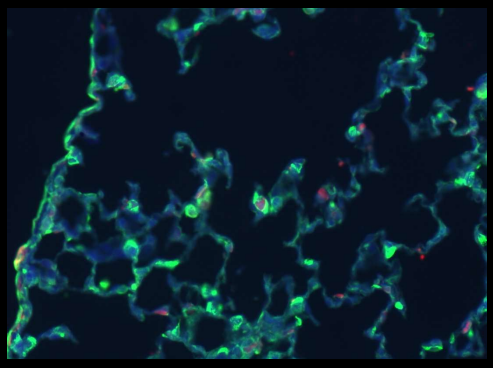


Department of Clinical Sciences Faculty Meeting – 4-14-11

THE BASICS OF IMMUNOHISTOCHEMISTRY

1

Common Methods of protein detection

- ELISA
- Gel Electrophoresis
- Western blot
- Immunoprecipitation
- Spectrophotometry
- Enzyme assays
- X-ray crystallography
- NMR
- Immunohistochemistry

2

Immunohistochemistry – what's good about it?

- Antibodies bind to antigen in **specific manner**
- Gives you a *spatial location*
- **Can be used to locate particular cells and proteins**
- **Can be used to identify cellular events – e.g.apoptosis**

3

Introduction

- Immunohistochemistry (IHC) combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue.

4

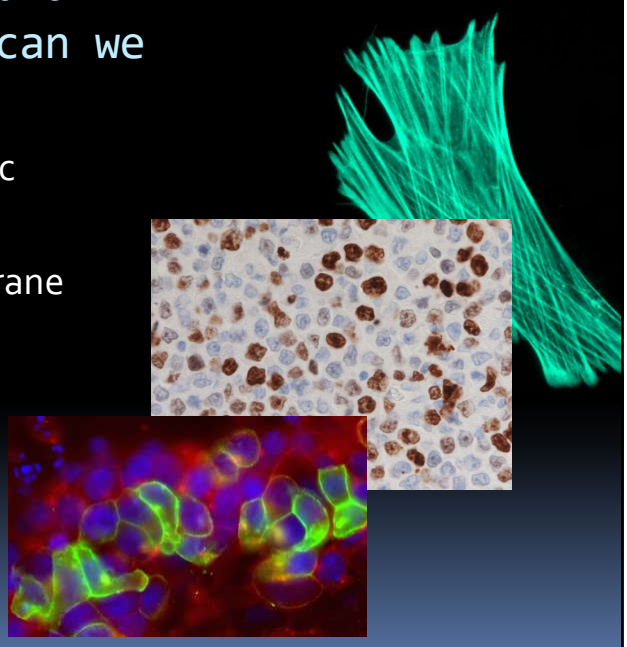
History

- The principle has existed since the 1930s.
- Started in 1941 when Coons identified pneumococci using a direct fluorescent method.
- Indirect method
- Addition of horseradish peroxidase
- Peroxidase anti-peroxidase technique in 1979
- Use of Avidin & Biotin complex in early 1980's

5

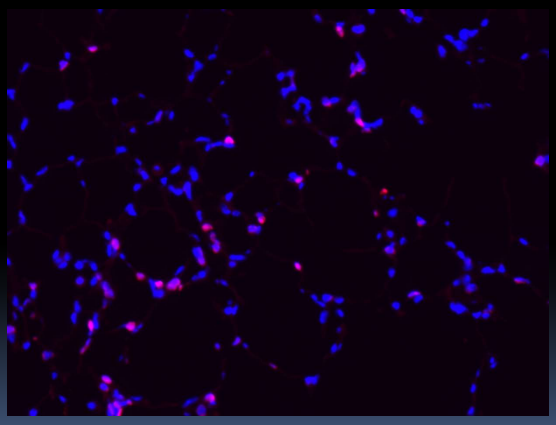
What cellular antigens can we target?

- Cytoplasmic
- Nuclear
- Cell membrane
- Lipids
- Proteins



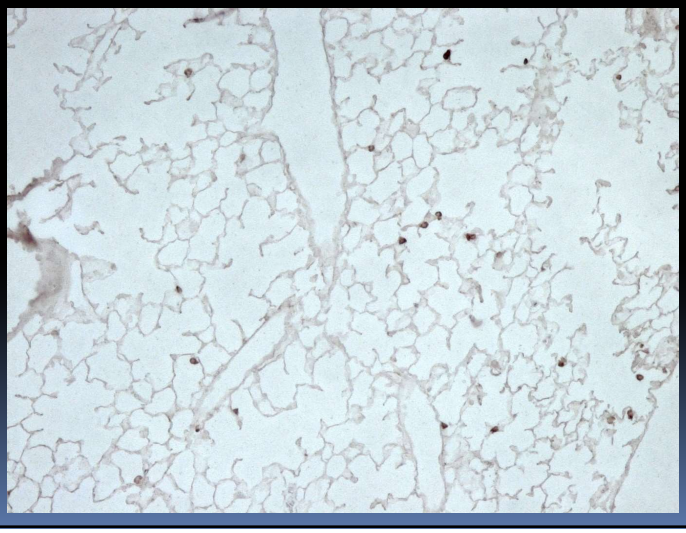
6

Identify replicating cells



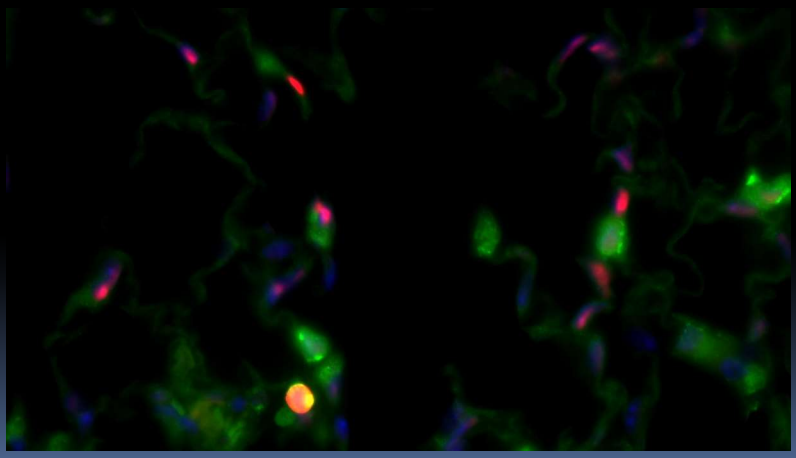
7

Locate cells that are signaling



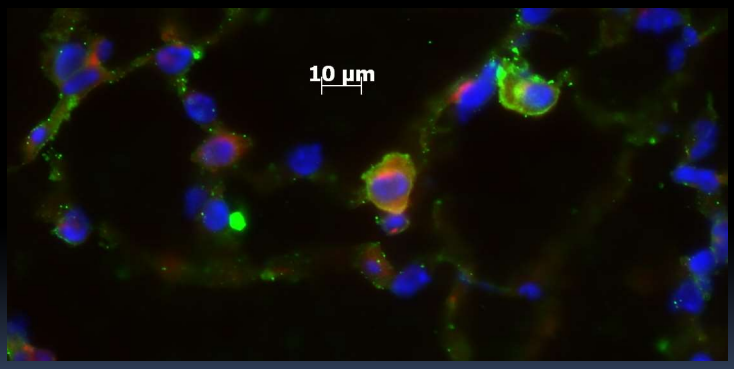
8

Locate apoptotic cells



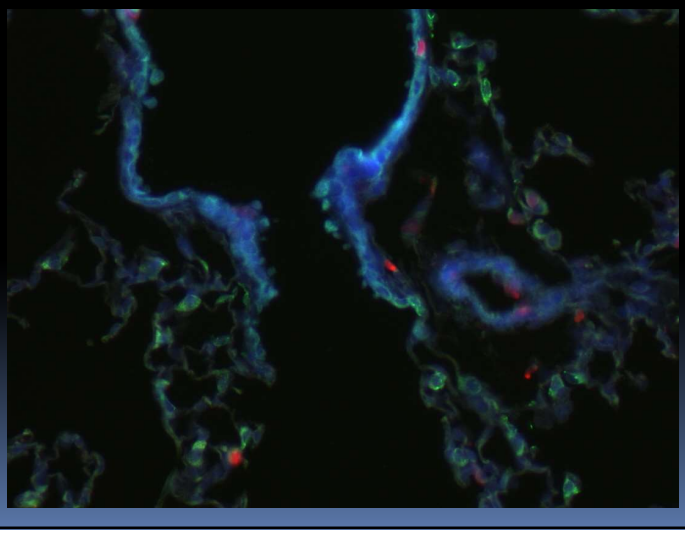
9

Identify activation states



10

Identify different types of cells in a tissue



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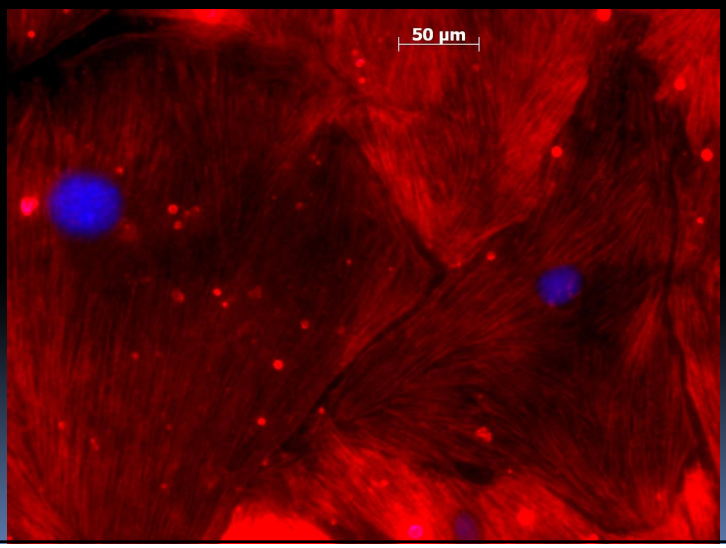
Examine cytoskeletal structure



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Important considerations for IHC

- Antibody selection
- Fixation
- Sectioning
- Antigen Retrieval
- Blocking
- Controls
- Direct method
- Indirect method
- Immunoenzyme
- Fluorescence
- Multiple labeling

You actually need to care about all this now because it may affect how you harvest your samples !

13

Options for antibodies that will affect your results

- Monoclonal v. Polyclonal
- Raised against whole molecule, N-terminus, C-terminus, specific amino acids
- Ascites, supernatant, serum

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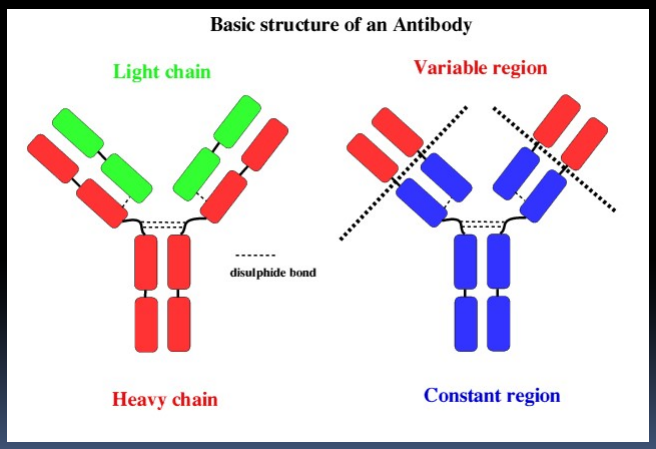
General antibody structure



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Monoclonal v. polyclonal

- Monoclonal
  - Mouse or rabbit hybridoma
  - Tends to be 'cleaner'
  - Very consistent batch-to-batch
  - More likely to get false negative results
- Polyclonal
  - Many different species
  - Tends to have more non-specific reactivity
  - Can have very different avidity/affinity batch-to-batch
  - More likely to have success in an unknown application

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Make sure your antibody is validated for your application!!!

- IF v. IHC with fluorescence
- WB, ELISA, IP, etc.

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Whole molecule or specific portion of epitope?

- Very dependent on individual assay

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Ascites, supernatant, serum?

- Differences in affinity/avidity
  - Ascites – highest affinity
  - Supernatant next
  - Serum lowest
  - Depends on concentration!

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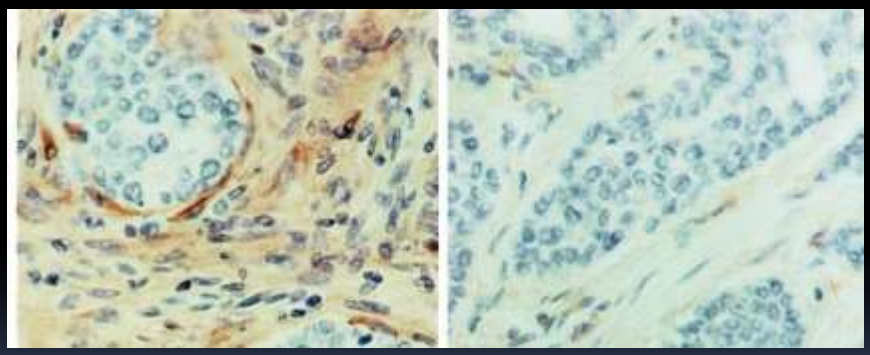
Fixation

- Aldehyde
  - 10% NBF
  - 4% formaldehyde with PBS buffer
  - 2% formaldehyde with picric acid and PBS
  - The paraformaldehyde paradox
  - Immersion v. transcardial perfusion
  - 24-72 hours
  - Many others
  - Best for good architecture
- Frozen
  - LN_2
  - With or without sucrose
  - OCT
  - Fix with acetone or methanol (fix by coagulation, also permeabilizes)
  - Best for cell membrane antigens, cytokines

20



Plasma urokinase inhibitor – 48 hours fixation v. 7 days fixation

21

Sectioning

- Paraffin
  - Must heat and process through xylenes and alcohols – ruins some antigens
  - Most commonly used
  - BEST if not stored more than two weeks – lose antigenicity after that time
- Frozen
  - Better survival of many antigens
  - Poor morphology
  - Poor resolution at higher mag
  - Special storage
  - Cutting difficulty

22

Antigen retrieval

- HIER
  - Use MW/steamer/pressure cooker ~ 20 minutes, slow cool
  - Citrate 6.0
  - Tris-EDTA 9.0
  - EDTA 8.0
  - Must determine for each new antibody/antigen target
- PIER
  - Proteinase K
  - Trypsin
  - Pepsin
  - Pronase,etc.
  - Destroys some epitopes
  - Bad for morphology

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Improving antibody penetration

- Need this for intracellular (cytoplasmic, nuclear) or membrane components when epitope is inside cell membrane
- Detergents most popular
  - Triton-X
  - Tween
  - Also decreases surface tension – better coverage
- Can't use for membrane proteins
- Acetone/Methanol
  - Precipitate proteins outside cell membranes- more accessible
- Saponin
  - Punches holes in cell membrane – holes close up when removed

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Blocking

- Background staining
- Specific
  - Polyclonal antibodies – impure antigen used
  - Inadequate fixation – diffusion of antigen – often worse in center of large block
- Non-specific
  - Non-immunologic binding – usually uniform
  - Endogenous peroxidases
  - Endogenous biotin

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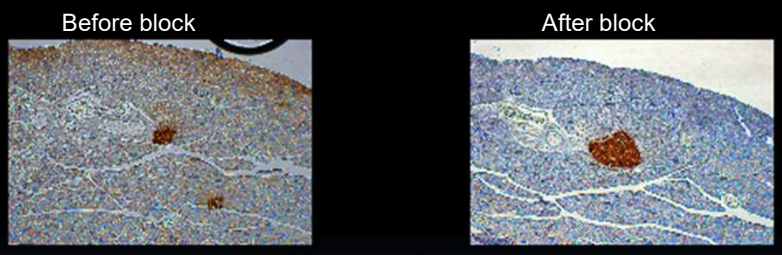
Non-specific staining



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Controls

- Positive control
  - Best is tissue with known specificity
- Negative control
  - Best is IgG from same species immunized against non-biologic molecule – e.g. BRDU when no BRDU is present in tissue
  - Can also use non-immunized serum from same species

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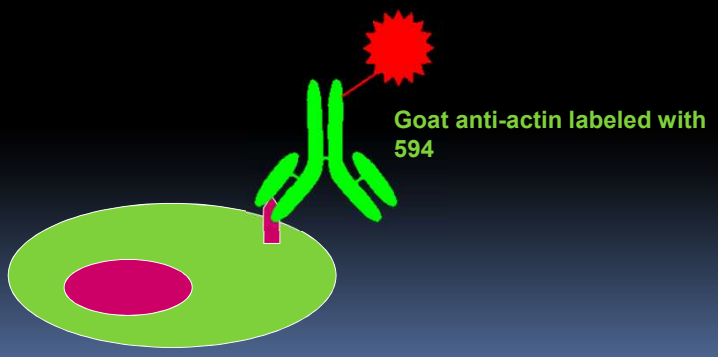
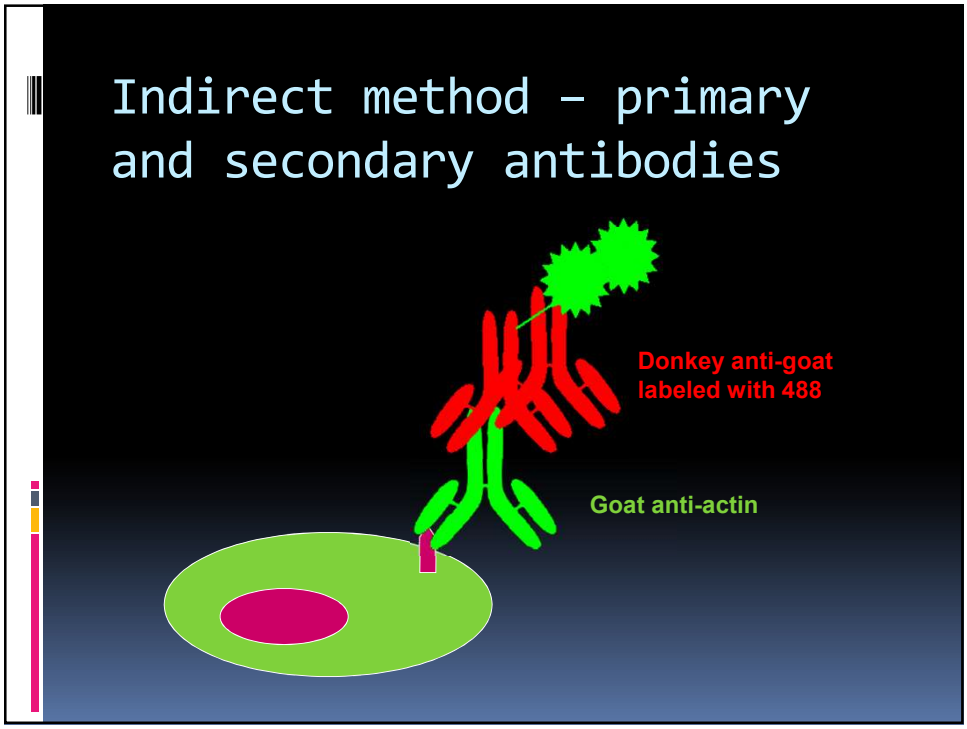
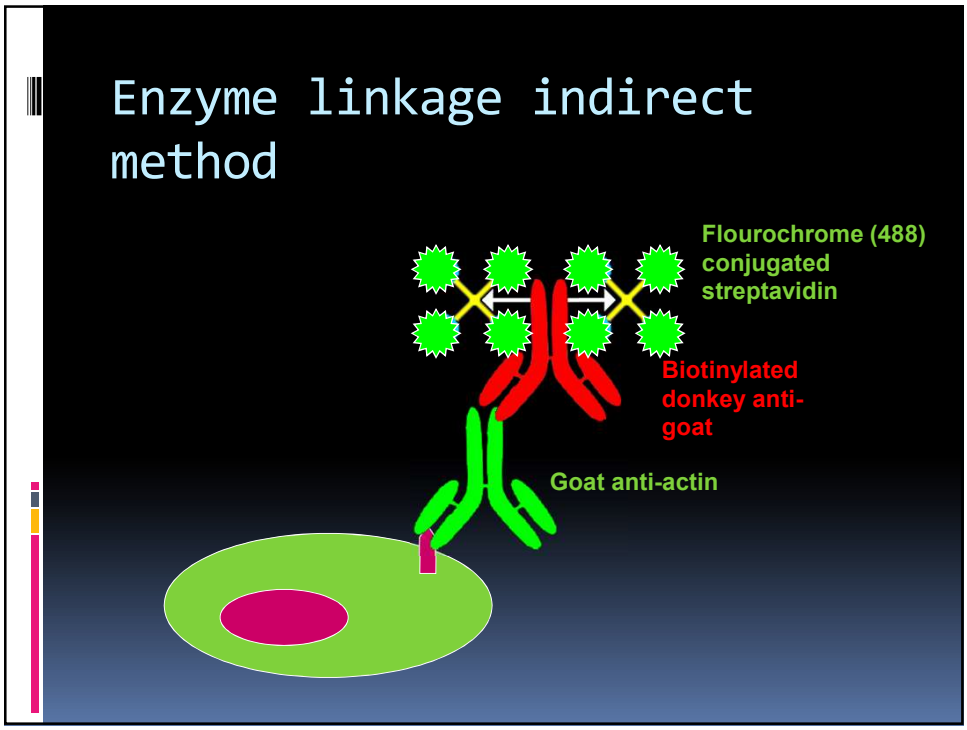
Direct method- primary antibody only



28

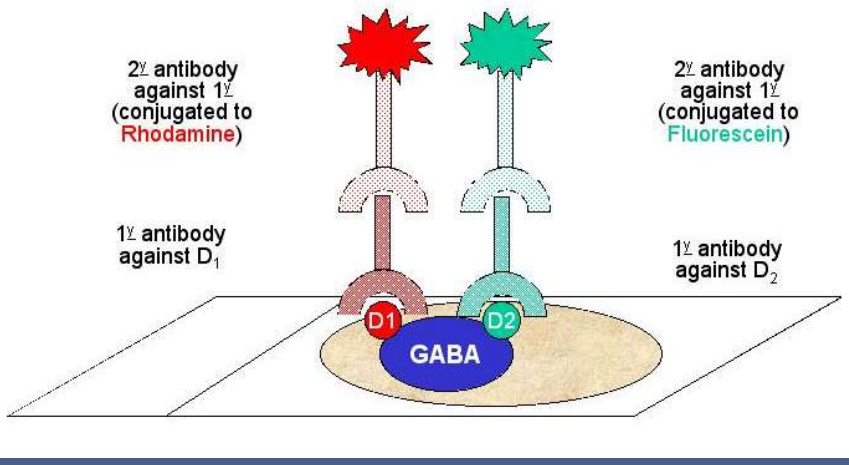

Indirect method – primary and secondary antibodies
Donkey anti-goat labeled with 488
Goat anti-actin

29


Enzyme linkage indirect method
Flourochrome (488) conjugated streptavidin
Biotinylated donkey anti-goat
Goat anti-actin

30

Multiple Immunofluorescence



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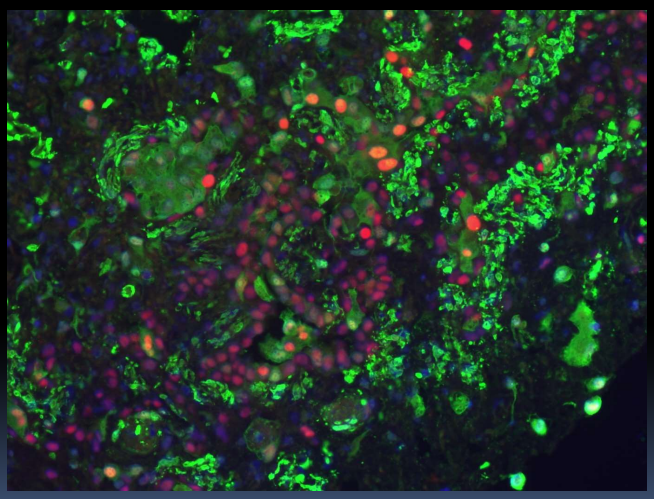
Multiple Labelling of a Tissue Section



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Enzymatic detection methods

- Brightfield microscope sufficient for analysis of specimens
- Suitable for tissue analysis at low magnification
- Resolution of subcellular structures not as good as with fluorescence methods, but can be combined with electron microscopy
- Unimited shelf life of labelled specimens
- Substrate reagents often toxic/carcinogenic

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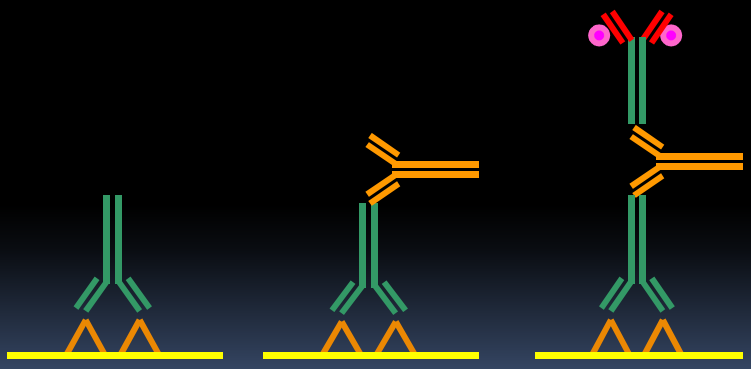
PAP Method
(peroxidase anti-peroxidase method)



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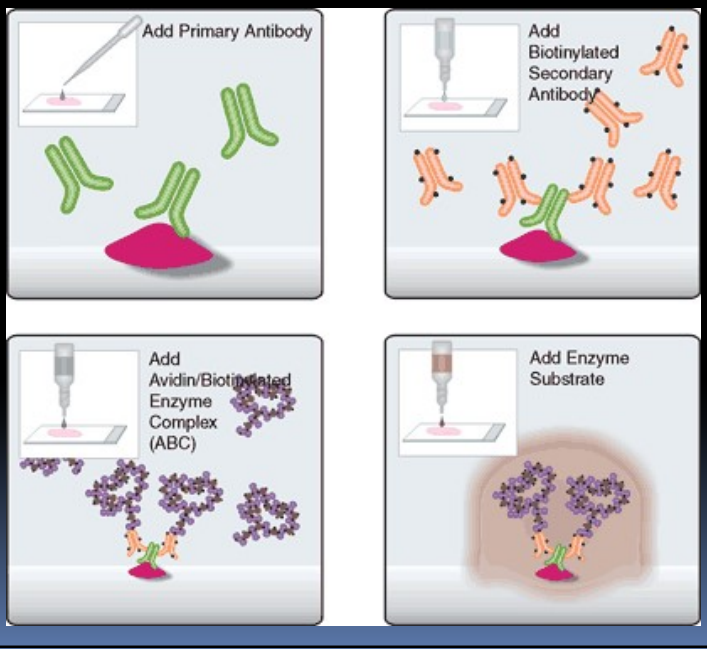
ABC method



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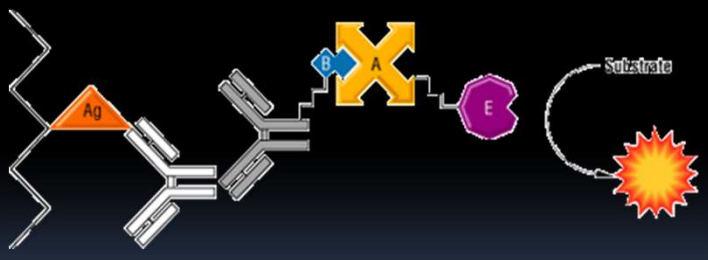
SP Method

(streptavidin peroxidase conjugated method)



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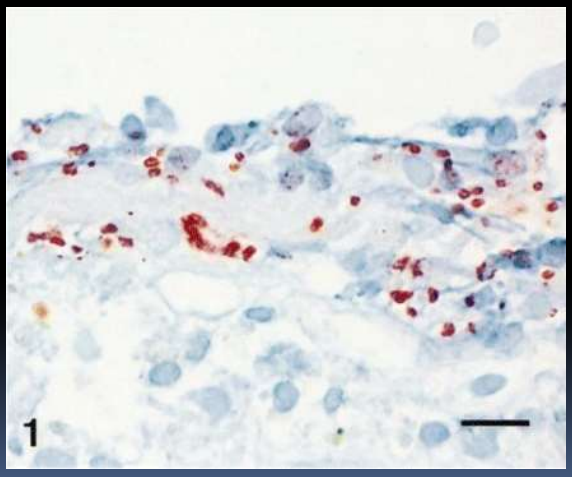
Beta-2 toxin for C. perf - DAB



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Summary

- IHC = immunology +histology + chemistry
- Has strengths and weaknesses
- Think about your planned assay before acquiring tissue
- Good block, appropriately fixed and sectioned can give you great data
- Bad block, inappropriately fixed and sectioned, can give you misleading data and waste money

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