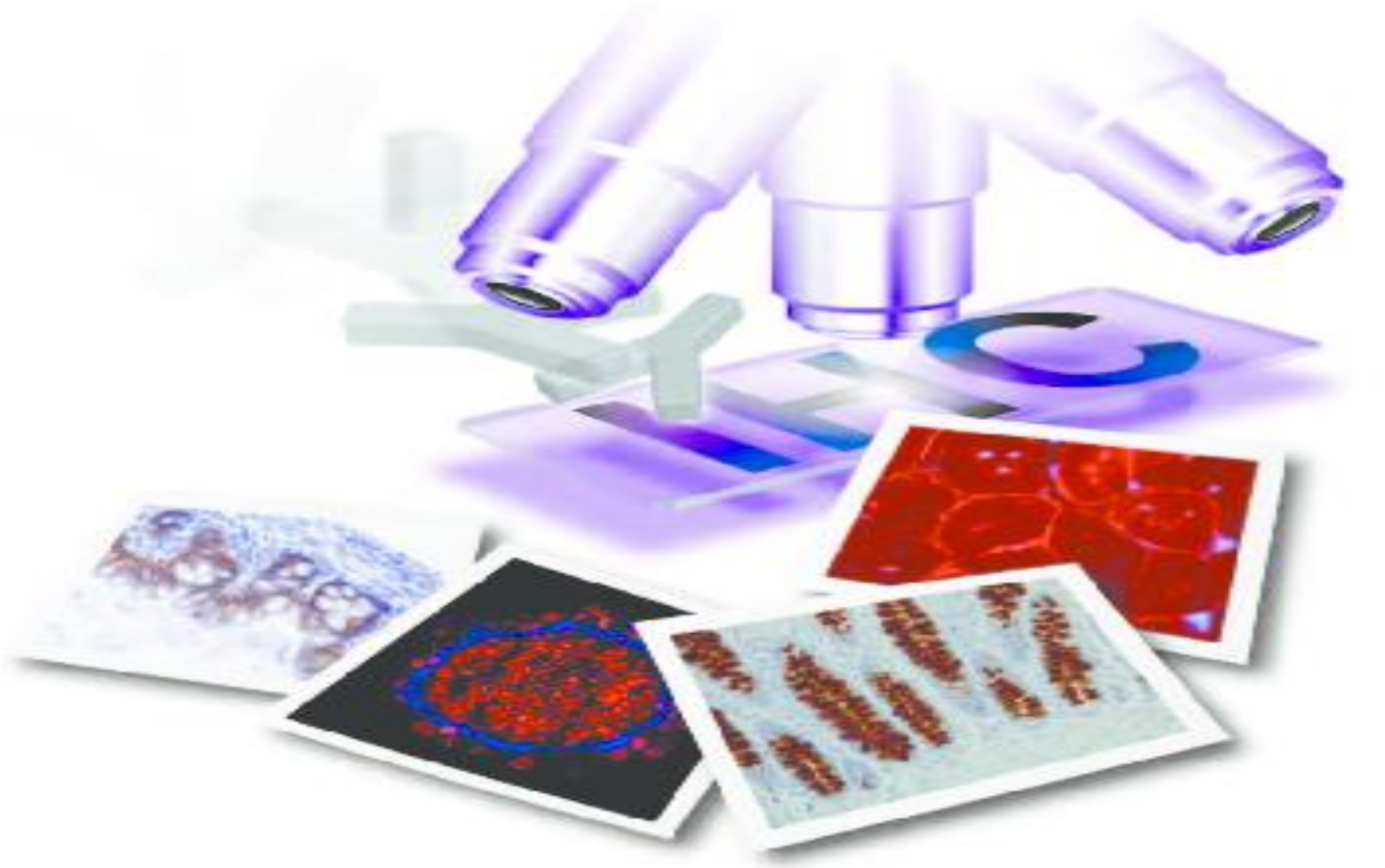
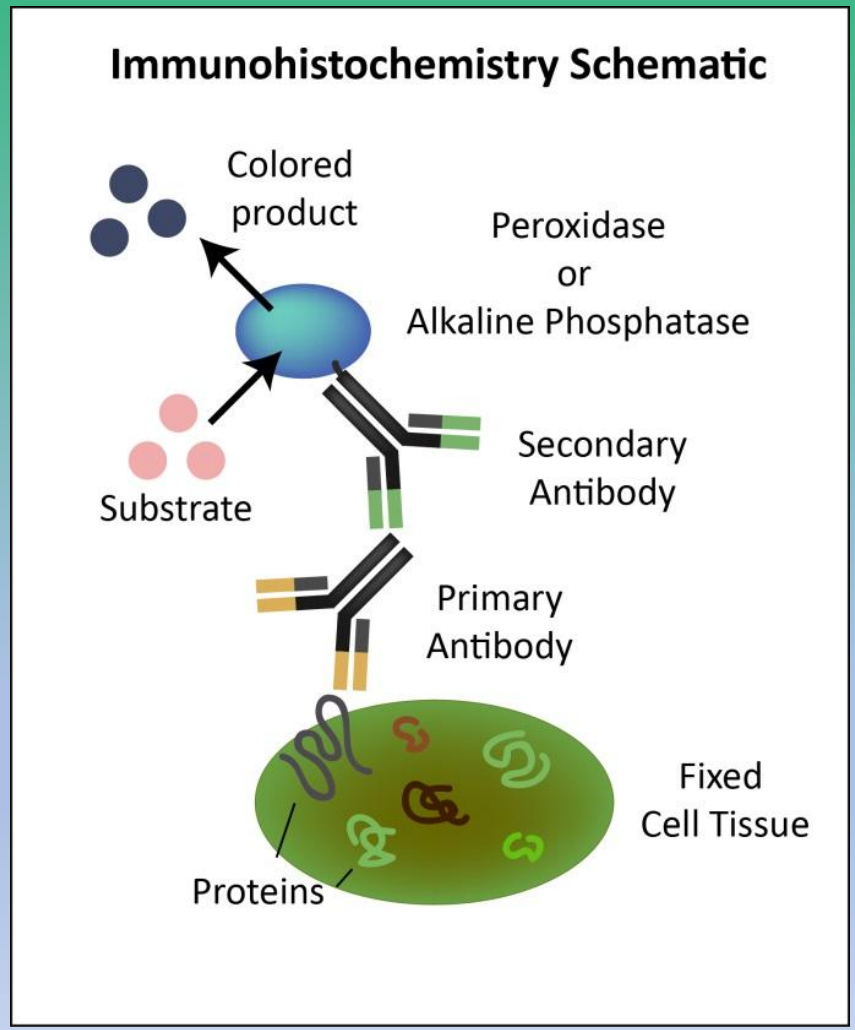
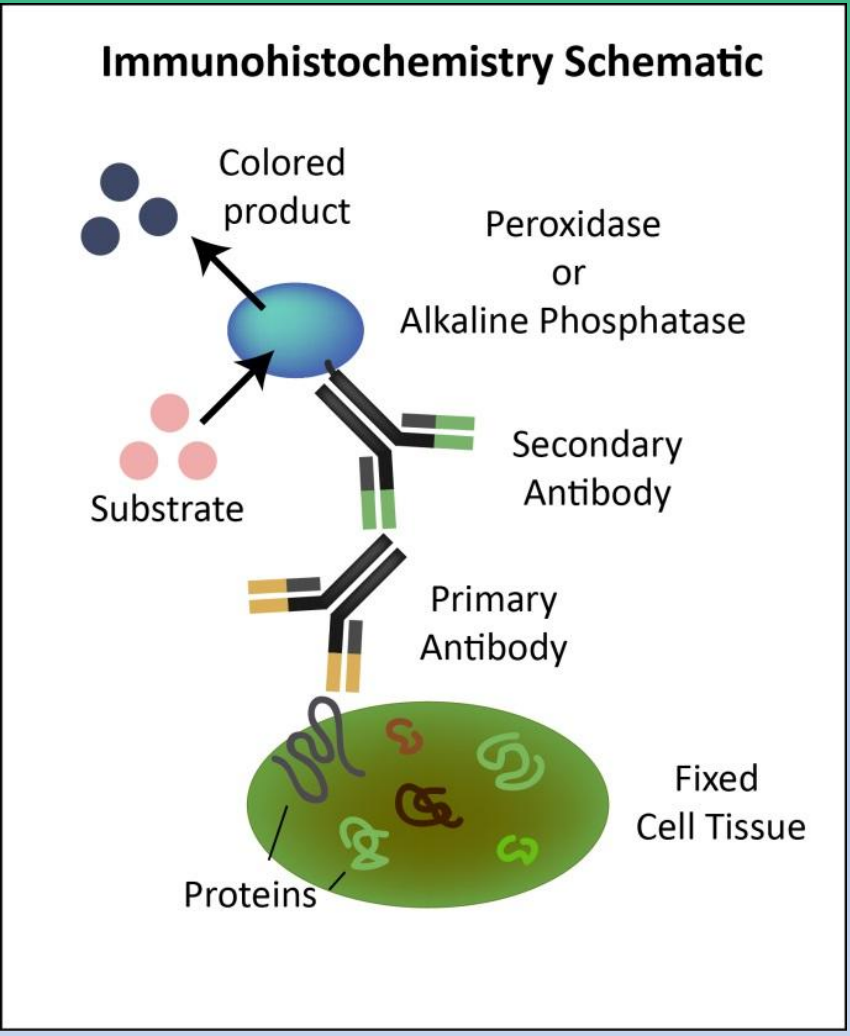
Immunohistochemistry (IHC)



- Immunohistochemistry (IHC) allows you to visualize proteins in tissue while retaining its microstructure.
- It helps to demonstrate the exact position and distribution of the protein of interest in the analyzed tissue section.
- The advantage of this visualization is that it allows for comparison between, for example, healthy and diseased tissues. Briefly, in an IHC experiment, the antigen of interest is localized by the binding of an antibody.



- The antibody-antigen interaction is then further visualized via chromogenic or fluorescent detection.
- The IHC protocol contains many steps that may require optimization to ensure specific antibody binding and optimal visualization of the target protein.
- As IHC protocol contains many different variable factors, it is challenging to find the best working conditions to obtain strong and specific staining.



- Antibodies are small protein molecules that are naturally expressed by the immune system of the body in response to the entry of a foreign molecule (antigen) and help in neutralizing it.

- Thus, antibodies act as a defense against potentially harmful foreign organisms and their products.

- The beauty of the antigen–antibody reaction is that each antibody is specific to only a portion of the antigen, called an epitope, and does not bind other molecules that do not match its target, including the body's own molecules.

- All immunostaining techniques, including IHC, utilize this important property of the antigen–antibody reaction specificity to ensure the detection of a single molecule type from a milieu of thousands of different ones.

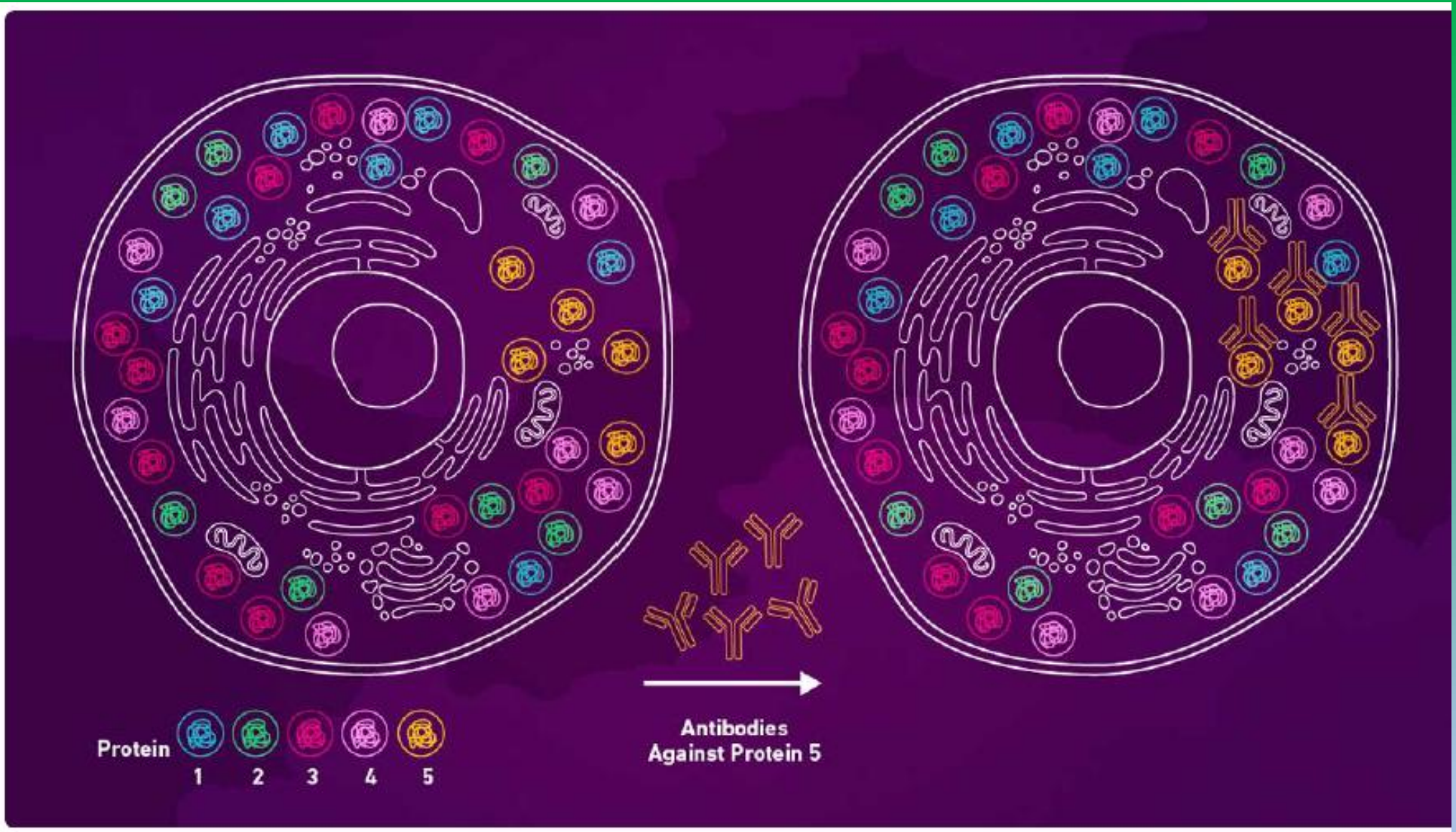
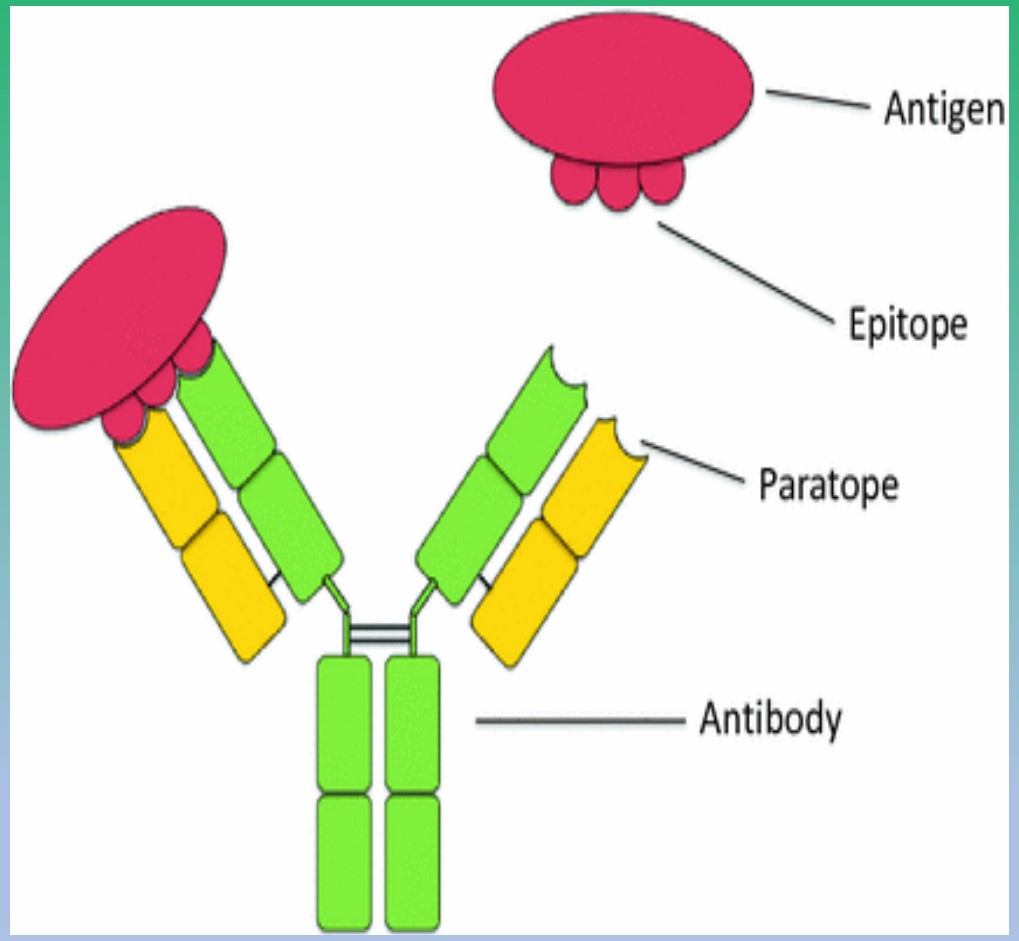


Figure 1: Diagrammatic representation of the specificity of the antigen–antibody reaction that enables detection and localization of a single target in a milieu of thousands of intracellular molecules. *Credit: Technology Networks.*

ANTIGEN

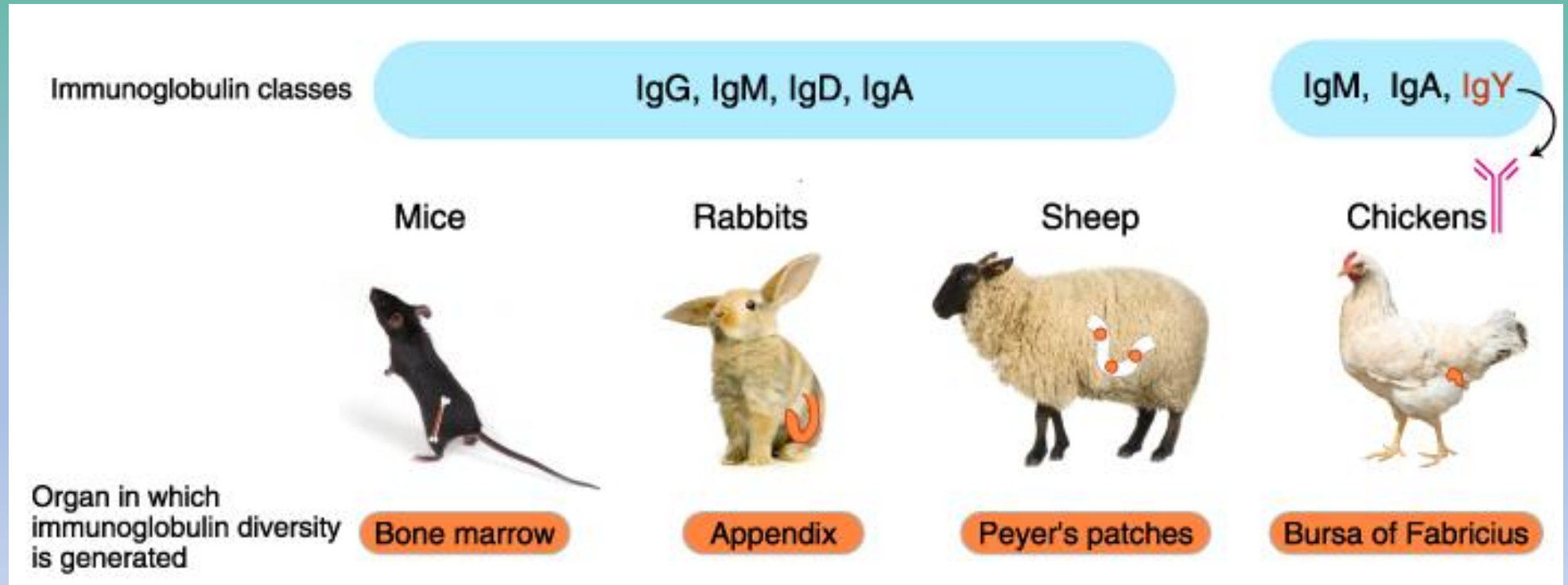
- An antigen is any kind of marker — like a protein or string of amino acids — that your immune system can recognize.
- Antigens are usually proteins or sugars (polysaccharides) found on the outside of things like cells or viruses.
- Antigens exist on viruses, bacteria, allergens, parasites, proteins, tumor cells and normal cells in your own body.
- An epitope is a localized region on the surface of an antigen that is recognized by the immune system, specifically the B- or T-cells.



ANTIBODY

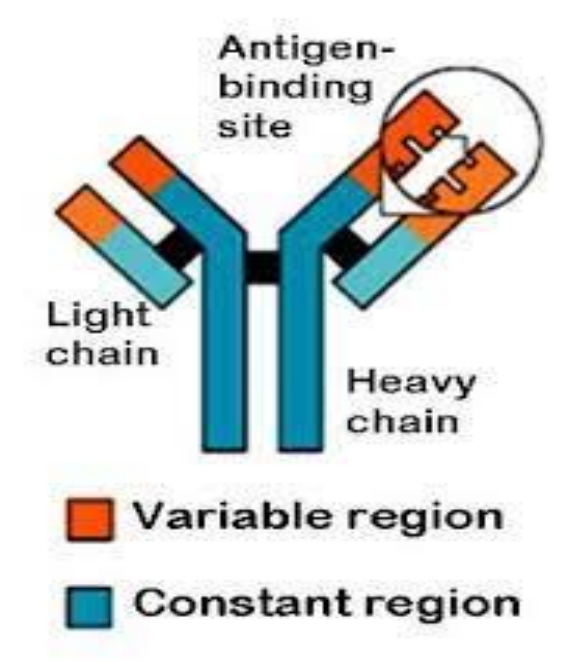
- Antibodies are glycoproteins secreted by specialized B lymphocytes known as plasma cells.

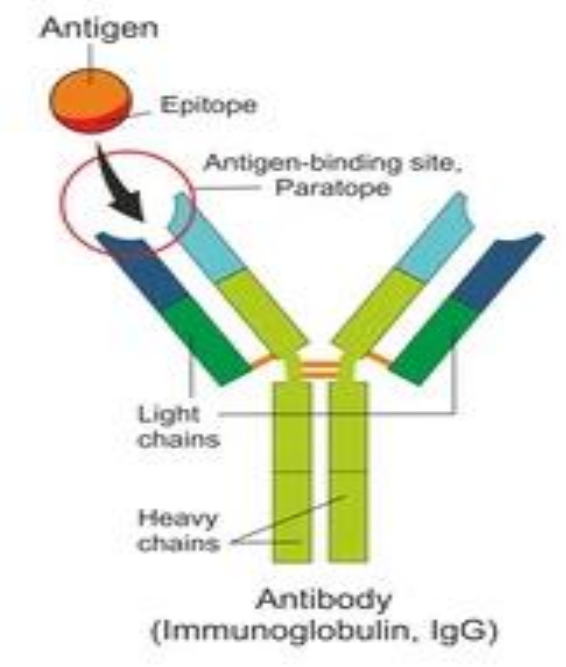
- Also referred to as immunoglobulin (Ig1), because they contain a common structural domain found in many proteins, antibodies are composed of four polypeptides.



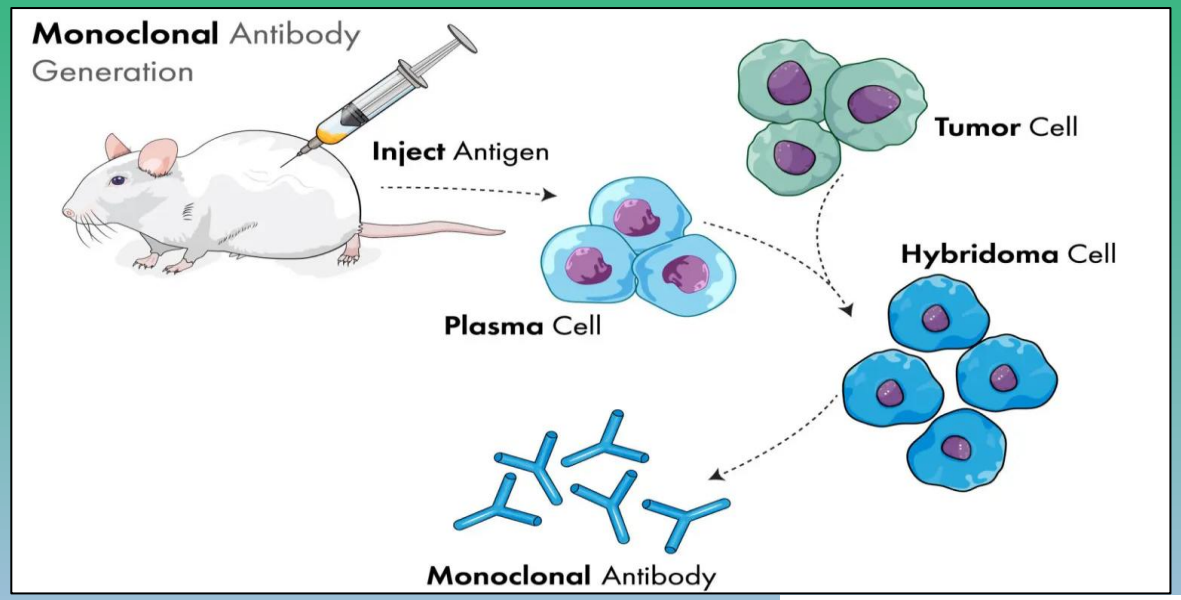
ANTIBODY

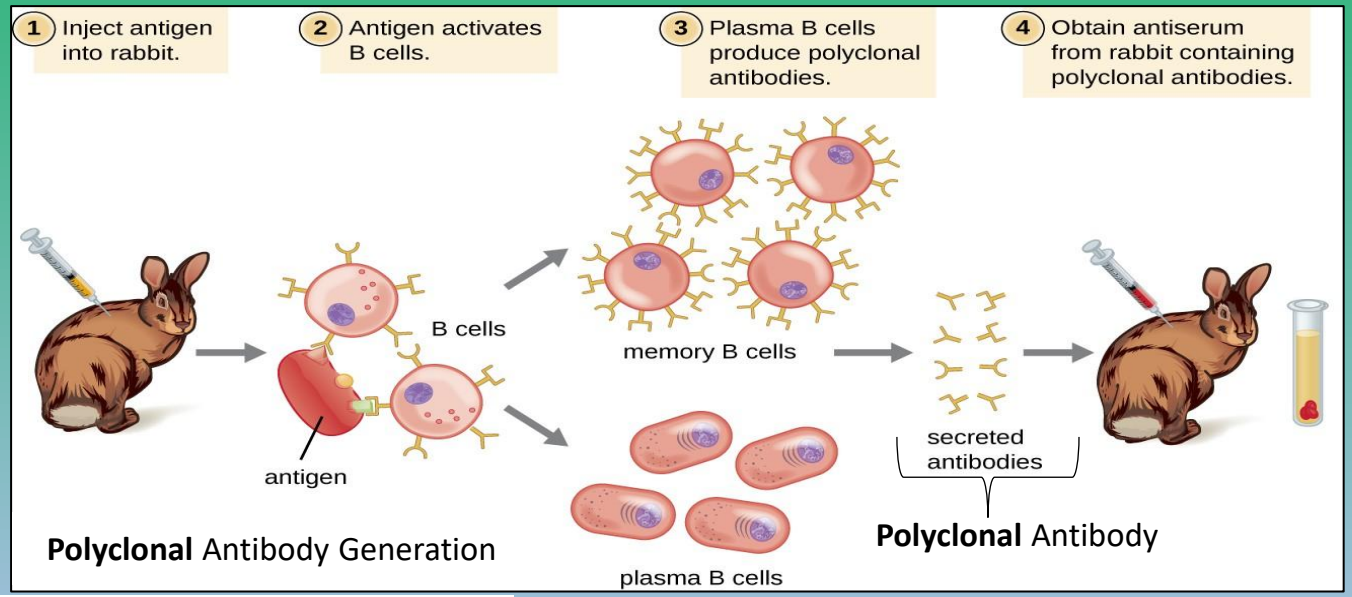
- Two identical copies of both a heavy (~55 kD) and light (~25 kD) chain are held together by disulfide bonds.
- An antibody is a protein component of the immune system that circulates in the blood, recognizes foreign substances like bacteria and viruses, and neutralizes them.
- A paratope is known as an antigen-binding site and is a part of an antibody (Ab) that recognizes and binds to an antigen.

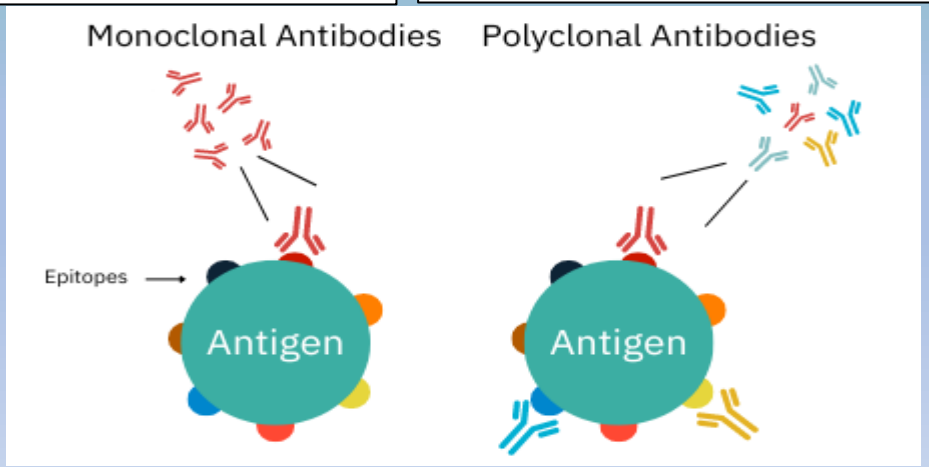




- Because most antigens are highly complex, they present numerous epitopes that are recognized by a large number of lymphocytes.
- Each lymphocyte is activated to proliferate and differentiate into plasma cells, and the resulting antibody response is **polyclonal**. In contrast, **monoclonal antibodies** (MAbs1) are antibodies produced by a single B lymphocyte clone.







- IHC employs a combination of histology, anatomy, immunology and biochemistry to detect the amount, distribution and localization of a specific target within a tissue.

- The antibodies against the molecule of interest, often a protein, are generated in an organism of a different species and are typically labeled or are aided by another set of labeled antibodies.

- The tissue samples are prepared following specialized techniques to enable the entry of the antibodies, and the label is detected using light or electron microscopy.

How does immunohistochemistry work?

- The success of immunohistochemistry depends on multiple parameters.
- Therefore, a scientific approach and careful experimental design is key to obtaining reliable and reproducible data.
- To give a brief summary of the steps involved, IHC is performed on thin slices of tissue obtained from the organism under study.
- These tissue sections are then processed to permit the entry of antibodies that will bind specifically to the antigen of interest.

How does immunohistochemistry work?

- Routinely, a second antibody will then be applied that binds specifically to the primary antibody and enables detection.

- In chromogenic detection methods, the secondary antibody is tagged with an enzyme that catalyzes a chromogenic reaction.

- In immunofluorescence detection methods, the secondary antibody is labeled with a fluorophore that can be directly observed under a fluorescence microscope.

- Let’s now consider the different parameters that need to be accounted for when performing IHC.

- **Tissue**

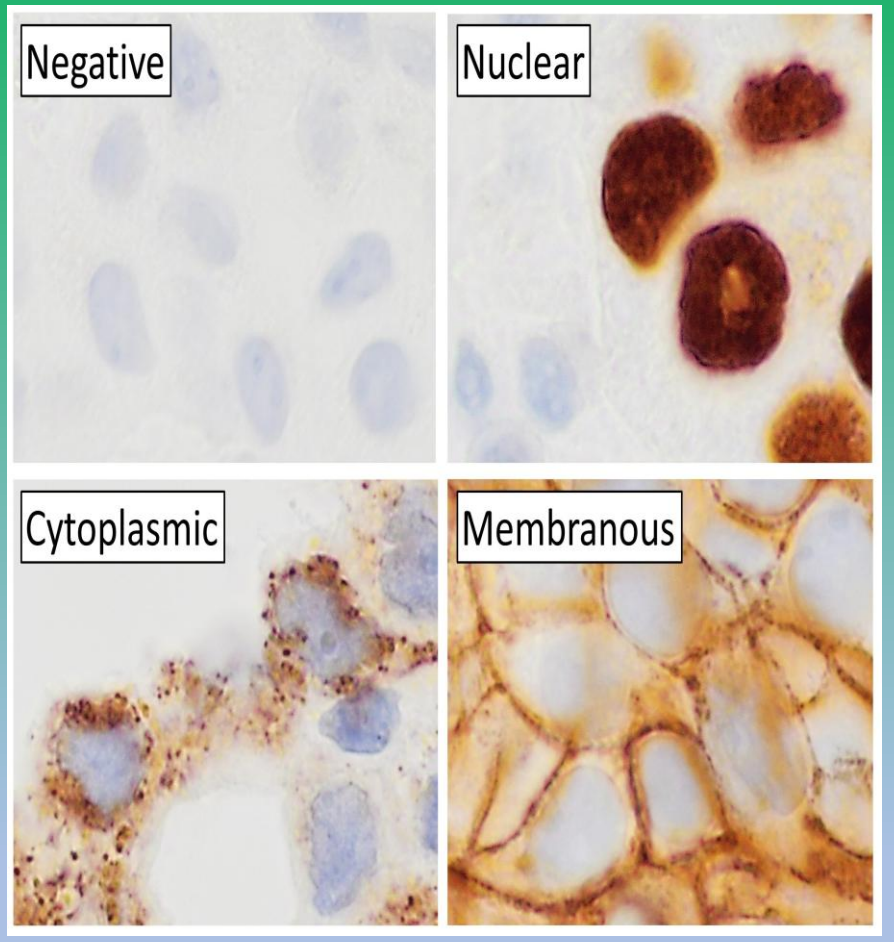
- The source of the tissue, i.e., the species of the organism of study and the organ of interest, will determine how the tissue is harvested and prepared for slicing.

- Prior to performing the experiments, it is vital to refer to literature pertaining to the specific organism to understand the techniques involved in harvesting the tissue and the special care that must be taken when performing IHC.

- Further, it is important to note that prior to fixation, all tissue must be handled in cold conditions and quickly to prevent rapid decay and drying.

- **Target**

- The levels and the subcellular localization of the target are extremely important in IHC experiment design.
- For example, a more abundantly expressed protein can be detected with less effort and a primary antibody tagged with a fluorophore can suffice for its detection.
- However, the detection of less abundant targets would require signal amplification methods.
- The localization of the target within the cell directly influences the degree of permeabilization required.
- Therefore, while a nuclear protein would require harsher surfactant-based permeabilization treatment, a cytoplasmic component may be detected using a comparatively milder treatment.
- Detection of intracellular membrane proteins may be achieved by freeze-thawing alone.

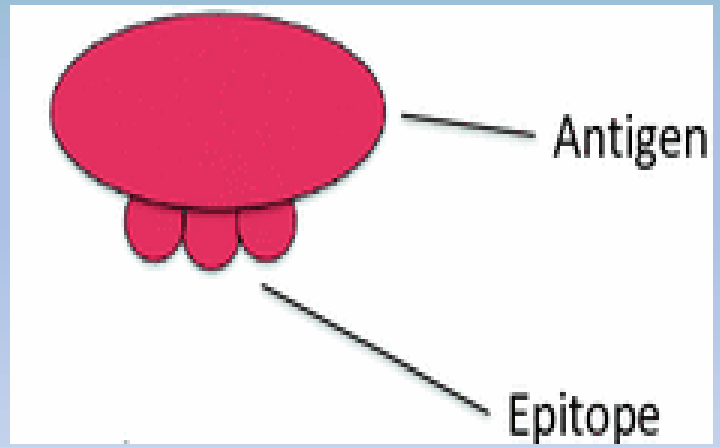


- **Epitope**

- The epitope is the small three-dimensional surface region of the antigen to which an antibody would specifically bind.

- It is important that the epitope to which the antibody would bind is exposed at the time of antibody addition during IHC.

- The epitope that may be recognized by the antibody can sometimes become masked during tissue processing and steps, such as antigen retrieval, may be necessary to expose the epitope.



- **Fixation methods**

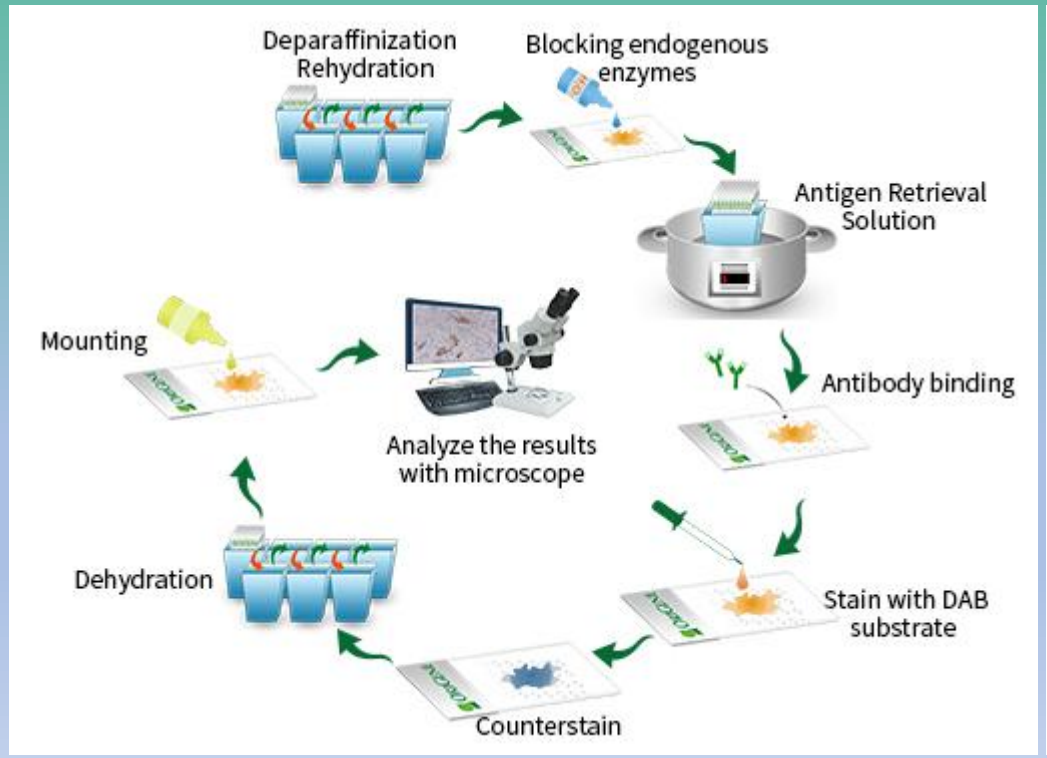
- Fixation refers to the preservation of the tissue morphology and cell structure in a stationary state by immobilization of the target. It prevents tissue degeneration and enables long-term storage.

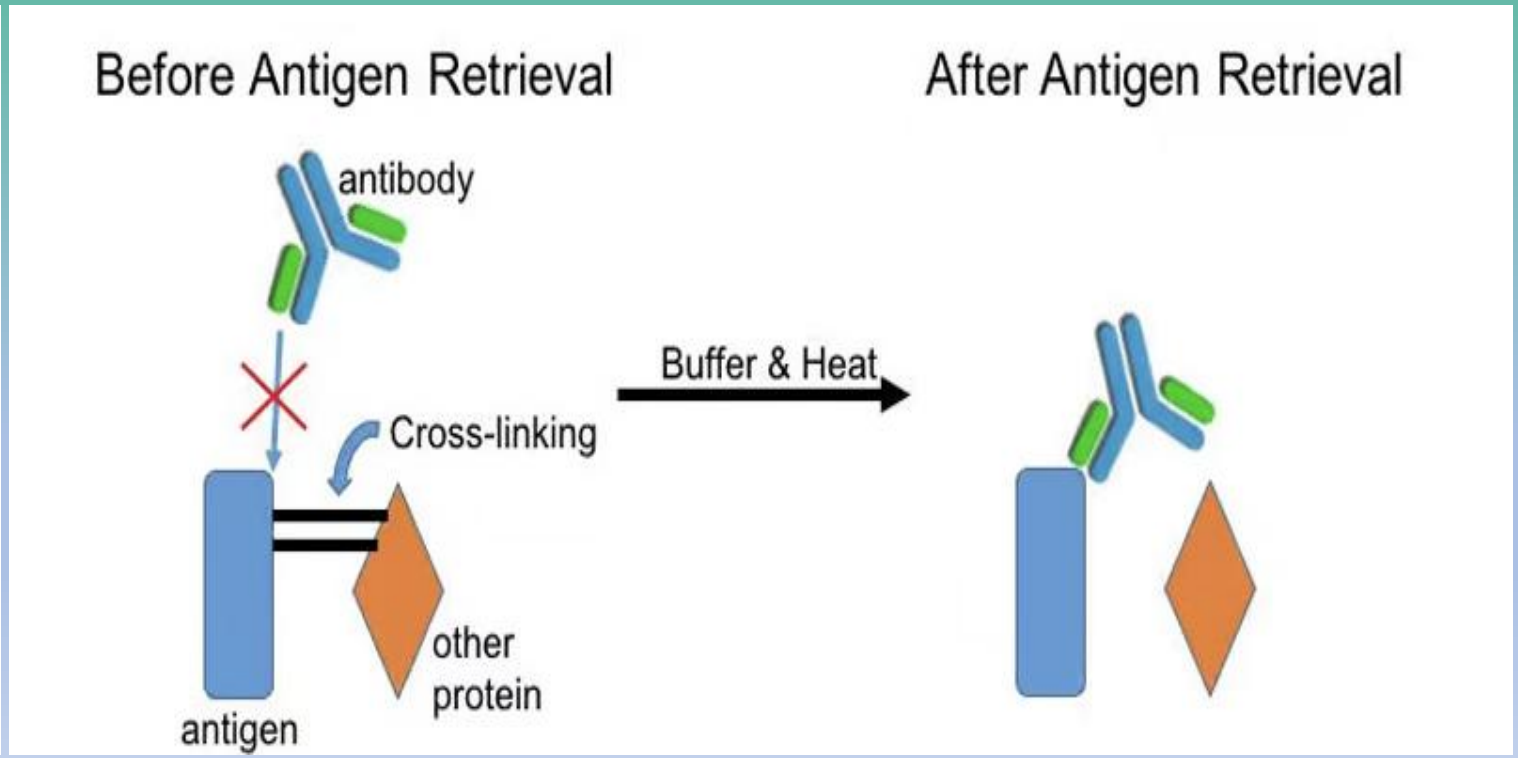
- Typically, soon after harvesting, the tissue is immersed in an appropriate fixative for several hours before it is further processed for sectioning.

- To minimize the time between tissue harvesting and fixation and to achieve uniform fixation, whole animal transcardial perfusion with the fixative is the preferred method of fixation in animal models, such as rodents.

- **Antigen retrieval methods**

- Formaldehyde-based fixation can often lead to masking of the antigen epitopes.
- Antigen retrieval is therefore required to unmask the epitopes and make them available for antibody binding and is often performed.
- This step is important when performing IHC for paraffin blocks, but can be too harsh for frozen tissue sections and may be omitted.
- Antigen retrieval can be achieved either by the application of heat (heat induced epitope retrieval: HIER) or through enzymatic degradation (proteolytic-induced epitope retrieval: PIER) in an appropriate buffer.



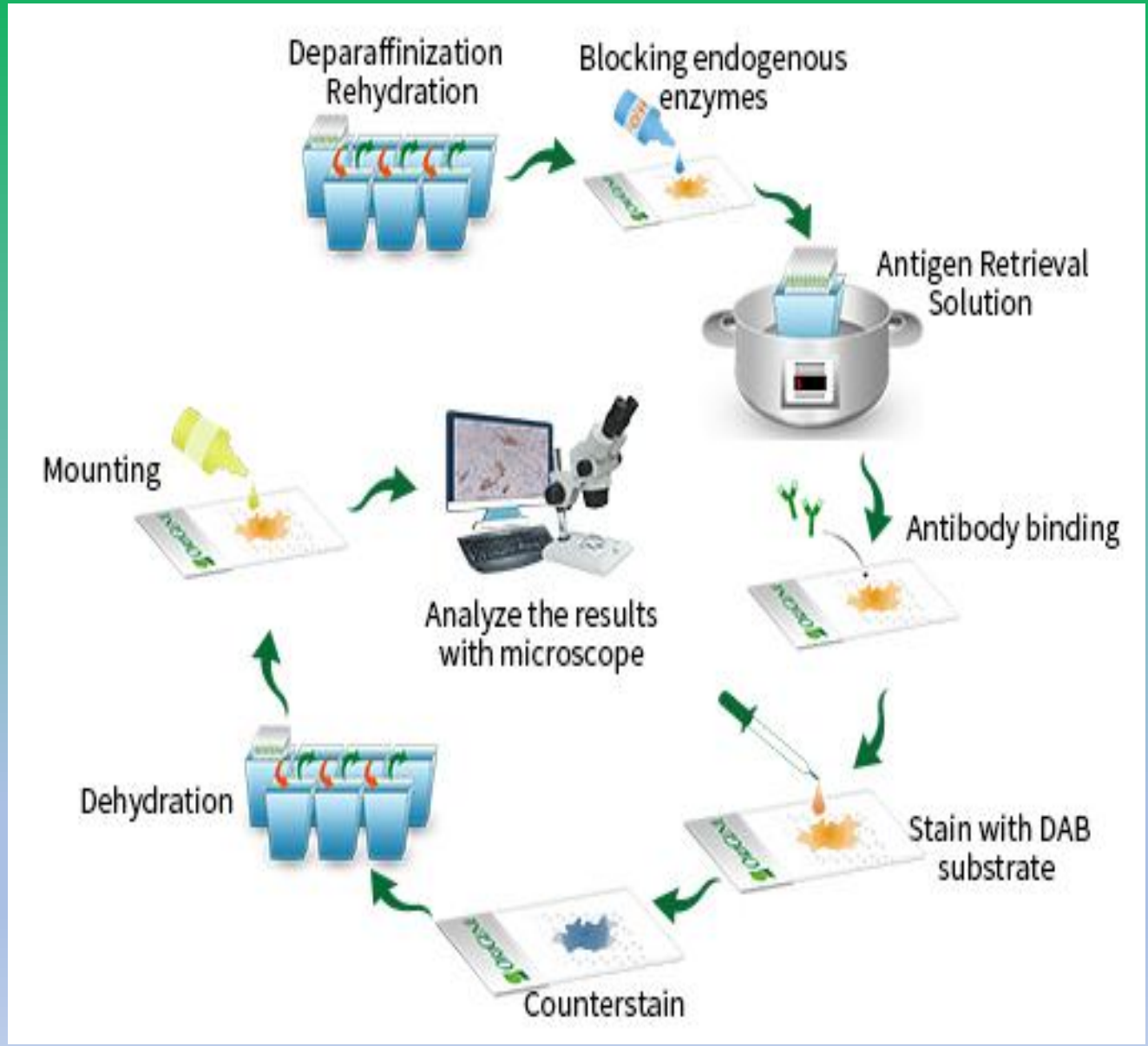


- **Permeabilization**

- Permeabilization is an essential first step that renders the plasma membrane of the cells in the tissue porous, thus allowing the entry of IHC reagents and antibodies.
- Routinely, surfactants such as Triton X-100, Tween-20, saponin and digitonin are used to achieve permeabilization.
- However, for a gentler permeabilization for preservation of intracellular membranes, the sections may be subjected to the freeze-thaw process.
- Fixatives, such as methanol and acetone, also permeabilize the tissue, and when using these fixatives this step may be omitted.
- The concentration of the surfactant and the time of incubation are determined based on factors such as the fixative used, thickness of the tissue section and the subcellular localization of the antigen of interest.

- **Blocking buffer**

- Although antibody–antigen binding can be very specific, some antibodies may adhere to certain non-specific cellular components due to various intramolecular forces at play.
- Incubation in blocking buffer before addition of the antibody helps to prevent the non-specific binding of the antibodies in the tissue.
- Commonly used blocking agents are normal serum and bovine serum albumin.
- When using chromogenic detection, blocking of endogenous enzyme activity is also required and can be achieved by using hydrogen peroxide to block endogenous peroxidase activity or levamisole to block endogenous alkaline phosphatase activity.



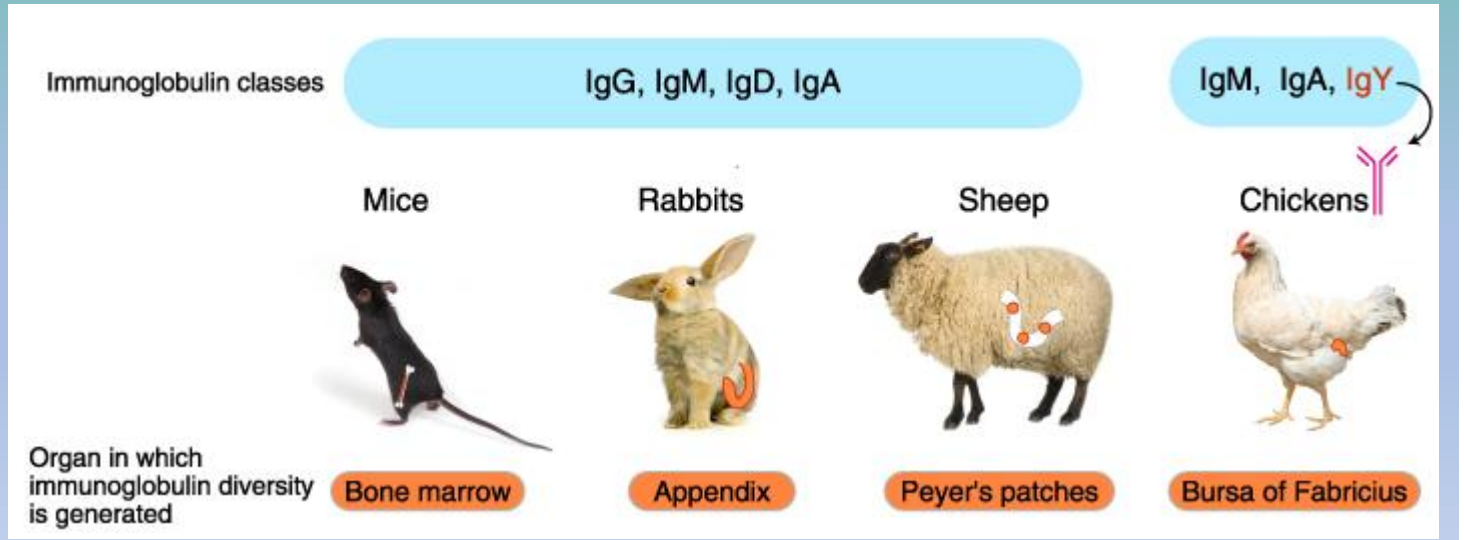
- **Detection method**

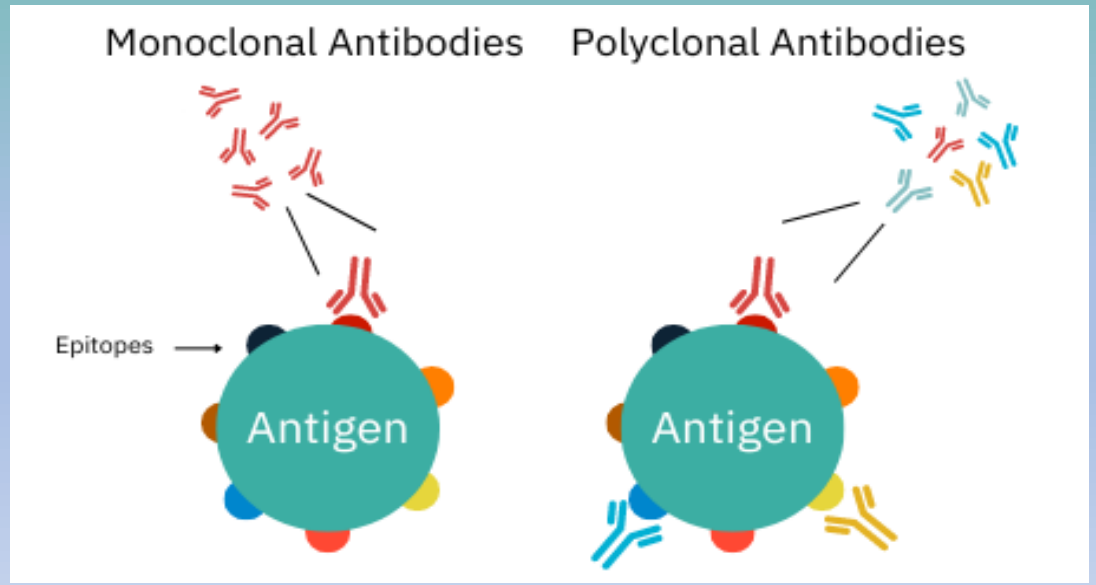
- The detection of the target antigen may be direct, where the label is directly attached to the primary antibody, or indirect, where the label or an enzyme that catalyzes a chromogenic reaction is attached to a secondary antibody.

- Further, signal amplification methods may be applied to enhance the sensitivity of signal detection.

- **Primary antibodies**

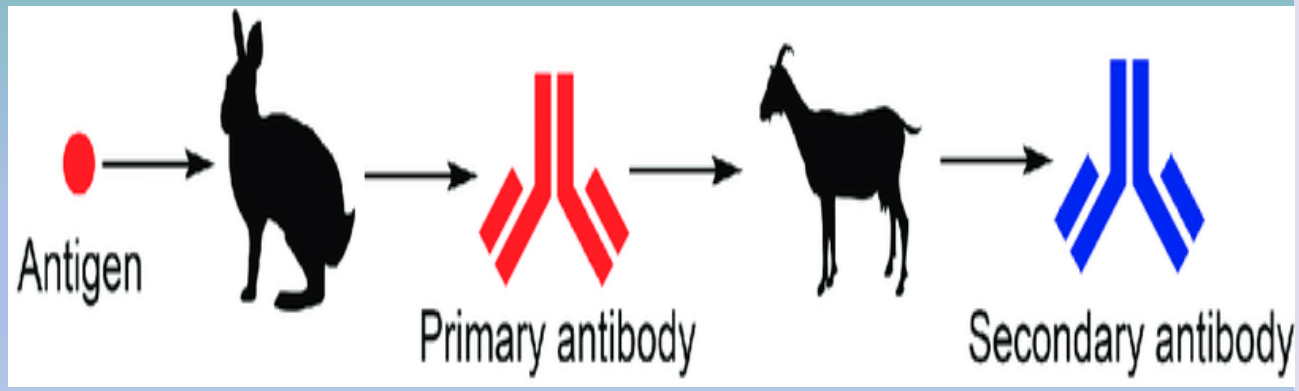
- The antibody that directly binds to the epitope of the antigen of interest is called the primary antibody.
- Primary antibody selection is an important step in IHC experimental design and one needs to ensure that the selected antibody is specific to the species under study.
- Specificity of the antibody against the target antigen also needs to be thoroughly evaluated. Primary antibodies may be polyclonal or monoclonal: while polyclonal antibodies consist of multiple individual antibody molecules that can recognize different epitopes of the same target, monoclonal antibodies all recognize the same single epitope.
- The concentration of the primary antibody needs to be determined through careful experimentation to achieve the best results.

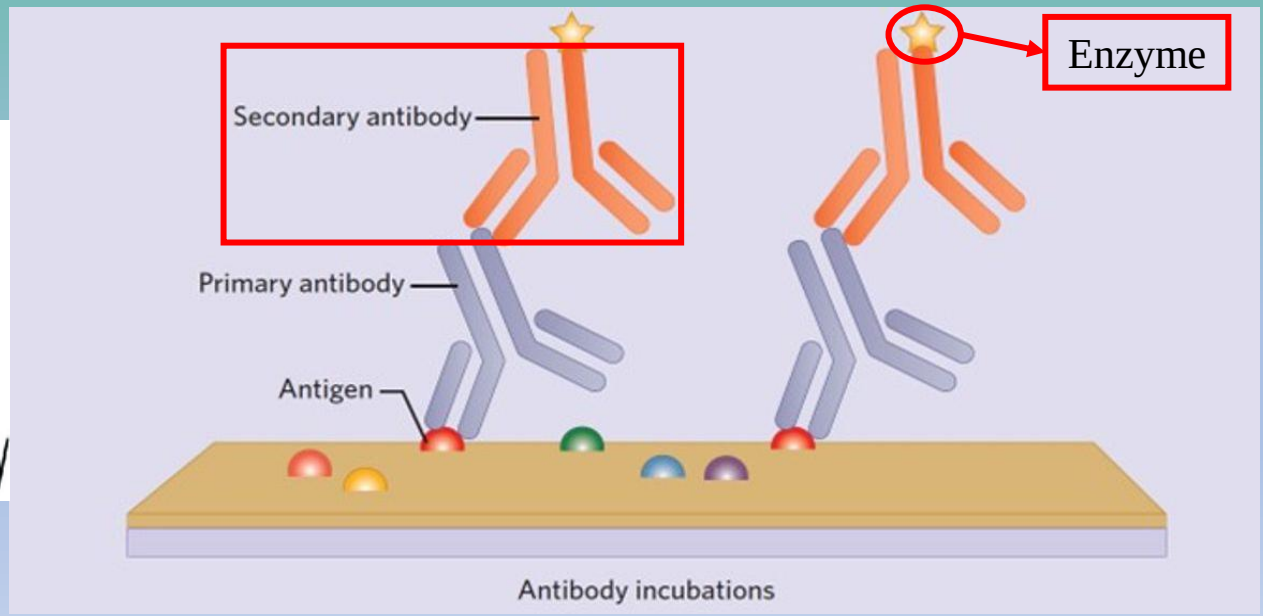




• Secondary antibodies

- The secondary antibodies are antibodies that recognize the primary antibody and thus enable detection of the target antigen.
- Secondary antibodies are often tagged with an enzyme to facilitate signal amplification for chromogenic detection, or they may be tagged with a fluorophore.





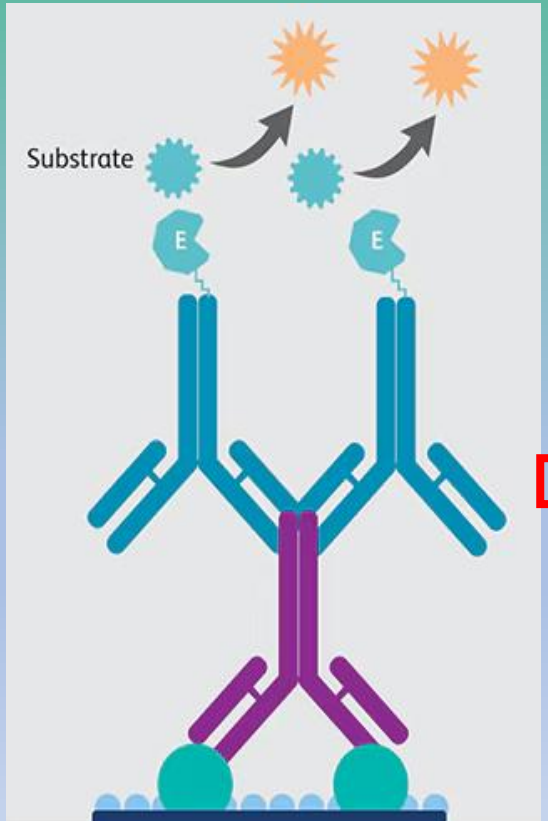
- **Signal amplification**

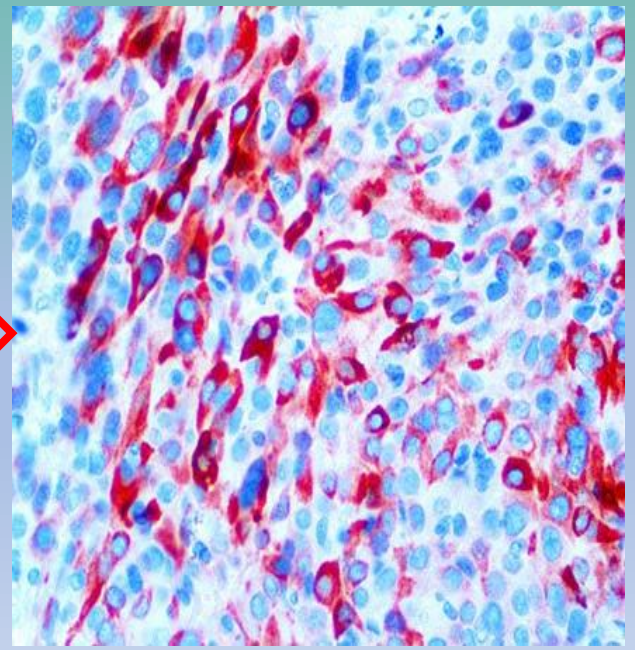
- For target antigens that are expressed at low levels, signal amplification may need to be performed to improve the sensitivity of the technique.

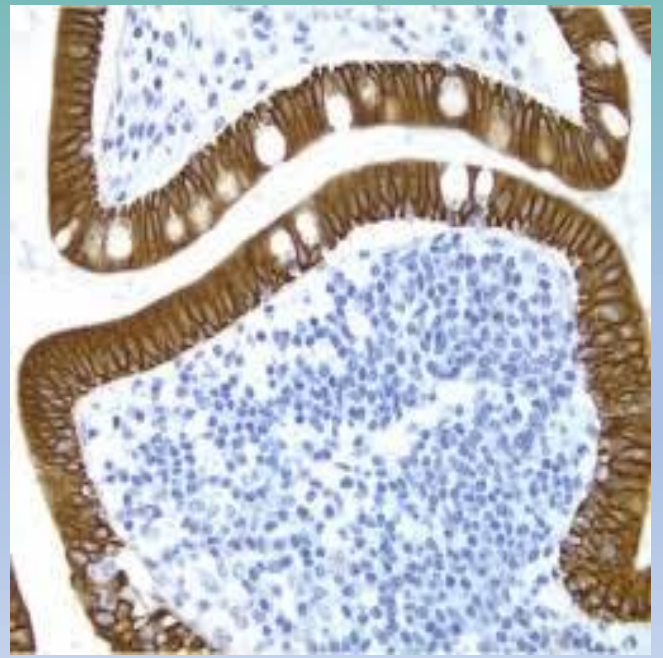
- Several signal amplification strategies that are used include, the avidin-biotin complex (ABC) method, labeled streptavidin-biotin (LSAB) method and tyramide signal amplification (TSA).

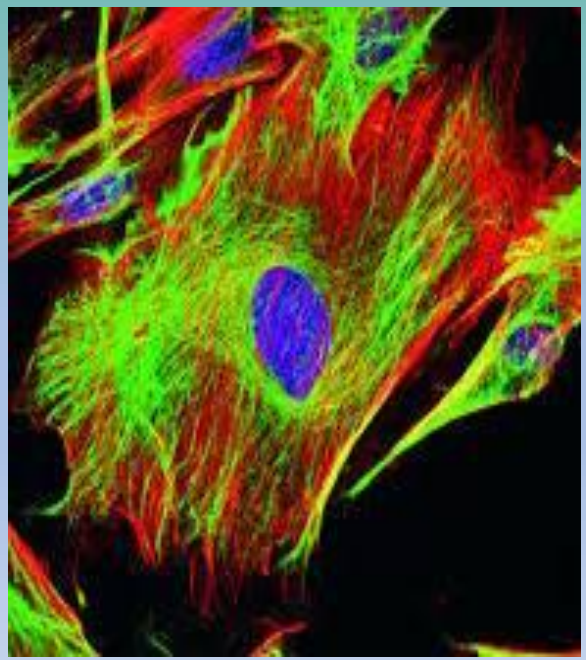
- **Label**

- Labels used to detect the target antibody are attached to the primary or secondary antibodies and may be either fluorogenic (as in the case of immunofluorescence) or chromogenic.
- Fluorogenic labels, such as fluorescein and tetramethylrhodamine (TAMRA), can be directly viewed under a fluorescence microscope.
- Chromogenic methods involve the conversion of a chromogenic substrate, such as 3,3’-diaminobenzidine (DAB) and 3-amino 9-ethylcarbazole (AEC), to a colored product in the presence of an antibody-conjugated enzyme such as, horseradish peroxidase (HRP) or alkaline phosphatase (AP).



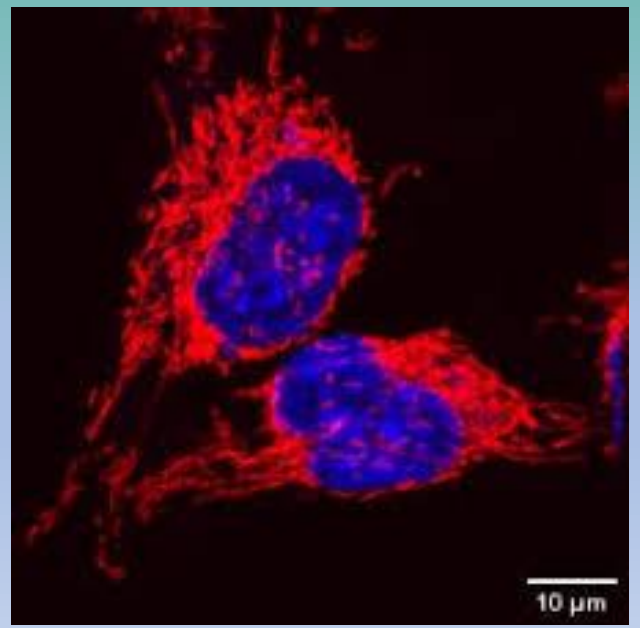


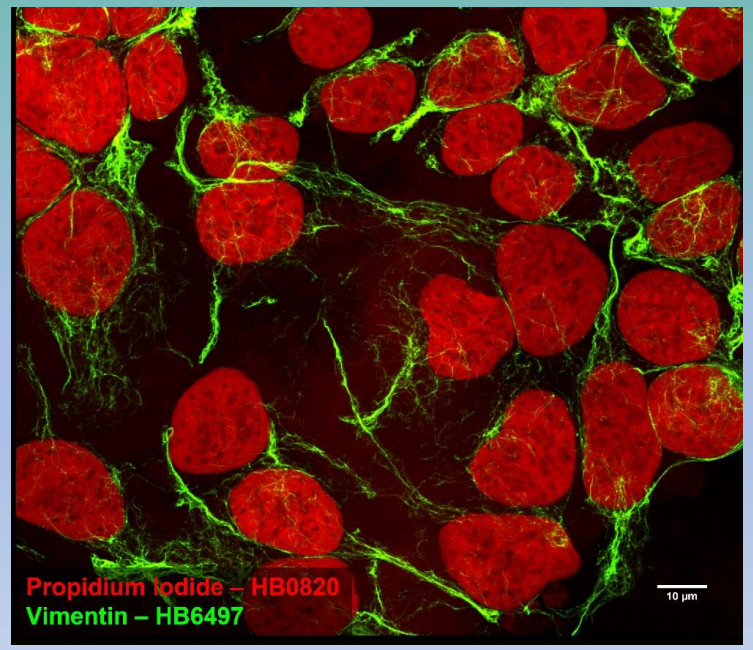


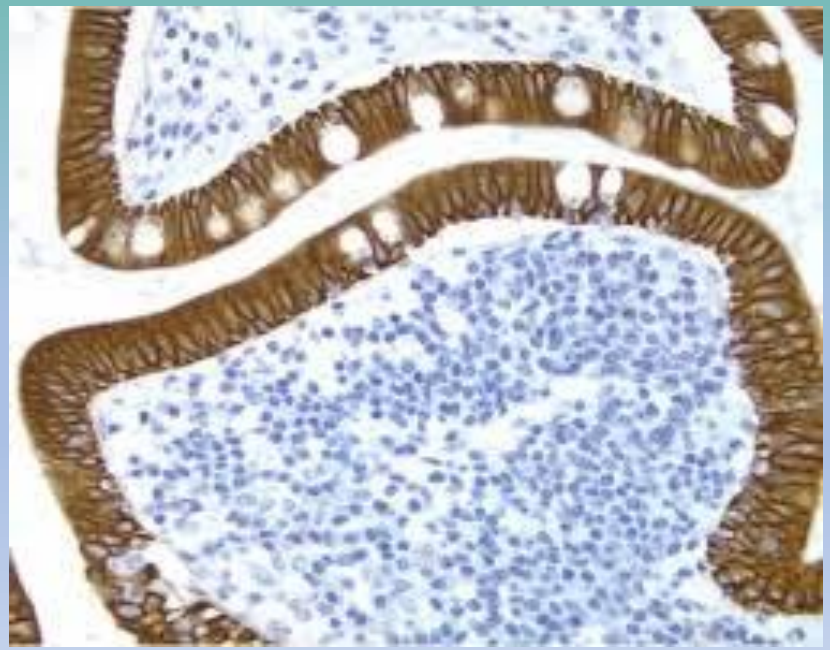


- **Counterstain**

- The tissue is often stained with a secondary nuclear or cytoplasmic stain that labels all the cells and helps visualize the IHC-labeled cells against the general morphology of the tissue, thus providing a contrast.
- Counterstains used with chromogenic IHC labels include hematoxylin, nuclear fast red and eosin; while those used with fluorogenic IHC labels include 4′,6-diamidino-2-phenylindole (DAPI), Hoechst33342 and propidium iodide.







- **Mounting**

- The prepared tissue sections are mounted in a medium with an appropriate refractive index that facilitates imaging under a microscope, protects the fluorescently labeled sections from photobleaching and prevents the section from drying.

- Commonly used mounting media include DPX, synthetic resins and glycerol-based mounting media containing antifade agents for fluorescently labeled sections.

- **Multiplexing**

- Multiplexing is performed when an experiment requires the concomitant investigation of more than one target antigen.

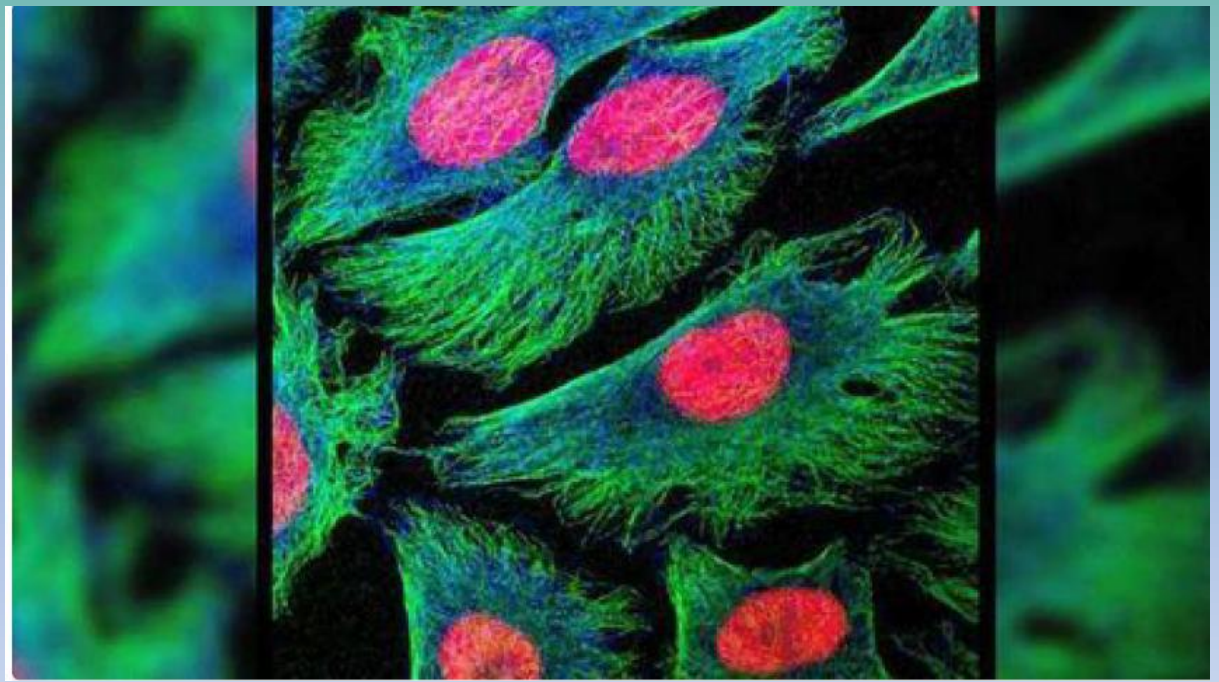
- Fluorophore labeling is the preferred method for multiplexing due to the availability of tags that fluoresce in the entire range of the visible spectrum and adjacent wavelengths.
-
- Special care must be taken to prevent the cross-reactivity of secondary antibodies when performing multiplexing.

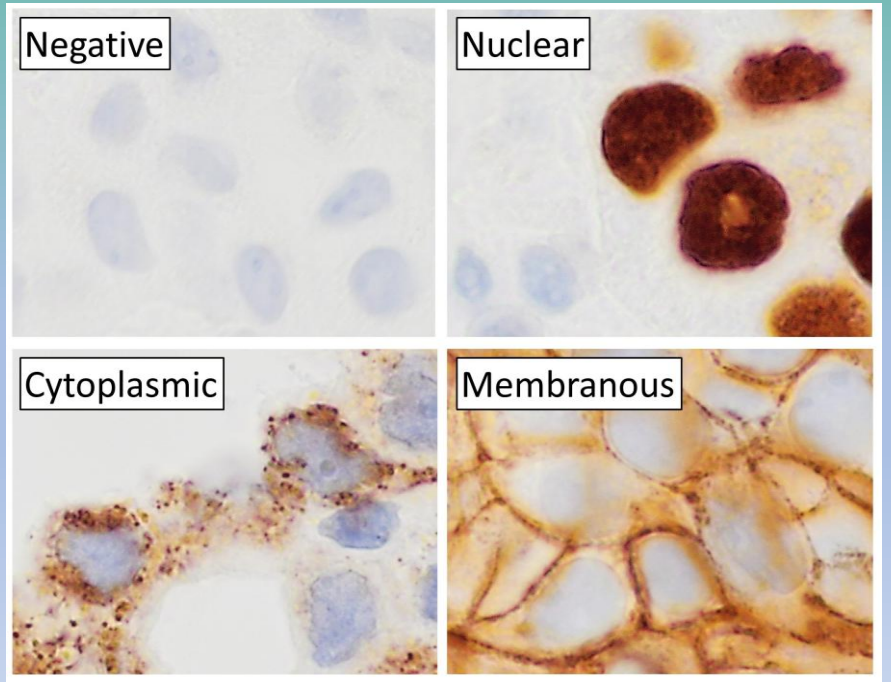
- **Imaging method**

- Chromogenic labels can be detected using a light microscope.

- Fluorescence or confocal microscopes are used for the detection of fluorophores.

- Electron microscopy may be used for imaging after immunohistochemical labeling with colloidal gold particles.





- **Controls**

- Proper controls are indispensable to ascertain the authenticity of the results from an IHC experiment.

- A tissue sample in which the expression of the target antigen is known can serve as a positive control.

- Similarly, a sample wherein the target antigen is known to be absent can be used as a negative control.

- Additionally, antibody controls need to be used to verify the specificity of the antibody.

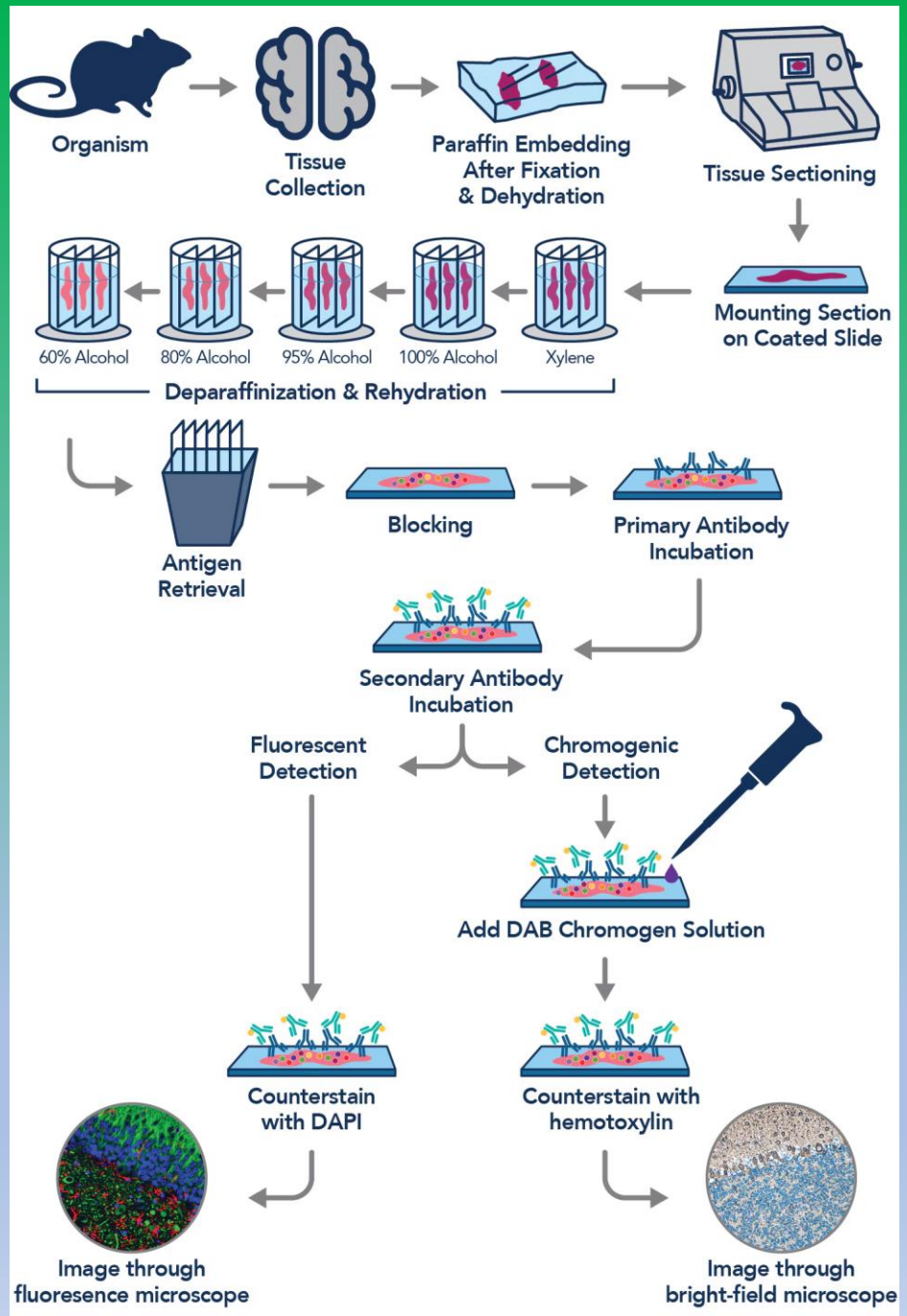

Organism
Tissue Collection
Paraffin Embedding After Fixation & Dehydration
Tissue Sectioning
Mounting Section on Coated Slide
60% Alcohol
80% Alcohol
95% Alcohol
100% Alcohol
Xylene
Deparaffinization & Rehydration
Antigen Retrieval
Blocking
Primary Antibody Incubation
Secondary Antibody Incubation
Fluorescent Detection
Chromogenic Detection
Add DAB Chromogen Solution
Counterstain with DAPI
Counterstain with hemotoxylin
Image through fluoresence microscope
Image through bright-field microscope

Table 1. Strengths and Limitations of IHC

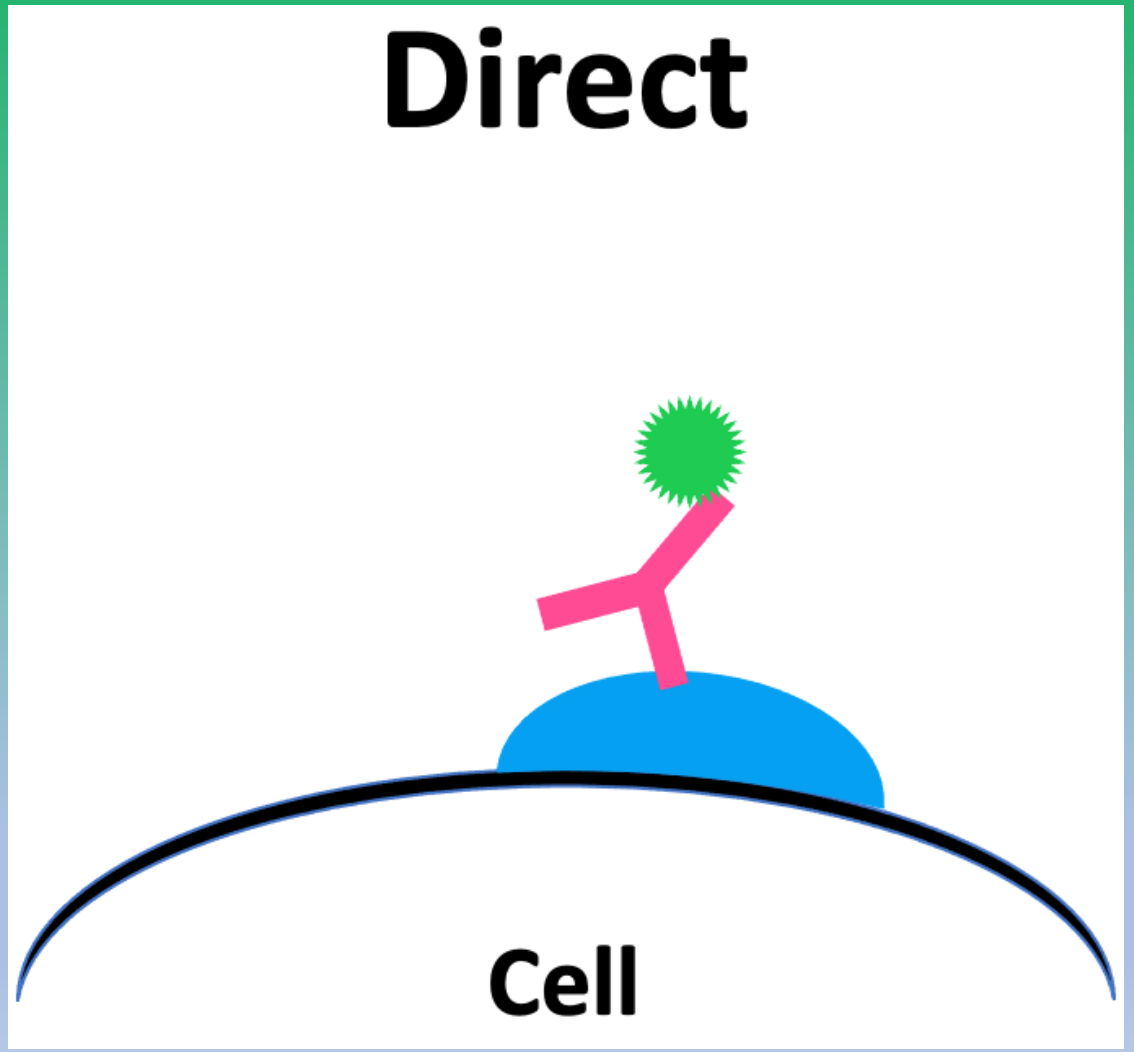
Strengths	Limitations
*Affordable and simple procedure that can be performed with few resources	➢ Specificity of antibodies can be variable and needs to be thoroughly checked using appropriate controls
*Powerful technique to study localization and presence/absence of a target at the tissue and cellular level	➢ The method is semi-quantitative, and the absolute abundance of the target cannot be reliably determined
*Paraffin embedded and frozen tissue samples can be stored and accessed when required	➢ Tissue is highly processed and may lead to loss of information of the natural state
*Stained tissue sections can be stored and referred to whenever required	➢ IHC is a multi-step procedure and variability can be introduced at any stage leading to poor reproducibility of results

IHC METHODS

1) DIRECT IHC	2) INDIRECT IHC

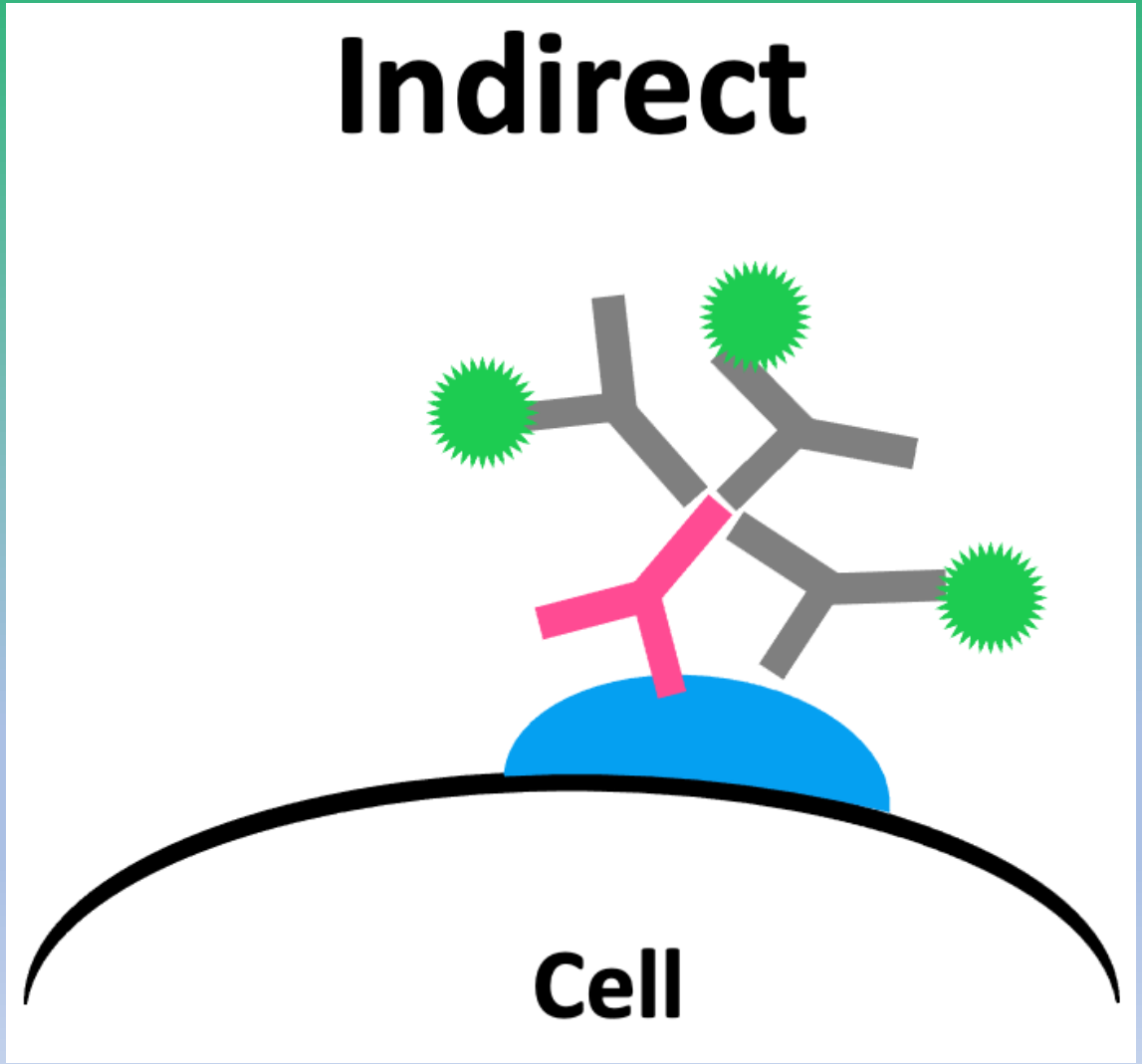
Direct IHC

- Direct methods are generally one step and involve the use of directly labelled antibodies to detect antigens of interest within the tissue.
- Whilst these techniques are straightforward and less time-consuming, they are usually less sensitive and lack the ability to amplify weak signals.
- Due to this fact, it is not as widely used as other methods.
- With direct IHC, your primary antibody is supplied already labelled.
- The range of labelled primary antibodies for use in direct IHC is also comparatively limited.

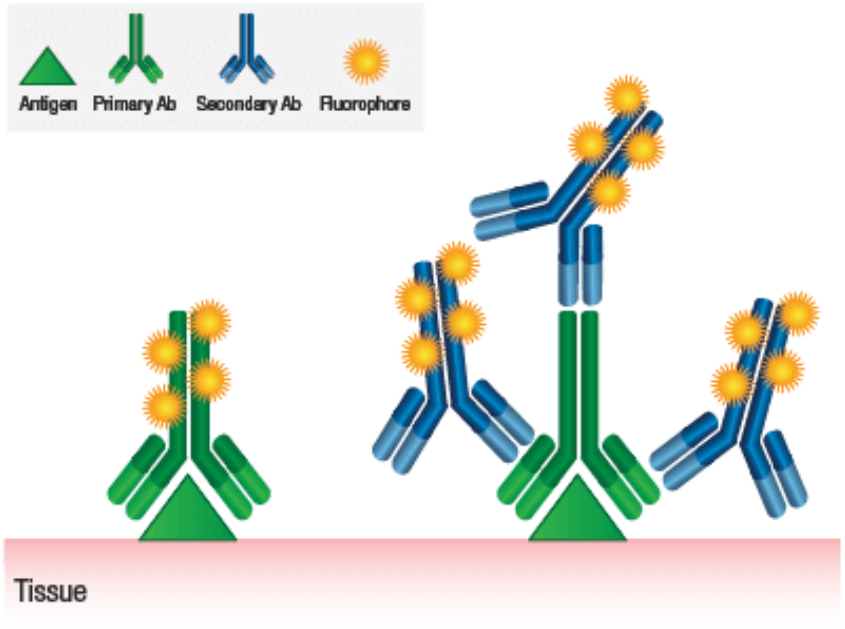


Indirect IHC

- The indirect method utilizes an unlabelled primary antibody to detect the antigen of interest in the tissue.
- A secondary labelled antibody is then used to bind to the primary antibody.
- This method is useful as it amplifies relatively weak antigen signals in tissue as many secondary antibodies can bind to different antigenic sites of the primary antibody.
- There is also a greater choice of labelled secondary antibodies and these can obviously be used with many different primary antibodies (species-dependent).



- **Blocking**
- Two things to remember with indirect IHC: the first is obvious- use a secondary antibody which is raised against the species of the primary antibody.
- The second- always use a normal serum to ‘block’ endogenous sites which is of the same species as the secondary antibody you are using (ie- if the secondary is a rabbit-anti-mouse, then you should use normal rabbit serum).
- The use of the normal serum prevents any false positive results in your staining as it blocks any non-specific sites which your secondary antibody may bind to. The blocking step is usually carried out before applying the primary antibody.
- Furthermore, if you are using a horseradish peroxidase (HRP) you should be aware that many tissue types contain endogenous peroxide sites. To check if your tissue of interest contains these sites, simply incubate a fixed slide with the HRP substrate (3,3′-Diaminobenzidine, or ‘DAB’).
- To block against endogenous peroxide sites, sections should be incubated with a solution of hydrogen peroxide prior to the application of the primary antibody



Antigen Detection	Direct immunofluorescence with primary antibody conjugated to a fluorophore	Indirect immunofluorescence with secondary antibody conjugated to a fluorophore
Protocol	Parallel staining	Parallel staining
Primary Antibody	Same host species can be used for multiple targets	Different host species or isotype for each target
Seconday Antibody	No	Yes
Signal Amplification	None	Moderate

What is immunohistochemistry used for?

- Immunohistochemistry is a simple and cost-effective technique that has become an important tool for scientists. Some applications of this technique are listed here:

  - **Biomarker assessment in oncology**

- IHC is a very popular tool for detection of biomarkers in cancer diagnosis as well as for the development of new biomarkers. IHC allows tumor detection, staging and classification, in addition to predicting tumor prognosis and understanding the response of tumor to treatment paradigms. IHC-based biomarkers have become vital for the diagnosis and treatment of breast cancer, prostate cancer, pancreatic cancer, lung cancer, bladder cancer, colorectal cancer and ovarian cancer.

What is immunohistochemistry used for?

- **Diagnoses of infectious diseases**

- IHC serves as an important tool for detection and identification of pathogenic antigens in tissue samples from infected individuals, which can help in the treatment of infectious diseases.

- Bacterial pathogens such as, *Bartonella quintana*, *Yersinia pestis*, *Treponema pallidum*, *Chlamydia trachomatis*; viral pathogens such as, human herpesvirus type 8 (HHV8), Epstein-Barr virus (EBV), human immunodeficiency virus (HIV); fungal pathogens such as, *Candida albicans* and *Cryptococcus neoformans* var. gattii; and protozoal pathogens such as, *Plasmodium falciparum* and *Trypanosoma cruzi* have been successfully identified using IHC.

- **Evaluating neurodegenerative disorders**

- Abnormal protein conformations and aggregations can be identified and evaluated using IHC and are a routine feature of many neurodegenerative disorders including, Alzheimer’s disease (AD), chronic traumatic encephalopathy (CTE), progressive supranuclear palsy (PSP), Pick’s disease, Lewy body disease, multiple system atrophy (MSA).

- **Human Protein Atlas**

- IHC has contributed to the age of big data by enabling the mapping of the human proteome. The Human Protein Atlas is a freely available and valuable resource that contains tissue level information of expression encompassing 90% of all protein encoding genes.