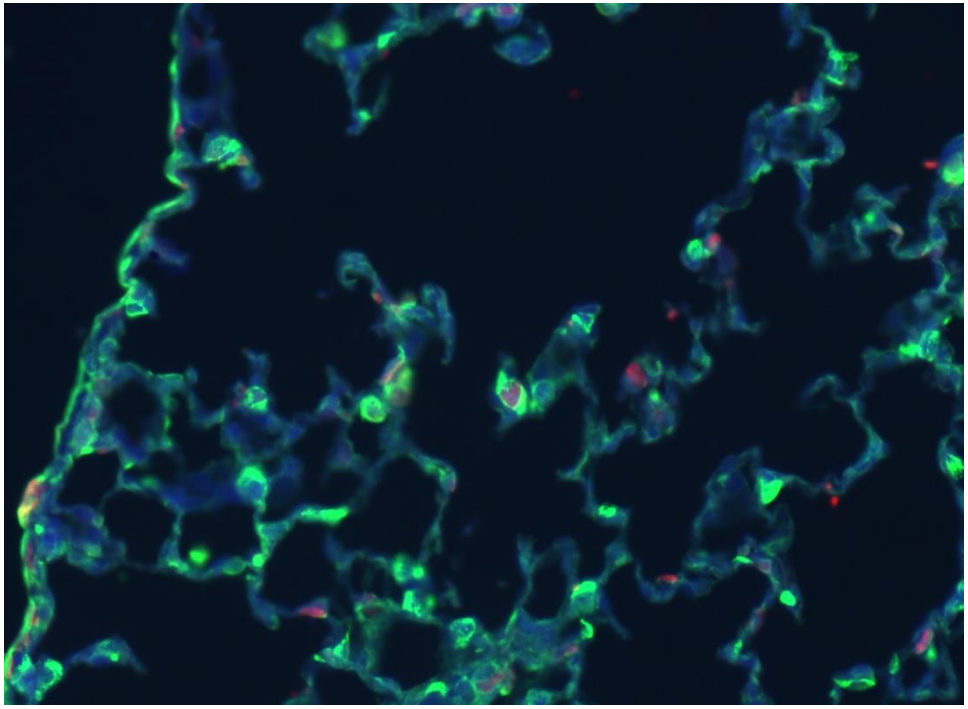


THE BASICS OF IMMUNOHISTOCHEMISTRY

Introduction

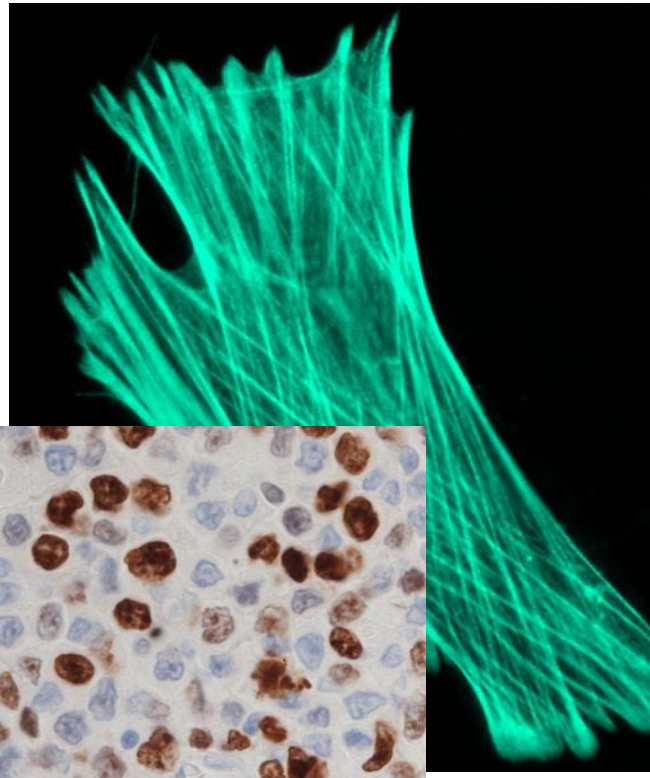
- Immunohistochemistry (IHC) identifies specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label.
- IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue.
- IHC utilizes labeled antibodies to localize specific cell and tissue antigens, and is among the most sensitive and specific histochemical techniques.

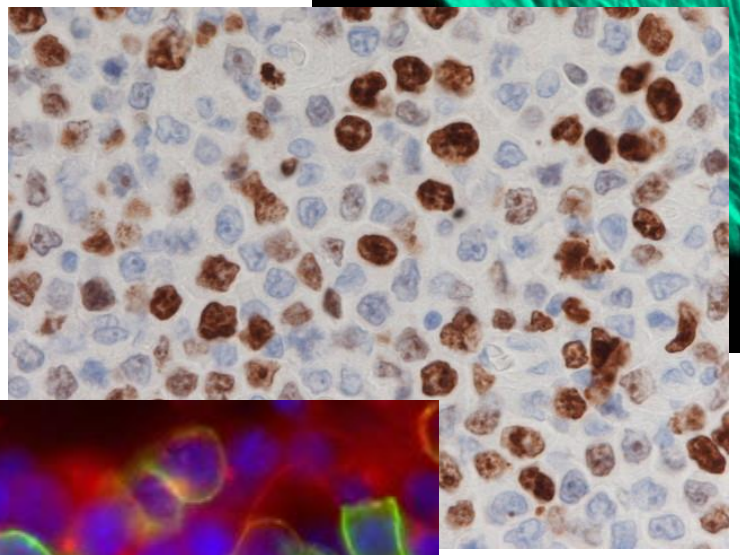
Immunohistochemistry – what's good about it?

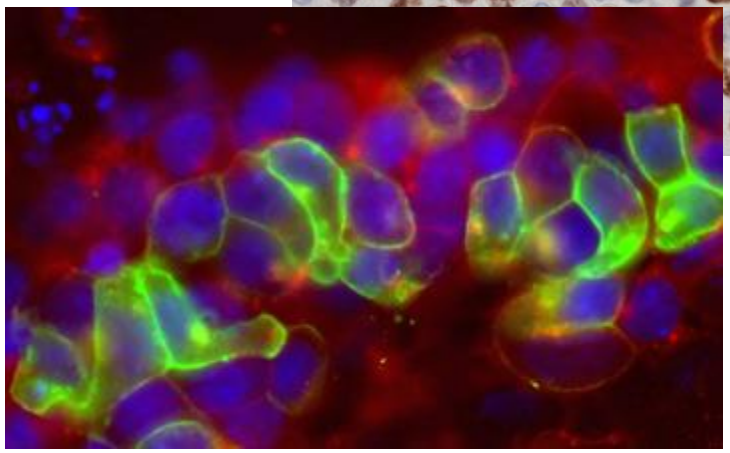
- Antibodies bind to antigen in **specific manner**
- **Gives you a *spatial location***
- **Can be used to locate particular cells and proteins**
- **Can be used to identify cellular events – e.g.apoptosis**

What cellular antigens can we target?

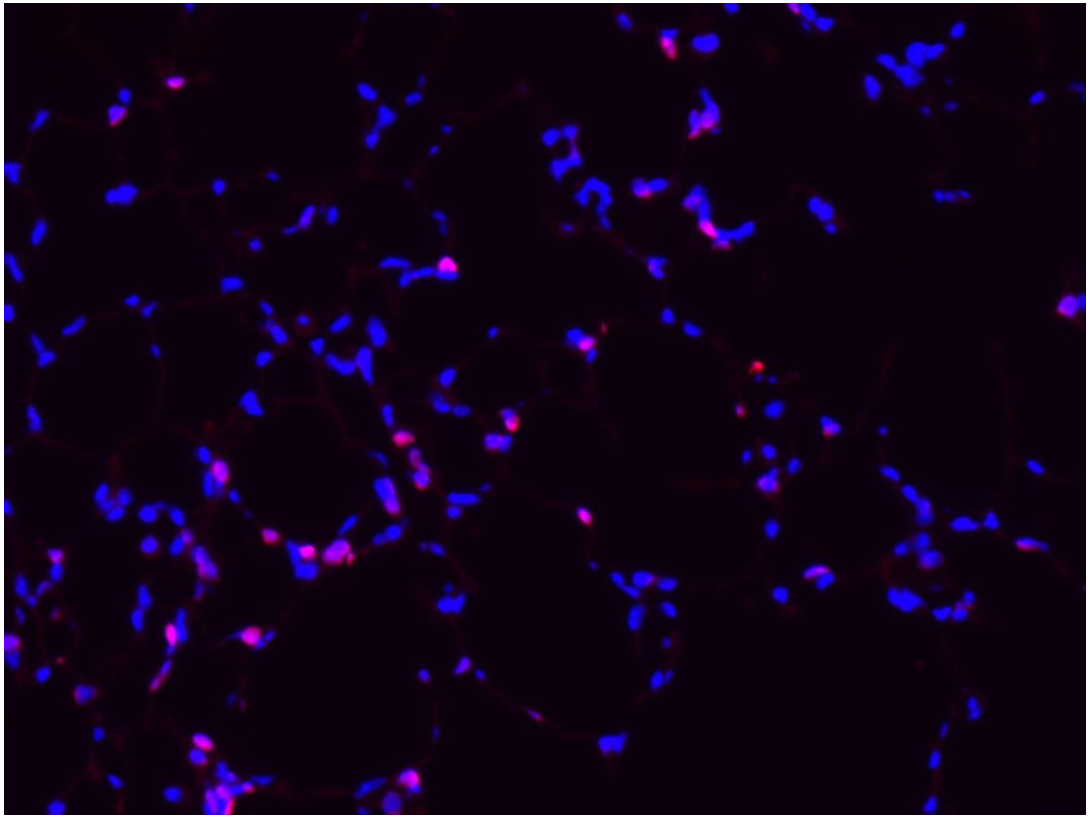
- Cytoplasmic
- Nuclear
- Cell membrane
- Extracellular matrix



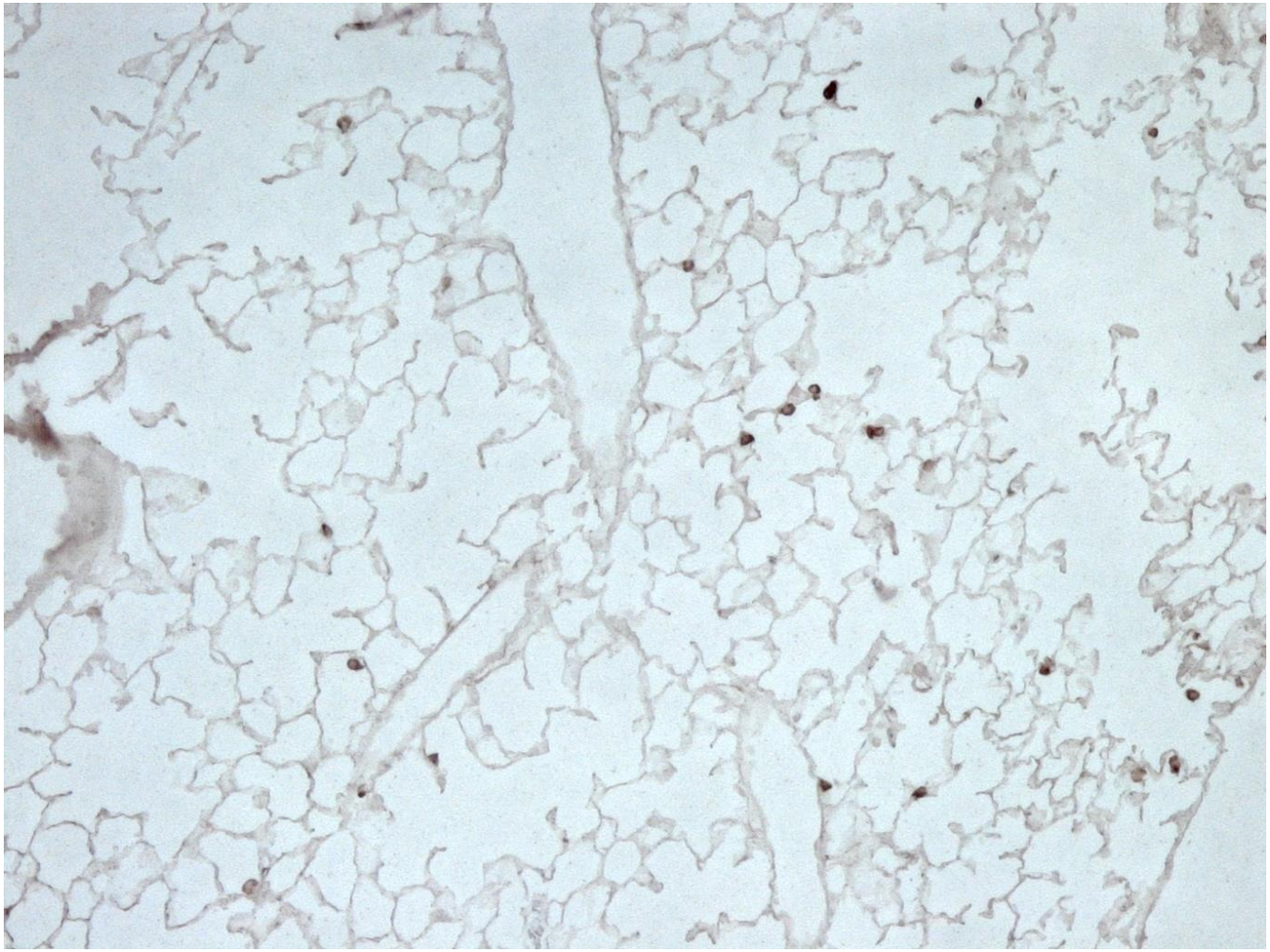




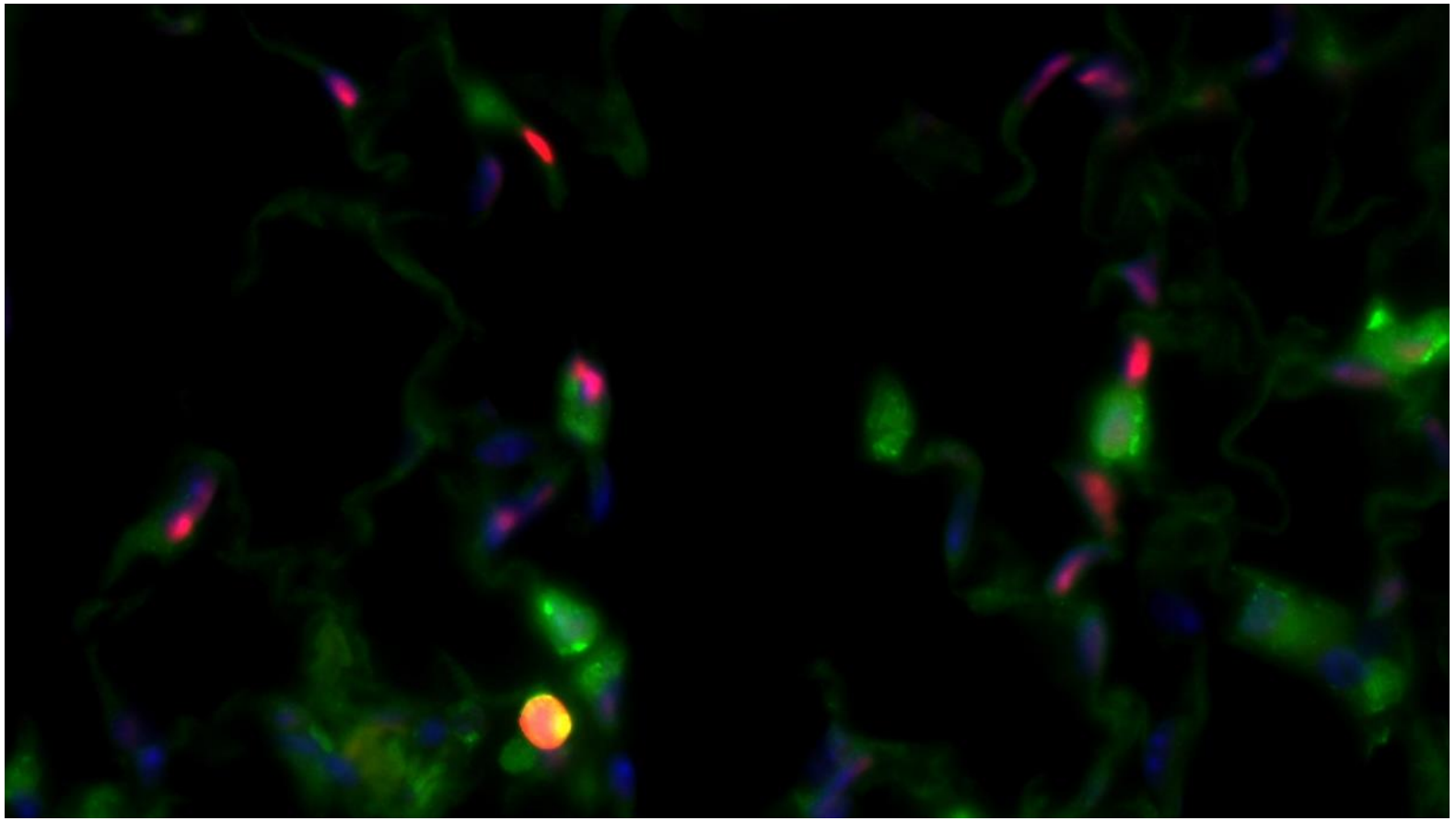
Identify replicating cells



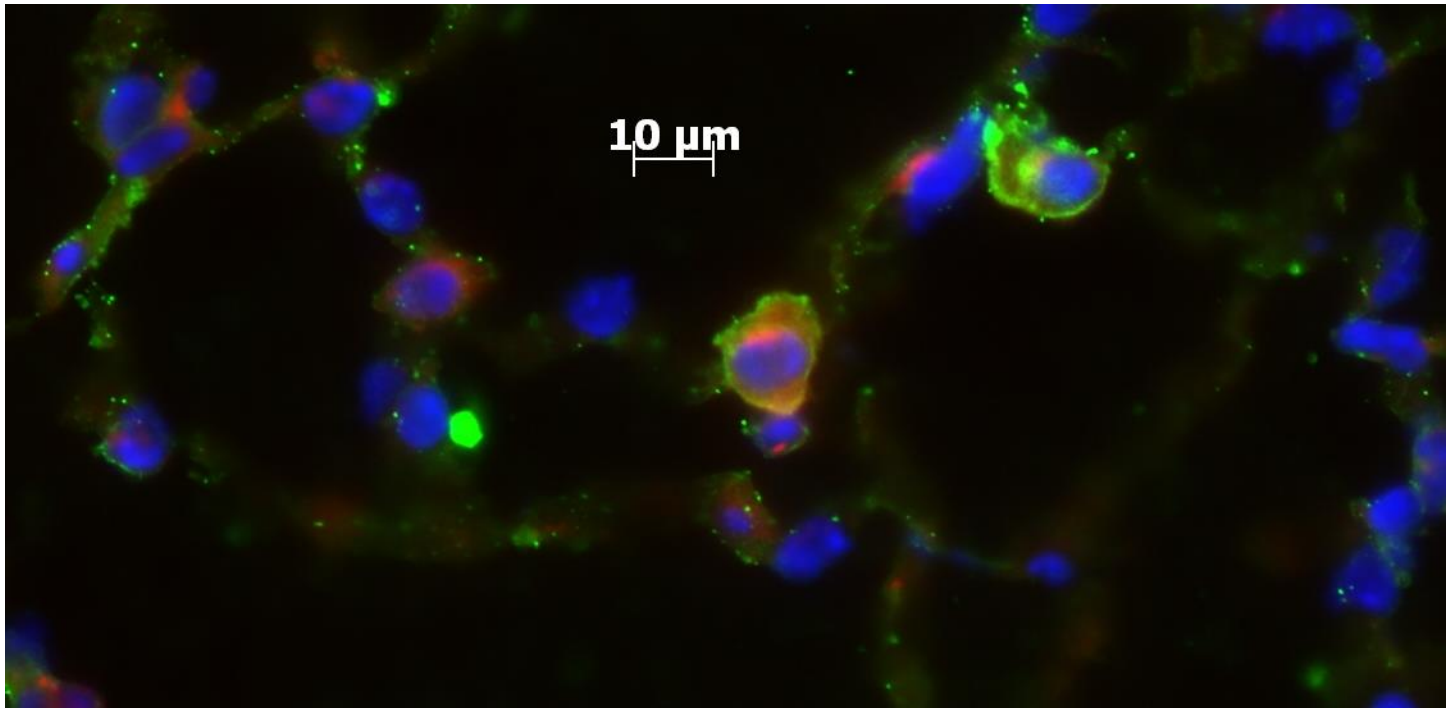
Locate cells that are signaling



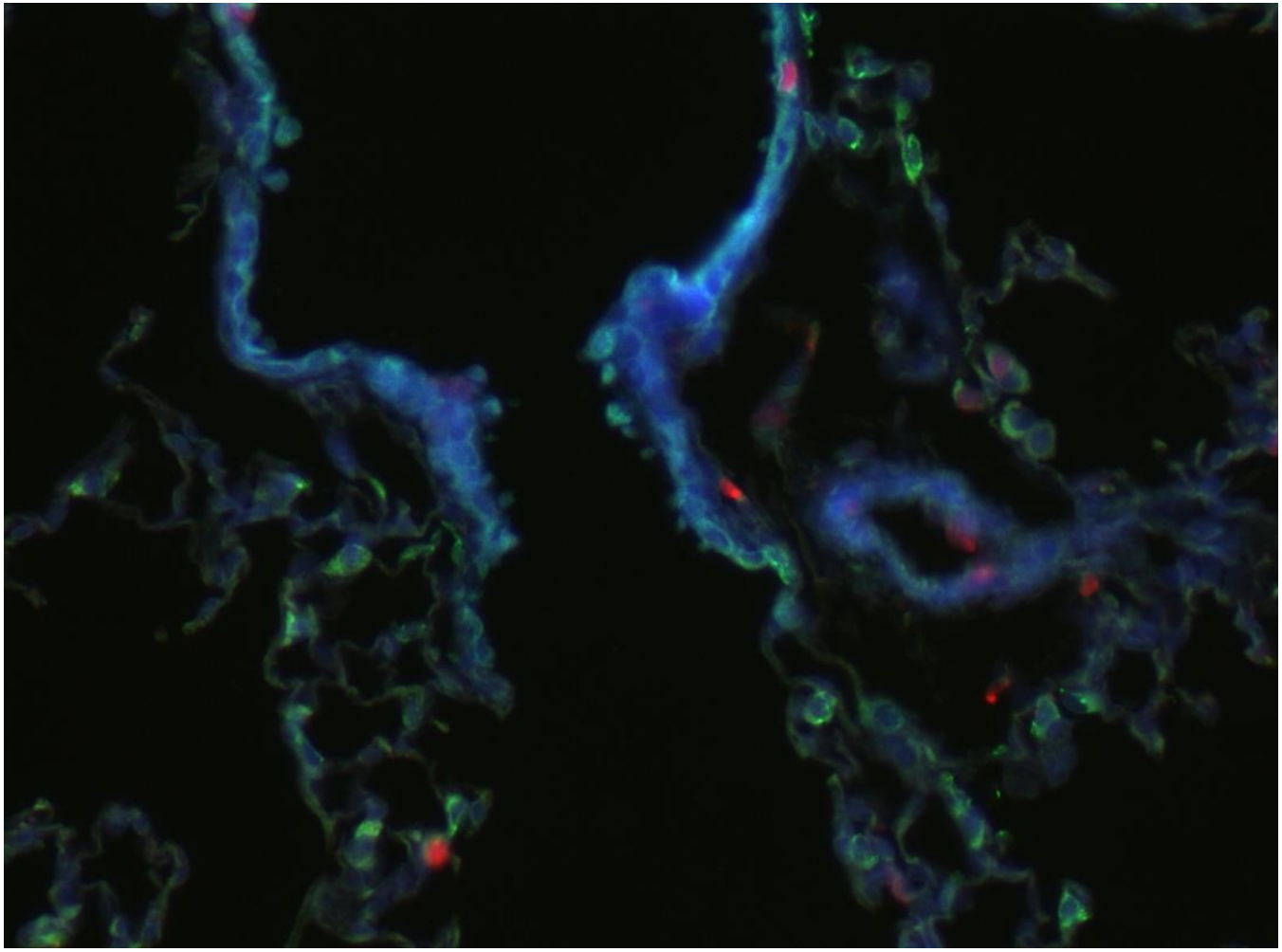
Locate apoptotic cells



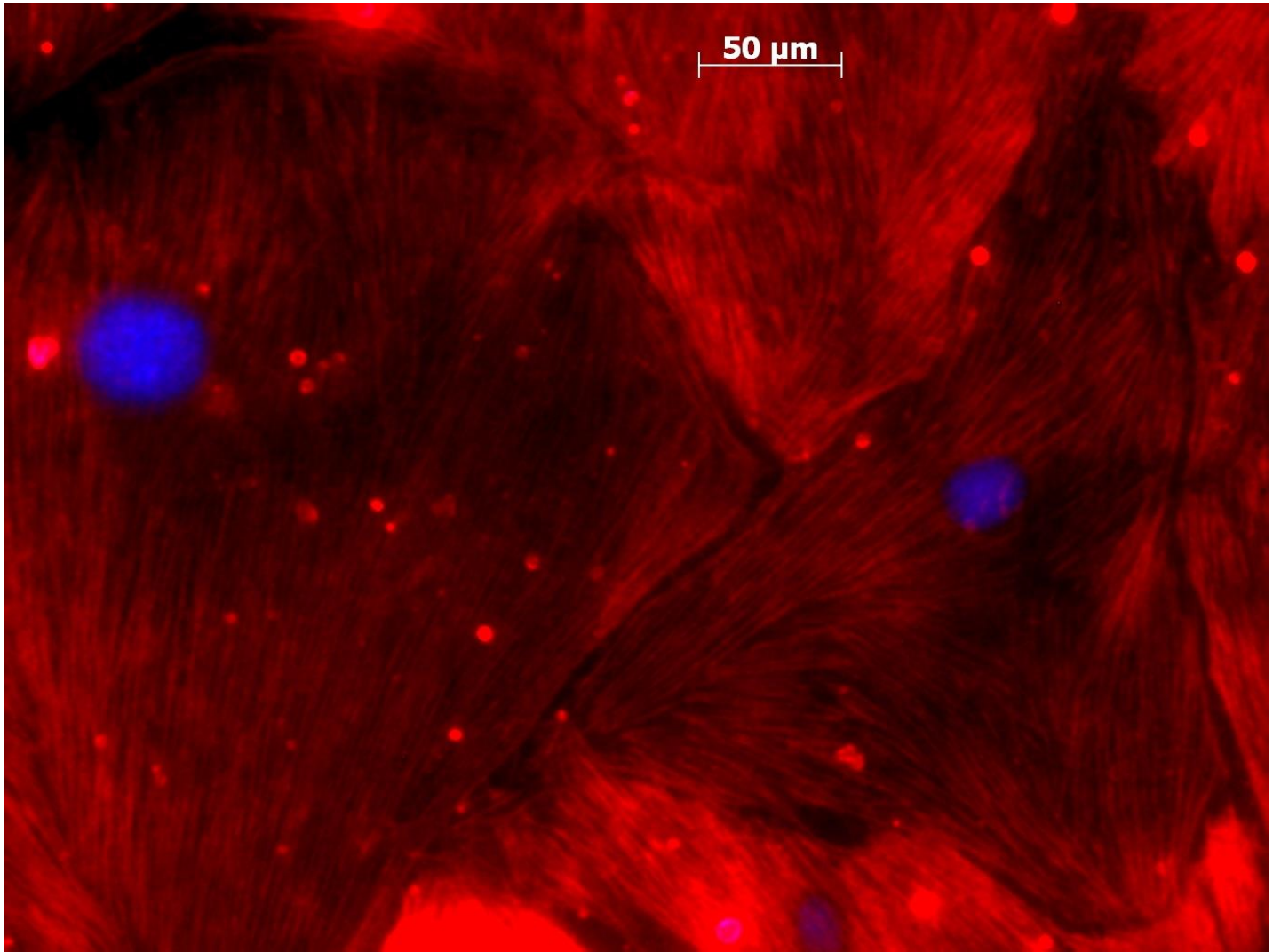
Identify activation states



Identify different types of cells in a tissue



Examine cytoskeletal structure



Important considerations for IHC

- Antibody selection
- Fixation
- Sectioning
- Antigen Retrieval
- Blocking

- Direct method
- Indirect method
- Immunoenzyme
- Fluorescence
- Multiple labeling
- Controls

IHC steps:

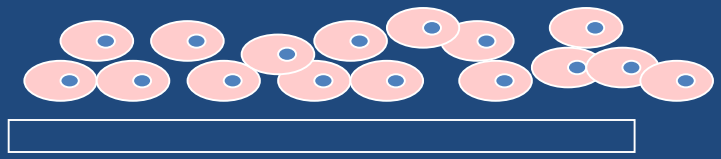
- Speciment
- Fixation
- Antigen retrieval / Permeabilization
- Blocking endogenous enzymes
- Blocking aspecific sites
- Incubation primary Ab
- Incubation secondary Ab
- Mounting

Controls

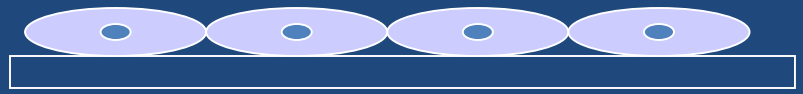
Microscopy Observation

Immunohistochemistry assays may use

cells on slides

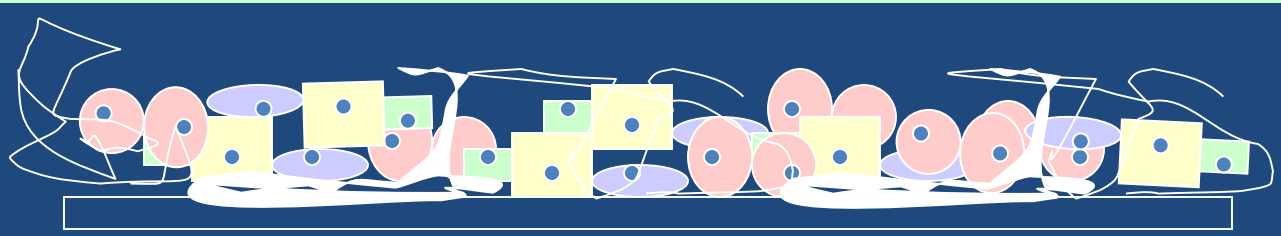


Cells grown, spun into a pellet, frozen or paraffin embedded and sectioned



Cells grown as a monolayer

or use tissue sections that are frozen or paraffin embedded



Sections from tissues contain many different kinds of cells

as well as extra-cellular matrix components

Fixation

Helps to prevent:

- Degradation
- Modification
- **Preserves the position of the Ag**
- Provides target for Ab molecules
- Formaldehyde is the preferred fixative
- Most of the Ab available are optimized for use with formaldehyde

Fixation

- Aldehyde
  - 10% NBF
  - 4% paraformaldehyde with PBS buffer
- Frozen
  - LN_2 + Isopentane

If the tissue is frozen

Advantage antigens are unaltered

Disadvantage Poor morphology

Poor resolution at higher magnification

Special storage

Cutting difficulty

Tissue section on glass slide: Frozen

If the tissue is paraffin embedded

- **Must fix. Remember the paraformaldehyde paradox (12 – 24 hours)**
- **Process through xylenes and alcohols ruins some antigens**
- **Deparaffinize (remove the infiltrated paraffin wax, by using organic solvents).**

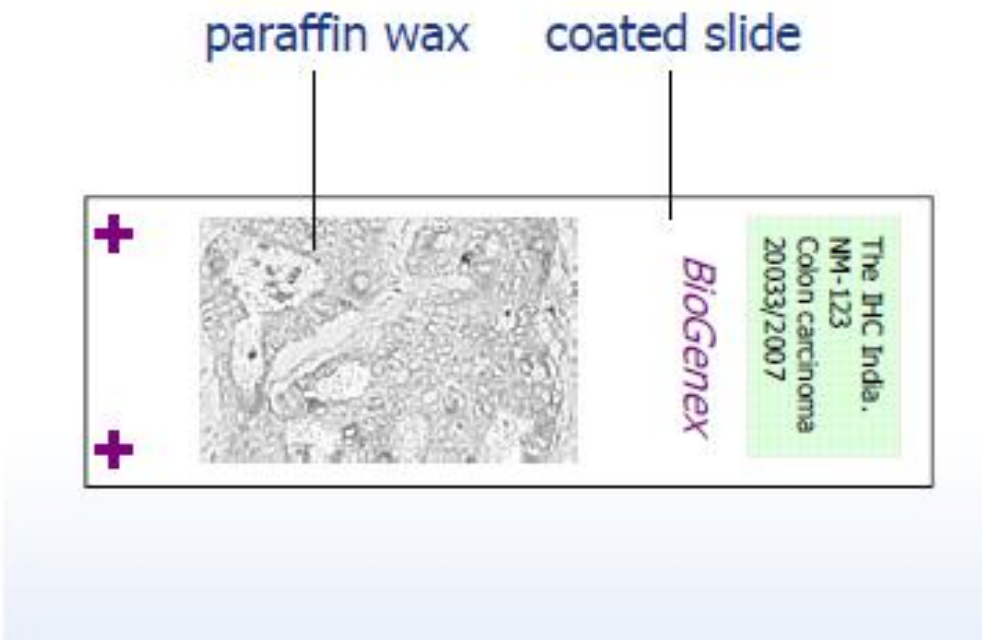
- The deparaffinized section have to be treated to expose buried antigenic epitopes with either proteases or by heating in low pH citrate buffer (Antigen Retrieval)

Best for good architecture

Slide preparation

- 5-8 micron tissue sections are cut onto slides
- Charged slides provide adhesion to tissue sections
- **Deparaffinization**

  Tissue is treated in a series of xylene and alcohol to remove paraffin.



Antigen retrieval

- **Enables the partial reversal of formaldehyde induced confirmational change of Ags.**
- **Increases the accessibility of the Ab to the Ag.**

- **Two methods:**
- **Heat**
- **Enzyme digestion**

- **Choice of Ag retrieval depends on the Ag to be demonstrated.**
- **Must determine for each new antibody/antigen target.**

Antigen retrieval

- **HIER**
- **(Heat Induced Epitope Retrieval)**
  - **Use MW/steamer/pressure cooker ~ 20 minutes, slow cool**
  - **Citrate 6.0**

- **PIER**
- **(Protein Induced Epitope Retrieval)**
  - **Proteinase K**
  - **Trypsin**
  - **Pepsin**
  - **Pronase, etc.**

Be careful:

- **Destroys some epitopes**
- **Bad for morphology**

Permabilization: Improving antibody penetration

- Need this for intracellular (cytoplasmic, nuclear) Ag
- Detergents most popular
  - Triton-X
  - Tween20
- Can't use for membrane proteins

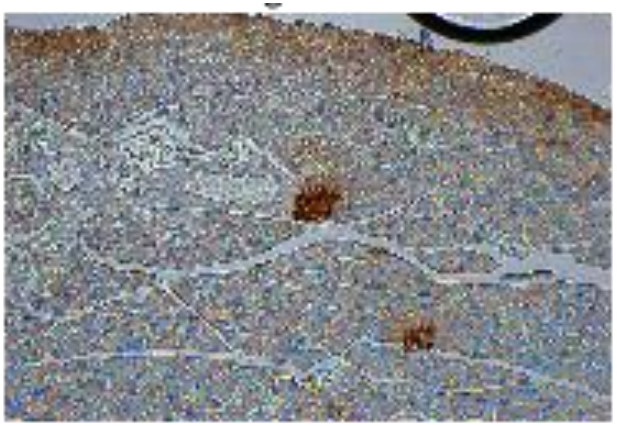
Cells grown as a monolayer

Blocking

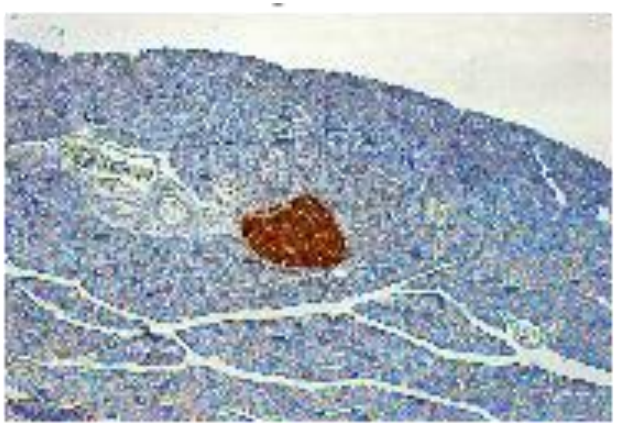
- Background staining
  - Non-specific immunologic binding – usually uniform
  - Endogenous peroxidases
  - Endogenous biotin

Non-specific staining

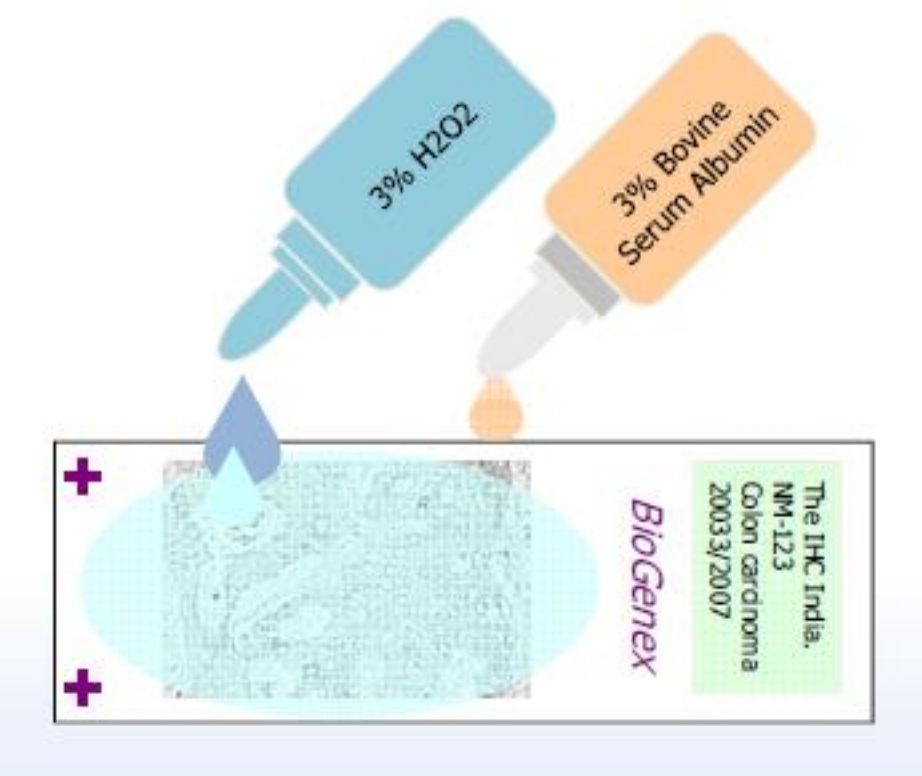
Before block



After block



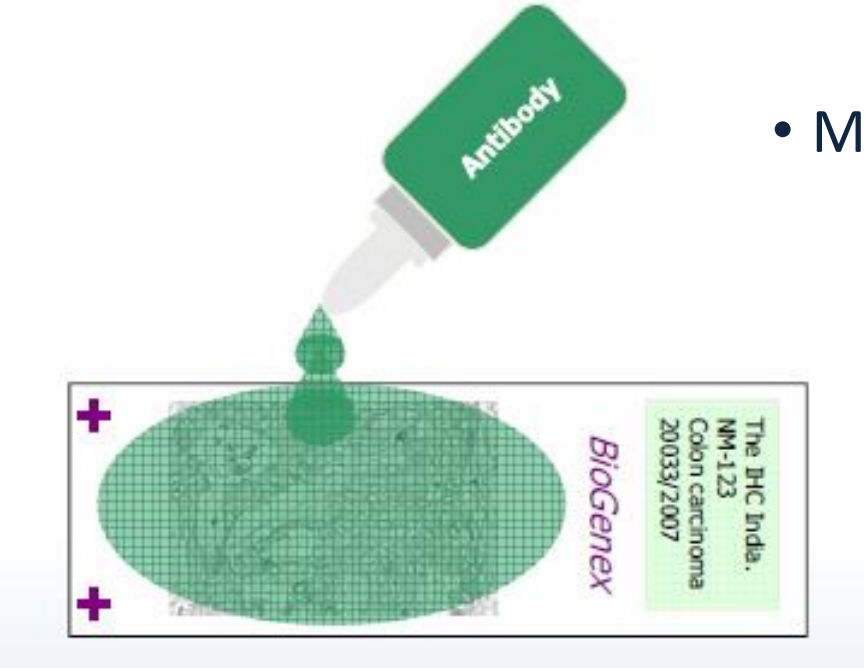
Blocking



Peroxide Block

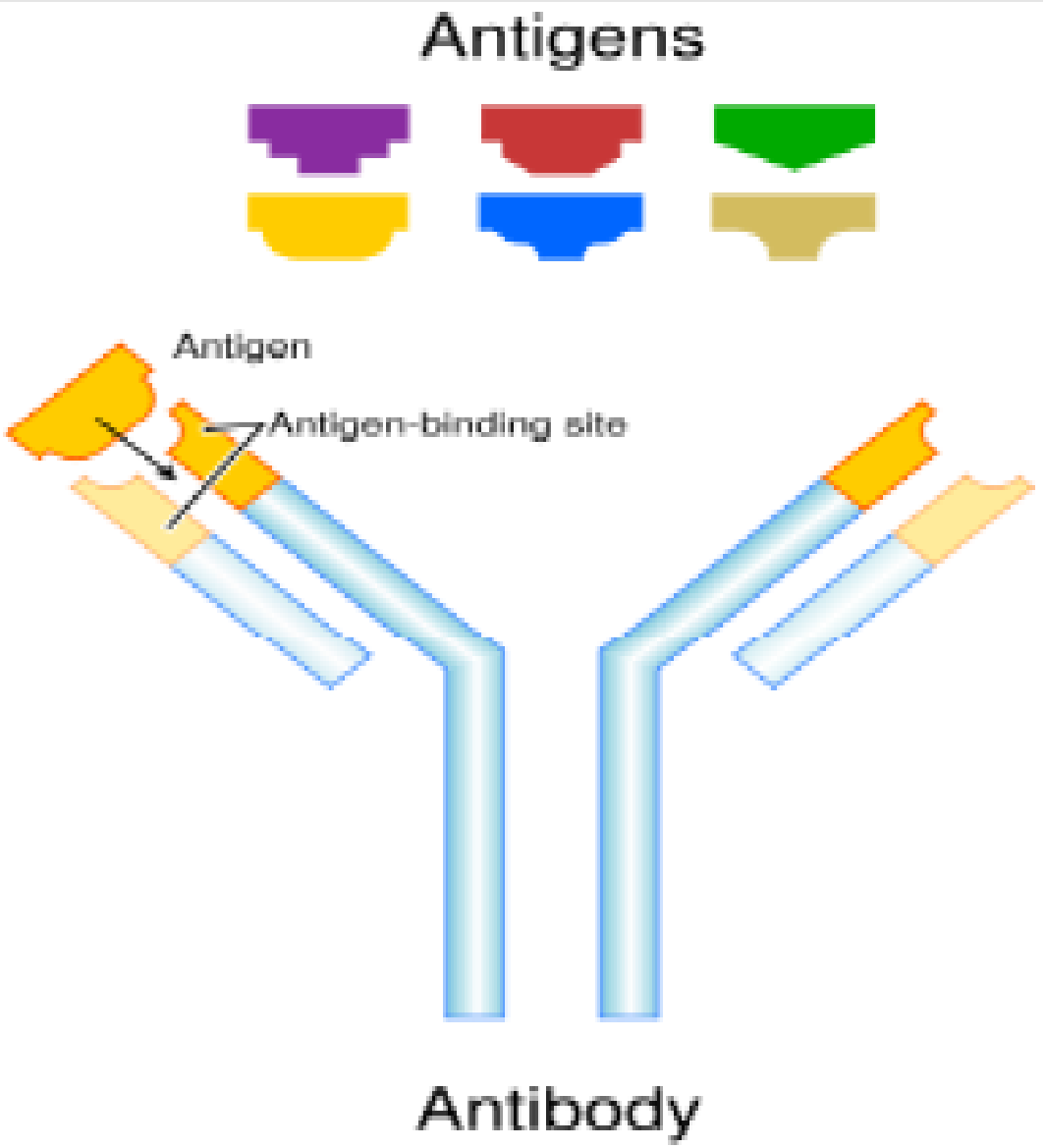
- Blocks endogenous peroxidases
- 3% H2O2
- **Protein Block**
- Blocks all non specific sites
- Reduces background
- 10% Normal serum or BSA is used

Primary Antibodies



Two types of Abs

- Polyclonal Abs
- Monoclonal Abs



Each antibody binds to a specific antigen; an interaction similar to a lock and key.

Monoclonal vs. polyclonal antibody

- Monoclonal
  - Mouse
  - 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 affinity batch-to-batch
  - More likely to have success in an unknown application

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

- IHC-P vs. IF
- WB, ELISA, IP, etc.

Labeling Antibodies:

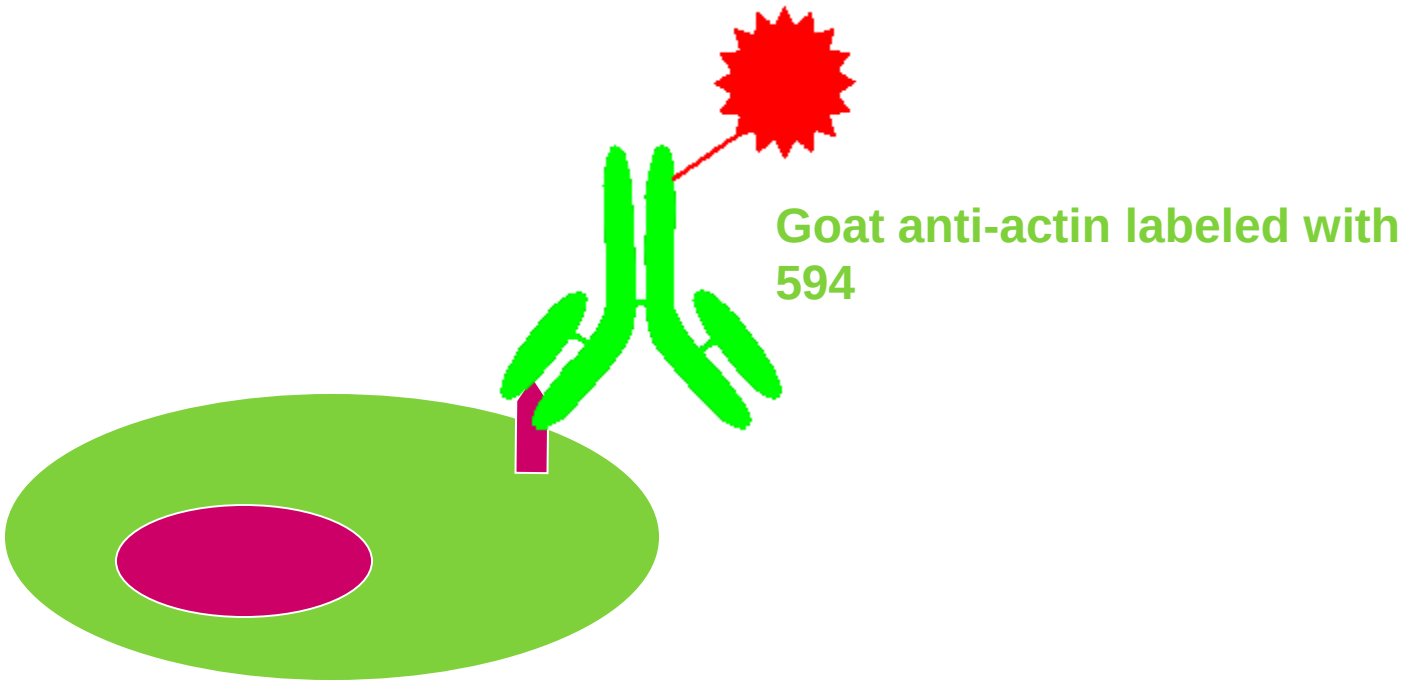
Antibodies are not visible with standard microscopy and must be labeled in a manner that does not interfere with their binding specificity.

Common labels include **fluorochromes** (eg, fluorescein, rhodamine), **enzymes** demonstrable via enzyme histochemical techniques (eg, peroxidase, alkaline phosphatase), and **electron-scattering compounds** for use in electron microscopy (eg, ferritin, colloidal gold).

Direct method- primary antibody only

Labelled Ab reacts directly with Ag in tissue sections

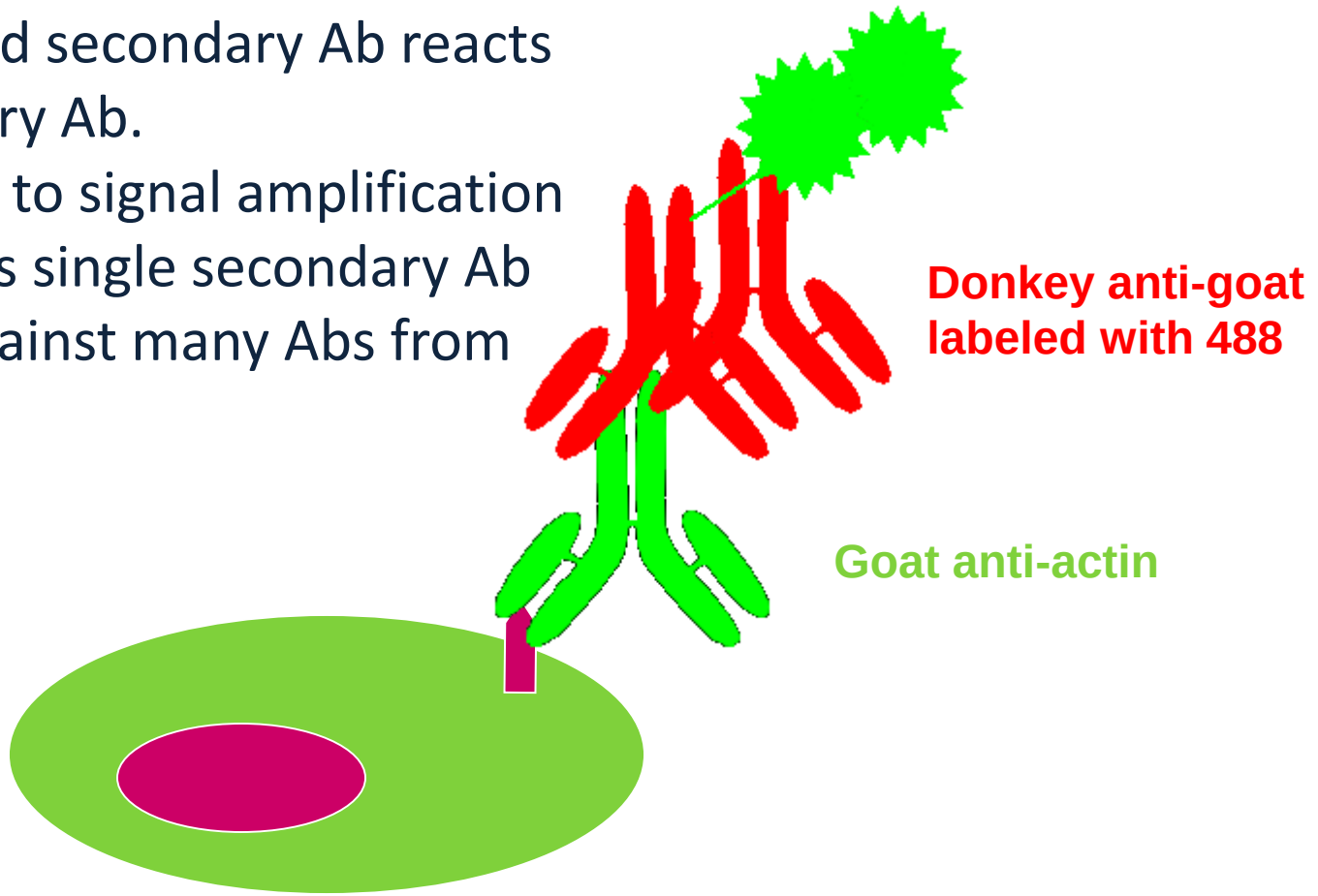
- Single step method
- Short and quick
- Insensitive due to little signal amplification



Indirect method – primary and secondary antibodies

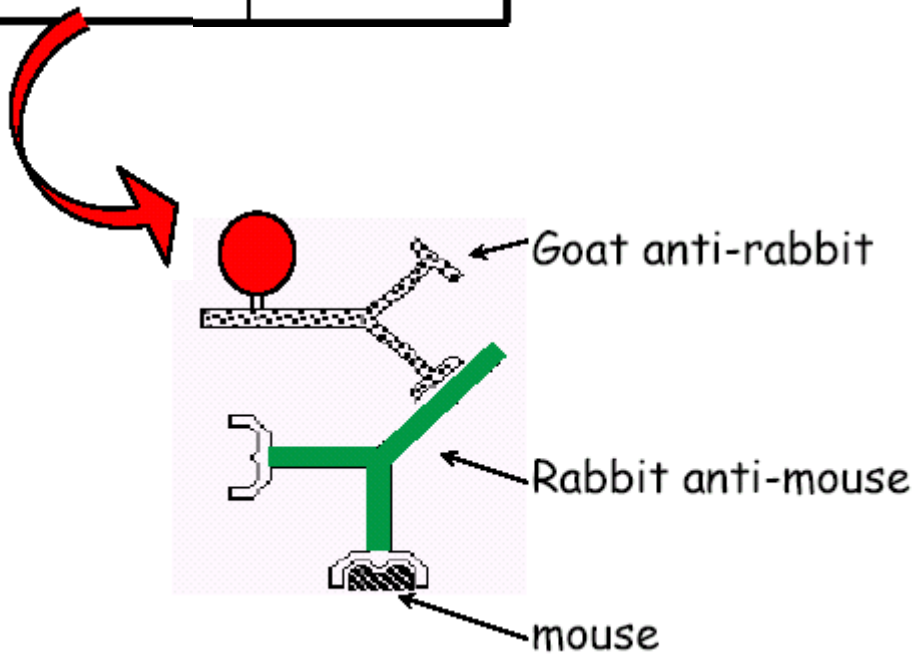
Unlabelled Primary Ab reacts with Ag and the labelled secondary Ab reacts with the primary Ab.

- Sensitive due to signal amplification
- Economical as single secondary Ab can be used against many Abs from same species

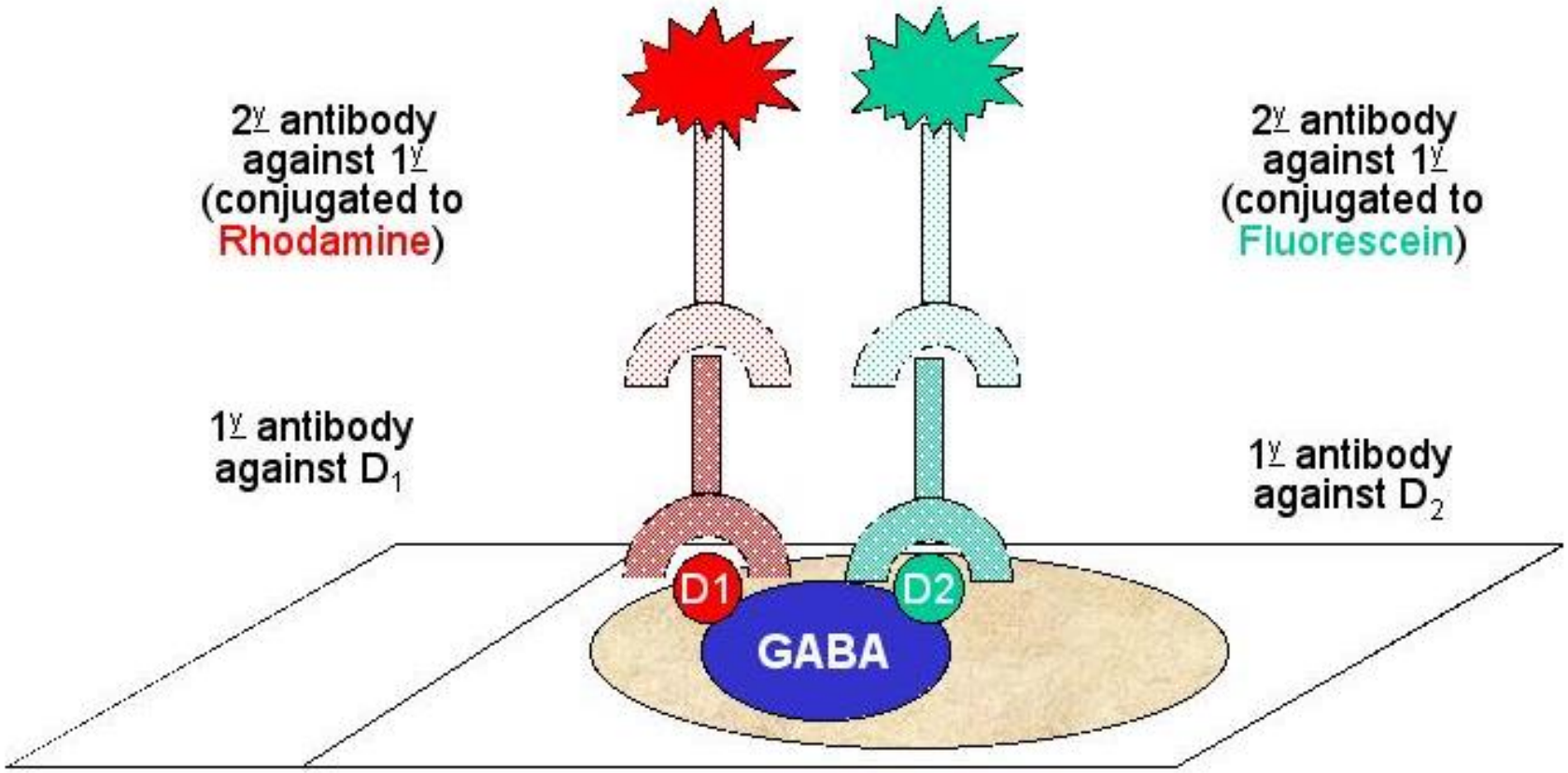


Indirect method – primary and secondary antibodies

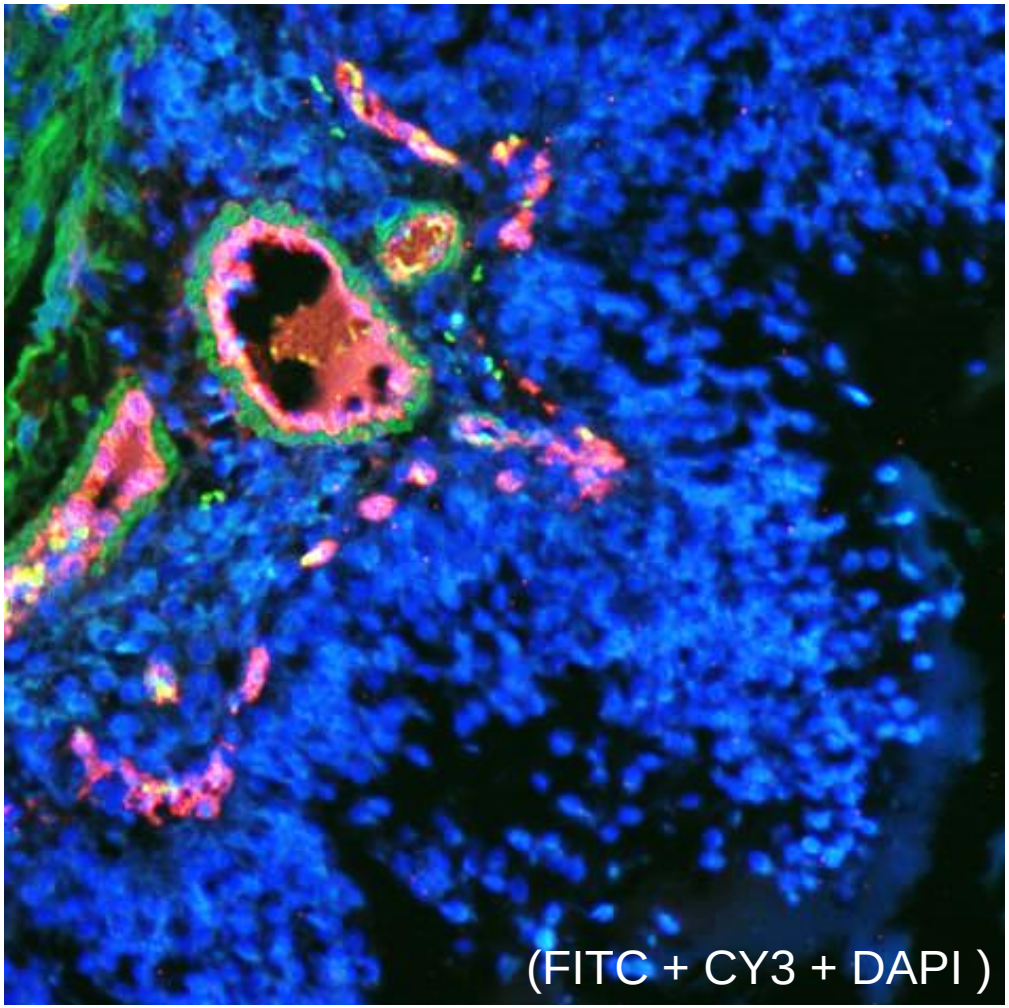
Ab	Classe	Host	Anti-
I°	IgG	Rabbit	Mouse
II°	IgG	Goat	Rabbit



Multiple Immunofluorescence



Multiple Labelling of a Tissue Section



Enzymatic detection methods

Light microscope sufficient for analysis of specimens

Resolution of subcellular structures not as good as with fluorescence methods

Unlimited shelf life of labelled specimens

Substrate reagents often toxic/carcinogenic

Enzyme indirect method

- Ag-Ab conjugates are visualized by the use of a label.
- Enzymes are used as labels that produce a colored precipitate in the presence of a substrate

Labels:

- Peroxidase
- Alkaline Phosphatase

Substrate:

- DAB

Enzyme indirect method

Enzyme labels produce a colored precipitate in the presence of a specific substrate

- **Most widely used label is Peroxidase**
- **Produces a dark brown precipitate when Diamino Benzidine (DAB) is added.**
- **Alkaline phosphatase is also used and produces either red or blue precipitates.**

Controls

- Positive control
  - Best is tissue with known specificity
  - Cells or tissues that are known to contain the specific Ag
  - Detects false negatives due to fixation and processing.

- Negative control
  - It is to test for the specificity of the antibody involved.
  - Omission of Primary Ab with the same tissue and procedure
  - Useful to detect endogenous biotin and peroxidase activity