fixative must fully diffuse through the tissues to preserve all cells, tissues are usually cut into small fragments before fixation to facilitate penetration.

Fixatives

- One widely used fixative for light microscopy is formalin, a buffered isotonic solution of 37% formaldehyde.
- Both this compound and glutaraldehyde, a fixative used for electron microscopy, react with the amine groups (NH2) of proteins, preventing their degradation by common proteases.
- Glutaraldehyde also cross-links adjacent proteins, reinforcing cell and ECM structures.
- Electron microscopy provides much greater magnification and resolution of very small cellular structures and fixation must be done very carefully to preserve additional “ultrastructural” detail.
- Typically in such studies glutaraldehyde treated tissue is then immersed in buffered osmium tetroxide, which preserves (and stains) cellular lipids as well as proteins.

Embedding & Sectioning

- To permit thin sectioning fixed tissues are infiltrated and embedded in a material that imparts a firm consistency.
- Embedding materials include paraffin, used routinely for light microscopy, and plastic resins, which are adapted for both light and electron microscopy.

Dehydration & Clearing

- Before infiltration with such media the fixed tissue must undergo **dehydration** by having its water extracted gradually by transfers through a series of increasing ethanol solutions, ending in 100% ethanol.
- The ethanol is then replaced by an organic solvent miscible with both alcohol and the embedding medium, a step referred to as **clearing** because infiltration with the reagents used here gives the tissue a translucent appearance.

Infiltration & Embedding

- The fully cleared tissue is then placed in melted paraffin in an oven at 52°-60°C, which evaporates the clearing solvent and promotes **infiltration** of the tissue with paraffin, and then **embedded** by allowing it to harden in a small container of paraffin at room temperature.
- Tissues to be embedded with plastic resin are also dehydrated in ethanol and then infiltrated with plastic solvents that harden when cross-linking polymerizers are added.
- Plastic embedding avoids the higher temperatures needed with paraffin, which helps avoid tissue distortion.

Sectioning

- The hardened block with tissue and surrounding embedding medium is trimmed and placed for sectioning in an instrument called a **microtome**
- Paraffin sections are typically cut at 3-10 μm thickness for light microscopy, but electron microscopy requires sections less than 1 μm thick.
- The sections are placed on glass slides and stained for light microscopy or on metal grids for electron microscopic staining and examination.

N.B.

- One micrometer (1 μm) equals 1/1000 of a millimeter (mm) or 10^{-6} m.
- Other spatial units commonly used in microscopy are the nanometer (1 nm = 0.001 μm = 10^{-6} mm = 10^{-9} m) and angstrom (1 A = 0.1 nm or 10^{-4} μm).

Medical Application

- Biopsies are tissue samples removed during surgery or routine medical procedures. In the operating room, biopsies are fixed in vials of formalin for processing and microscopic analysis in a pathology laboratory.
- If results of such analyses are required before the medical procedure is completed, for example to know whether a growth is malignant before the patient is closed, a much more rapid processing method is used.
- The biopsy is rapidly frozen in liquid nitrogen, preserving cell structures and making the tissue hard and ready for sectioning. A microtome called a **cryostat** in a cabinet at subfreezing temperature is used to section the block with tissue, and the frozen sections are placed on slides for rapid staining and microscopic examination by a pathologist.

Major differences in sample preparation for TEM

- Uses hard epoxy resin for embedding instead of paraffin wax
- To improve contrast and resolution in TEM, compounds with **heavy metal ions** are often added to the fixative or dehydrating solutions used for tissue preparation. These include osmium tetroxide, lead citrate, and uranyl compounds, which bind cellular macromolecules, increasing their electron density and visibility.
- Sectioning is done through an ultramicrotome producing ultra-thin sections
- Sections are mounted on copper grids instead of glass slides

Staining

- Most cells and extracellular material are completely colorless, and to be studied microscopically tissue sections must be stained (dyed).
- Methods of staining have been devised that make various tissue components not only conspicuous but also distinguishable from one another.
- Dyes stain material more or less selectively, often behaving like acidic or basic compounds and forming electrostatic (salt) linkages with ionizable radicals of macromolecules in tissues.

Basophilic & acidophilic

- Cell components such as nucleic acids with a net negative charge (anionic) have an affinity for basic dyes and are termed **basophilic**; cationic components, such as proteins with many ionized amino groups, stain more readily with acidic dyes and are termed **acidophilic**.

Light microscopy stains

- The most commonly used stain in histological preparation is Hematoxylin and Eosin (H&E) stain (a water soluble stain).
- Other special satins are common, such as:
  - Periodic Acid Sciff (PAS) stain for carbohydrates
  - Silver (Ag) for reticular fibers (collagen type III)
  - Masson's Trichrome to distinguish collagen (Blue) from muscle (Red)
  - Orcein for elastic fibers

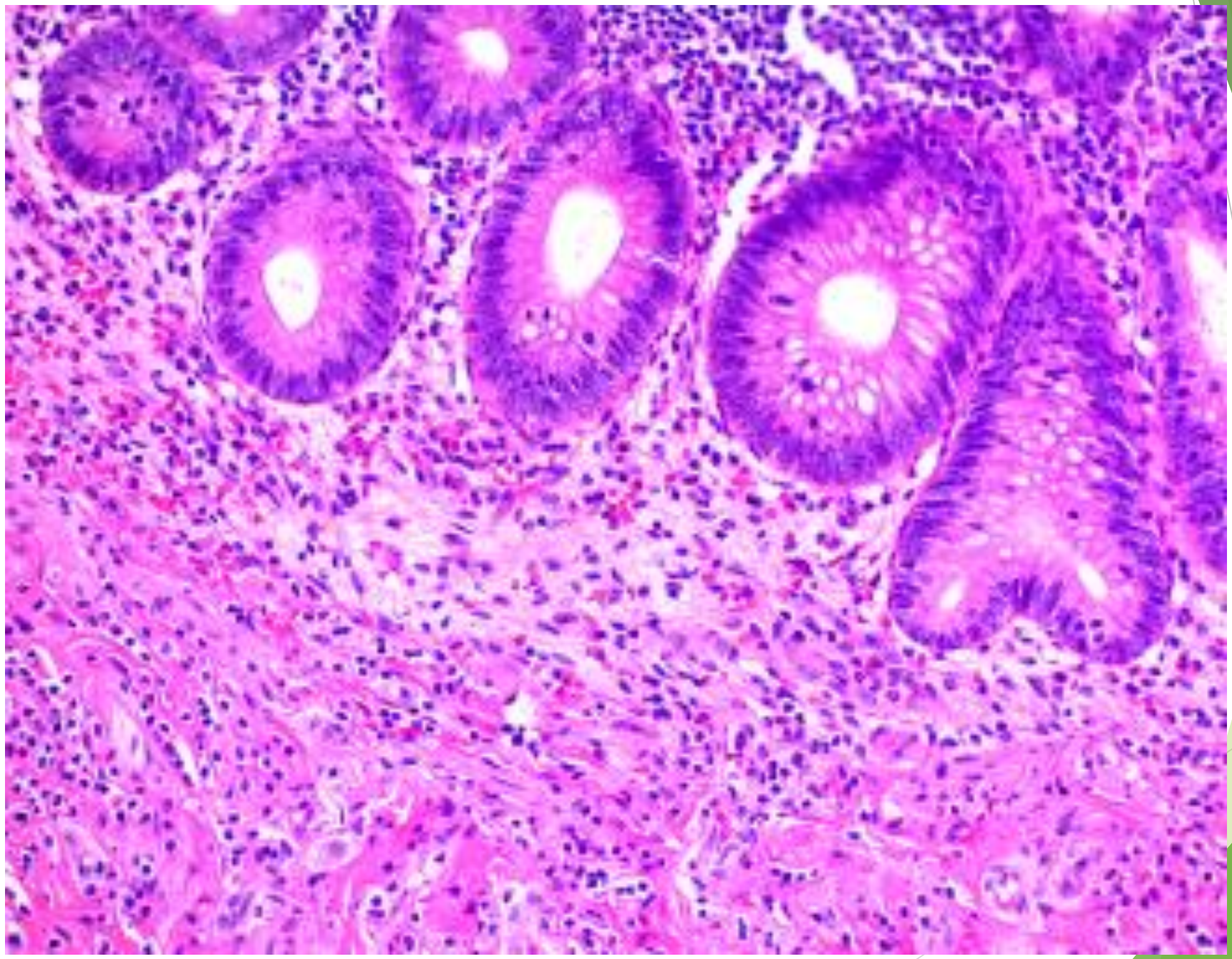
H&E

Hematoxylin

- Basic dye
- Has positive charge
- Will stain negative (basophilic) structures BLUE
- Examples: DNA, RNA, ribosomes, rER, GAGs

Eosin

- Acidic dye
- Has negative charge
- Will stain positive (acidophilic, eosinophilic) structures PINK
- Examples: proteins, collagen, cytoplasm, mitochondria, secretory granules



H&E

Lipid-rich compounds

- Lipid-rich structures of cells are revealed by avoiding the processing steps that remove lipids, such as treatment with heat and organic solvents, and staining with **lipid-soluble dyes** such as **Sudan black,** which can be useful in diagnosis of metabolic diseases that involve intracellular accumulations of cholesterol, phospholipids, or glycolipids.

Visualizing specific molecules

- A specific macromolecule present in a tissue section may also be identified by using tagged compounds or macromolecules that bind *specifically* with the molecule of interest. The compounds that interact with the molecule must be visible with the light or electron microscope, often by being tagged with a detectible label.
- The most commonly used labels are fluorescent compounds, molecules of peroxidase or other enzymes that can be detected with histochemistry, and metal (usually gold) particles that can be seen with light and electron microscopy.

Examples

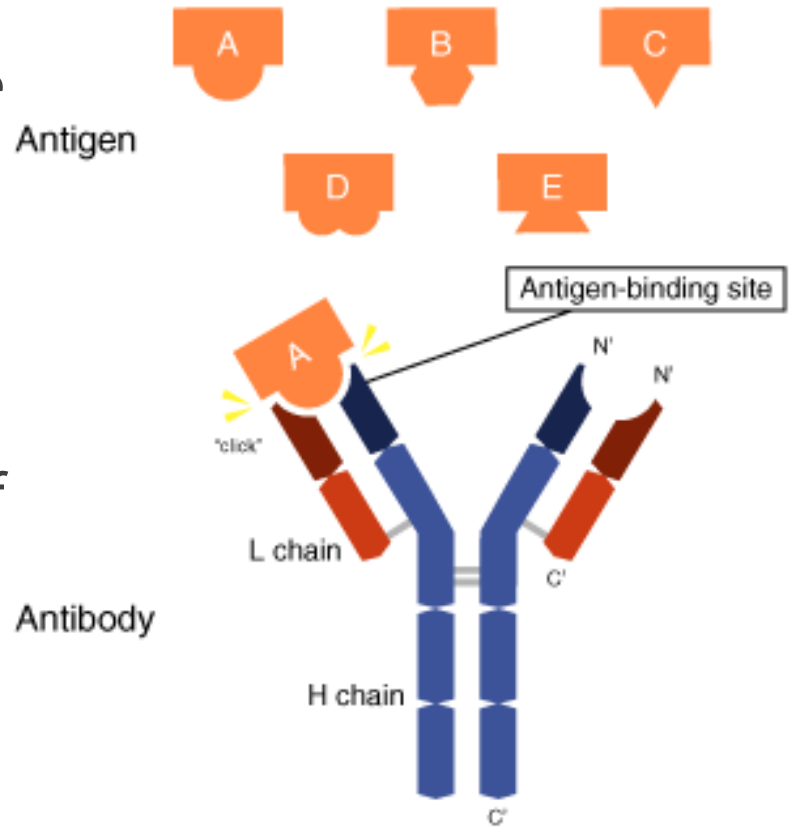
- **Phalloidin**, a compound extracted from a mushroom, interacts strongly with the actin protein of microfilaments.
- **Protein A**, purified from *Staphylococcus aureus* bacteria, binds to the Fc region of antibody molecules, and can therefore be used to localize naturally occurring or applied antibodies bound to cell structures.
- **Lectins**, glycoproteins derived mainly from plant seeds, bind to carbohydrates with high affinity and specificity. Different lectins bind to specific sugars or sequences of sugar residues, allowing fluorescently labeled lectins to be used to stain specific glycoproteins or other macromolecules bearing specific sequences of sugar residues.

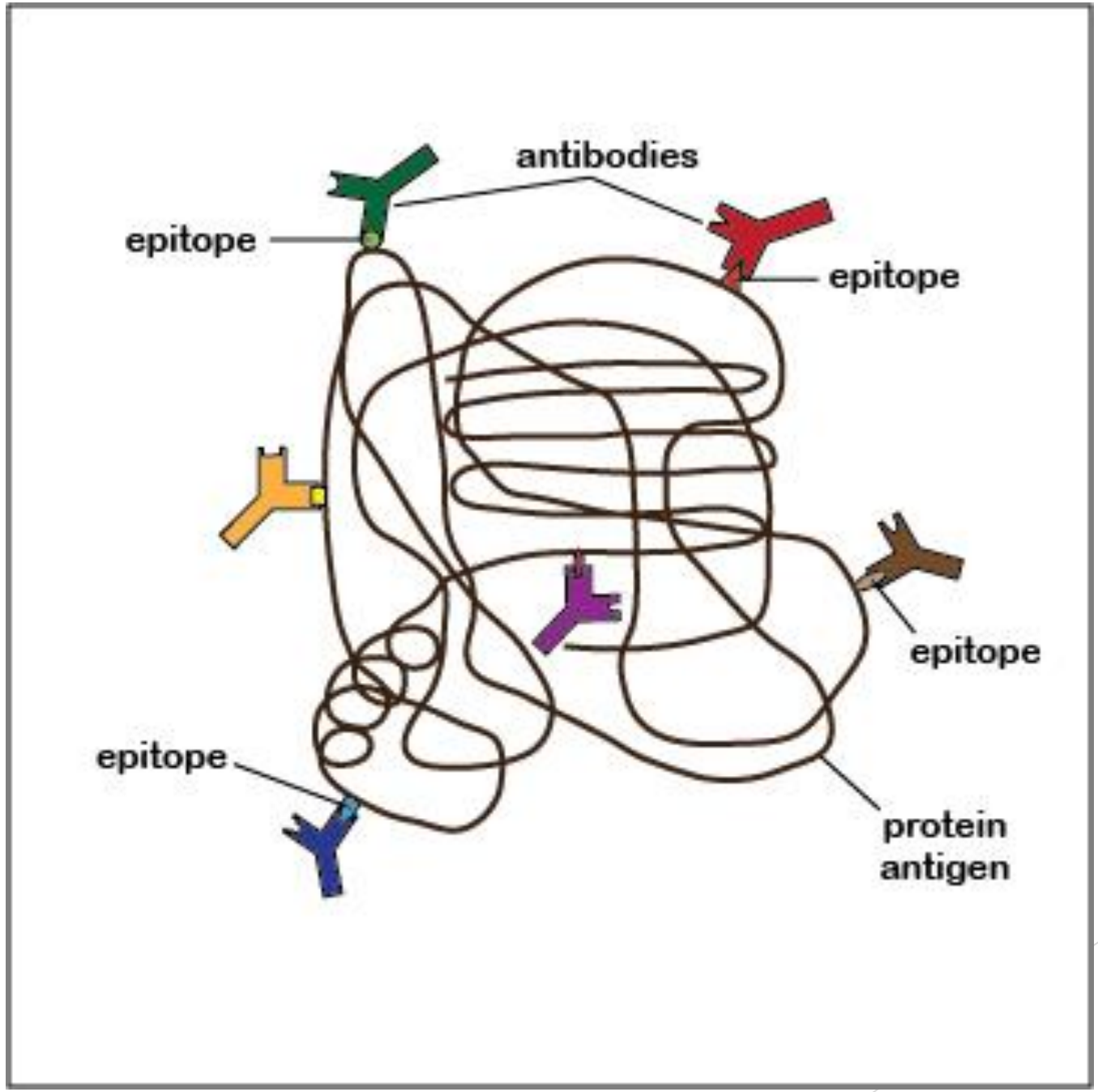
Immunohistochemistry

- A highly specific interaction between macromolecules is that between an antigen and its antibody.
- For this reason labeled antibodies are routinely used in **immunohistochemistry** to identify and localize many specific proteins
- Every immunohistochemical technique requires an antibody against the protein that is to be detected.
- This means that the protein must have been previously purified using biochemical or molecular methods so that antibodies against it can be produced.

What is an antibody?

- The body's immune cells interact with and produce **antibodies** against other macromolecules—called antigens—that are recognized as "foreign," not a normal part of the organism, and potentially dangerous.
- Antibodies belong to the **immunoglobulin** family of glycoproteins and are secreted by lymphocytes.
- These molecules normally bind specifically to their provoking antigens and help eliminate them.




antibodies
epitope
epitope
epitope
epitope
protein
antigen

How are antibodies produced?

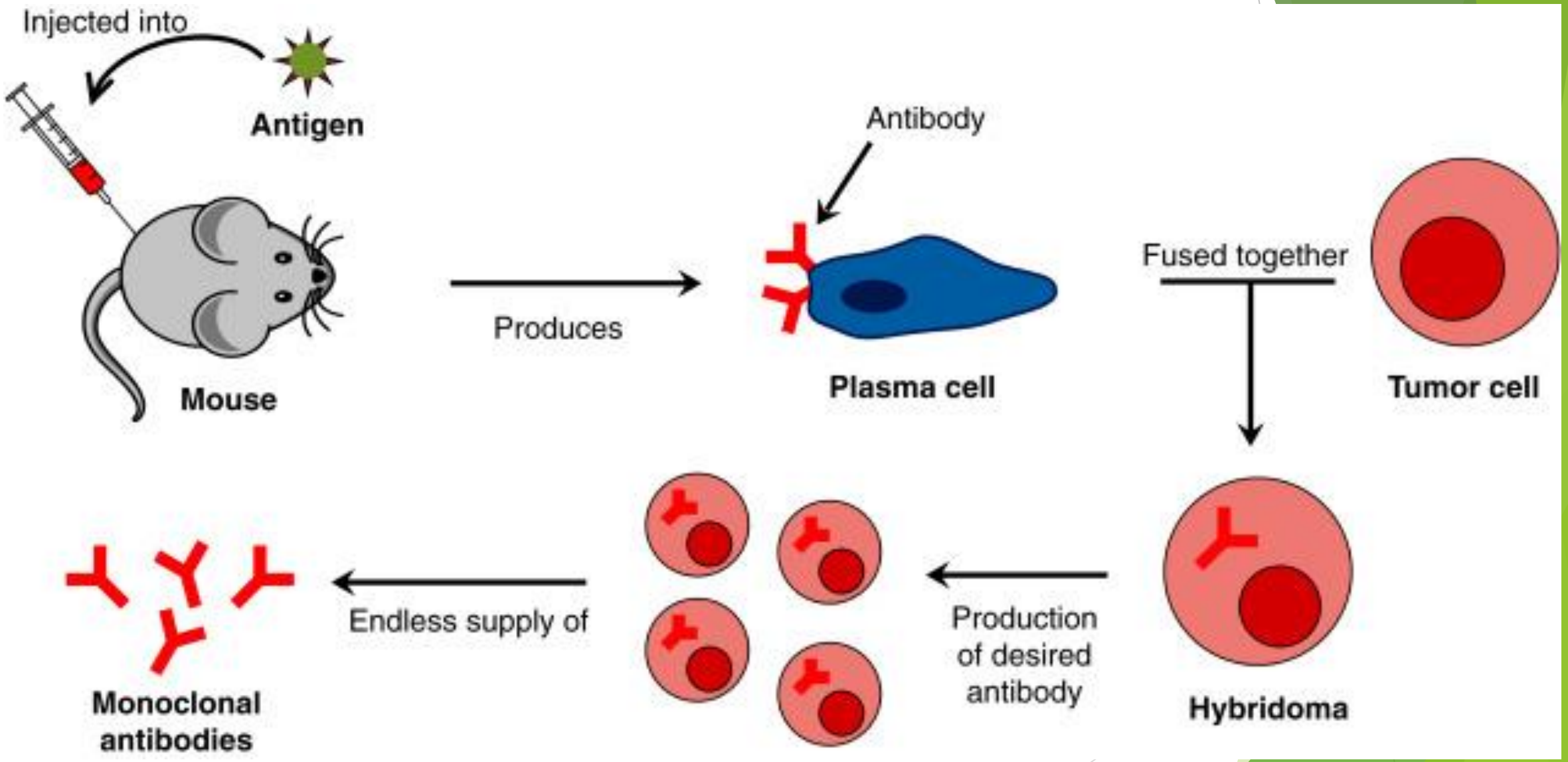
- To produce antibodies against protein *x* of a certain animal species (eg, a human), the isolated protein is injected into an animal of another species (eg, a rabbit or a goat).
- If the protein's amino acid sequence is sufficiently different for this animal to recognize it as foreign—that is, as an antigen—the animal will produce antibodies against the protein.

Polyclonal antibodies

- Different groups (clones) of lymphocytes in the injected animal recognize different parts of protein *x* and each clone produces an antibody against that part.
- These antibodies are collected from the animal's plasma and constitute a mixture of **polyclonal antibodies**, each capable of binding a different region of protein *x*.

Monoclonal antibodies

- It is also possible, however, to inject protein *x* into a mouse and a few days later isolate the activated lymphocytes and place them into culture.
- Growth and activity of these cells can be prolonged indefinitely by fusing them with lymphocytic tumor cells to produce hybridoma cells.
- Different hybridoma clones produce different antibodies against the several parts of protein *x* and each clone can be isolated and cultured separately so that the different antibodies against protein *x* can be collected separately.
- Each of these antibodies is a **monoclonal antibody**.
- An advantage to using a monoclonal antibody rather than polyclonal antibodies is that it can be selected to be highly specific and to bind strongly to the protein to be detected, with less nonspecific binding to other proteins that are similar to the one of interest.


Injected into
Antigen
Mouse
Produces
Antibody
Plasma cell
Fused together
Tumor cell
Hybridoma
Production of desired antibody
Endless supply of
Monoclonal antibodies

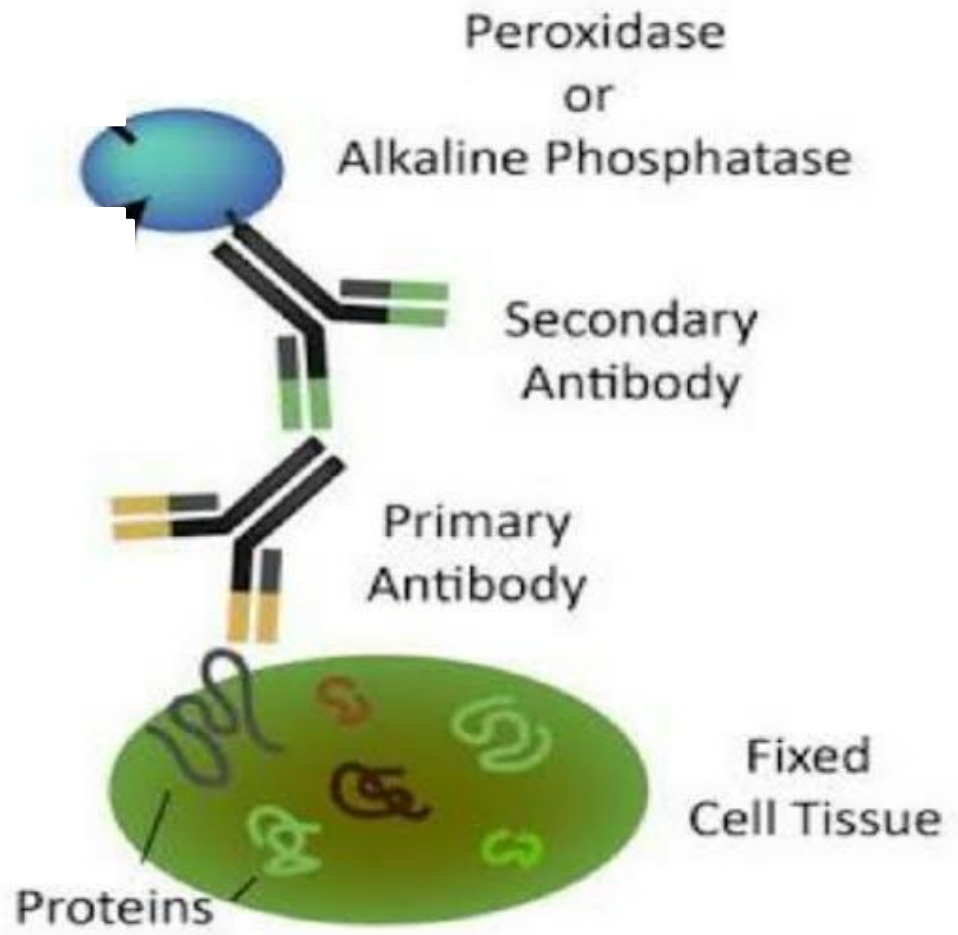
Immunohistochemistry technique

- In immunohistochemistry, a tissue section that one believes contains the protein of interest is incubated in a solution containing antibody (either monoclonal or polyclonal) against this protein.
- The antibody binds specifically to the protein and after a rinse the protein's location in the tissue or cells can be seen with either the light or electron microscope by visualizing the antibody.

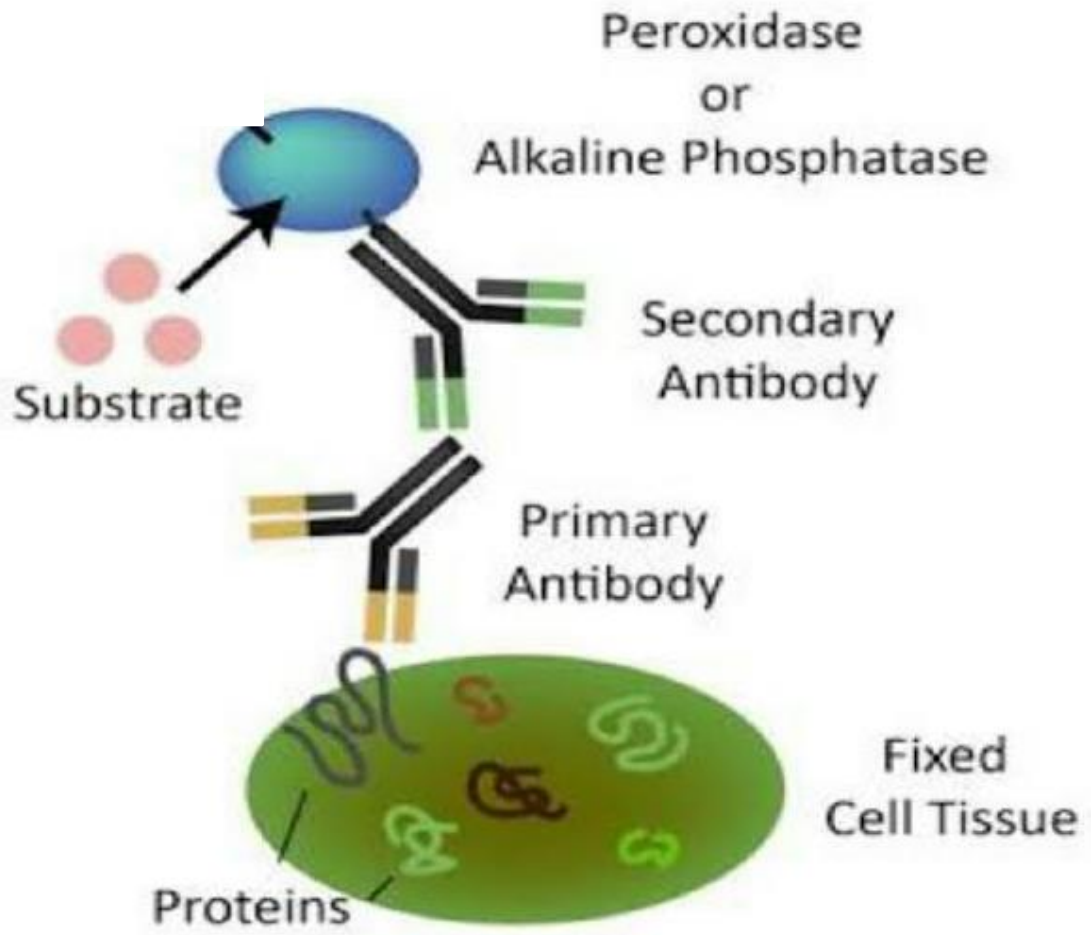
Visualizing bound antibodies

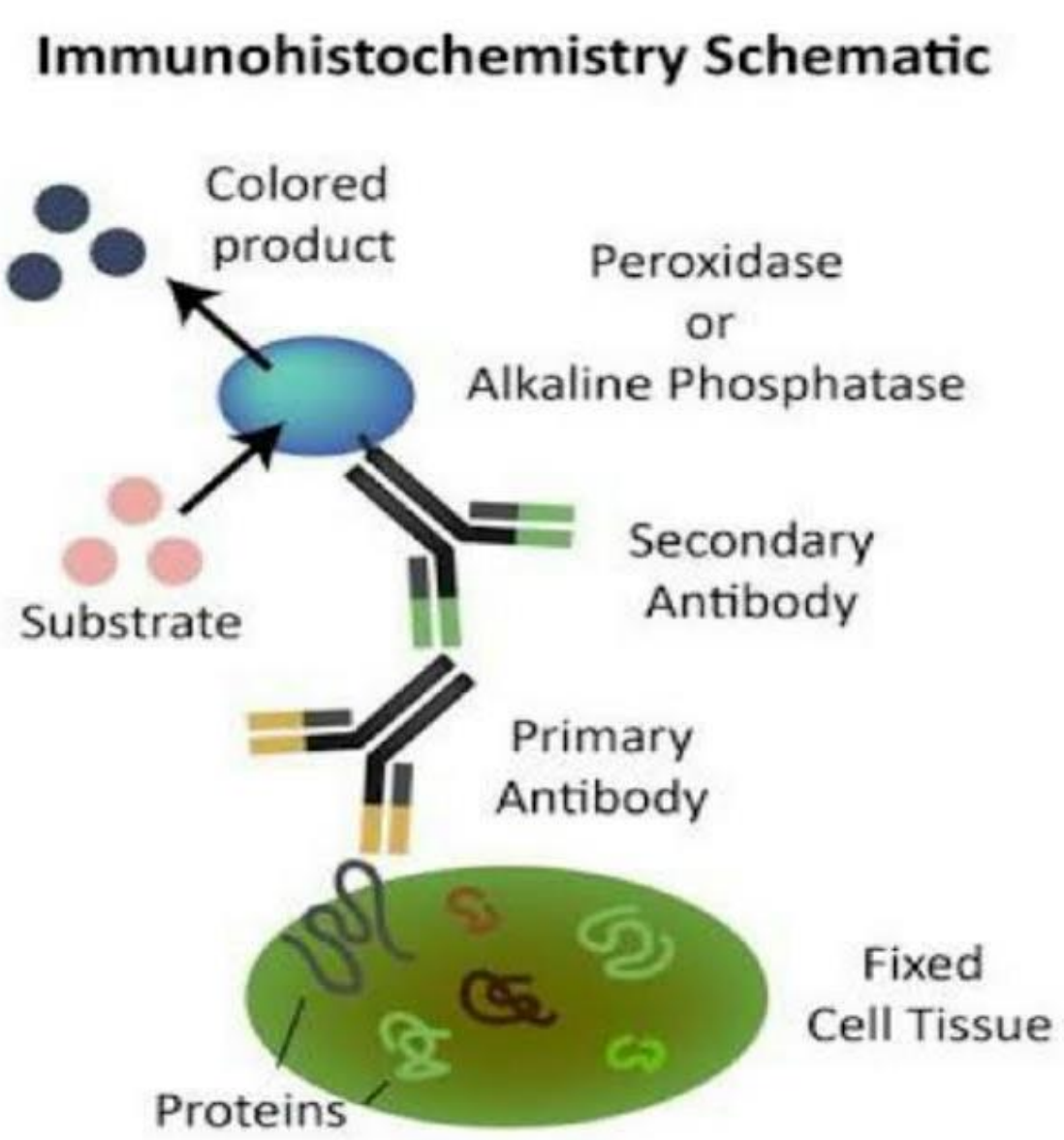
- Antibodies are commonly tagged with one of the following:
  - fluorescent compounds
  - peroxidase or alkaline phosphatase (enzymes) for histochemical detection
  - electron-dense gold particles for TEM.

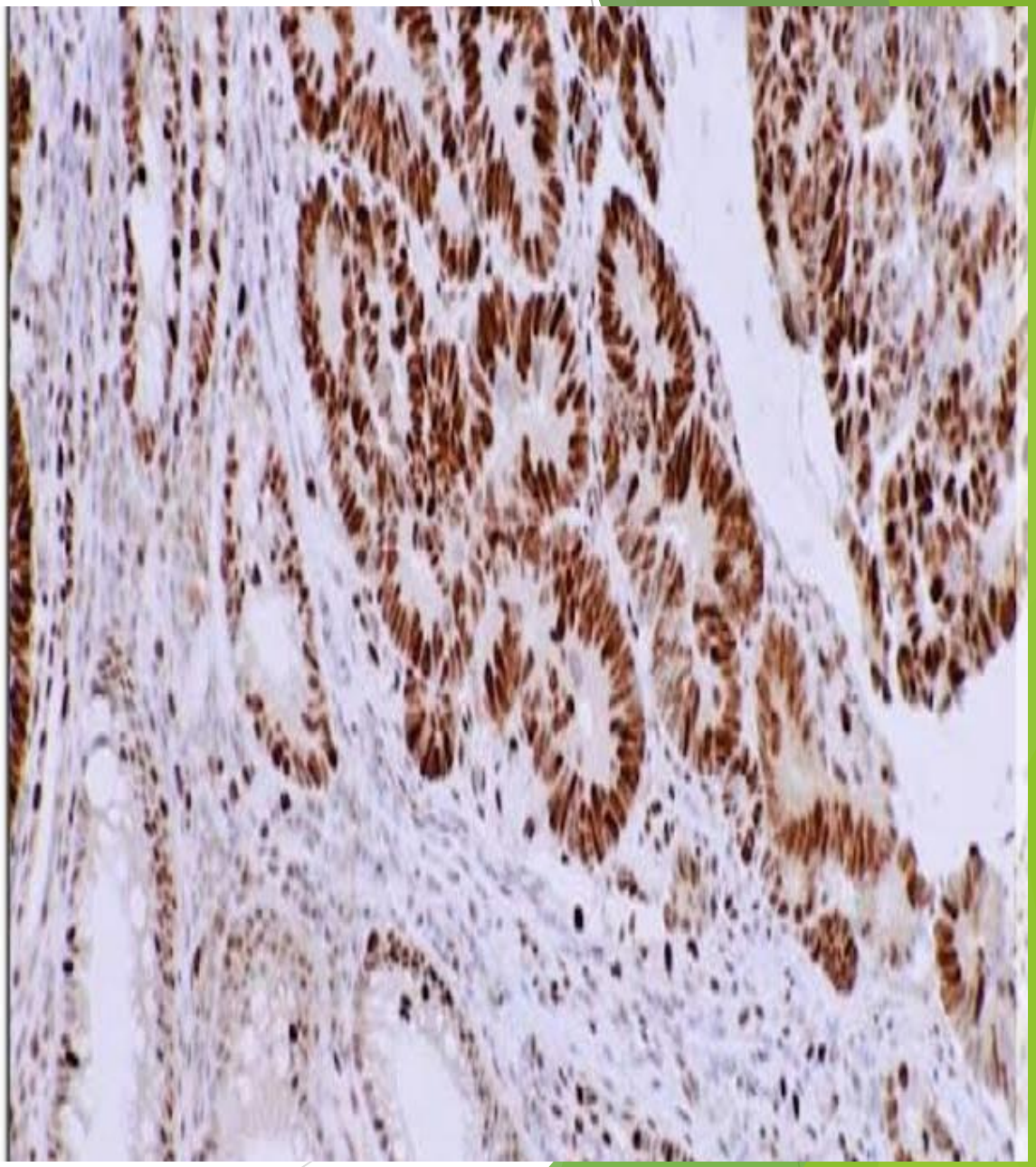
Immunohistochemistry Schematic



Immunohistochemistry Schematic

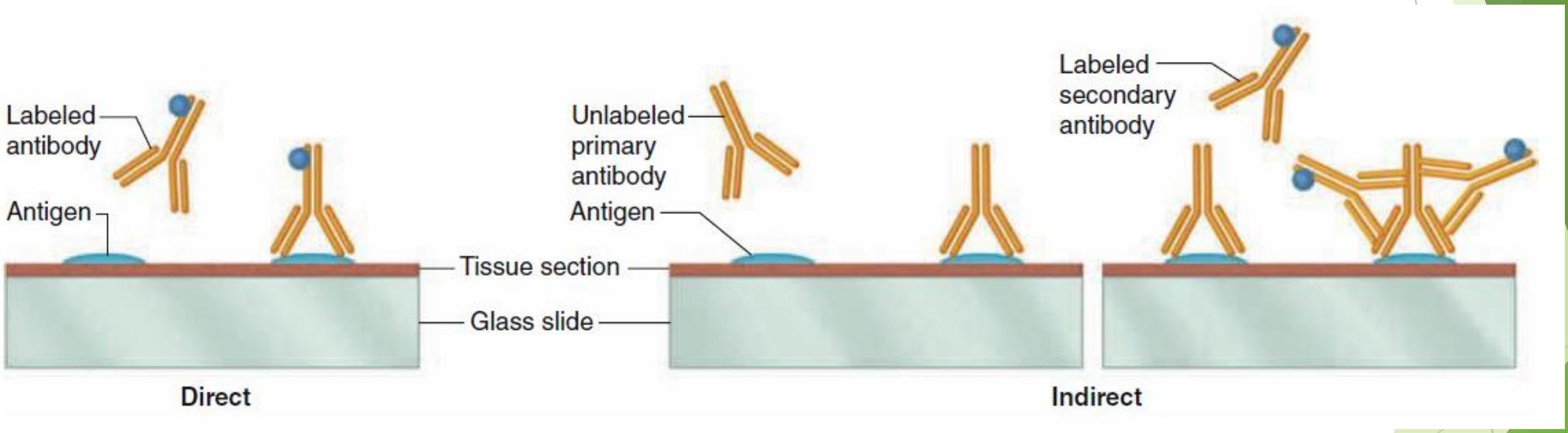


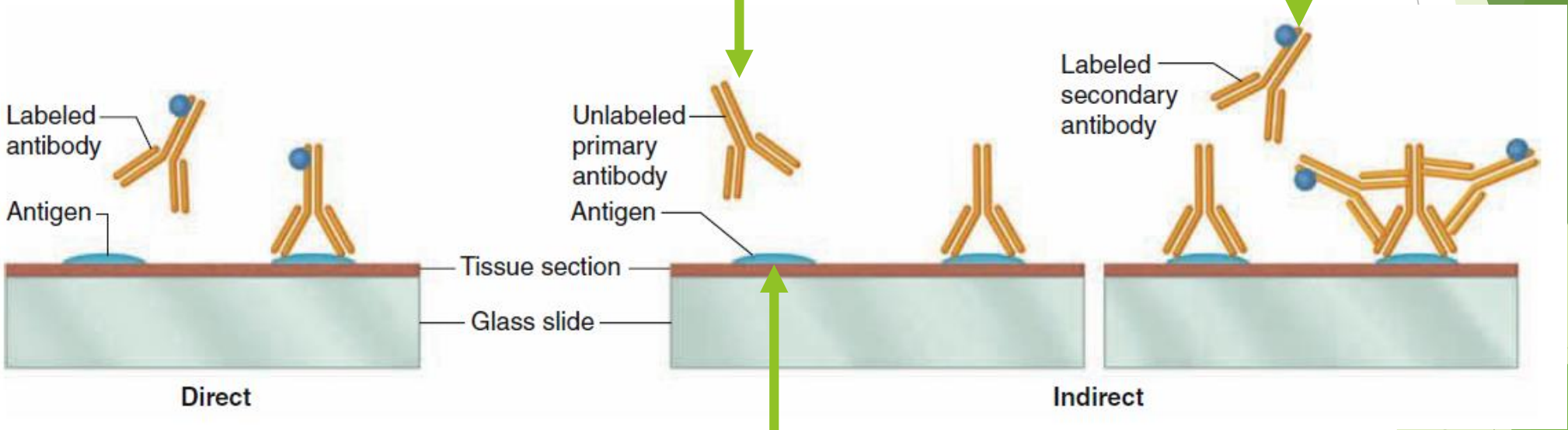

Immunohistochemistry Schematic
Colored product
Peroxidase or Alkaline Phosphatase
Substrate
Secondary Antibody
Primary Antibody
Fixed Cell Tissue
Proteins



Direct & Indirect methods

- The direct method just involves a labeled antibody that binds the protein of interest.
- Indirect immunohistochemistry involves sequential application of two antibodies and additional washing steps.
- The (primary) antibody specifically binding the protein of interest is not labeled.
- The detectible tag is conjugated to a **secondary antibody** made in an animal species different ("foreign") from that which made the primary antibody.
- For example, primary antibodies made by mouse lymphocytes are specifically recognized and bound by antibodies made in a rabbit or goat injected with mouse antibody immunoglobulin.


Labeled antibody
Antigen
Tissue section
Glass slide
Direct
Unlabeled primary antibody
Antigen
Labeled secondary antibody
Indirect



mouse anti-human

goat anti-mouse

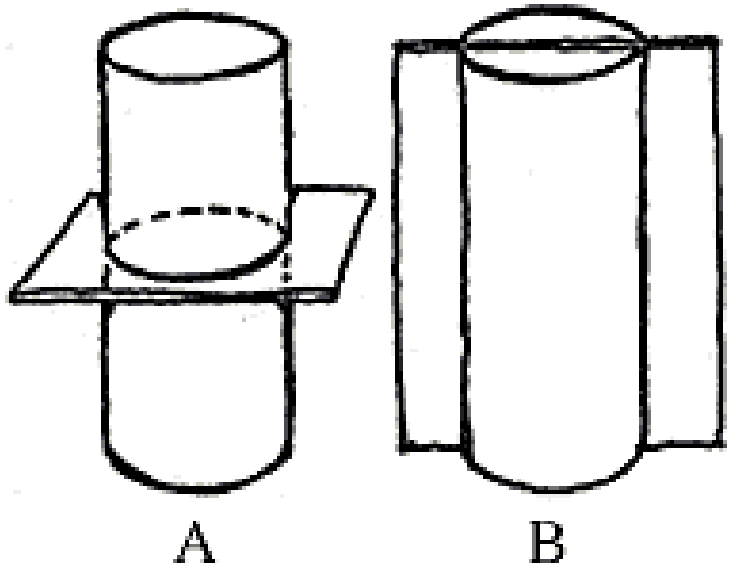
human

Artifacts

- In studying and interpreting stained tissue sections, it is important to remember that microscopic preparations are the end result of a series of processes that began with collecting the tissue and ended with mounting a coverslip on the slide.
- Certain steps in this procedure may distort the tissues slightly, producing minor structural abnormalities called **artifacts** not present in the living tissue.

Types of sections

- Cross section: perpendicular to the long axis of a tissue/organ (A)
- Longitudinal: along the long axis of the tissue/organ (B)



Interpretation of 3D structures in 2D sections.

In thin sections 3D structures appear to have only two dimensions. Such images must be interpreted correctly to understand the actual structure of tissue and organ components. For example, blood vessels and other tubular structures appear in sections as round or oval shapes whose size and shape depend on the transverse or oblique angle of the cut. A highly coiled tube will appear as several round and oval structures.

In TEM sections of cells, round structures may represent spherical organelles or transverse cuts through tubular organelles such as mitochondria. It is important to develop such interpretive skill to understand tissue and cell morphology in microscopic preparations.