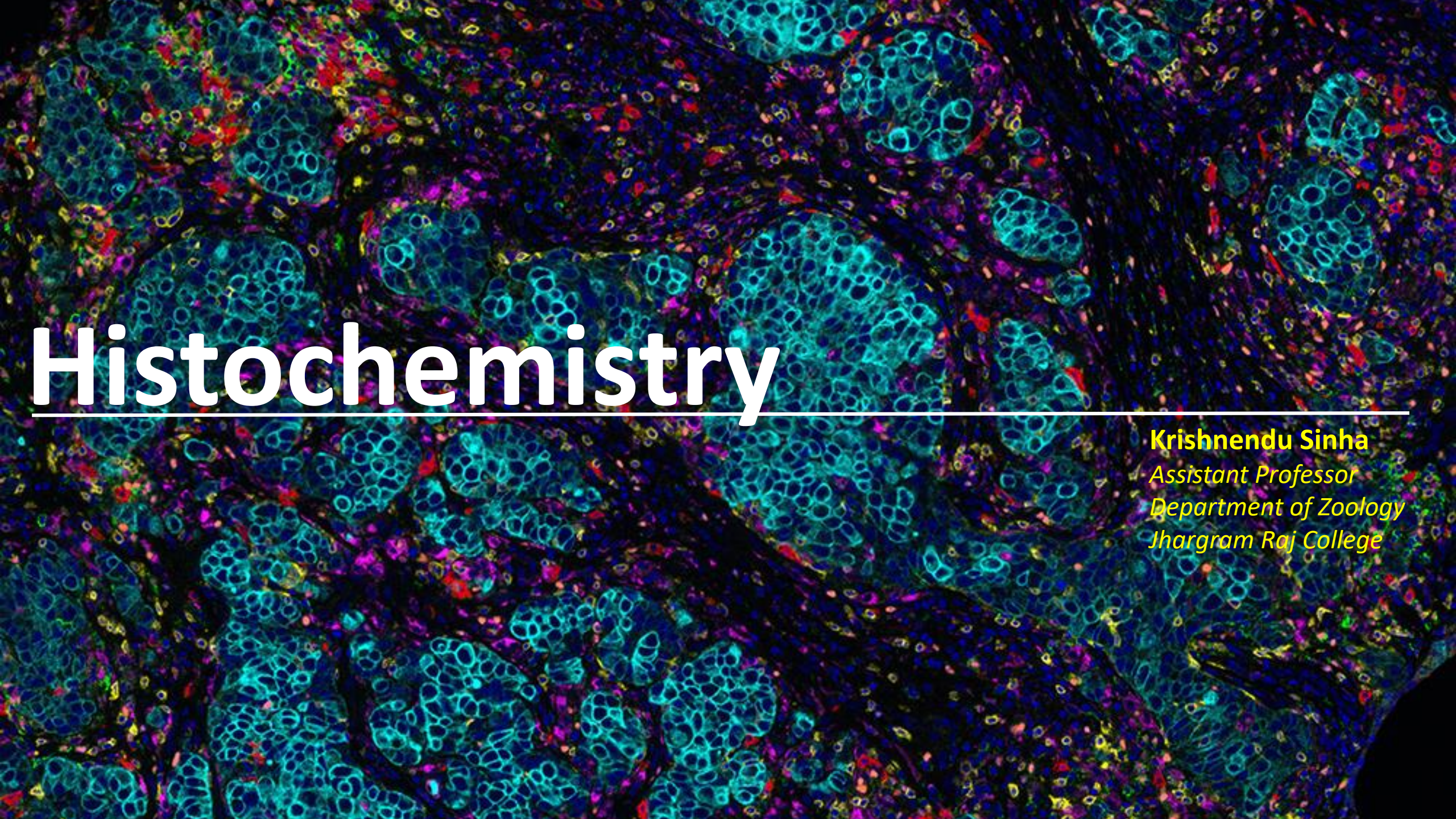


Histochemistry

Krishnendu Sinha
Assistant Professor
Department of Zoology
Jhargram Raj College

Histochemistry is a science that combines the techniques of *biochemistry* and *histology* in the study of the chemical constitution of (cells and) tissues

Enzyme Histochemistry

Enzymes are labile and their preservation is important

Fixation for enzyme histochemistry

Cryofixation

freezing and subsequent thawing of blocks or sections damages **lysosome**

Hydrolytic enzymes show considerable diffusion if demonstrated on frozen, unfixed sections

Chemical fixation

Chemical fixation

Fixation will destroy many of the oxidative enzymes

Cryofixation

Cryofixation will preserve many of the oxidative enzymes

Cryosectioning and staining

Tissues to be used for the demonstration of the majority of hydrolytic enzymes may be subjected to controlled fixation

for the demonstration of hydrolytic enzymes, histochemical techniques are usually applied to sections that have been prefixed in **cold (4°C) formal calcium**

decreasing the amount of demonstrable enzyme within the section, does allow for a **considerably sharper localization** of the **remaining enzyme activity**

Mitochondria → Contains most of the oxidative enzymes and damaged immediately after blood supply cut

Lysosome → Contains most of the hydrolytic enzymes and damaged on freeze thaw

Smears

- ✓ In enzyme histochemistry, the use of smears for cytochemical identification and evaluation of cells is used
- ✓ Preparation may be achieved in various ways, include **blood, bone marrow and tissue cell suspensions**
- ✓ **Three** of the most useful enzymes are **non-specific esterase, acid phosphatase and chloroacetate esterase**
- ✓ It is usual to **fix smears before histochemical staining** to preserve cell structure and enzyme localization

Enzyme types

Oxidoreductases

- **Oxidases:** catalyze oxidation of a substrate in the presence of oxygen.
- **Peroxidases:** catalyze oxidation of a substrate by removing hydrogen, which combines with hydrogen peroxide.
- **Dehydrogenases:** catalyze oxidation of a substrate by removal of hydrogen.
- **Diaphorases:** catalyze oxidation of NADH and NADPH by removal of hydrogen.

Transferases

These catalyze the transfer of the radicals of two compounds without the loss or uptake of water.

Hydrolases

These catalyze the introduction of water or its elements into specific substrate bonds, although in some instances water may be removed. These enzymes include:

- Phosphatases (acid, alkaline and specific)
- Esterases
- Lipases
- Glycosidases
- Peptidases
- Pyrophosphatases.

Classical histochemical reactions are generally based on one of the 4 principles:

1.Simple ionic interactions

2.Reactions of aldehydes with Schiff's reagent or silver compounds

3.Coupling of aromatic diazonium salts with aromatic residues on protein

4.Conversion acting on a substrate to form a colored ppt

Types of histochemical reactions

1. Simultaneous capture

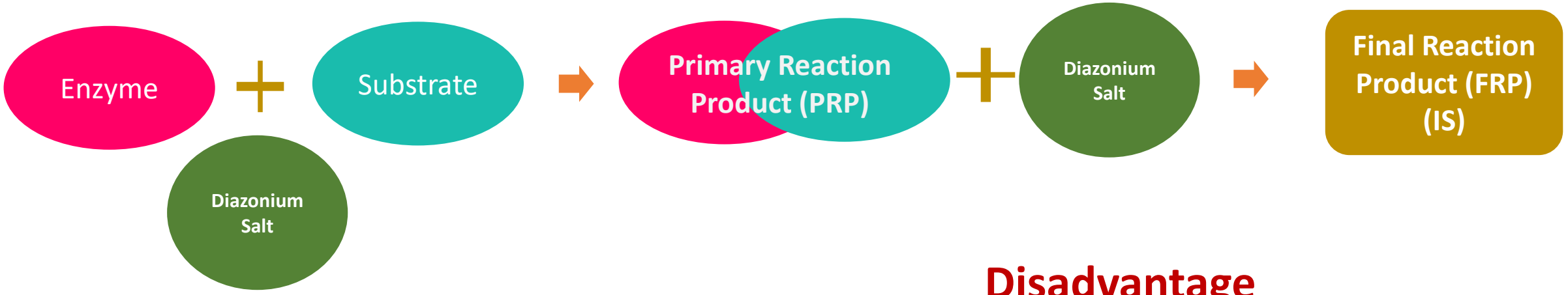
2. Post incubation coupling

3. Self coloured substrate

4. Intramolecular rearrangement

1. Simultaneous capture

-Gomori's Metal ppt. technique
-Azo dye method



Advantage

1. A great repository of alternative substrates
2. Highly sensitive

Disadvantage

1.Diffusion of PRP

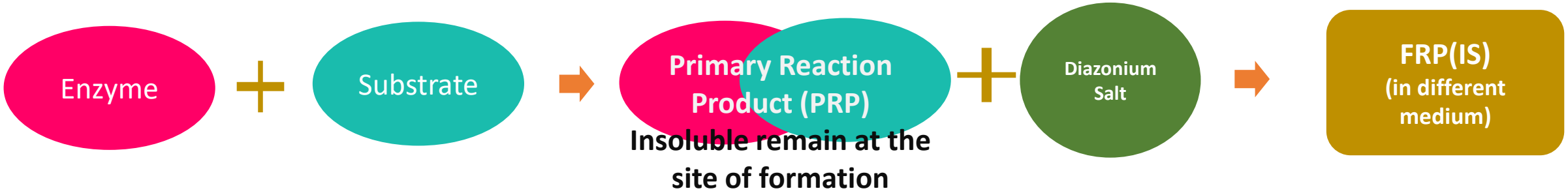
2.Rate of hydrolysis of substrate

3.Diffusion coefficient of the PRP for the buffer

4.Rate of coupling of the PRP and diazo salt

5.Diazo salt and Enzyme same pH

2. Post incubation coupling



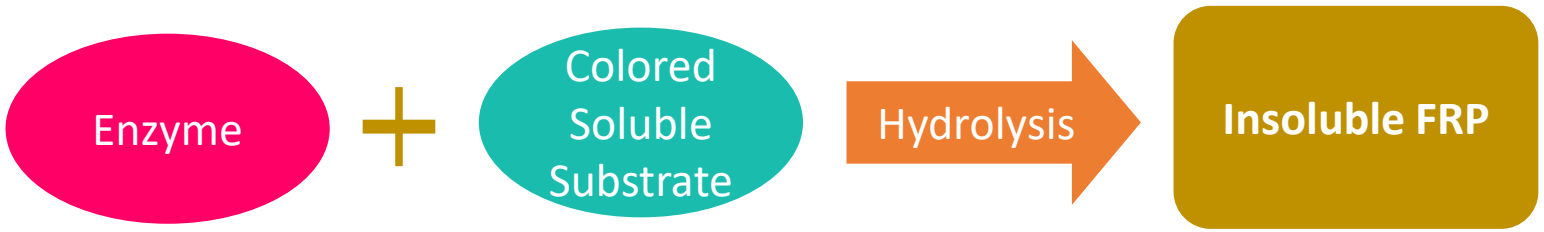
Advantage

1. Case where long first incubation stage is necessary
2. Optimum pH for enzyme and for diazonium salt separately

Disadvantage

1. PRP is not completely insoluble
2. Diffusion is always there

3. Self coloured substrate



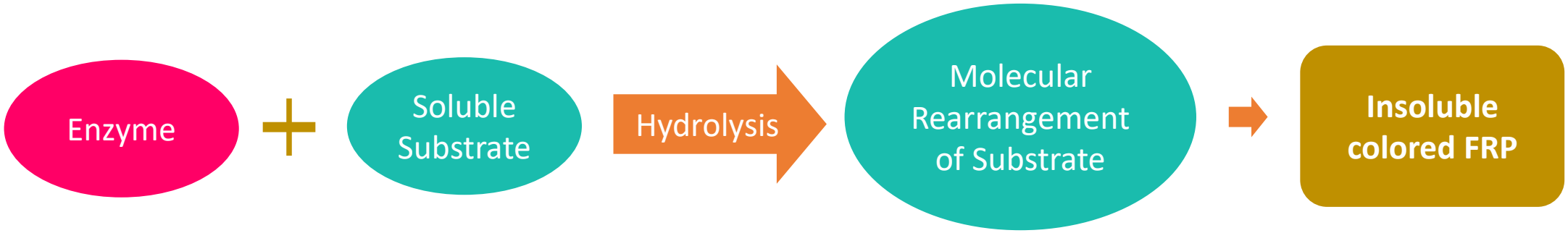
Advantage

1. Diazonium coupling not required

Disadvantage

1. Limited number of substrates are available

4. Intramolecular rearrangement



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Disadvantage

1. Limited number of substrates are available

Diagnostic applications

The current common uses of enzyme histochemistry in surgical histopathology laboratories can be summarized:

- **skeletal muscle biopsy**
- **colonic biopsy in cases of suspected Hirschsprung's disease**

Skeletal muscle biopsy

The application of enzyme histochemical methods to **cryostat sections** of **unfixed skeletal muscle** shows the presence of **different fiber types, and changes in the number, size and relative proportions** of the different fibers are valuable in establishing the diagnosis

Methods in common use for muscle biopsy diagnosis:

- **Adenosine triphosphatase (ATPase)**
- **Cytochrome oxidase (COX)**
- **NADH diaphorase**
- **Phosphorylase (after Meijer 1968**)

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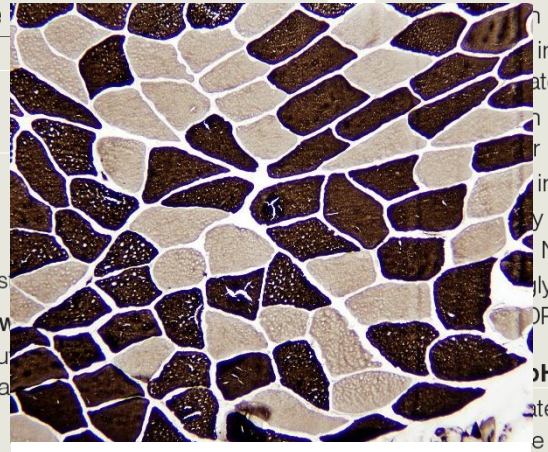
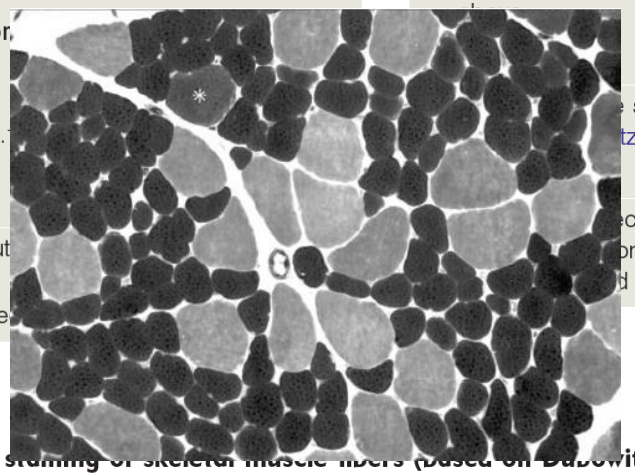


Normal skeletal muscle, ATPase stain x 100



Muscle biopsy of **Congenital fibre type disproportion (CFTD)**. ATPase at pH 4.63, showing predominance and smallness of type 1 fibres (darker fibres). Clear fibres are of type 2. Note that only one of the type 2 fibres belongs to subtype 2B (asterisk).

Colonic biopsy in cases of suspected Hirschsprung's disease

- In the normal colon and rectum, there are ganglion cells in both the submucosa and the so-called myenteric plexus between the circular and longitudinal muscle of the outer bowel wall

- These ganglia, and their associated nerves, are responsible for colonic motility

- In Hirschsprung's disease in children, a variable segment of the rectum and colon is devoid of ganglion cells ('aganglionic segment')

- In the affected segment peristalsis is impossible and the large bowel becomes obstructed

- The diagnosis may be suspected clinically and radiologically but requires histological confirmation, usually by the examination of one or more suction biopsy specimens of rectal mucosa and submucosa with the aid of the enzyme histochemical method, acetylcholinesterase.

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Acetylcholinesterase *(from Filipe & Lake 1983)*

Preparation of tissue

Cryostat sections of snap-frozen tissue cut at 10 µm are air-dried and fixed for 30 seconds in 4% formaldehyde in 0.1 M calcium acetate (formal calcium).

Frozen sections of formal calcium-gum sucrose treated blocks of tissue.

Incubation medium

Acetylthiocholine iodide	5 mg
0.1 M acetate buffer, pH 6.0	6.5 ml
0.1 M sodium citrate	0.5 ml
30 mM copper sulfate	1 ml
Distilled water	1 ml
4 mM iso-octamethyl pyrophosphoramide (iso OMPA)	0.2 ml

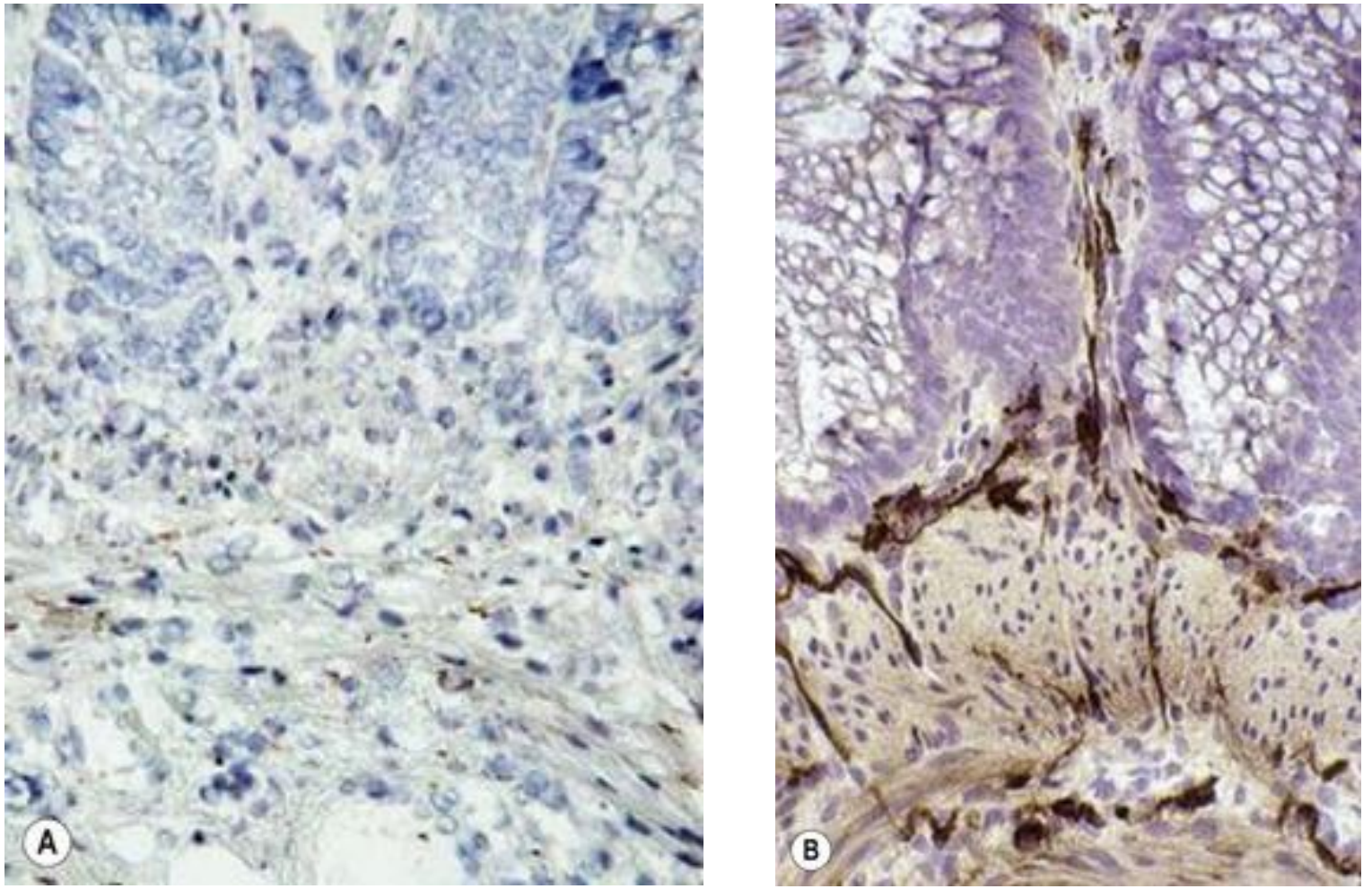
Add 1.0 ml 5 mM potassium ferricyanide just before use.

Method

1. Rinse the fixed sections for 10 seconds in tap water.
2. Incubate at 37°C for 1 hour in the above medium.
3. Wash briefly in tap water.
4. Treat with 0.05% *p*-phenylene diamine dihydrochoride in 0.05 M phosphate buffer, pH 6.8 for 45 minutes at room temperature.
5. Wash in tap water.
6. Treat with 1% osmium tetroxide for 10 minutes at room temperature.
7. Wash well in tap water, counterstain lightly (10 seconds) in Carrazzi hematoxylin (or Mayer's hemalum), wash, dehydrate, clear and mount in DPX.

Results

Nerve fibers and cells containing acetylcholinesterase are stained dark brown to black.



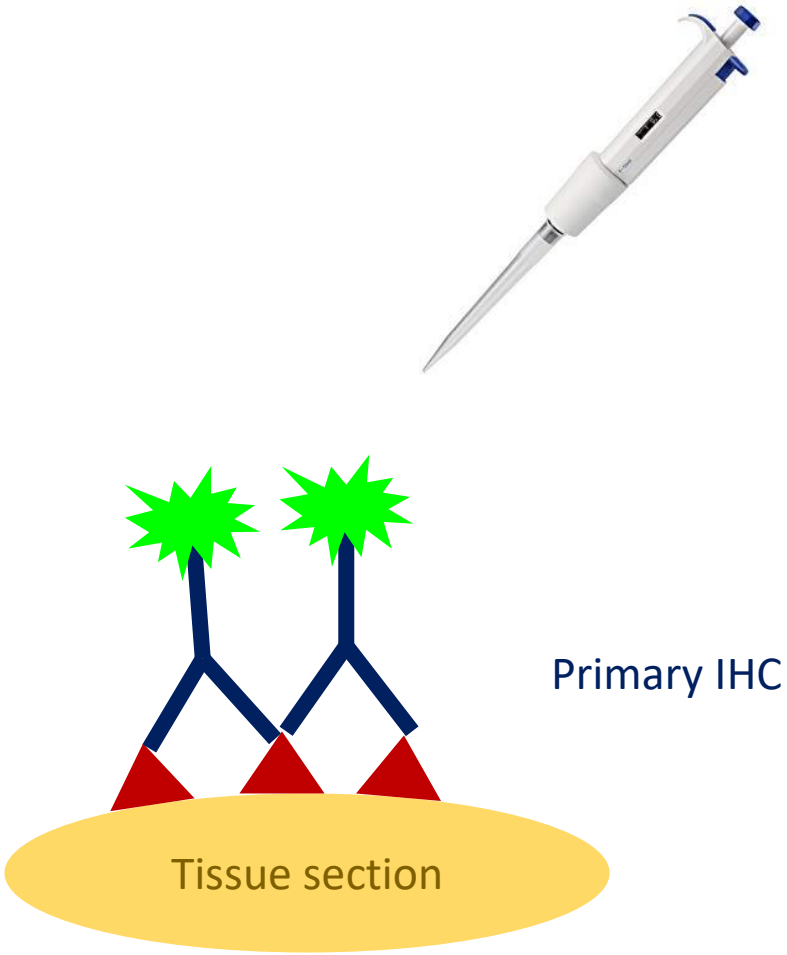
Cholinesterase staining in **(A) normal colon** and **(B) colon affected** by Hirschsprung disease

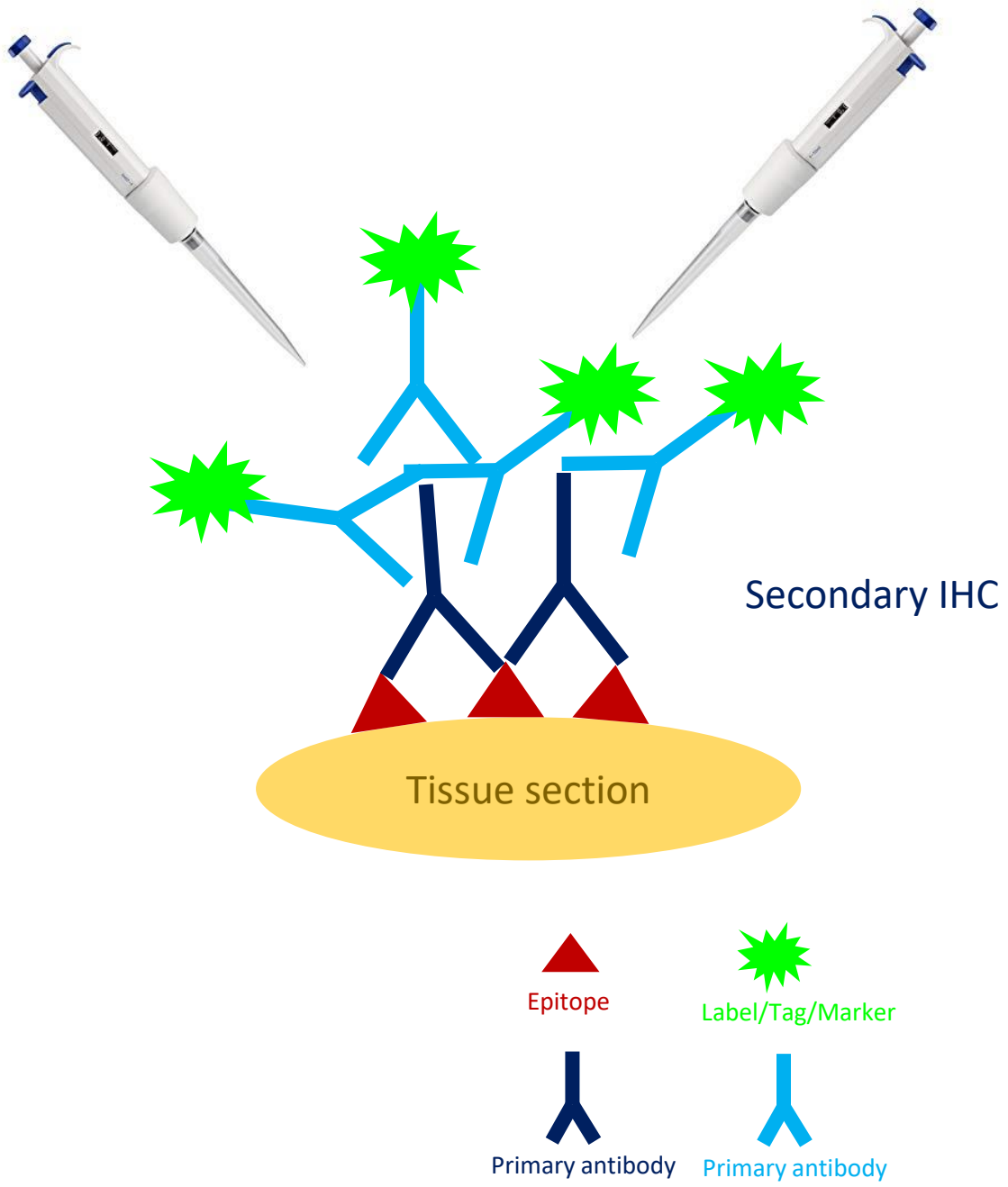
Immunohistochemistry

Immunohistochemistry

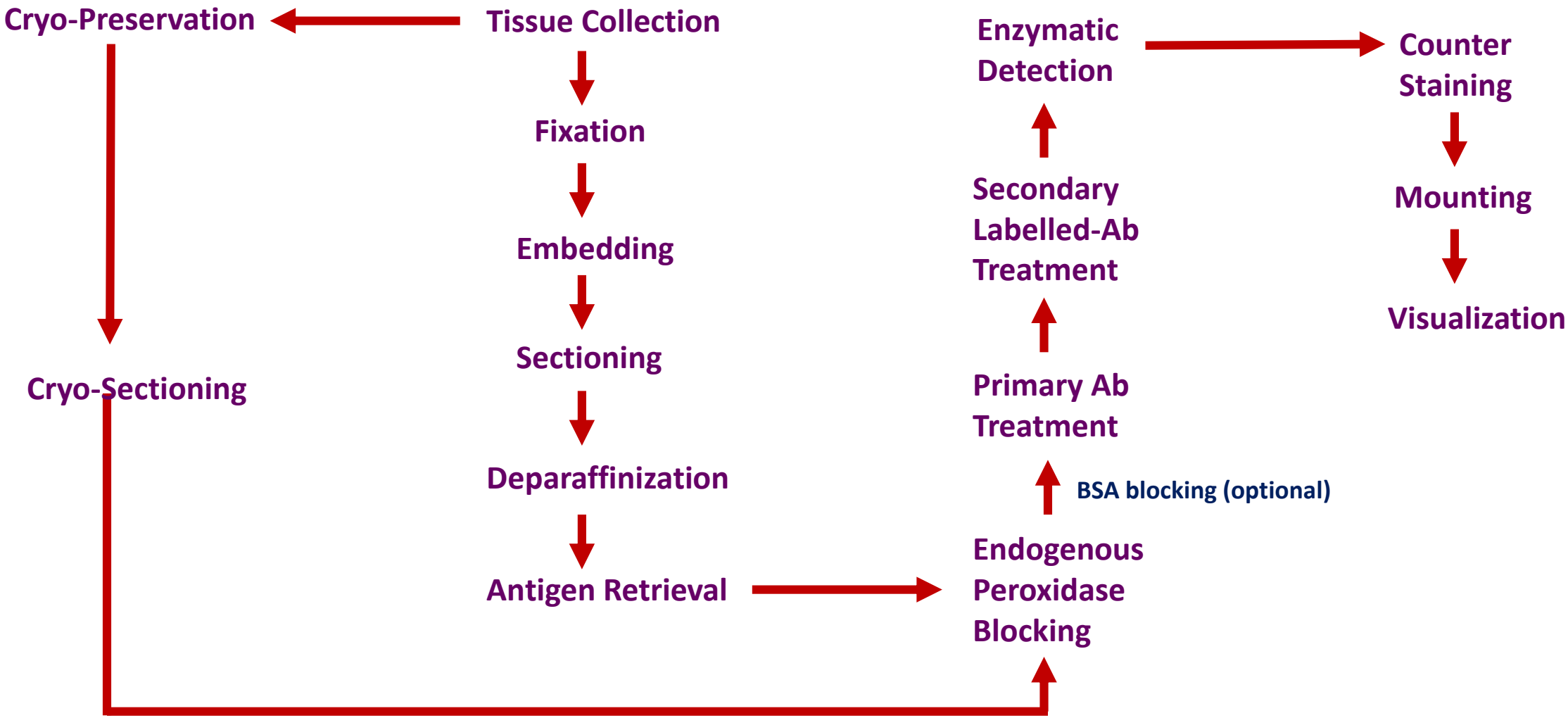
This is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions and the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labelling method

Brief overview of IHC





Steps of IHC (in brief)



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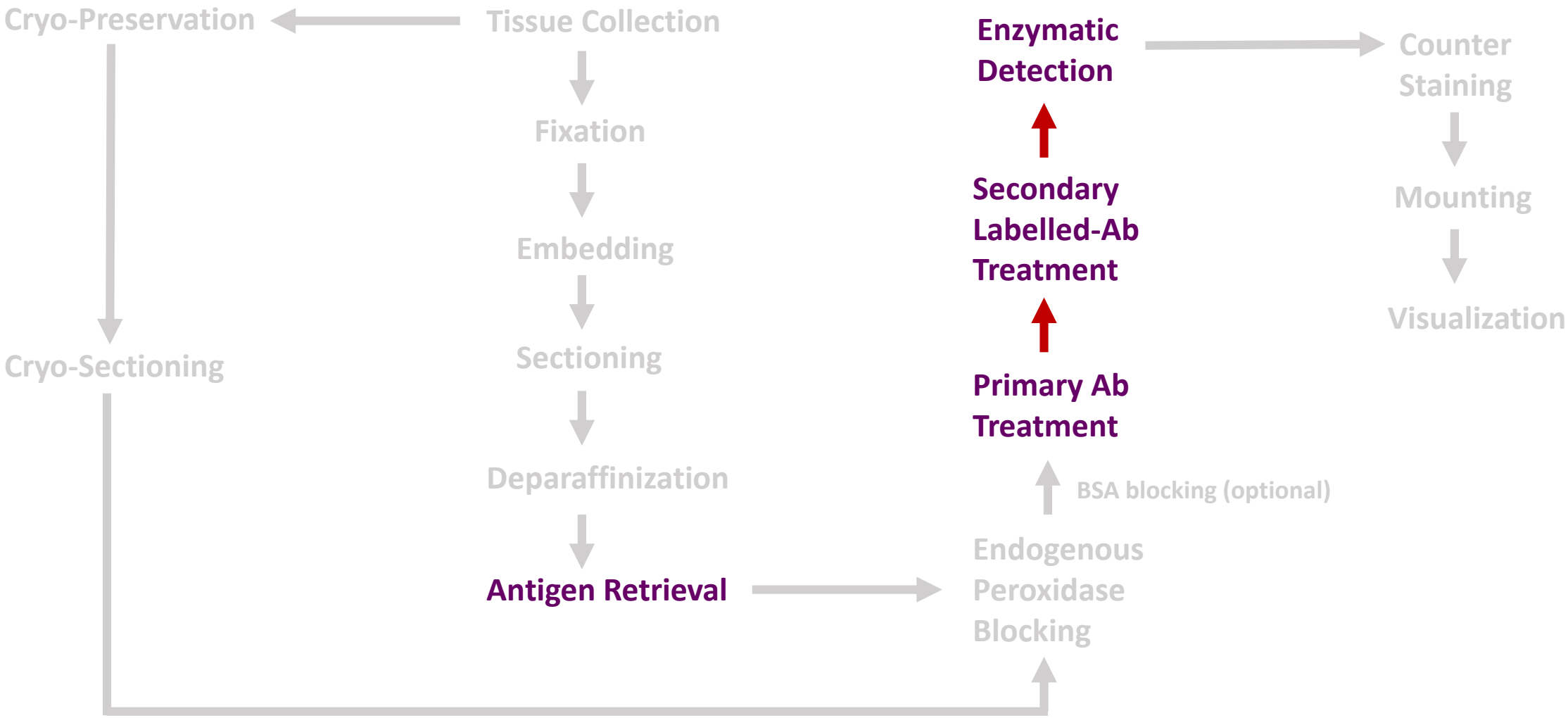
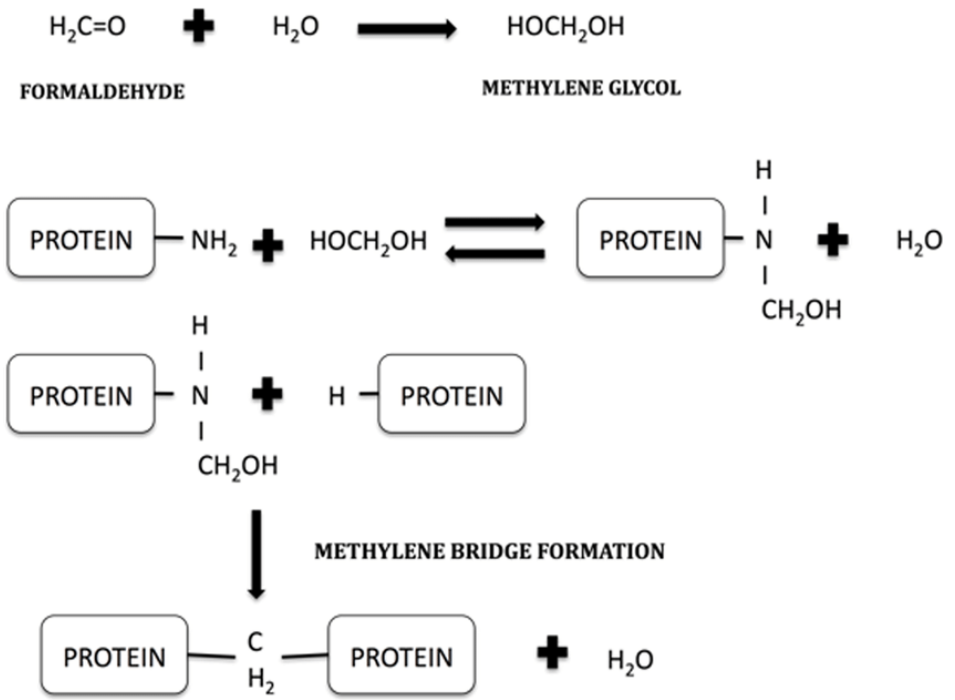


Antigen Retrieval (AR) for IHC-P

❖ Most formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining can proceed due to the formation of **methylene bridges** during fixation, which **cross-link proteins and therefore mask antigenic sites**



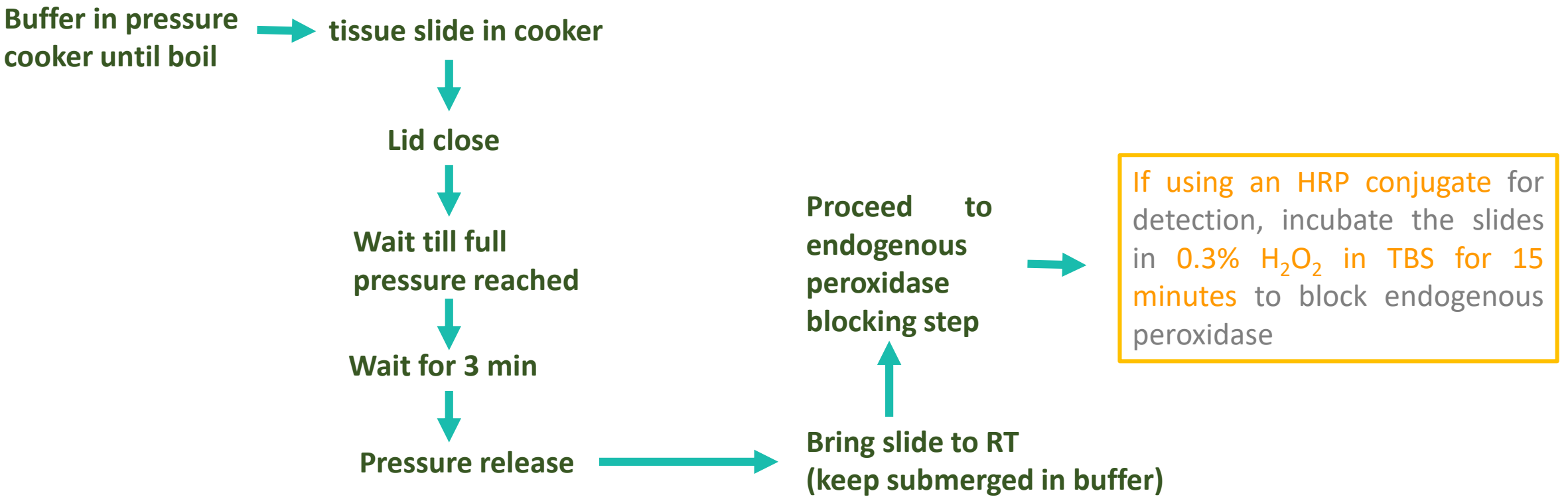
❖ The two methods of antigen retrieval are

Heat-mediated (also known as heat-induced epitope retrieval, or **HIER**) and **enzymatic**

- HIER is most often performed using a pressure cooker, a microwave, or a vegetable steamer

- Buffer solutions for heat induced epitope retrieval

  1. Sodium Citrate Buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0)
  2. 1 mM EDTA, adjusted to pH 8.0
  3. Tris/EDTA Buffer (10mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0)



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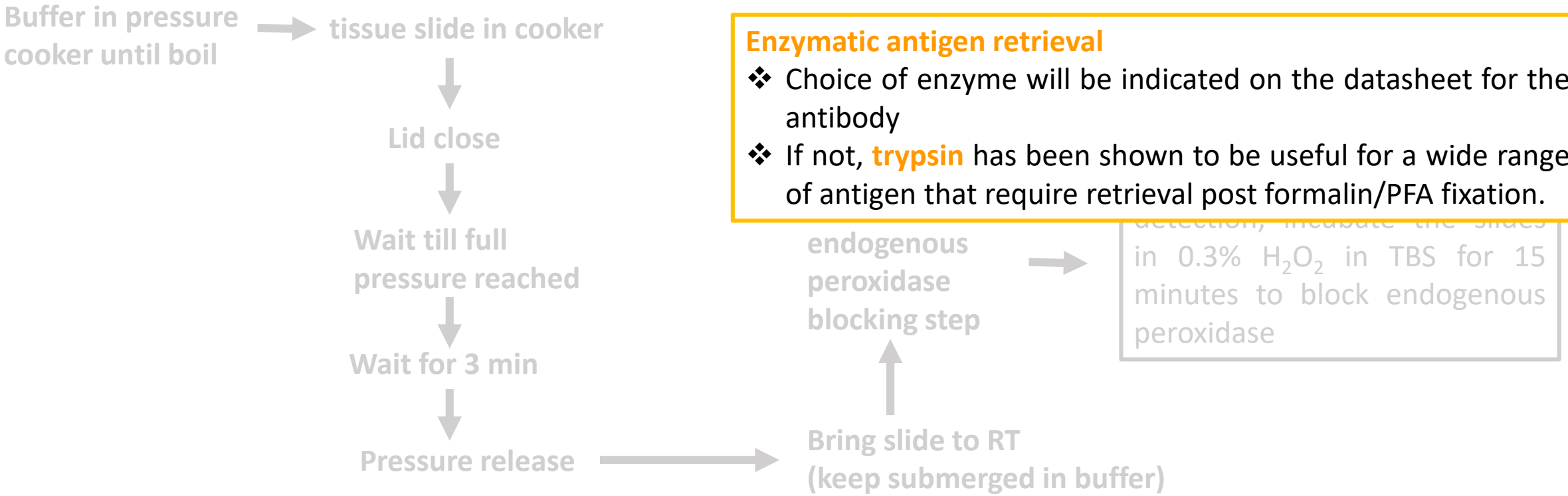
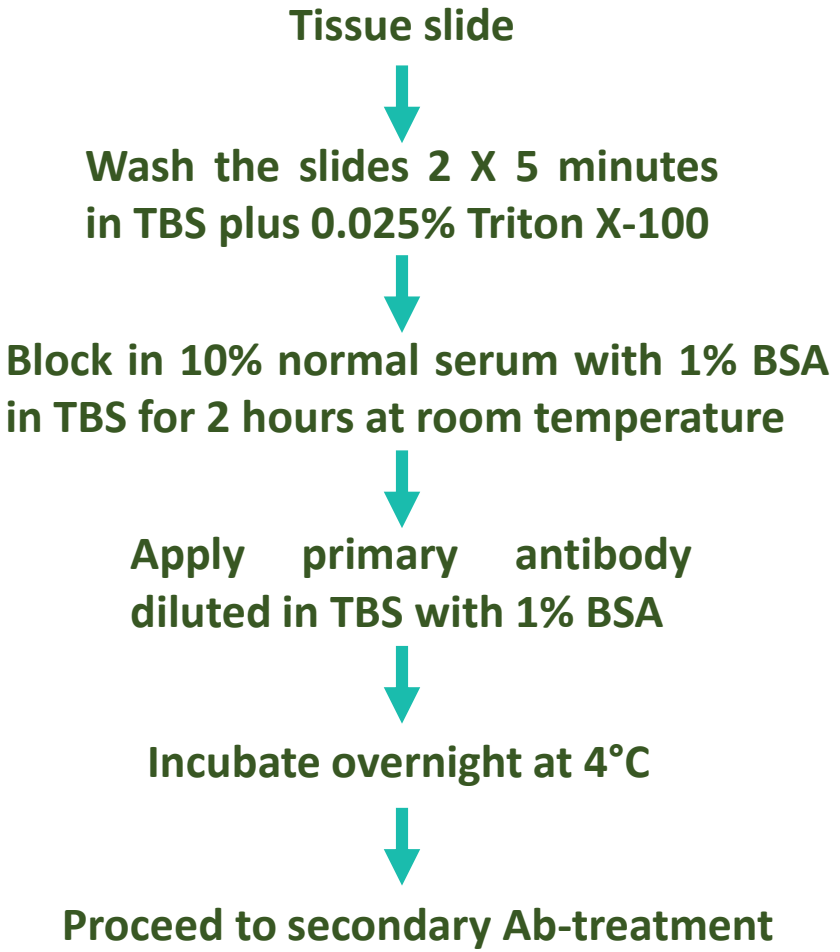


Enzymatic antigen retrieval

- Choice of enzyme will be indicated on the datasheet for the antibody
- If not, **trypsin** has been shown to be useful for a wide range of antigen that require retrieval post formalin/PFA fixation.

Primary Ab-treatment


Tissue slide
Wash the slides 2 X 5 minutes in TBS plus 0.025% Triton X-100
Block in 10% normal serum with 1% BSA in TBS for 2 hours at room temperature
Apply primary antibody diluted in TBS with 1% BSA
Incubate overnight at 4°C
Proceed to secondary Ab-treatment

Secondary Ab-treatment

Tissue slide

↓

Wash the slides 2 X 5 minutes in TBS plus 0.025% Triton X-100

↓

Apply secondary antibody diluted in TBS with 1% BSA

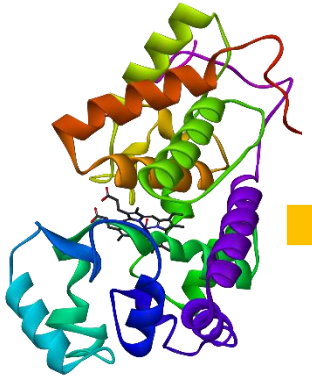
↓

Incubate for 2h at 4°C

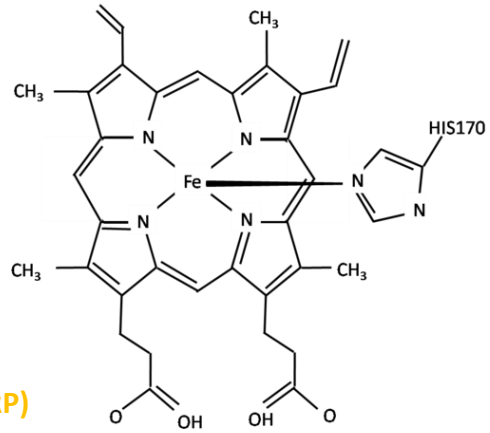
↓

Wash and proceed to enzymatic detection

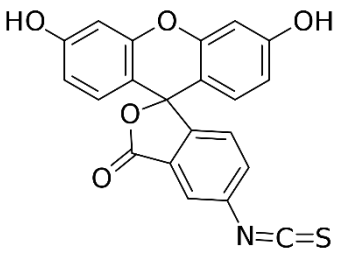
Enzymatic Detection



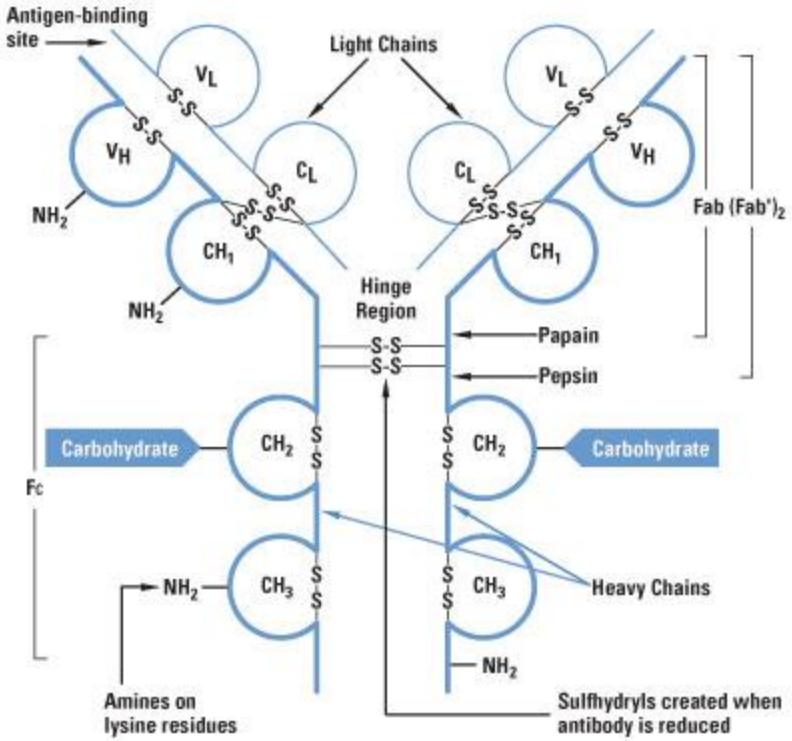
Horseradish Peroxidase (HRP)

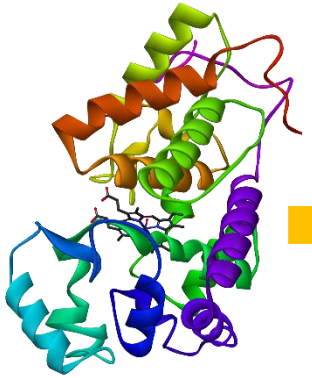


Fe-heme group of the HRP



Fluorescein isothiocyanate (FITC)





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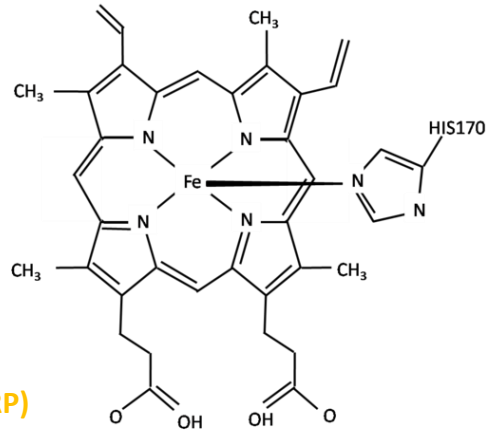

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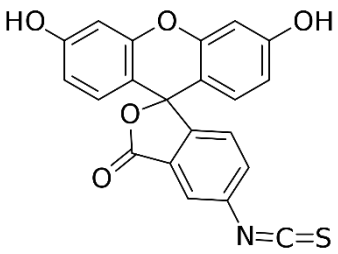

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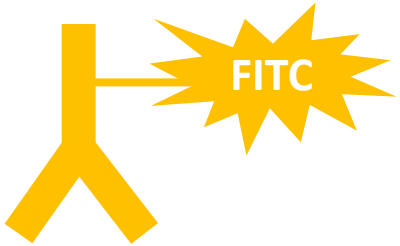
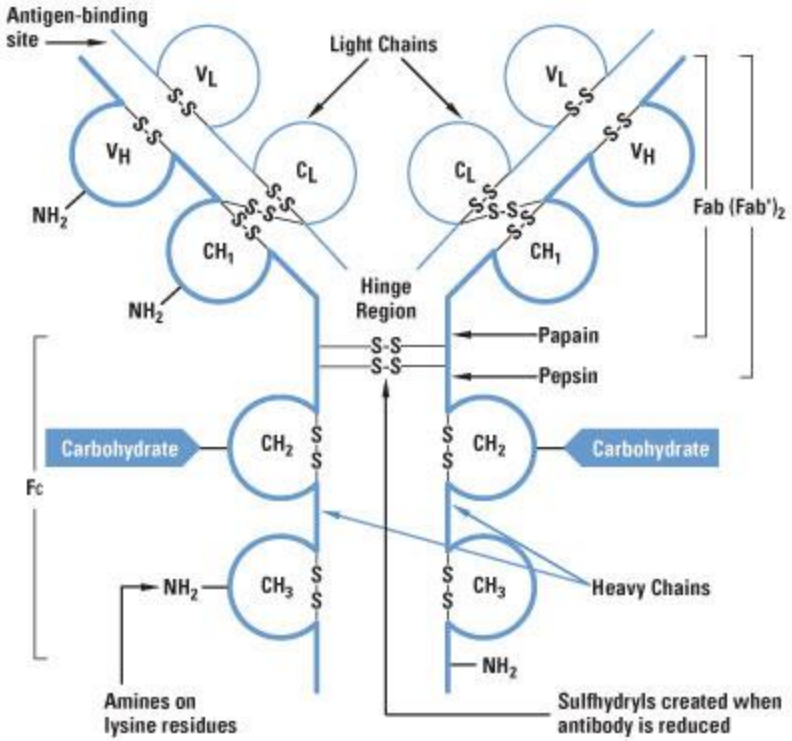




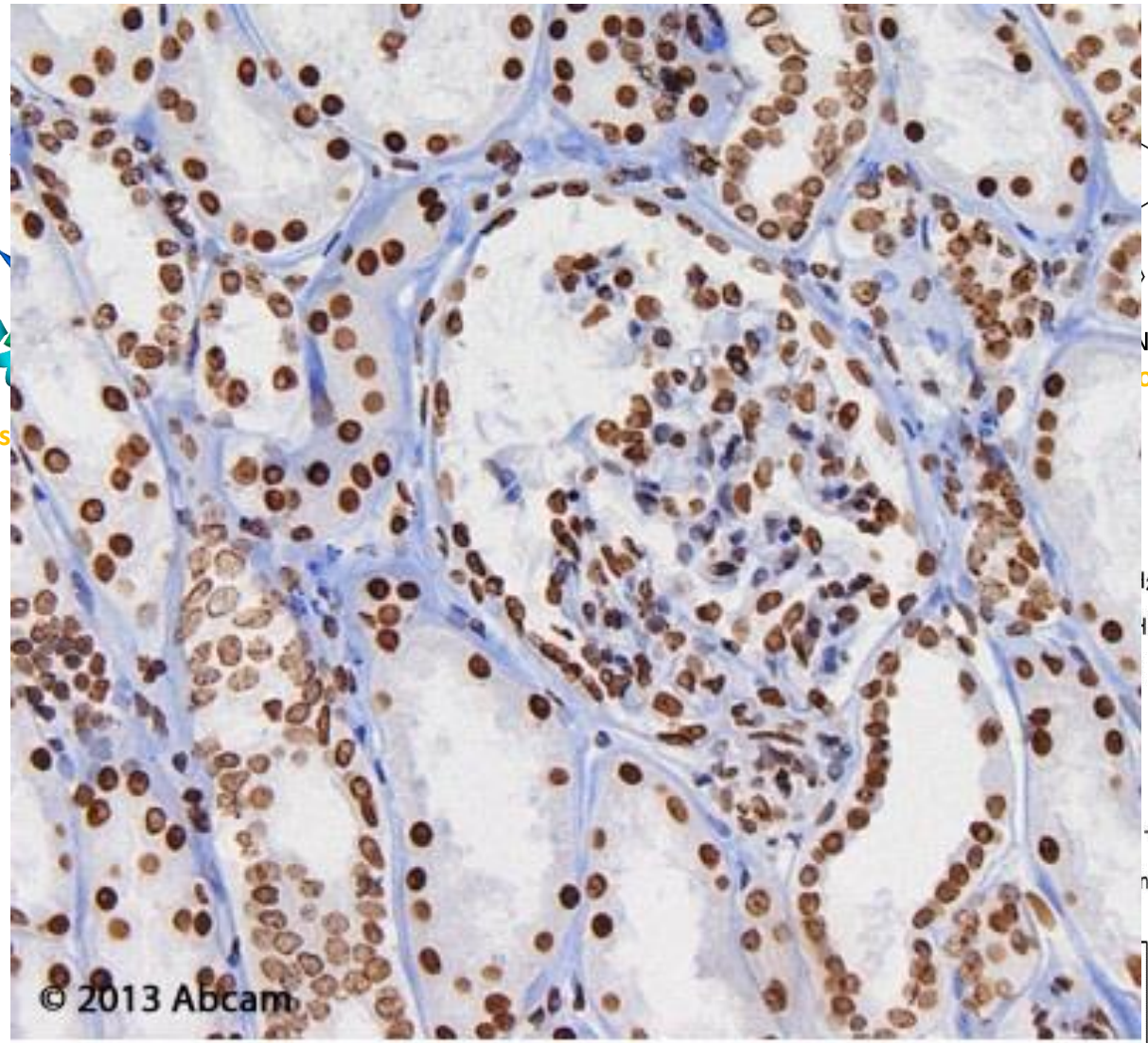
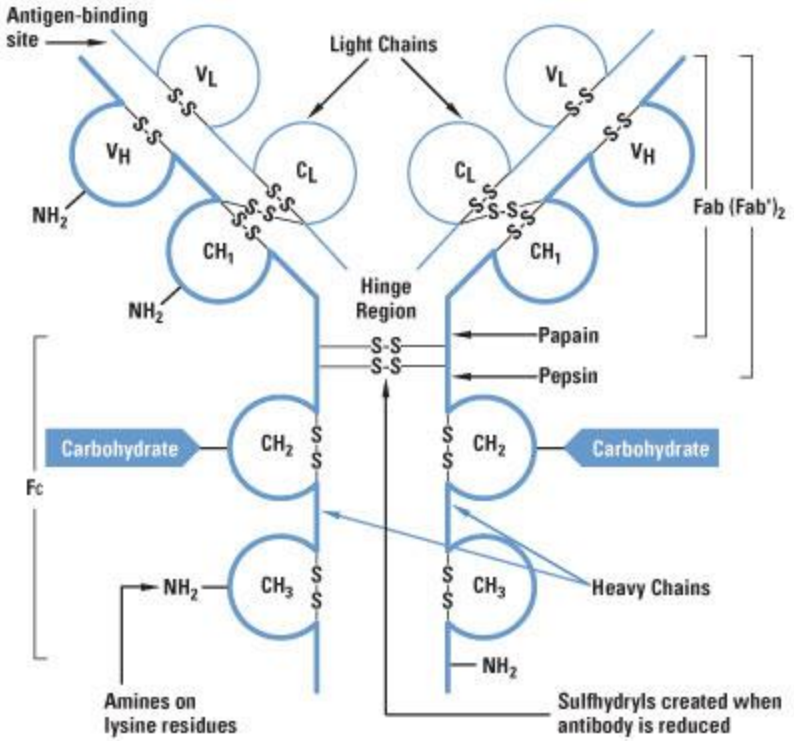
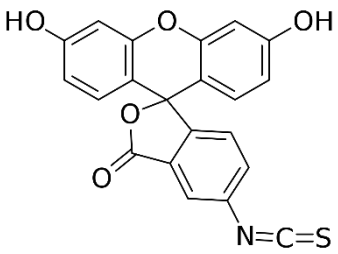


Image using ab80436 (previous version of this kit)

Histone, Kidney (Abcam)





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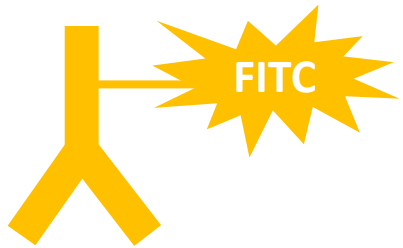
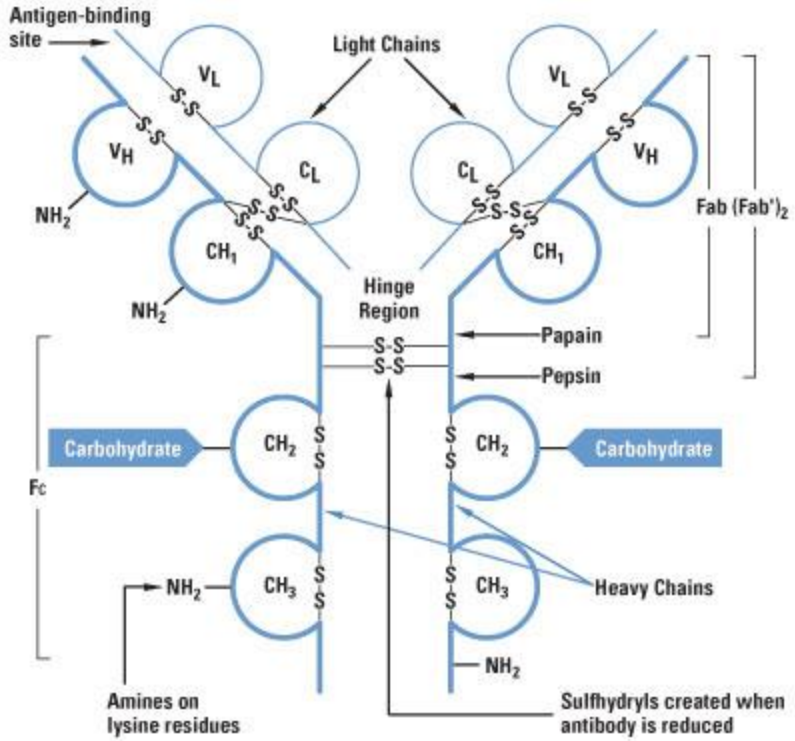
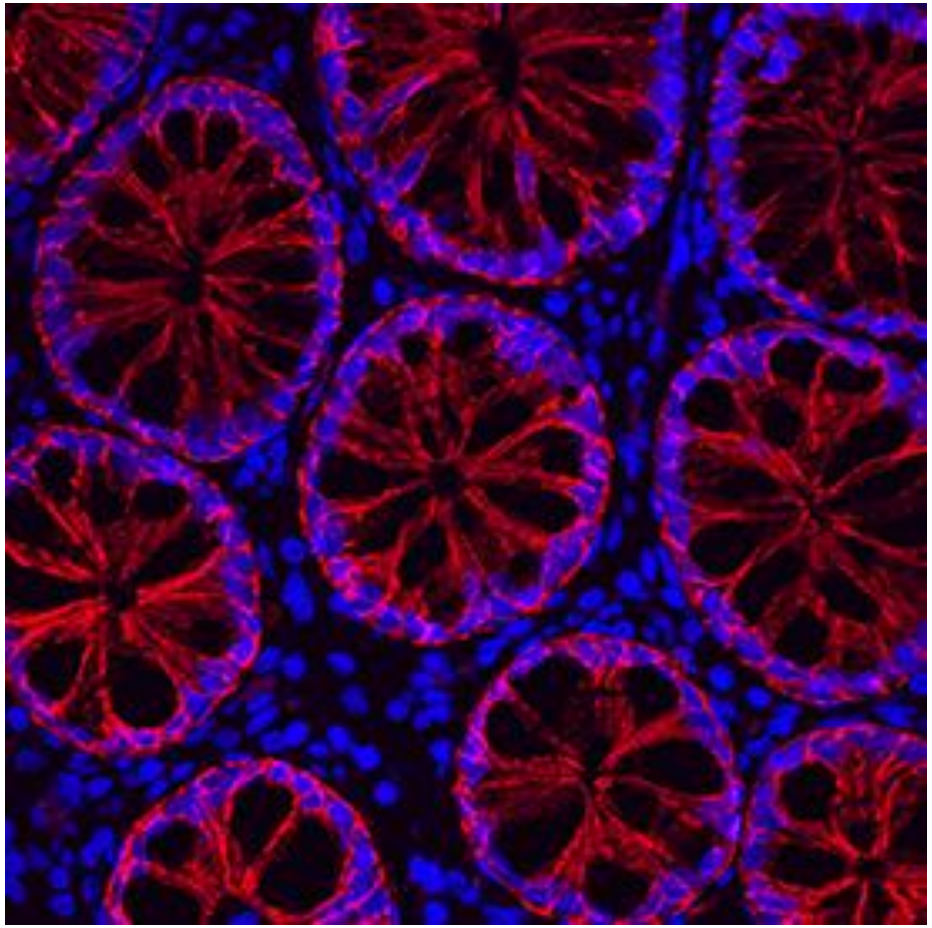
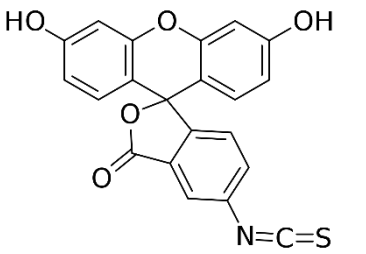
(FITC)







Cadherin-17 in Human Ascending Colon, Hu (Cadherin, DAPI)
Source: https://www.rndsystems.com/products/human-cadherin-17-antibody-141713_mab1032



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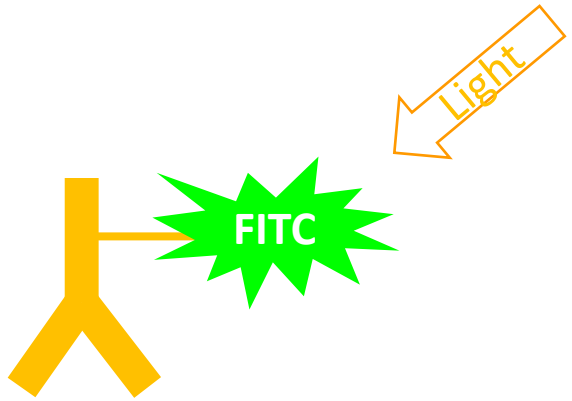
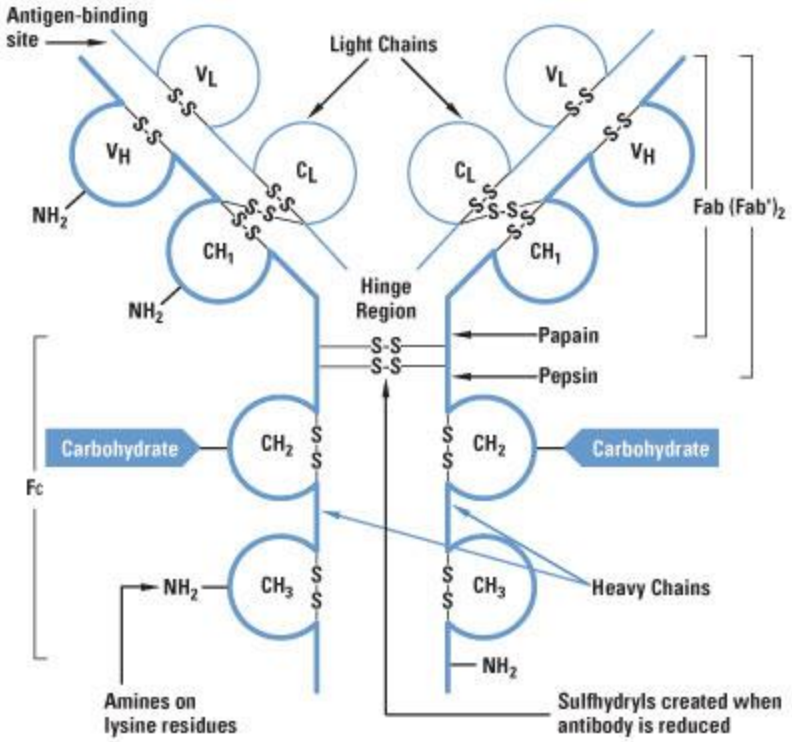
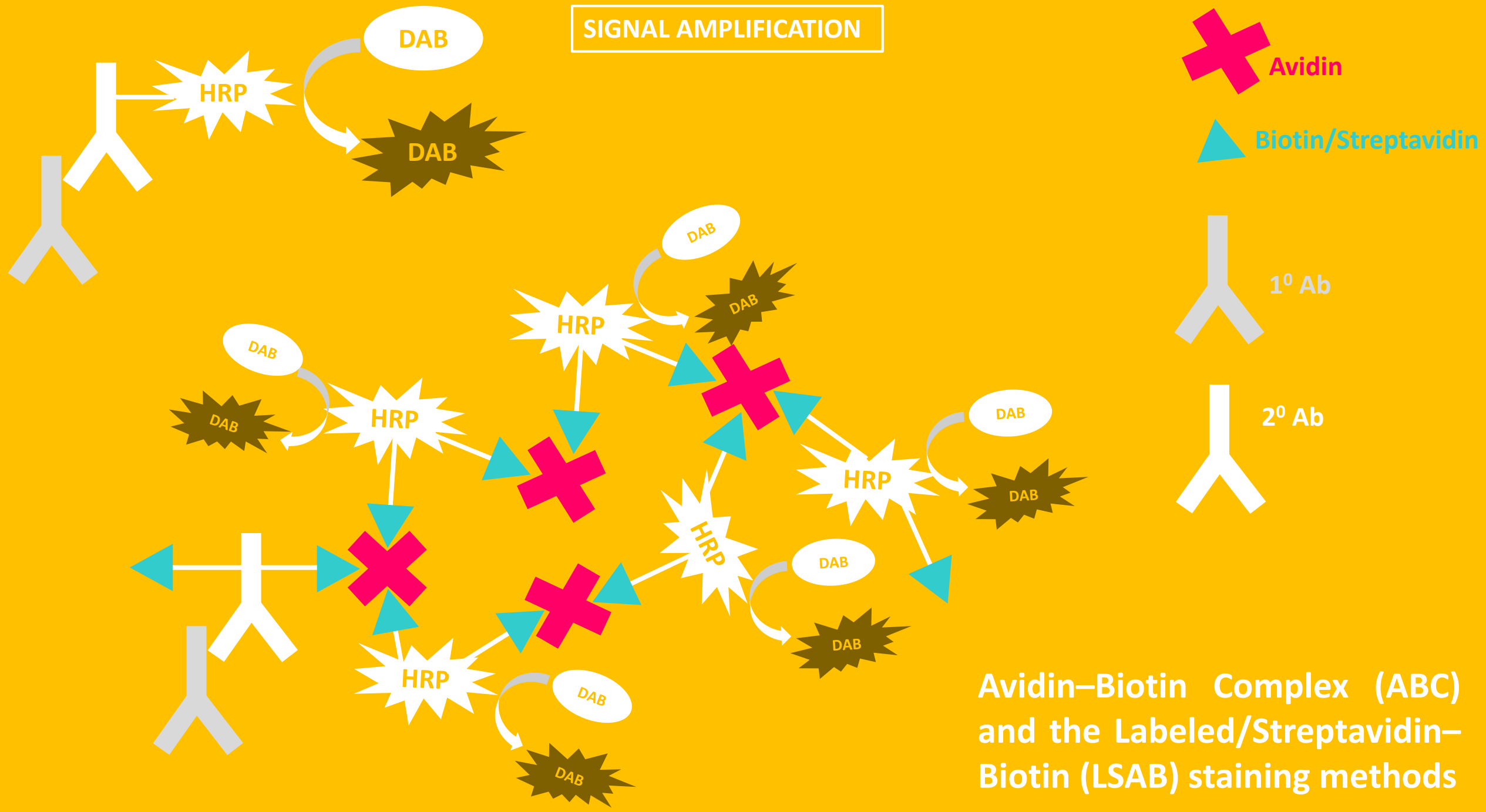




Enzyme	Substrate	Color	Advantages	Disadvantages
Horseradish peroxidase (HRP)	3,3'-Diaminobenzidine (DAB)	Brown	Intense color; permanent	Endogenous peroxidase activity in the tissue can lead to false positive staining. AEC is alcohol soluble and incompatible with organic mounting media.
	3-Amino-9-ethyl carbazol (AEC)	Red	Intense color; contrasts well with blue for double staining	
Alkaline phosphatase (AP)	5-bromo-4-chloro-3-indoyl phosphate; Nitroblue tetrazolium (BCIP/NBT)	Blue	Intense color	Endogenous alkaline phosphatase activity in the tissue can lead to false positive staining.
	Vector Blue	Blue	Less intense color, but better for double staining	
Glucose oxidase	Nitroblue tetrazolium (NBT)	Blue	No endogenous enzyme activity	Low staining intensity (high concentration of primary and secondary antibodies required for effectiveness)


SIGNAL AMPLIFICATION
DAB
HRP
DAB
Avidin
Biotin/Streptavidin
1⁰ Ab
2⁰ Ab
Avidin–Biotin Complex (ABC) and the Labeled/Streptavidin–Biotin (LSAB) staining methods

Biotin

Biotin, also known as vitamin H, is a small molecule (MW 244.3) that is present in tiny amounts in all living cells and is critical for a number of biological processes. The *valeric acid* side chain of the *biotin molecule can be derivatized in order to incorporate various reactive groups* that are used to attach biotin to other molecules. In the context of IHC, biotin is conjugated to antibodies or to the enzyme reporters use to detect target antigens.

Avidin (AV)

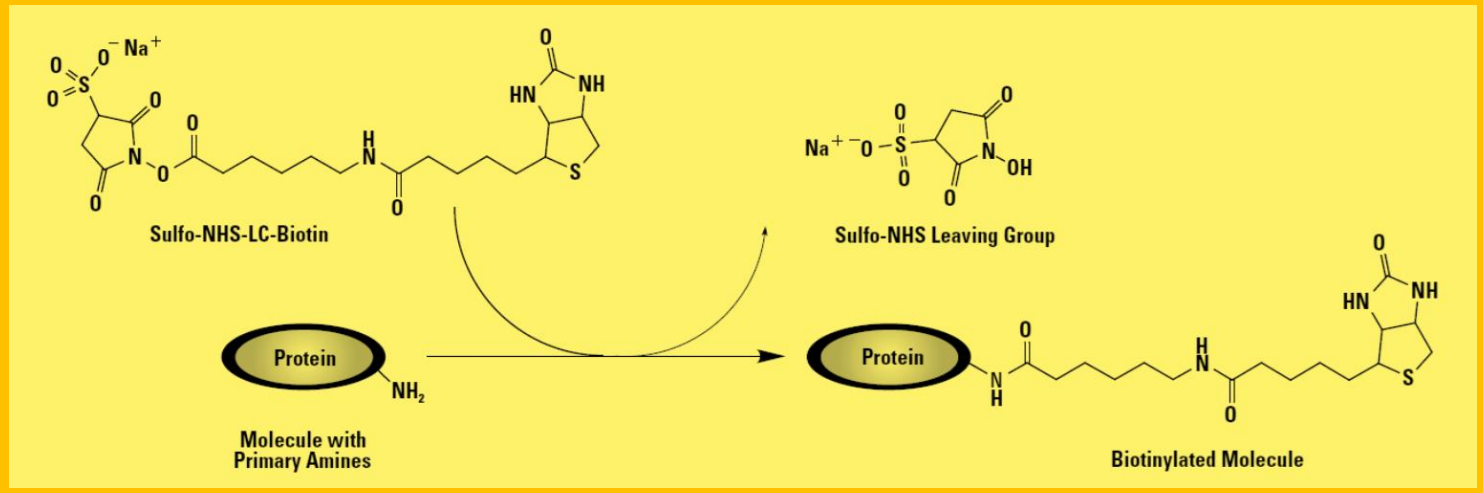
The extraordinary affinity of avidin (AV) for biotin allows biotin-containing molecules in a complex mixture to be specifically bound to avidin. Avidin is a glycoprotein found in *the egg white and tissues of birds*, reptiles and amphibia. It contains four identical subunits having a combined mass of 67 to 68 kDa. Each subunit consists of 128 amino acids and binds one molecule of biotin; thus, a total of four biotin molecules can bind to a single avidin molecule. *The extent of glycosylation on avidin is very high*; carbohydrates account for about 10% of the total mass of the tetramer. *Avidin has a basic isoelectric point (pI) of 10 to 10.5* and is stable over a wide range of pH and temperatures. Extensive chemical modification has little effect on the activity of avidin, making it especially useful for protein purification. However, because of its carbohydrate content and basic pI, avidin exhibits relatively high nonspecific binding properties. Avidin–biotin binding is the strongest known non-covalent interaction between a protein and ligand. The bond between biotin and avidin is formed very rapidly, and once formed, is unaffected by extremes in pH, temperature, organic solvents and other denaturing agents. These features of avidin make detecting or purifying biotin-labeled proteins or other molecules particularly useful for a number of biomedical applications.

Streptavidin (SA)

Streptavidin (SA) is a *biotin-binding protein isolated from Streptomyces avidinii*, and is similar in size and affinity for biotin. In contrast to avidin, though, streptavidin is not glycosylated, which makes the protein less prone to nonspecific binding in IHC applications.

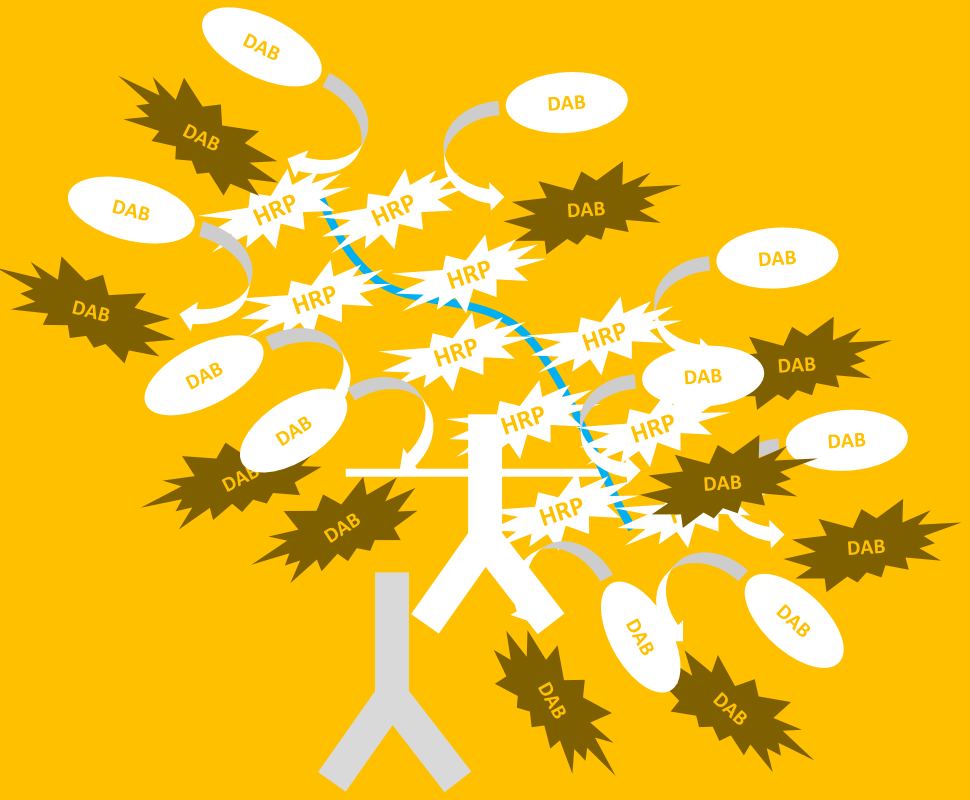
Side-by-side comparison of biotin-binding proteins

	Avidin (chicken)	Streptavidin (recombinant)	NeutrAvidin (from avidin)
Molecular weight (kDa)	67	53	60
Biotin-binding sites	4	4	4
Isoelectric point (pI)	10	6.8 to 7.5	6.3
Specificity	Low	High	Highest
Affinity for biotin (K_d)	~1.3×10^{-15} M	~0.04×10^{-15} M	~1.3×10^{-15} M
Nonspecific binding	High	Low	Lowest*



HRP-polymer Secondary Antibodies

- ✓ HRP-polymer secondary antibodies use micropolymer technology to form smaller detection complexes that allow better tissue penetration and sensitivity
- ✓ In addition, HRP-polymer secondaries bind more horseradish peroxidase than standard HRP secondary antibodies, increasing signal.



	Biotin	HRP-polymer	Advantages
Protocol	Additional steps for biotin blocking may be required	On average **1 hour less**	Simplified protocol means **quicker results** with HRP-polymer secondaries.
Sensitivity	♦♦♦	♦♦♦♦	**Higher number of HRP** molecules bound to HRP-polymer antibodies increase their sensitivity
Specificity	Potential background from endogenous biotin	**No background** from endogenous biotin	Using HRP-polymer secondary antibodies **eliminates biotin background.** *Great for tissues with high biotin.*

Multiplex IHC (mIHC)

Fluorescent multiplex immunohistochemistry (**mIHC**) is a method that enables simultaneous detection of multiple proteins of interest in formalin-fixed paraffin-embedded (FFPE) tissue sections. There are various approaches to fluorescent multiplexing:

1. **Direct immunofluorescence:** involves the use of multiple antigen-specific primary antibodies conjugated to distinct fluorophores. The disadvantage of this approach is limited sensitivity for targets of low abundance due to lack of signal amplification.

2. **Indirect immunofluorescence**: antigen detection is mediated via conjugated secondary antibodies specific to the species of the host in which each primary antibody was raised. This approach provides modest signal amplification but is limited by the number of available host species, e.g. rabbit, mouse, rat, and others.

3. **Deposition assays:** involve the use of enzyme-labeled antibodies and tyramide-fluorophore conjugates. This approach is unhindered by host species and isotype concerns, while providing ample signal amplification.

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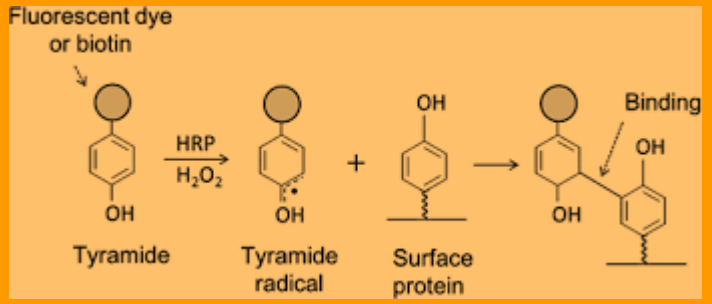
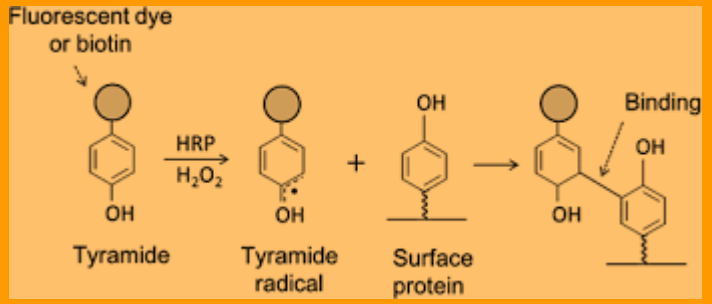
Fluorescent Multiplex Immunohistochemistry with Tyramide Signal Amplification



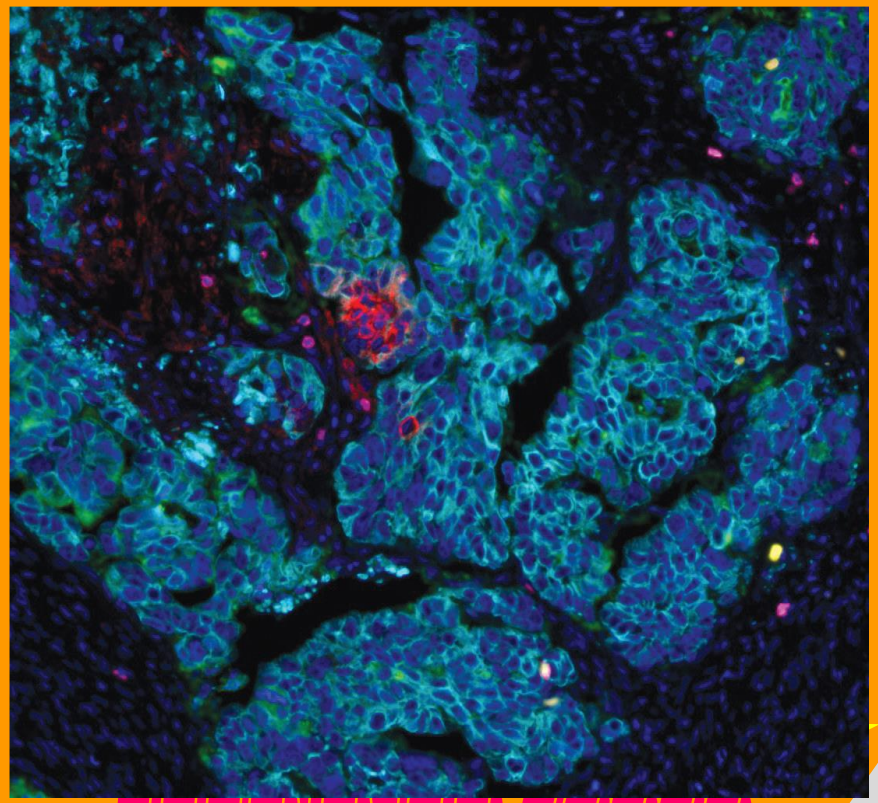
mIHC procedure (outlined)

Inactive tyramide

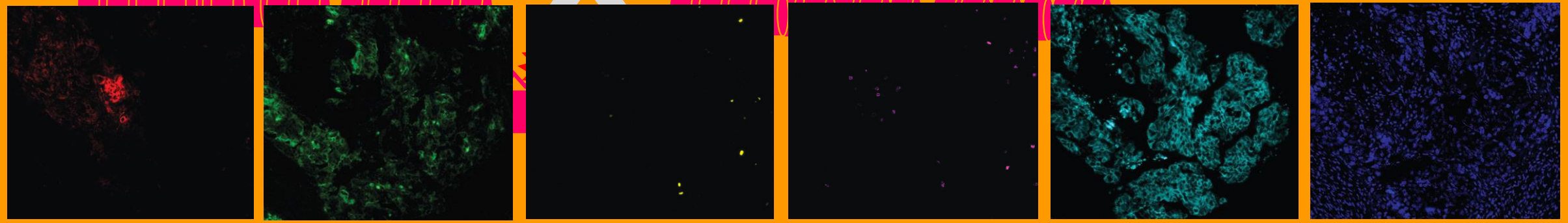
Active tyramide



mIHC procedure (outlined)



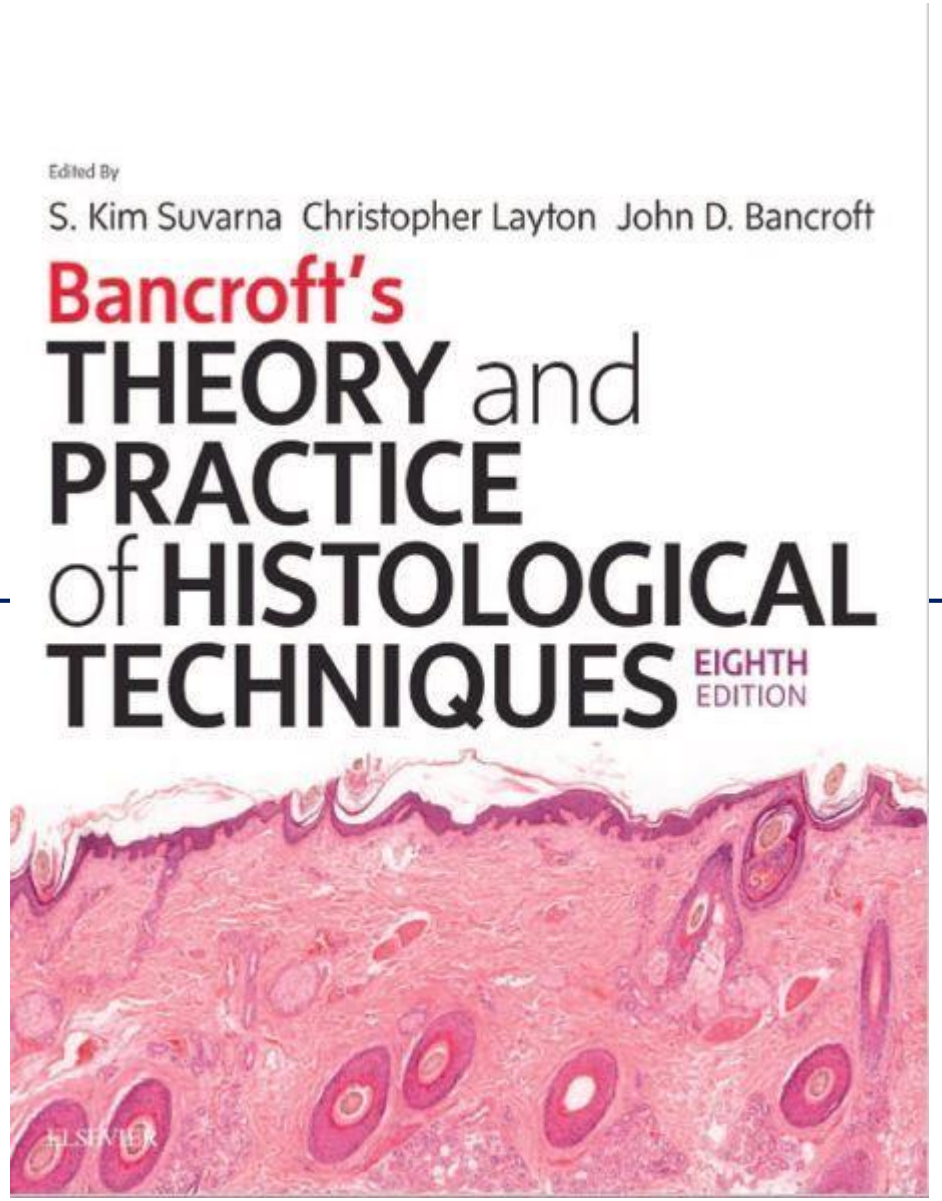
mIHC procedure (outlined)



PD-L1 B7-H4 FoxP3 CD8α CK DAPI

Fluorescent Multiplex IHC Analysis of a 5-Plex Panel (5 targets + a nuclear counterstain) (Cell Signalling Technology®)

Reference


Edited By
S. Kim Suvarna Christopher Layton John D. Bancroft
Bancroft's
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of HISTOLOGICAL
TECHNIQUES
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That's all Folks!