Immunohistochemical staining and staining protocol; the scientific basis

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Introduction

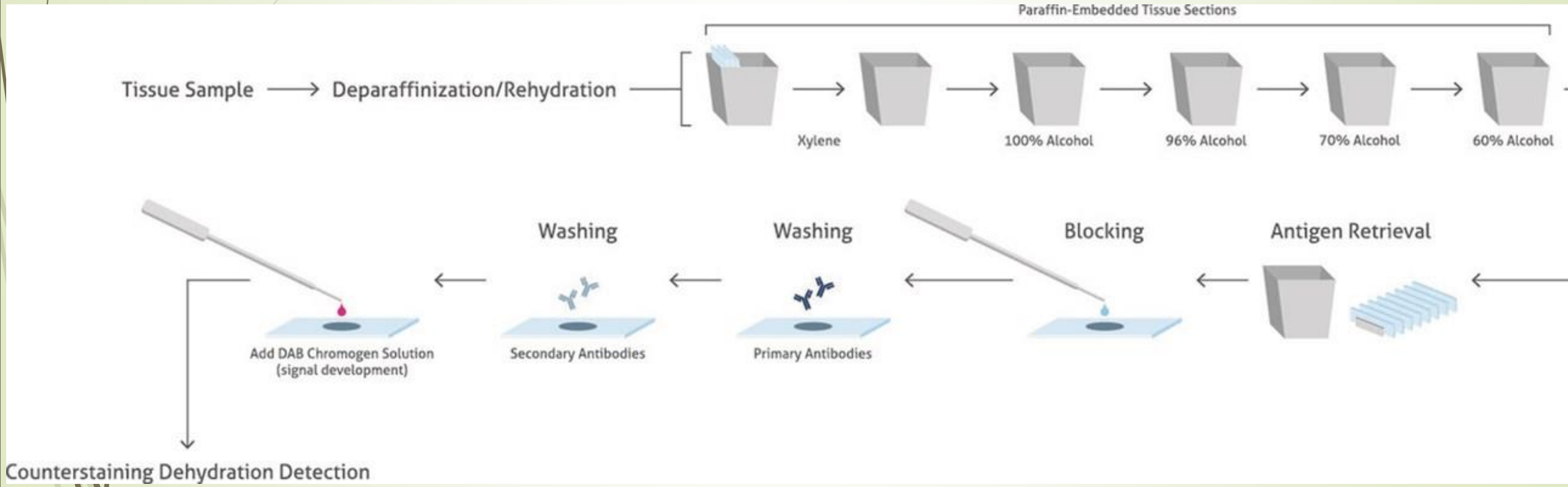
- IHC is a commonly used molecular test
- important and essential ancillary test in histopathology practice
- Use of IHC has expanded vastly as many new molecules involved in pathogenesis, diagnosis, and treatment of diseases are discovered
- **IHC is the detection of an antigen based on an antibody-antigen reaction where the end point is visible microscopically**
- performed without destruction of histologic architecture – a unique feature
- The **presence or absence** of the antigen and its **distribution** within cells and tissues can be identified providing clinically useful information

Uses of immunohistochemistry

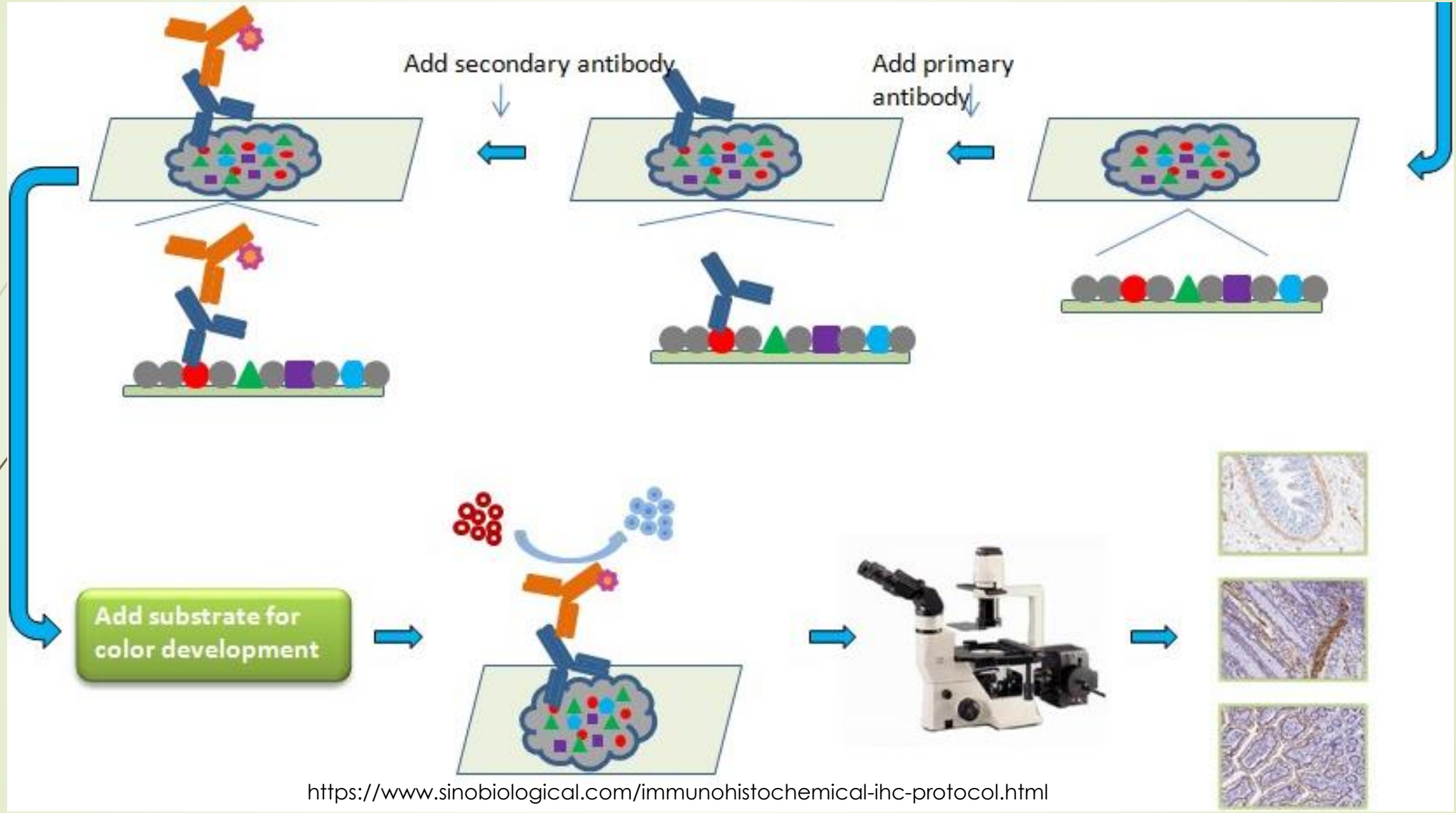
- Tumour pathology
  - Classification of tumours
  - Diagnosis of malignancy
  - Determine prognostic markers
  - Predict response to treatment
  - Screen for inherited cancer syndromes
  - Detect occult metastases
  - Research

- Non-tumour pathology
  - Inflammatory skin diseases
  - Infectious diseases
  - Transplant and native renal disease
  - Amyloidosis
  - Dementias

Basic steps in IHC staining



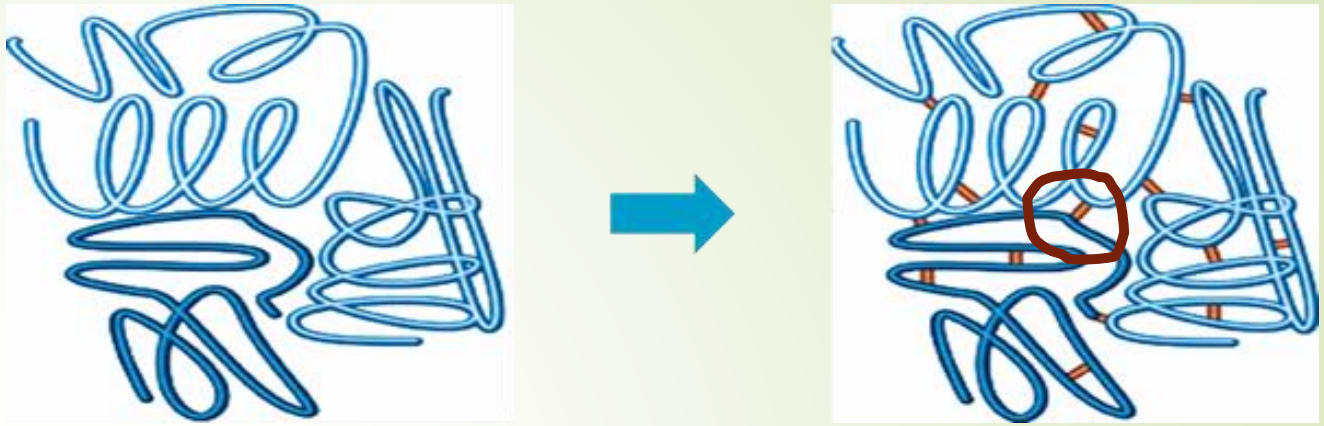
https://www.ptglab.com/support/immunohistochemistry-protocol/immunohistochemistry-overview/



https://www.sinobiological.com/immunohistochemical-ihc-protocol.html

Fixation

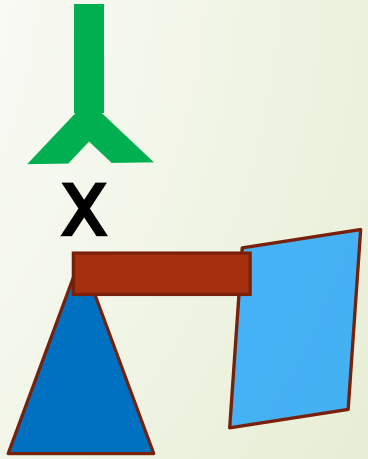
- Very important to IHC
- Fixation immobilizes antigens while retaining cellular and subcellular structures
- Poor fixation cannot be reversed



https://www.nationaldiagnostics.com/histology/article/aldehyde-fixatives

Types of fixatives

- Cross linking fixative (eg Formaldehyde)
  - Cause conformational changes in the protein structure
  - Modify the presentation of different epitopes
  - prevent access to specific antibodies



- Coagulative fixatives (eg Alcohol)
  - modify the tertiary structure of the protein by interacting with hydrophobic protein components

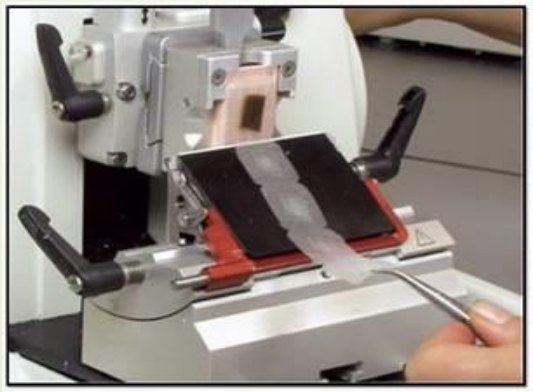
- Formaldehyde fixation is preferred
- Other fixatives-Ethanol, Methanol, Acetone
- no specific minimum fixation time for most Abs
  - For large tissue blocks – slicing, overnight fixation
  - For small biopsies - 3-4 hours
  - For ER and PR a minimum of 6 hours
- the tissue must be fixed evenly to achieve even staining
- Excessive fixation time (greater than 48 hours) may cause loss of antigenicity for some stains, but can be largely overcome with HIER

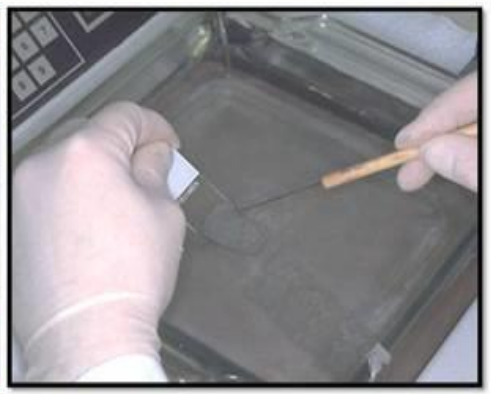
Decalcification and IHC

- When fixed adequately and then gently decalcified, effect on IHC is minimal
- With poor fixation, acid decalcification may reduce antigenicity for some Abs

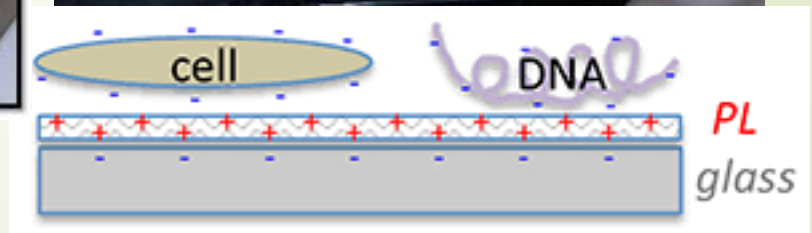
Processing and sectioning

- 4 micron sections are cut onto slides
- Electrostatic charged slides provide better adhesion to the tissue - enable sections to survive HIER
- Drying slides overnight at 37°C or baking slides at 60° C for 1 hour also assist adhesion
- Paraffin-embedded sections need to be dewaxed first to replace the wax with water as most staining solutions are aqueous
- If paraffin is not completely removed sections will not stain properly



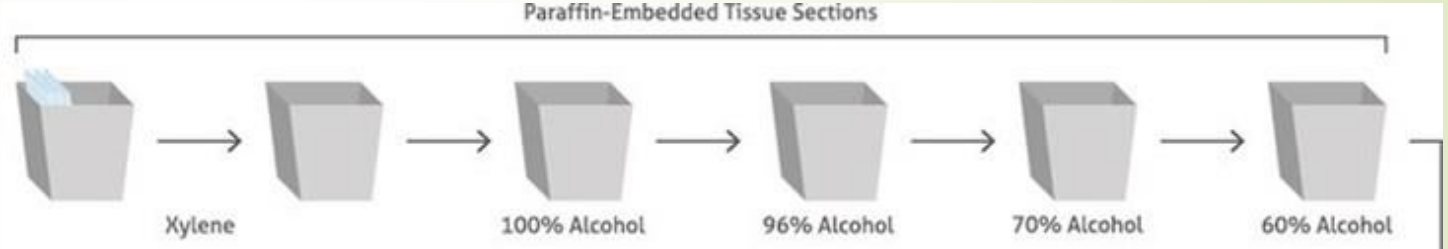


https://tissuesampling.weebly.com/processing.html



De-paraffinization and rehydration

- steps
  - Xylene: 2 x 3 min
  - Xylene 1:1 with 100% ethanol: 3 min
  - 100% ethanol: 2 x 3 min
  - 95% ethanol: 3 min
  - 70 % ethanol: 3 min
  - 50 % ethanol: 3 min
  - Running cold tap water to rinse
- Keep the slides in the tap water until ready to perform antigen retrieval
- From this point onwards slides should not be allowed to dry



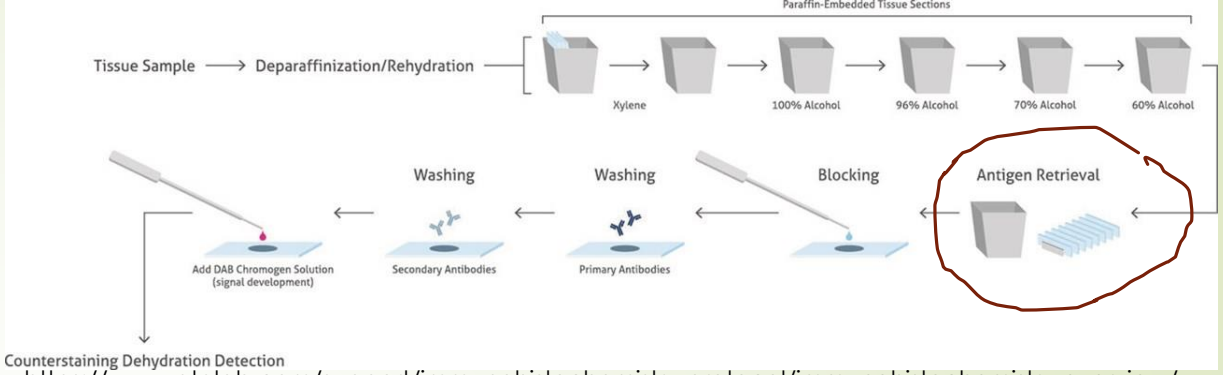
https://www.ptglab.com/support/immunohistochemistry-protocol/immunohistochemistry-overview/

Humidifying chamber

- After rehydration all other steps must be performed in a humidifying chamber
- Drying of tissues result in non-specific antibody binding leading to high background staining

https://www.polysciences.com/default/

Antigen retrieval



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- Process of partially reversing formaldehyde induced conformational change to unmask antigenic sites for Ab binding
- Essential for optimum IHC staining
- The main methods of Ag retrieval
  - Heat-induced epitope retrieval (HIER)
  - Proteolytic/enzyme-induced epitope retrieval (PIER)
  - Combined methods

Heat induced epitope retrieval (HIER)

- Standard method used
- Enables use of FFPE tissue for many Abs
- Must use coated/charged slides to prevent sections from falling off
- Dewaxed sections are immersed in a retrieval solution (citrate pH 6,EDTA pH 8-9)
- Sections are heated to approximately 100° Celsius or more for 10-20 minutes
- Heating methods
  - Microwave oven
  - Pressure cooker
  - Water baths
  - Vegetable steamers
  - Automated immunostainers
  - Autoclave

Heating by microwave

- Disadvantages of domestic microwave
  - Hot and cold spots are common, leading to uneven antigen retrieval
  - Antigen retrieval times are usually longer
  - absence of a pressurized environment lead to section dissociation
- A scientific microwave is more appropriate
  - have onboard pressurized vessels
  - keep the temperature at a constant 98°C
- Retrieval buffer can boil and evaporate
  - watch the buffer level of the slide vessel, and add more buffer if necessary
  - Do not allow the slides to dry out
- Slides should be placed in a plastic rack and vessel
  - Standard glass histology staining racks and vessels will crack when heated

https://basicmedicalkey.com/immunohistochemical-techniques/

http://www.scopem.ethz.ch/instruments-services/instruments-alphabetical/histology-microwave-owen.html

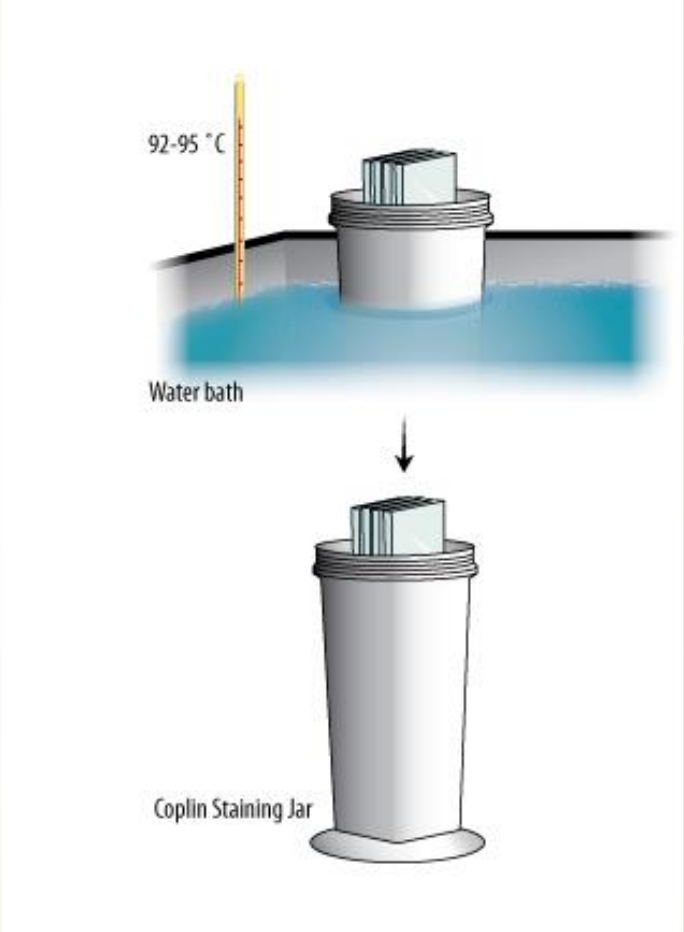
Heating by pressure cooker

https://basicmedicalkey.com/immunohistochemical-techniques/

http://www.dbaitalia.it/SchedaNews.aspx?id=403

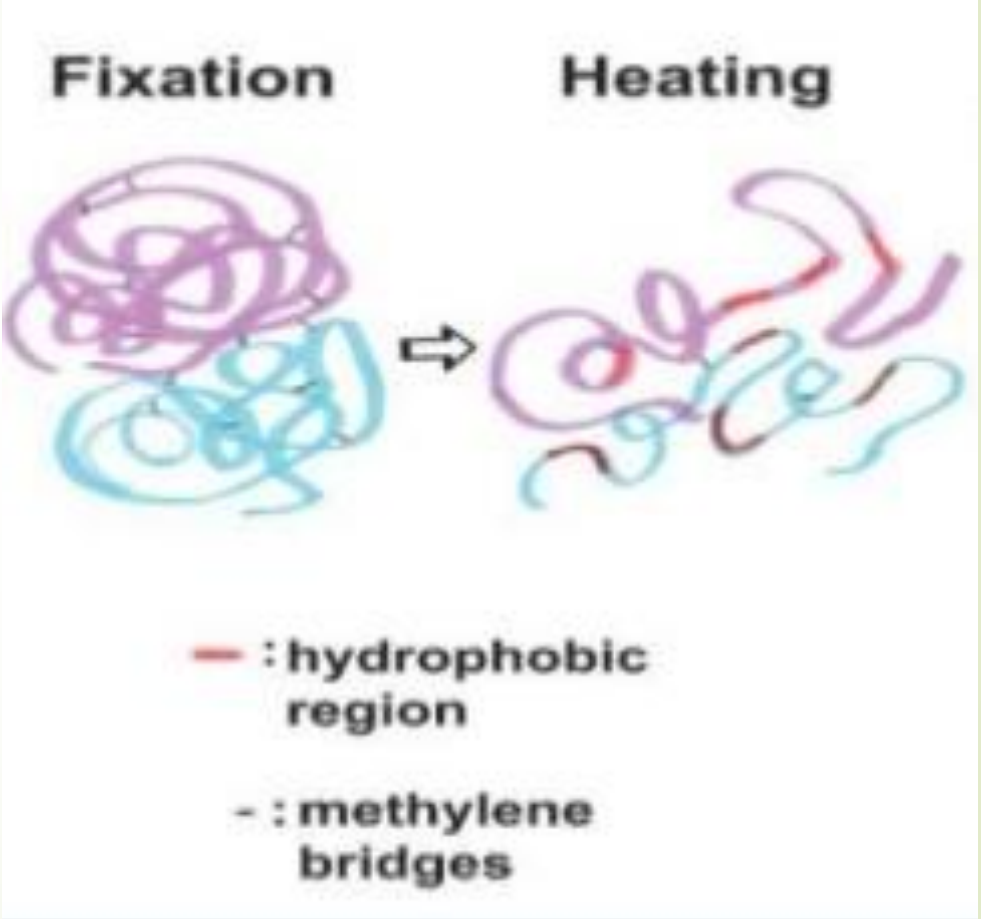
Heating by Water bath

- For tissues that tend to fall off easily with heating use a water bath at 60°C and incubate the slides in retrieval solution overnight
  - Bone
  - Cartilage
  - skin



https://www.rndsystems.com/resources/protocols/protocol-heat-induced-epitope-retrieval-graphic

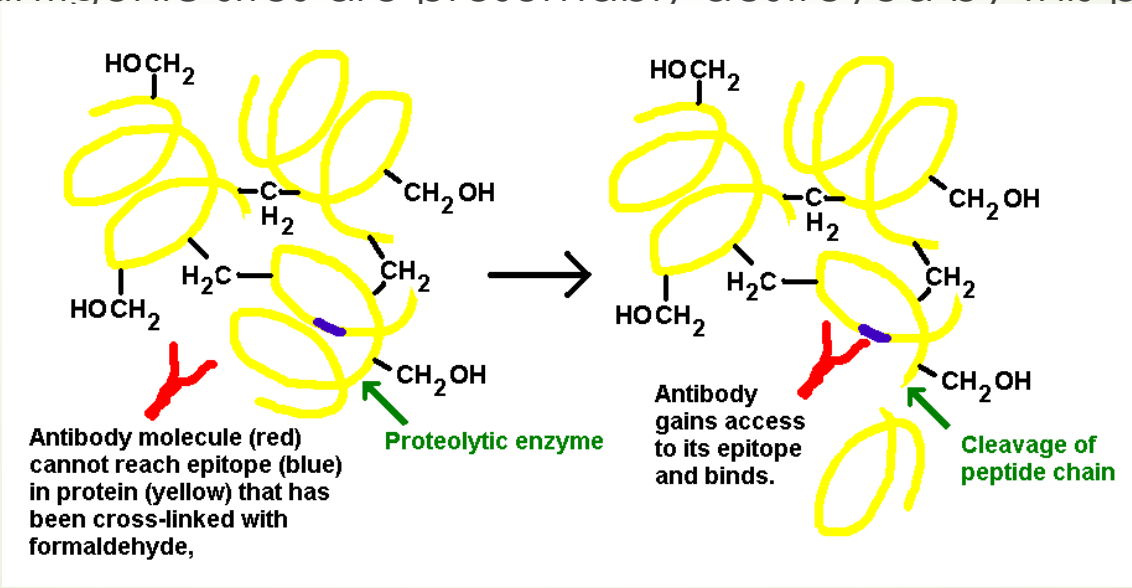
- The sections are then slowly cooled over approximately 20 minutes---- important for proper refolding of the proteins
- Cooling allows protein to return close to its pre-fixed conformation
- Excessive HIER destroys the tissue sections and results in poor morphology
- insufficient HIER results in false negative staining. A balance is required.
- exact mechanism of action is unknown
- Probably breaks/hydrolyses aldehyde induced protein and enzyme crosslinks



https://journals.sagepub.com/doi/pdf/10.1369/jhc.5C6627.2005

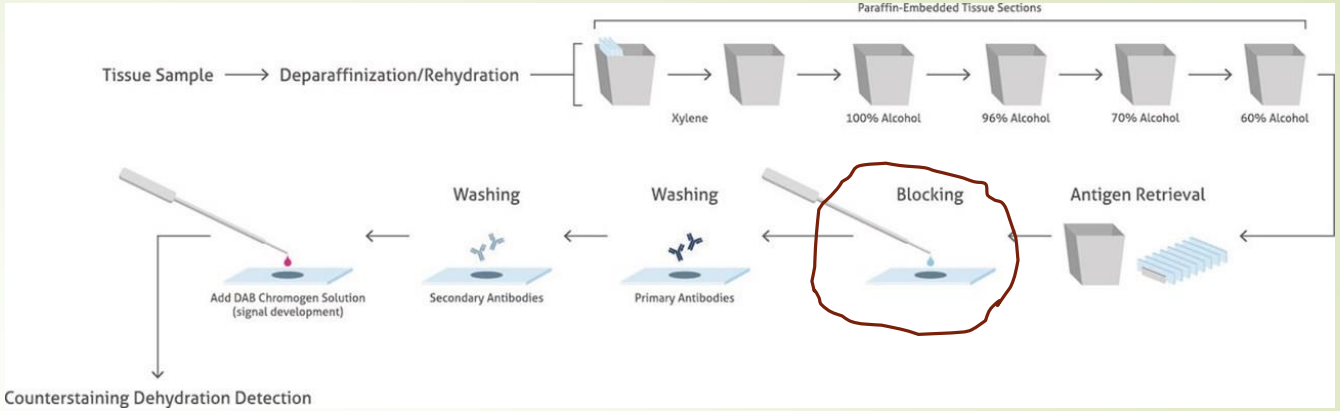
Proteolytic/enzyme induced epitope retrieval

- Used for retrieval of some antigens
- Not commonly used
- Sections are incubated with a proteolytic enzyme such as protease, pepsin
- Proteolytic enzymes cleave proteins at specific locations, releasing at least some antigenic sites intact
- Other antigenic sites are presumably destroyed by this process



http://publish.uwo.ca/~jkiernan/FixAnti2.pdf

Endogenous blocking

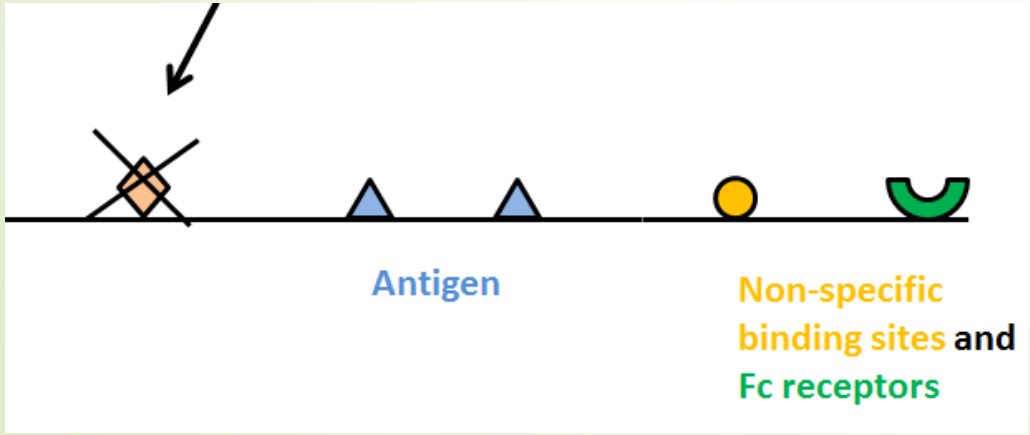


https://www.ptglab.com/support/immunohistochemistry-protocol/immunohistochemistry-overview/

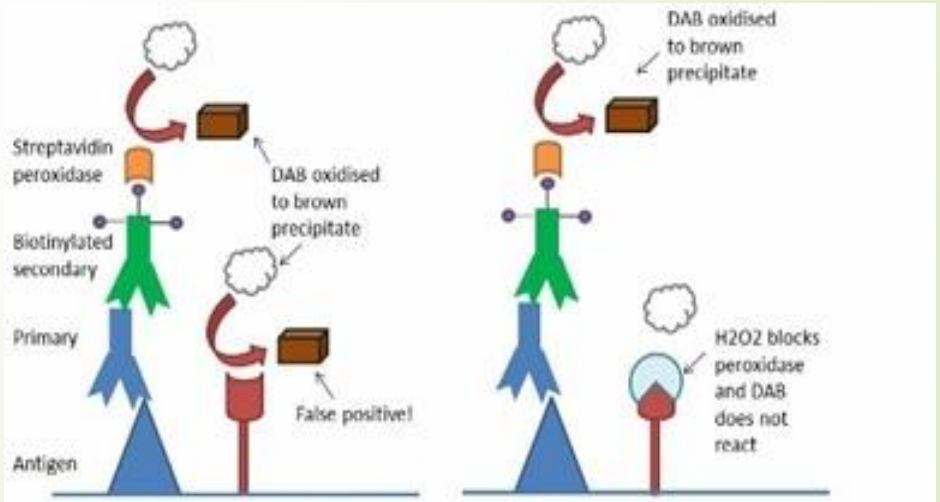
- Tissues may have endogenous substances that react with the substrate or antibodies used in the detection methods
  - Endogenous enzymes – Peroxidase & Alkaline phosphatase
  - Endogenous Biotin
  - Non-specific proteins/reactive sites - Antibodies show preferential avidity for specific epitopes but they may partially or weakly bind to sites on nonspecific proteins/reactive sites that are similar to the target antigen
- Need to block these to prevent non specific reactions leading to background staining and false positives
- Done prior to antibody staining

Blocking of endogenous peroxidase

- Endogenous peroxidase activity is present in RBC, neutrophils, eosinophils, hepatocytes
- Necessary if HRP conjugate is used for detection. Not for AP or fluorescent detection methods
- using excess of the enzyme substrate(hydrogen peroxide), at a much higher concentration will block activity of endogenous peroxidase
- Some epitopes are modified by peroxide, leading to reduced antibody-antigen binding. Incubation with peroxide after the primary incubation can overcome this



https://www.eurocytology.eu/en/course/894



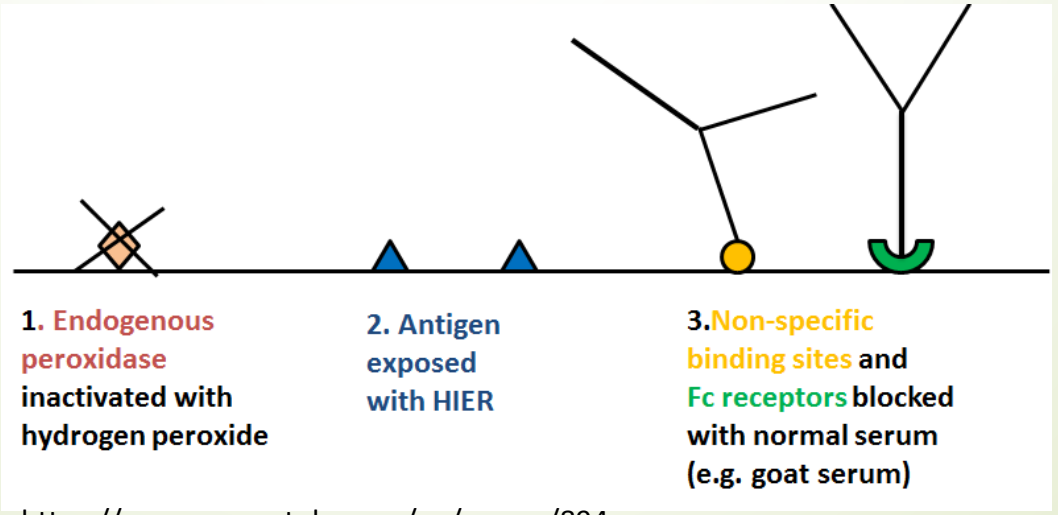
https://www.creative-diagnostics.com/Immunohistochemistry-IHC.htm

Blocking of endogenous AP and biotin

- endogenous alkaline phosphatase (AP)
  - Found mainly in frozen tissue
  - blocked with levamisol
- endogenous biotin
  - Found in tissues with abundant mitochondria – liver, kidney
  - blocked by incubating tissue section in avidin and biotin solution

Protein blocking

- Antibodies can bind to Fc receptors on the cells, collagen or proteins non-specifically via electrostatic charges
- These will react in the same way as specific/target antigen and give false positive results
- This can be blocked by a layer of non-immune serum (from the same species as the secondary antibody) before application of the primary antibody
- When the non-specific combining sites are occupied by normal immunoglobulin, the primary and secondary antibodies cannot bind to them
- Common blocking buffers include normal serum, non-fat dry milk, Bovine serum albumin(BSA), or gelatin



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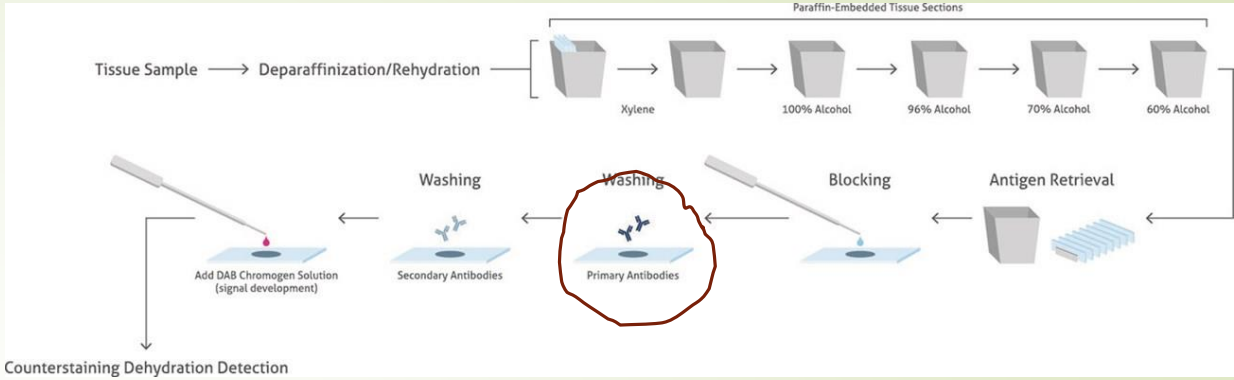
Primary antibody

- The primary antibody should be raised in a species different from the tissue being stained
- Apply primary antibody diluted in TBS with 1% BSA.
- Incubate to allow Ab to bind to Ag
- overnight incubation at 4°C - allows use of antibodies of lower titer or affinity more time to bind
- Two major types of primary Ab are used
  - Polyclonal Abs - produced by injecting an animal with antigen, then harvesting fluid from the animal
  - Monoclonal Abs - are mostly produced by hybridomas, created by fusion of a splenic B-cell from an immunised mouse, with an immortalised myeloma cell line



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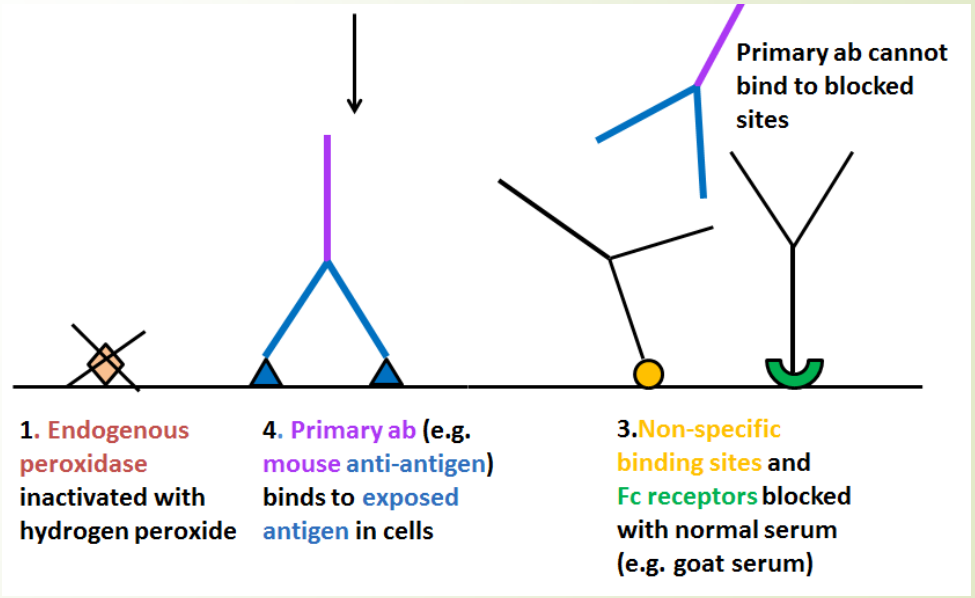
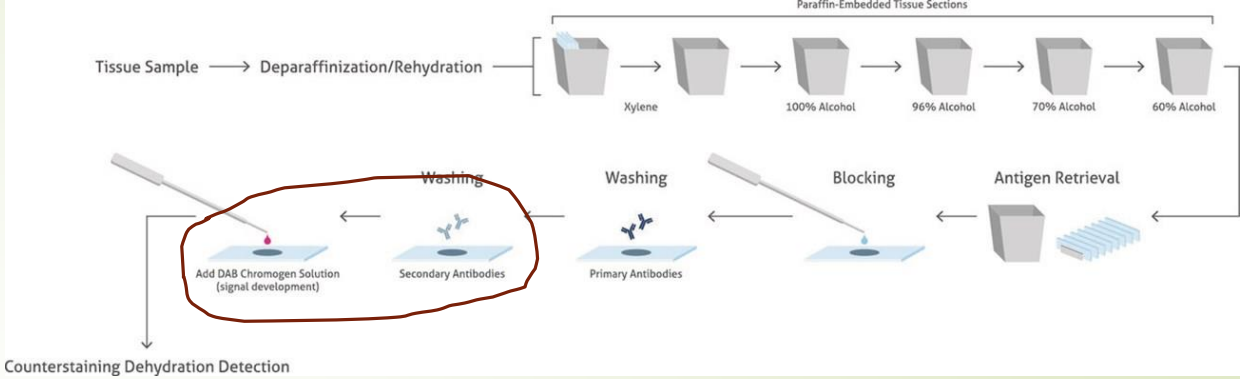


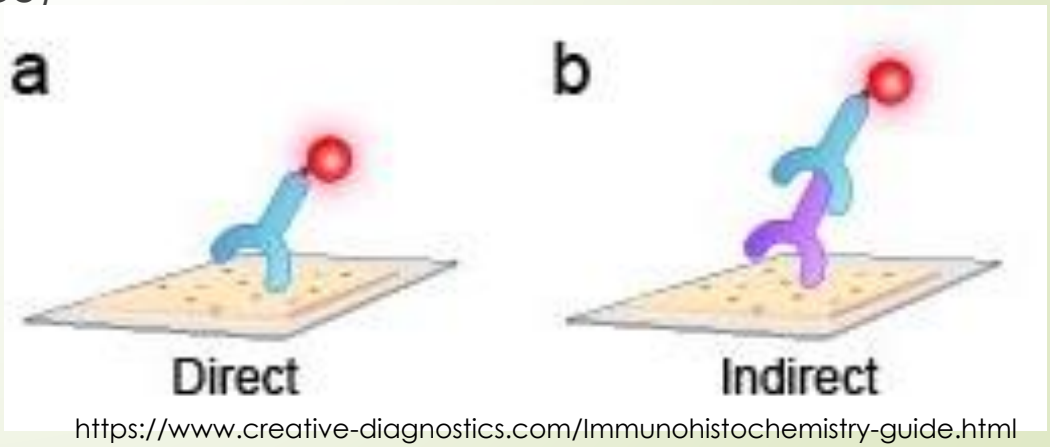
https://www.eurocytology.eu/en/course/894

Detection methods



https://www.ptglab.com/support/immunohistochemistry-protocol/immunohistochemistry-overview/

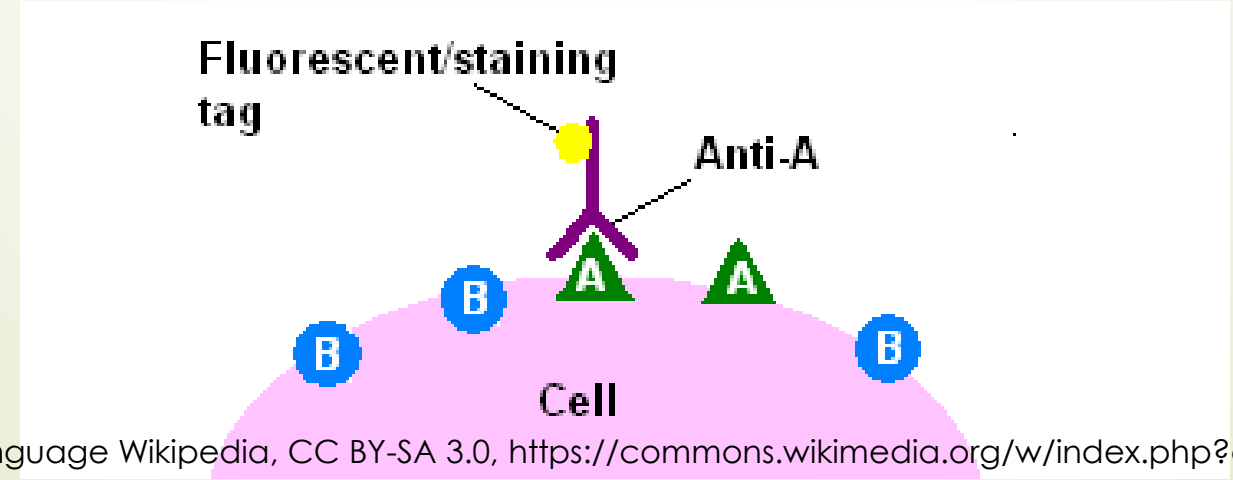
- Antigen-Ab conjugates are not visible by light microscopy
- A "label" is attached to help visualize the conjugate
- Labels are mostly
  - Enzymes - act on specific substrate chromogens to produce strongly coloured precipitates
    - Peroxidase-most commonly used
    - Alkaline phosphatase
  - Fluorescent molecules (immunofluorescence)
- Detection systems can be
  - Direct
  - Indirect



https://www.creative-diagnostics.com/Immunohistochemistry-guide.html

Immunofluorescence Method

- performed commonly on inflammatory skin biopsies and renal biopsies
- Use fresh frozen tissue without antigen retrieval
- Primary Ab is labelled with a fluorescent molecule (immunofluorescence)
- Produces light when excited by a laser (e.g. argon-ion laser).
- Specific antibody binding determined by the production of a visible light
- Allows rapid identification of an antigen
- Detected by a fluorescence microscope
- Fluorescence is only temporary so sections cannot be stored



Enzymatic Method

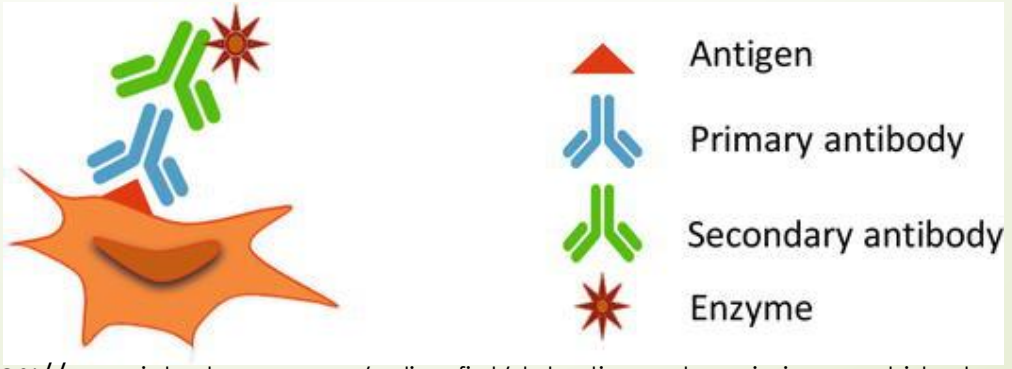
- antibody used for antigen detection is labeled with the enzyme before the reaction
- Antibody binds specifically to the target antigen
- The enzyme label is reacted with a substrate to yield an intensely coloured, insoluble product that can be visualized
- Labeled-enzyme method can be direct or indirect

- The direct method-one-step staining method where a labeled antibody (e.g. HRP-conjugated antibody) reacts directly with the antigen. The antigen-antibody-HRP complex is then allowed to react with a substrate for staining

- The indirect method is a two-step process

  1. unlabeled primary antibody (first layer) binds to the target

  2. enzyme-labeled secondary antibody (second layer) reacts with the primary antibody and then allowed to react with a substrate



https://www.intechopen.com/online-first/detection-systems-in-immunohistochemistry

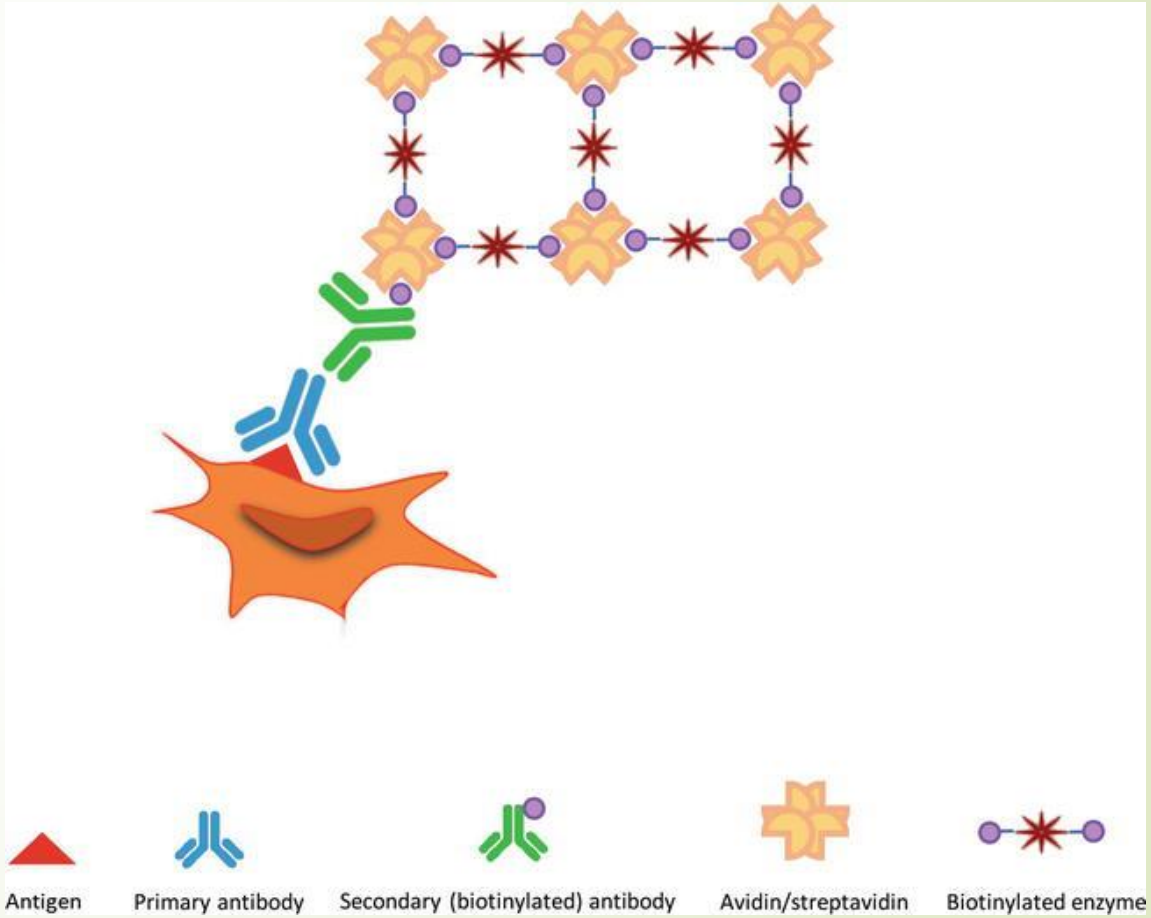
Affinity Method – signal amplification

- improve IHC sensitivity by binding higher number of molecules

- widely used affinity methods for amplifying the target antigen signal
  - Avidin-Biotin Peroxidase Complex (ABC)
    - Avidin, an egg white protein, has four binding sites for the low-molecular-weight vitamin biotin to form a large lattice-like complex
  - Labeled Streptavidin Binding (LSB)
    - Streptavidin is a tetrameric biotin-binding protein that is isolated from Streptomyces avidinii
  - Polymer method

ABC The method – Avidin Biotin Peroxidase complex

- Incubation of primary antibody with tissue sample to allow binding to target antigen
- Incubation of biotinylated secondary antibody (which has specificity against primary antibody) with tissue sample to allow binding to primary antibody
- Pre-incubation of biotinylated enzyme (HRP or AP) with free avidin to form large ABC complexes (Biotinylated enzyme and avidin are mixed together in a pre-determined ratio to prevent avidin saturation)
- Incubation of the above pre-incubated solution to tissue sample



https://www.intechopen.com/online-first/detection-systems-in-immunohistochemistry

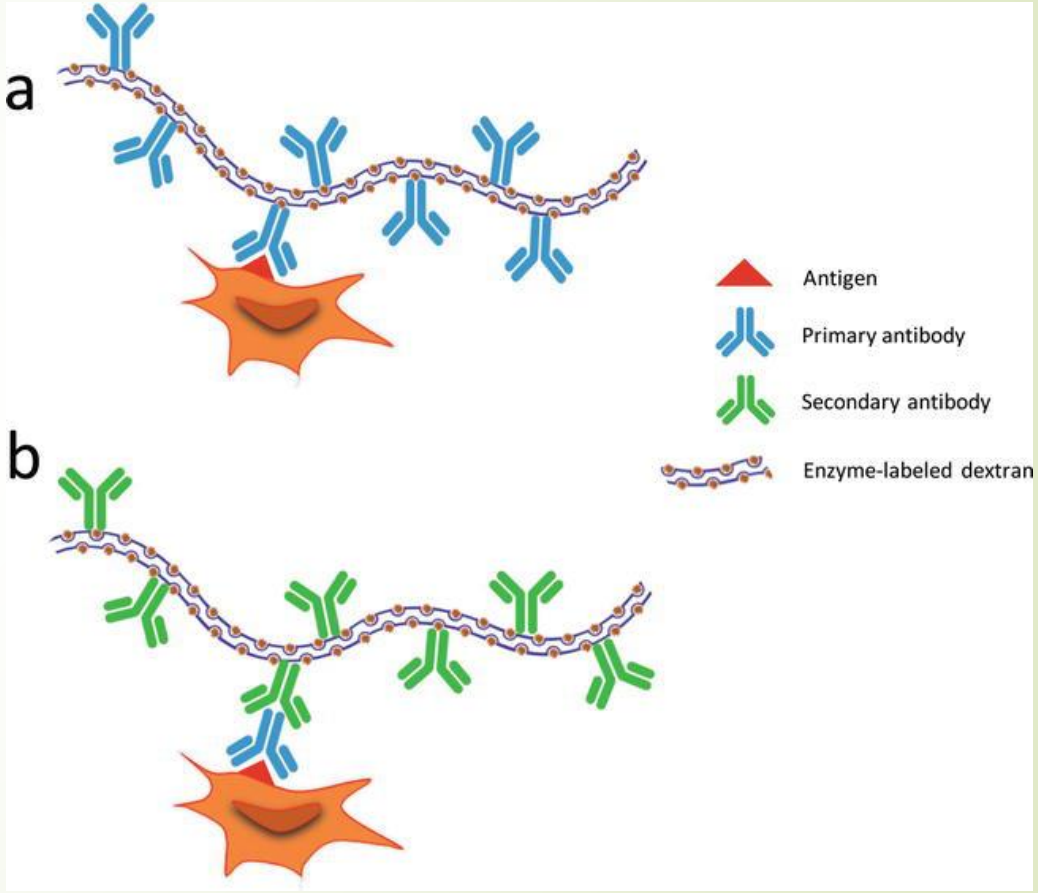
Labelled streptavidin Biotin system

- uses an enzyme-labeled streptavidin to detect the bound biotinylated primary antibody
- Due to its smaller size, the enzyme-labeled streptavidin enables better tissue penetration than ABC

https://www.intechopen.com/online-first/detection-systems-in-immunohistochemistry

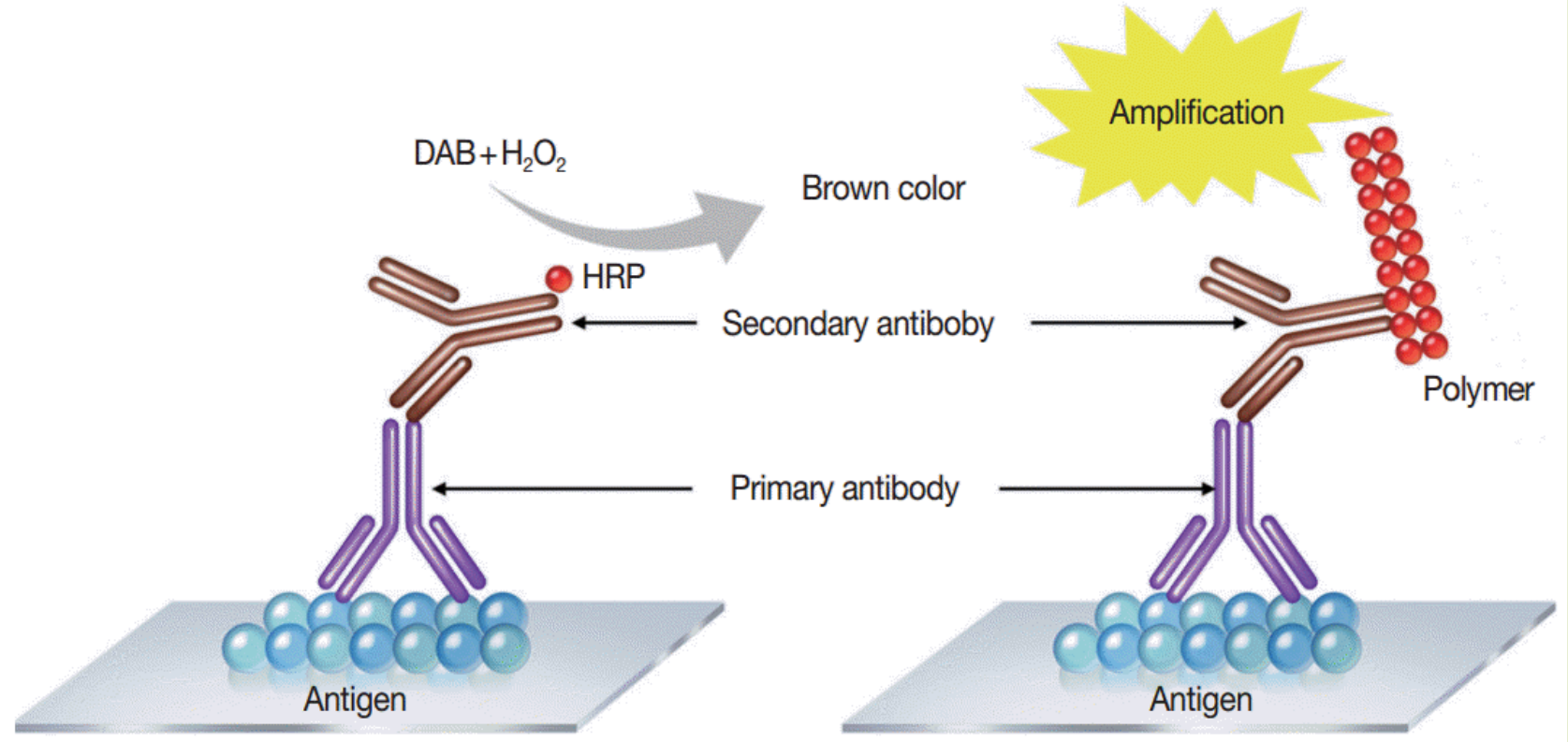
Polymer method

- A backbone of an inert polymer molecule (dextran) carries both antibodies and multiple enzymes
- Increases sensitivity
- As the labelled polymer does not contain avidin or biotin nonspecific staining resulting from endogenous avidin-biotin activity is eliminated or significantly reduced
- useful for the detection of antigens present in low concentrations or for low titer primary antibodies
- Used in Envision system



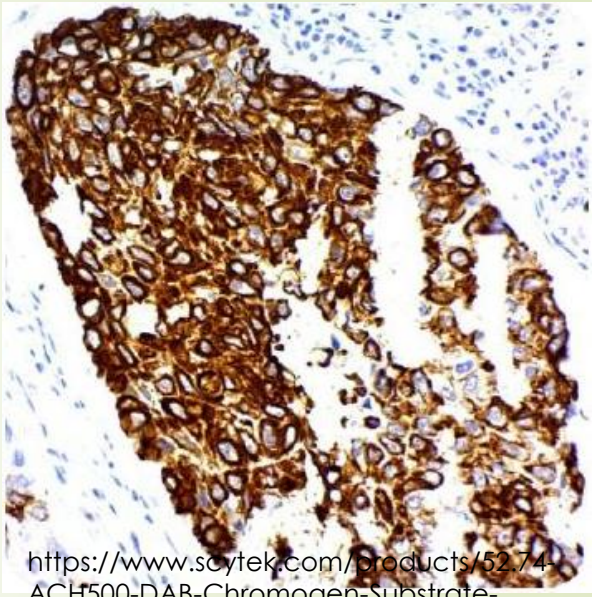
https://www.intechopen.com/online-first/detection-systems-in-immunohistochemistry

Chromogens

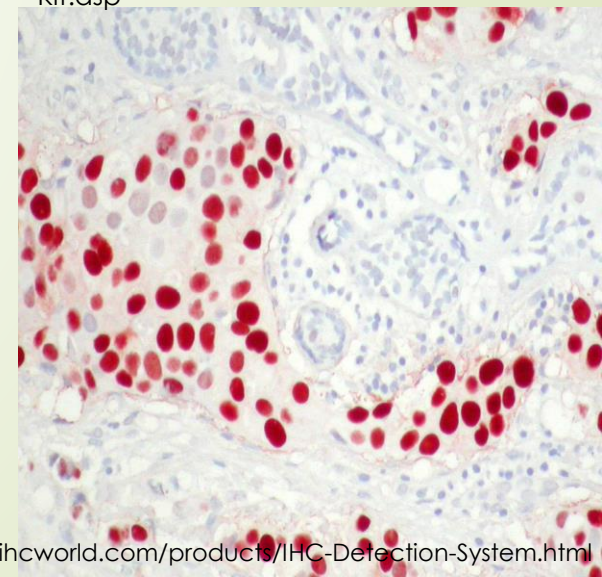


https://www.jpatholtm.org/journal/view.php?number=16651

- **Chromogens for HRP**
  - DAB (3,3'-Diaminobenzidine).
    - dark brown end-product
    - insoluble in water and alcohol
    - stable and suitable for long-term storage
    - end-product could be observed under a light microscope or processed with OsO4 for observation under electron microscopy
    - Hematoxylin, methyl green and methyl blue are the compatible counterstains
  - AEC (3-Amino-9-Ethylcarbazole)
    - End product is dark red
    - soluble in organic solvent
    - cannot be stored long-term
    - Glycerin gelatin should be used as the mounting medium
    - Hematoxylin, methyl green and methyl blue are the compatible counterstains

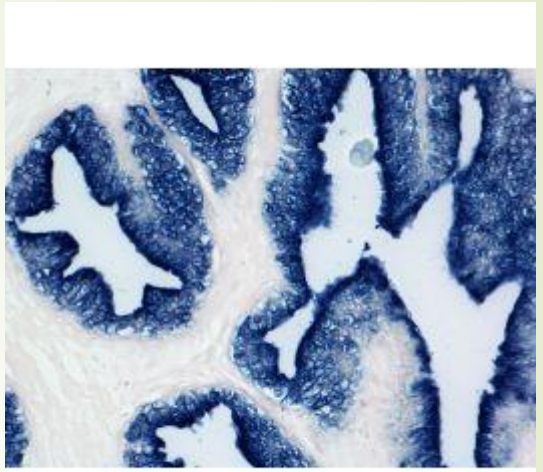


https://www.scytek.com/products/52.74-ACH500-DAB-Chromogen-Substrate-Kit.asp

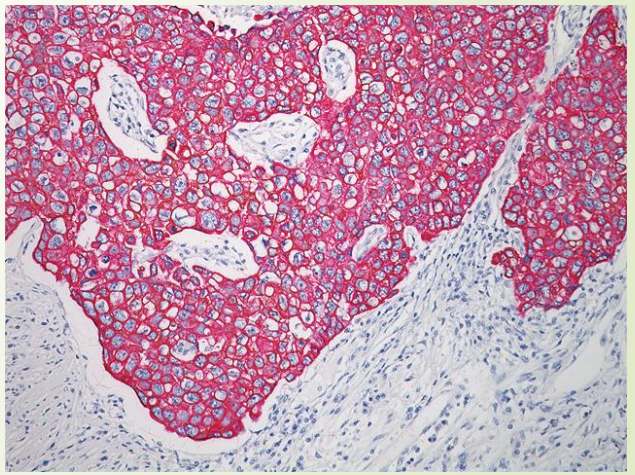


http://www.ihcworld.com/products/IHC-Detection-System.html

- **Chromogens for AP**
  - BCIP (5-Bromo-4-Chloro-3-Indolyl-Phosphate)/NBT (Nitro Blue Tetrazolium)
    - End product is bluish violet or black violet
    - insoluble in alcohol.
    - Nuclear fast red and brilliant green are the suitable counterstains
  - Fast Red
    - end-product has a rose color
    - soluble in alcohol
    - counterstains are : methyl green, brilliant green and soluble hematoxylin.



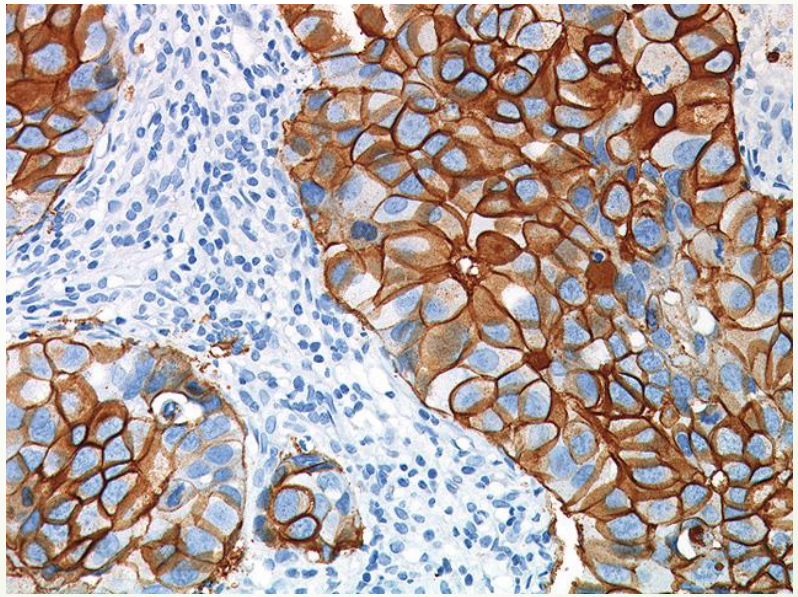
https://vectorlabs.com/bcip-nbt-ap-substrate-kit-5-bromo-4-chloro-3-indolyl-phosphate-nitroblue-tetrazolium.html



https://biocare.net/product/warp-red-chromogen-kit/

Counterstains

- provides contrast to the chromogens for better discrimination of the target signal
- Hematoxylin is the most commonly used counterstain



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Thank you