Light microscopy in Cellular Biology

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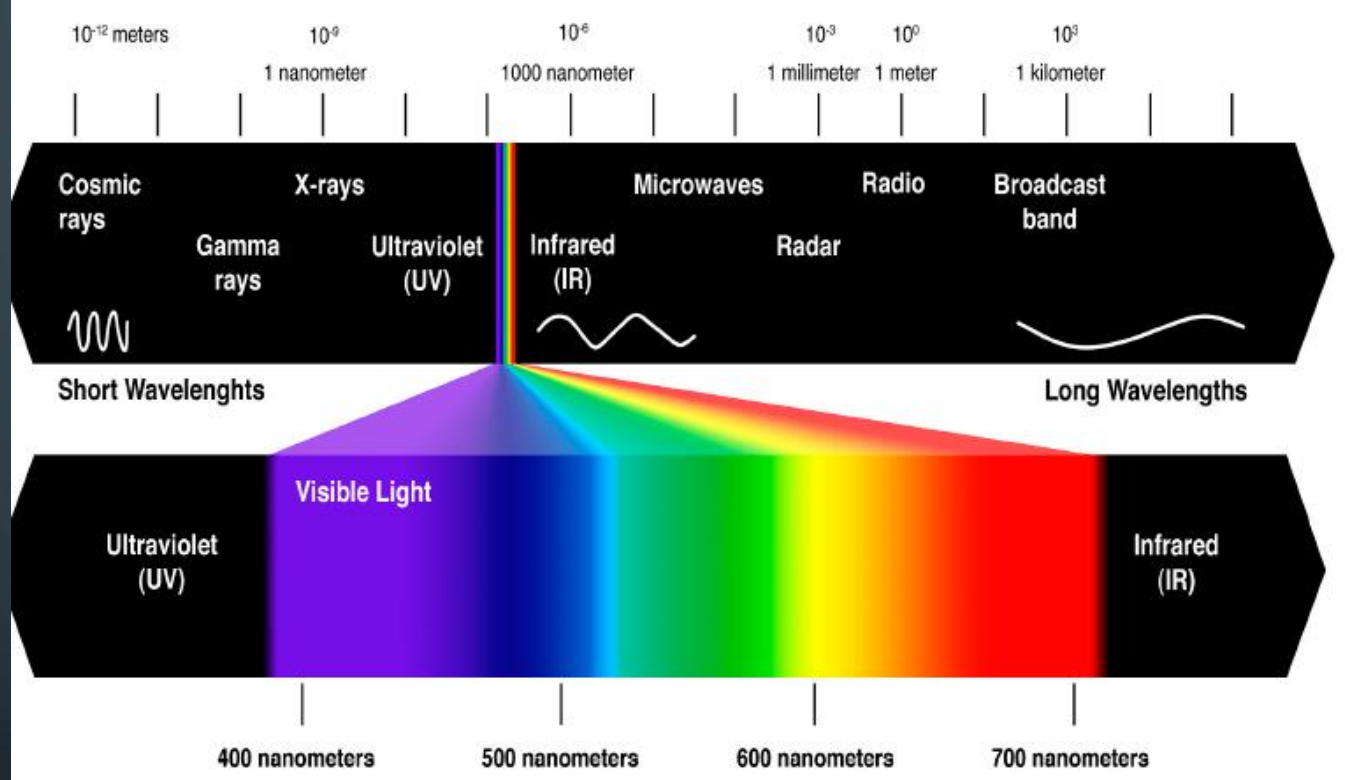
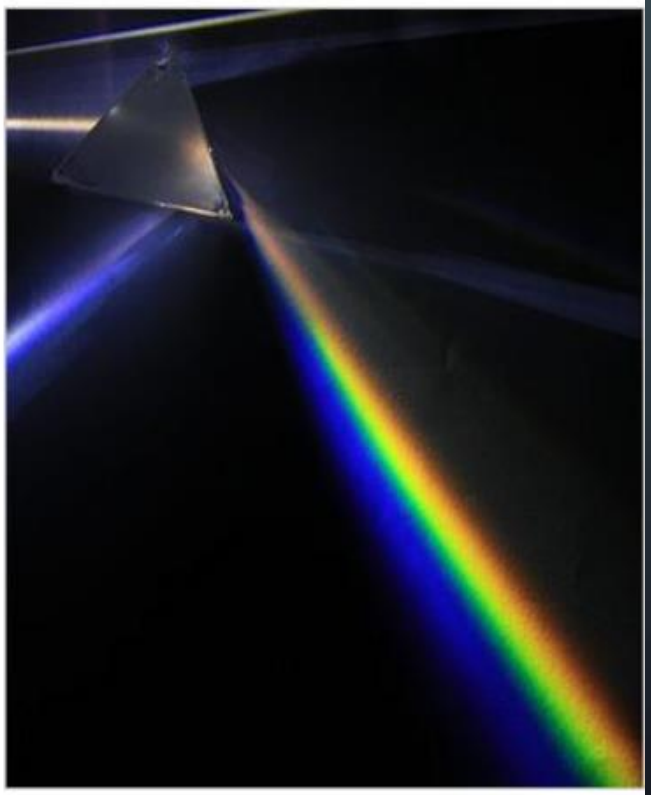
Visible Light



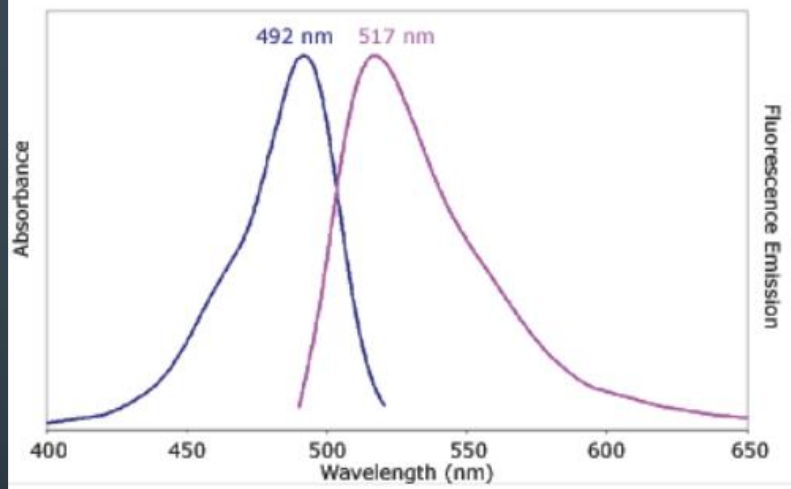


390 – 700 nm visible to the human eye
White light is split into its components through a prism
Reason: different λ refract at different angles

What is fluorescence?

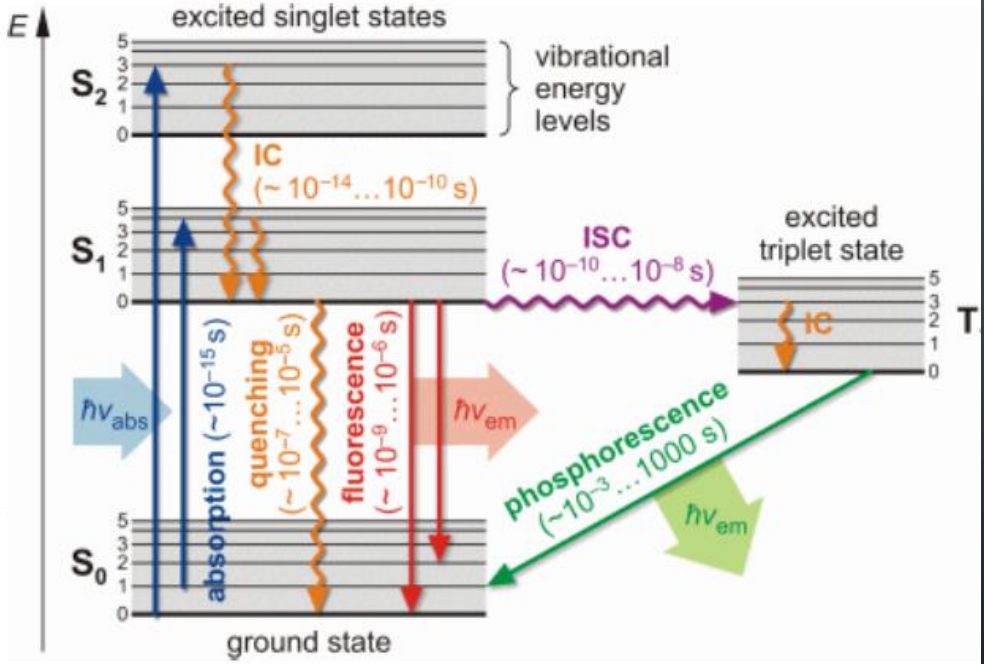
George Gabriel Stokes (1819-1903)

Stokes Shift



Alexa Fluor 488

Jablonski diagram



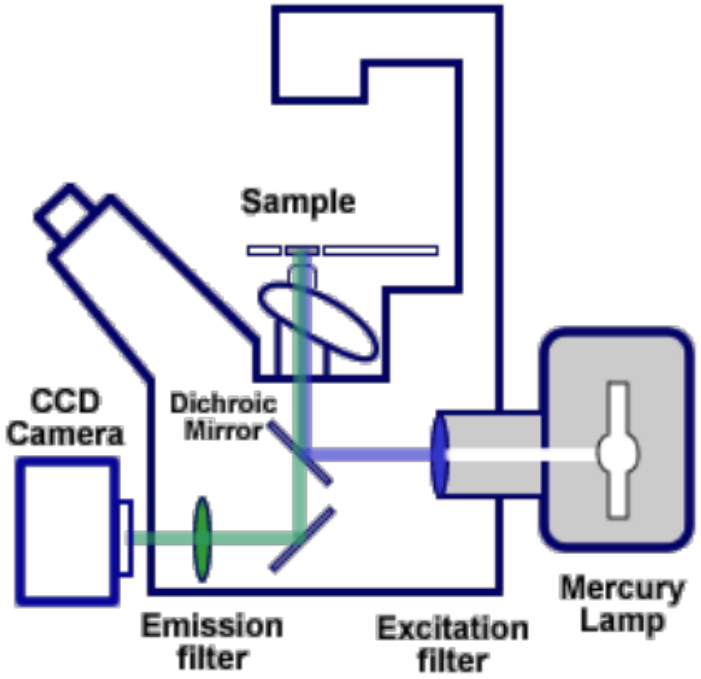
Photoluminescence:

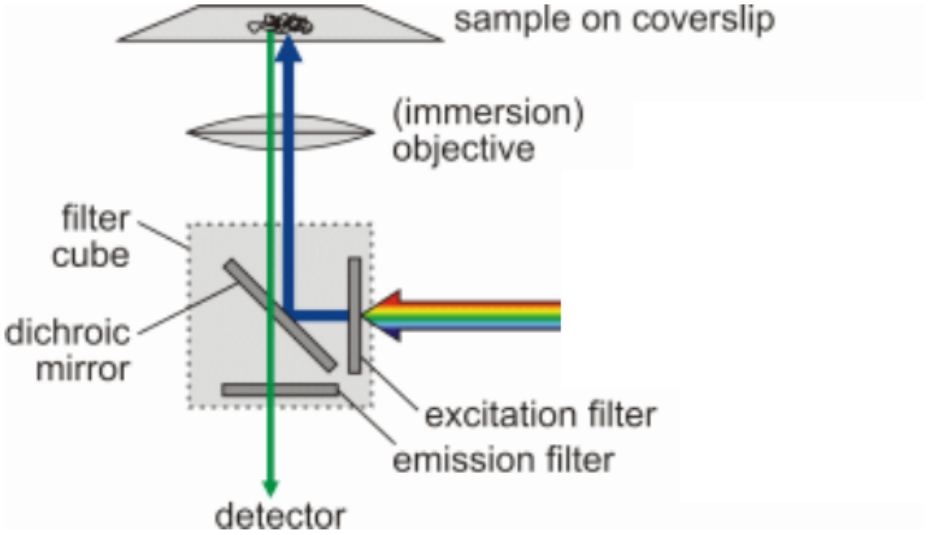
Fluorescence - spontaneous emission of light during transition of the system from its lowest vibrational energy level of an excited singlet state S_1 back to the ground state S_0 (10^{-9} to 10^{-6} s)

Phosphorescence – a non-radiative transition into an isoenergetic vibrational level of a triplet state T_1 , which lasts for 10^{-3} to 1000 s before it decays to the ground state

IC: internal conversion
ISC: intersystem crossing

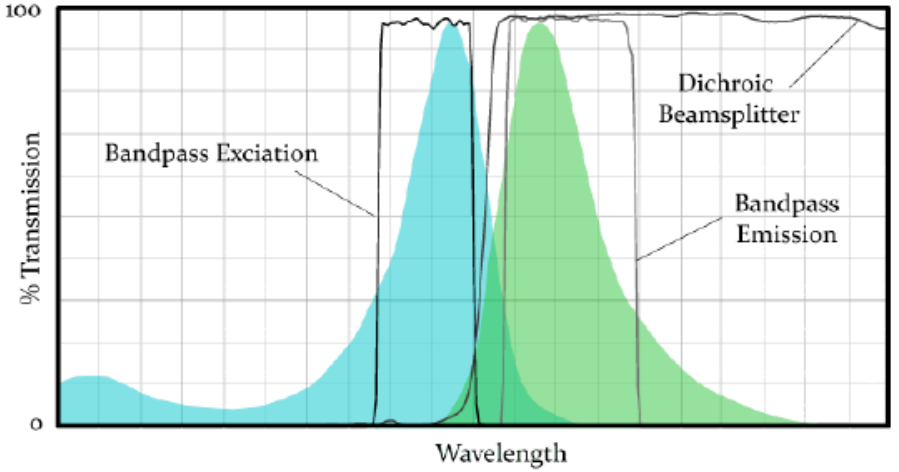
Basic principle of an (Inverted) Fluorescence Microscope





Fluorescence microscope:
Fluorescence light source
Excitation filter
Dichroic mirror
Objective
Emission filter
Camera/eye pieces

Ideal filter cube properties



Excitation
Dichroic
Emission

Upright Fluorescence Microscope

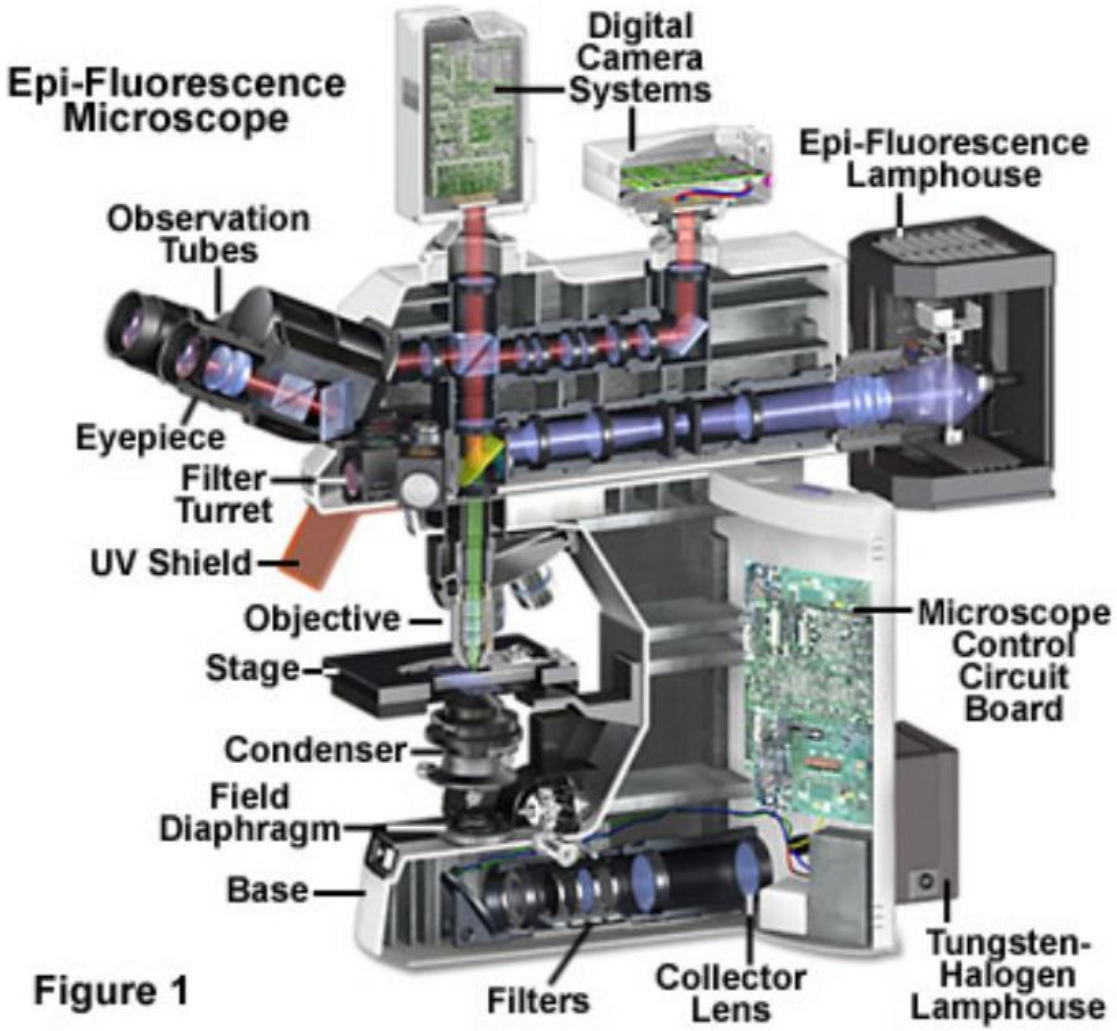


Figure 1

Used for fixed samples on slides and for live imaging where the objective is immersed in the medium

Inverted Fluorescence Microscope

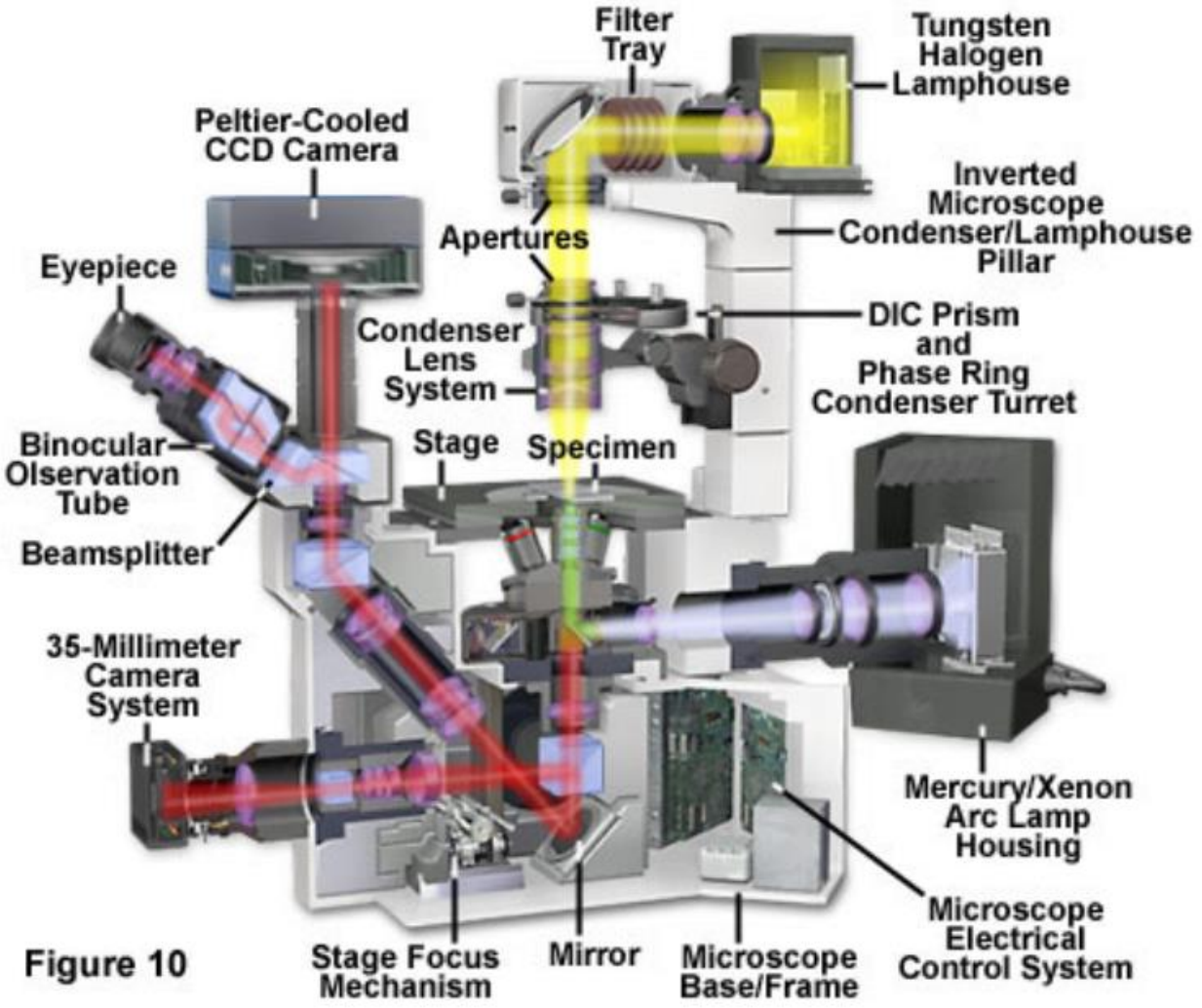
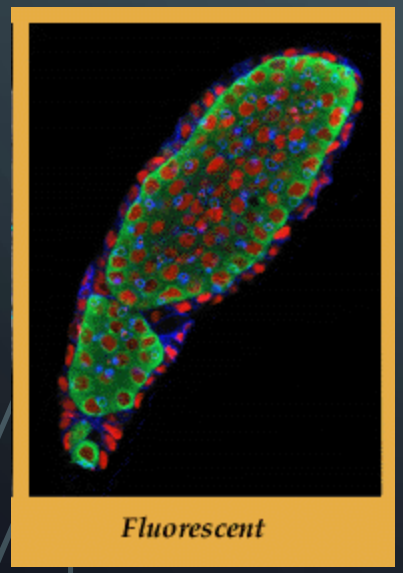
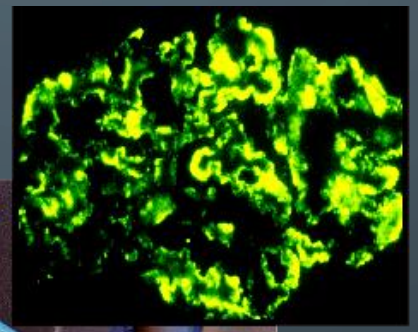


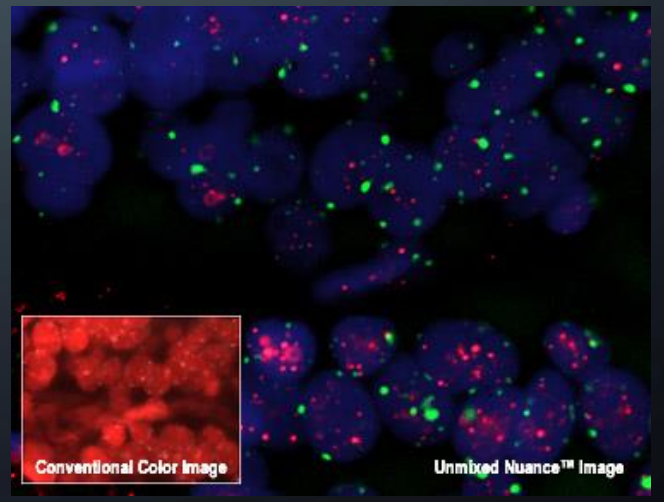
Figure 10

Used for live imaging through a coverslip and for fixed samples on slides
More versatile but danger of oil running down the objective

IMMUNOFLUORESCENCE MICROSCOPY







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**

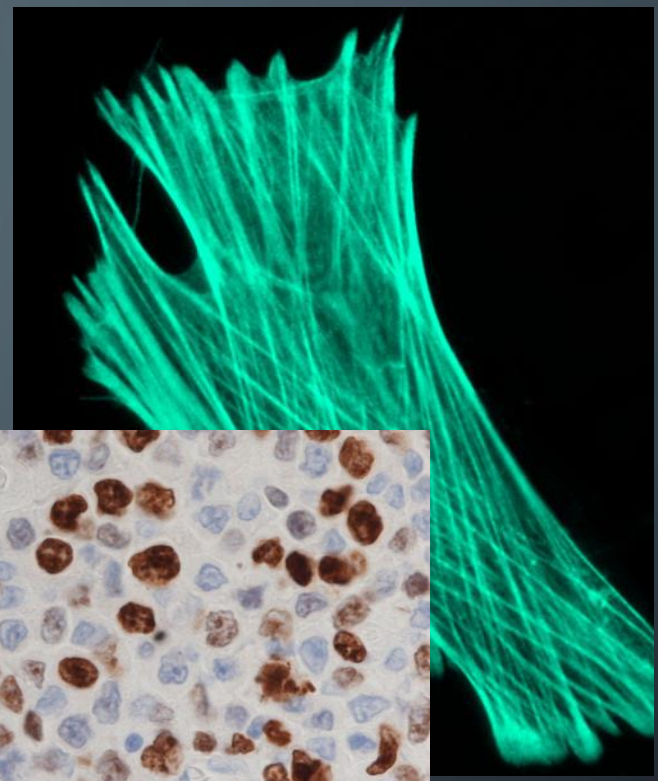
- Immunohistochemistry (IHC) combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific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

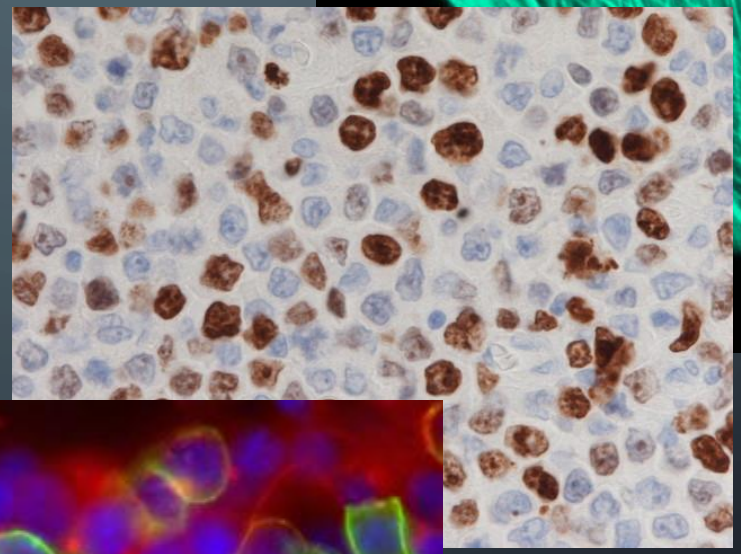
History

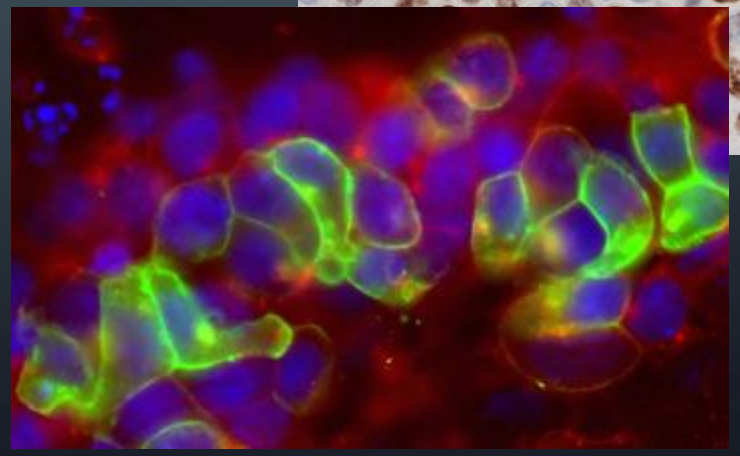
- The principle has existed since the 1930s.
- Started in 1941 when Coons identified pneumococci using a direct fluorescent method.
- Indirect method
- Addition of horseradish peroxidase
- Peroxidase anti-peroxidase technique in 1979
- Use of Avidin & Biotin complex in early 1980's

What cellular antigens can we target?

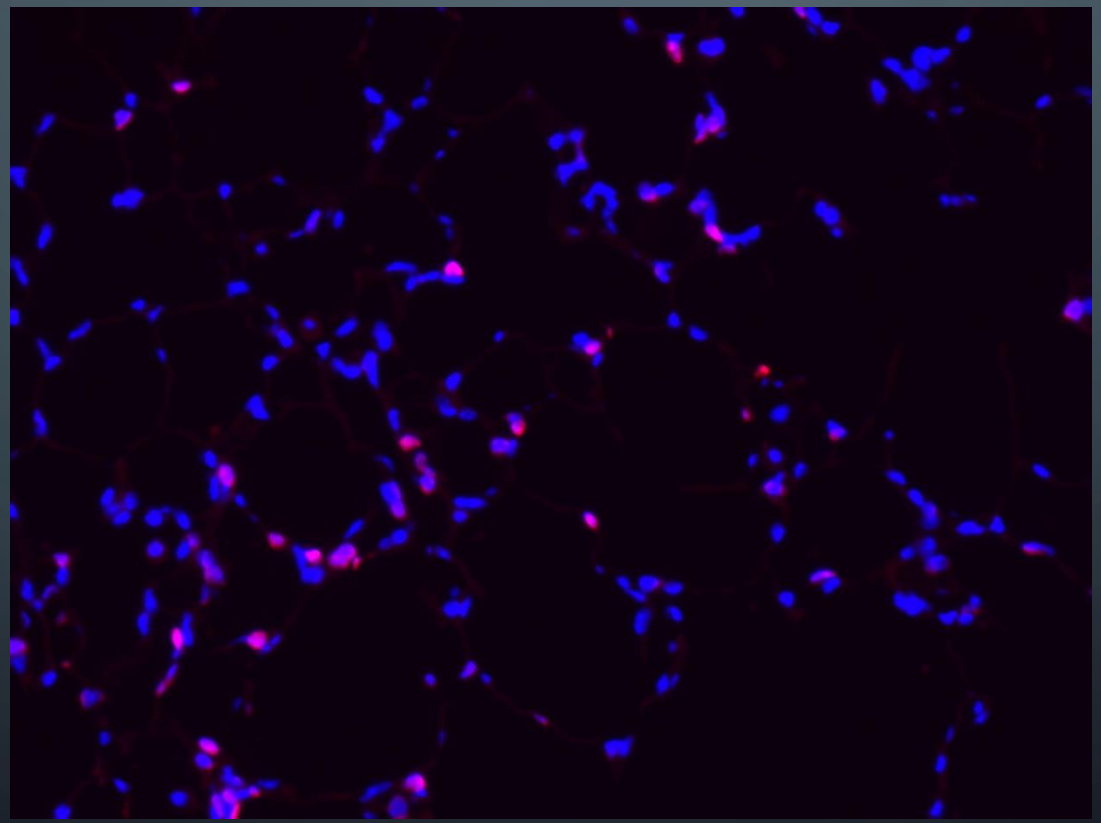
- Cytoplasmic
- Nuclear
- Cell membrane
- Lipids
- Proteins



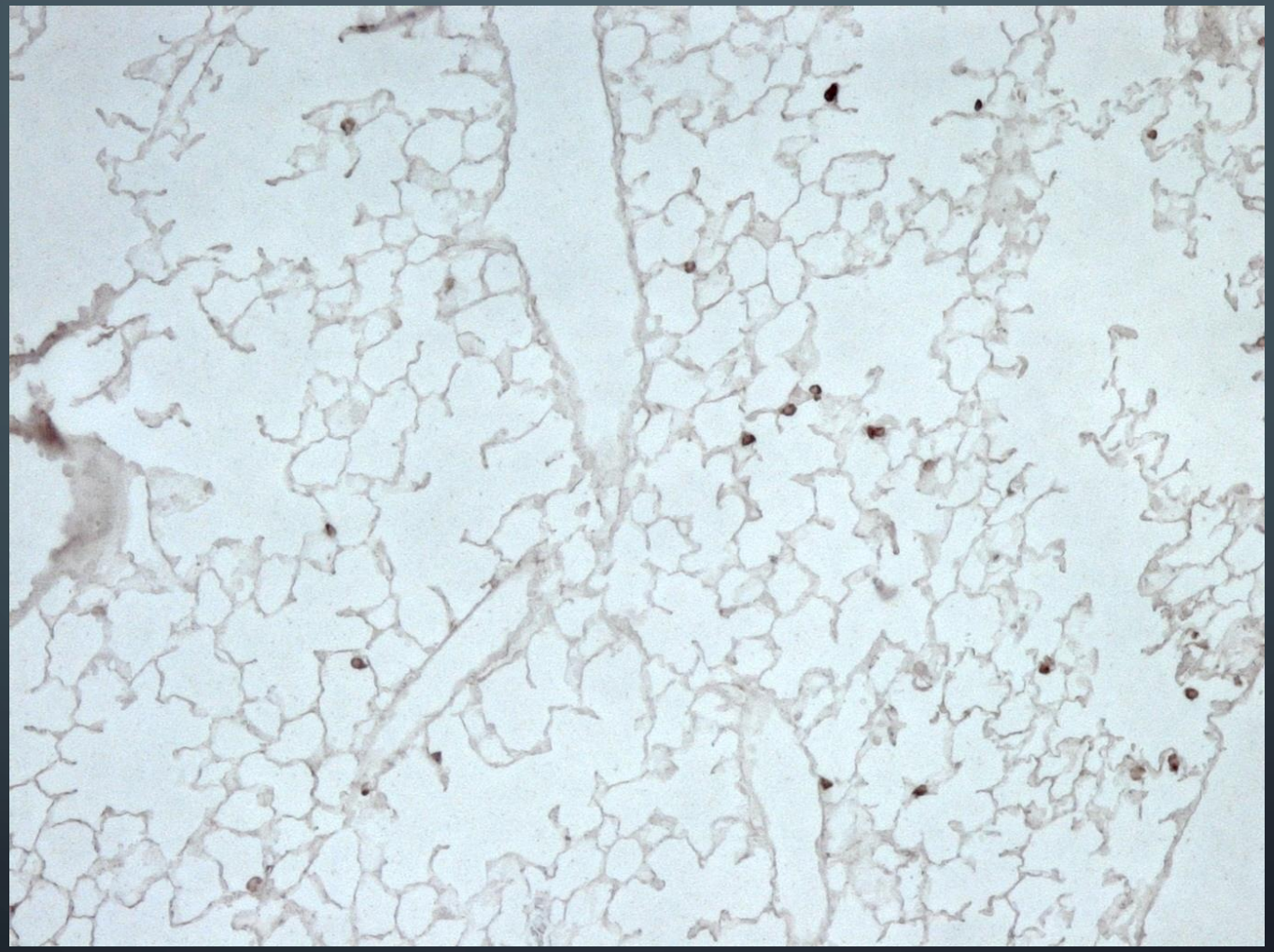




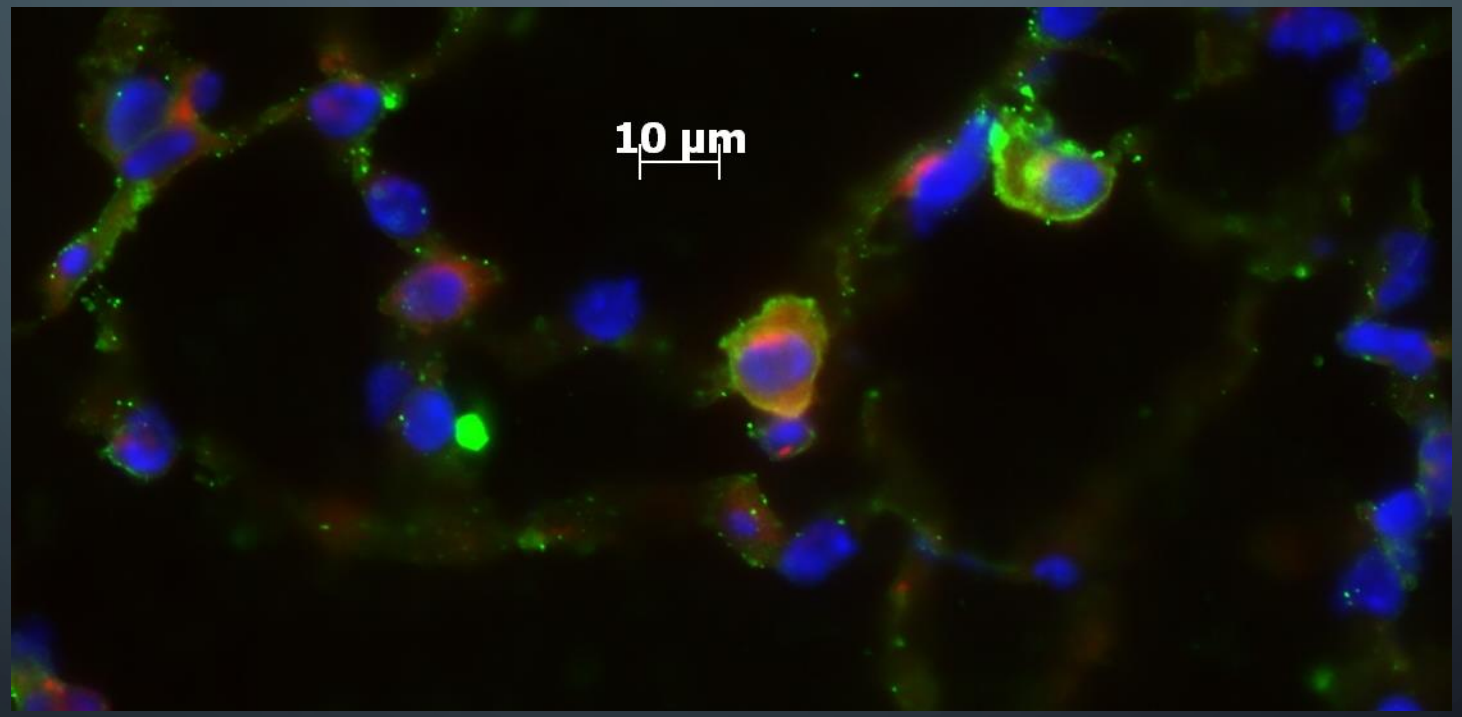
Identify replicating cells



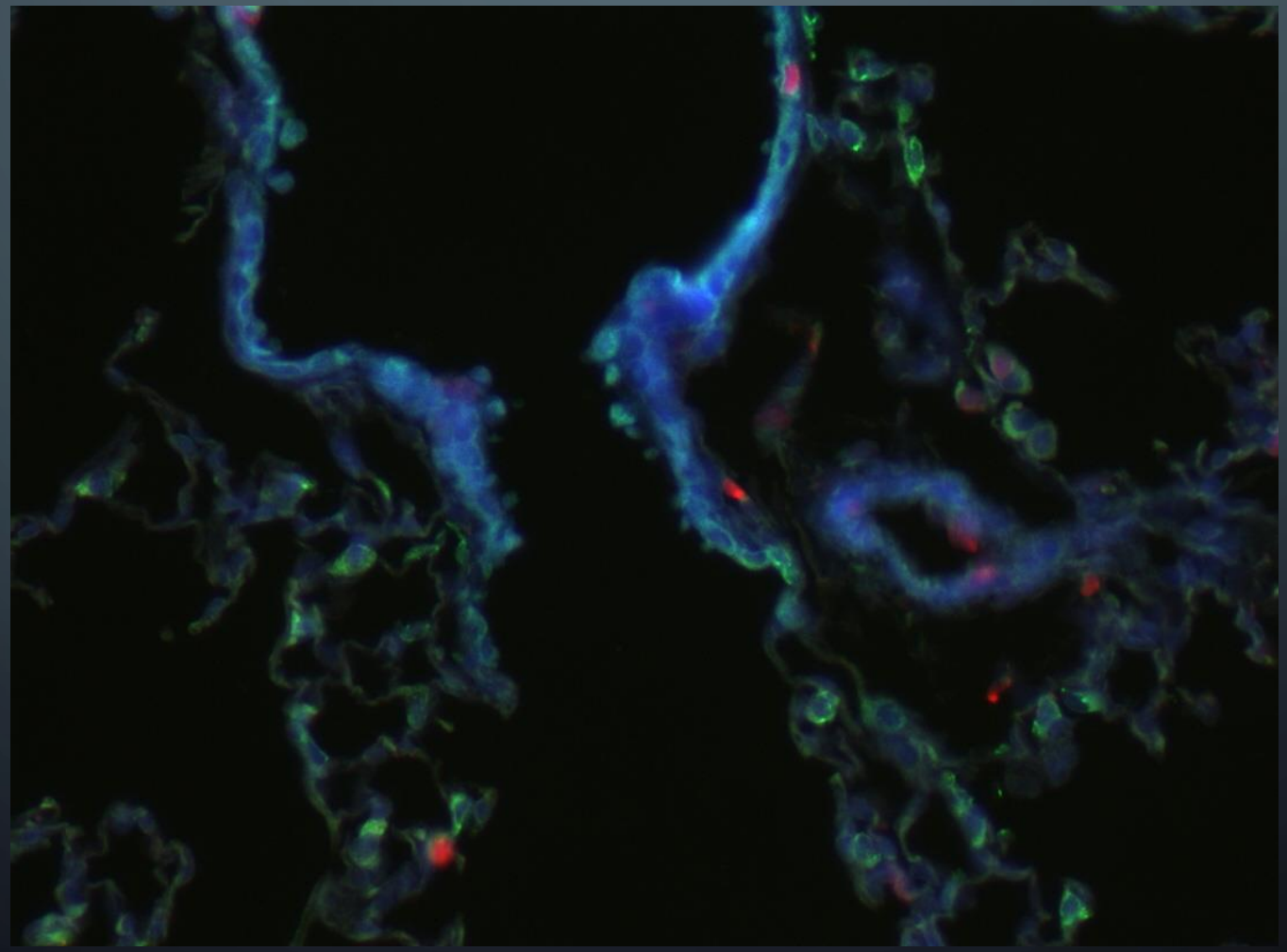
Locate cells that are signaling



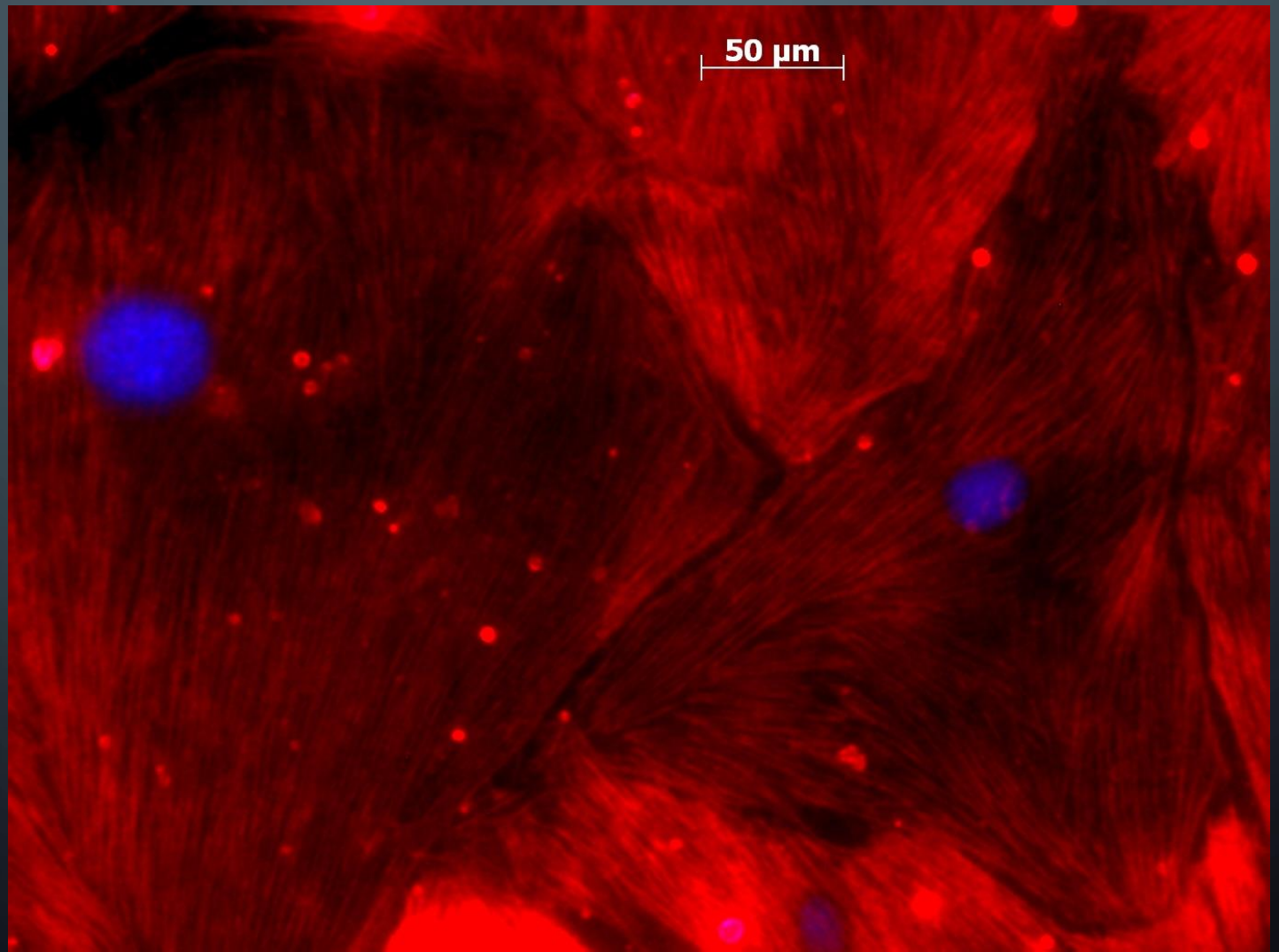
IDENTIFY ACTIVATION STATES



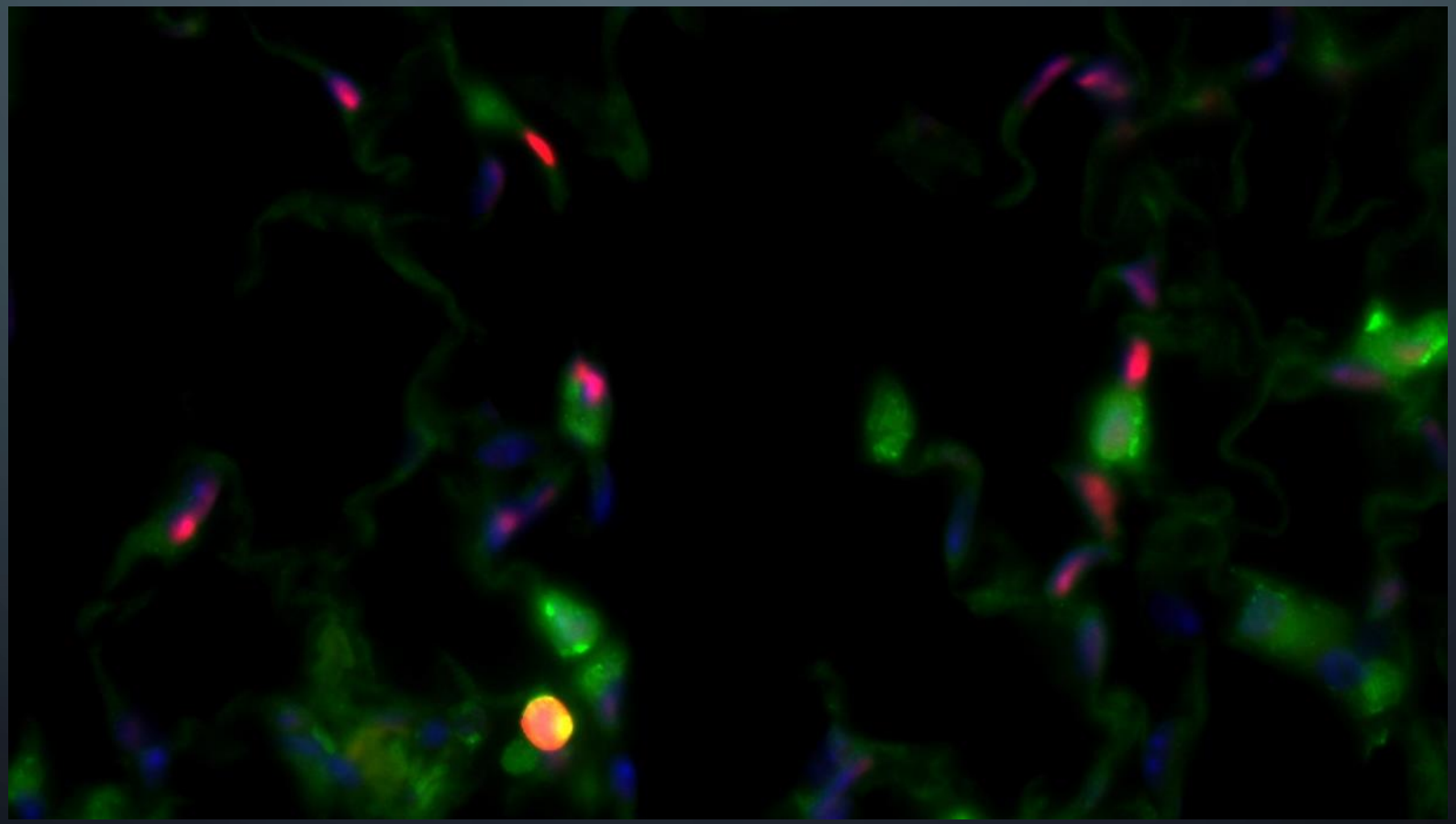
IDENTIFY DIFFERENT TYPES OF CELLS IN A TISSUE



EXAMINE CYTOSKELETAL STRUCTURE



Locate apoptotic cells



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

Important considerations for IHC

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

PREPARATION AND STAINING OF SPECIMENS

- increases visibility of specimen
- accentuates specific morphological features
- preserves specimens

FIXATION

- process by which internal and external structures are preserved and fixed in position
- process by which organism is killed and firmly attached to microscope slide
  - heat fixing
    - preserves overall morphology but not internal structures
  - chemical fixing
    - protects fine cellular substructure and morphology of larger, more delicate organisms

Preparation of Fixed Samples

Why use fixed samples at all?

- Primary cells cannot easily be transfected and transgenic animals are time-consuming to produce and not always possible

- Brighter than fluorescent fusion proteins

- Injection of antibodies only possible with big cells (e.g. oocytes)

- Can detect four different labels or even more at the same time

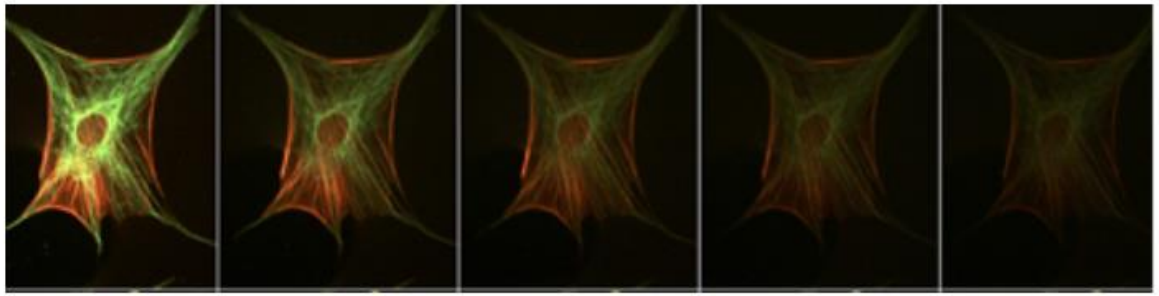
- High-throughput screening

DYES AND SIMPLE STAINING

- dyes
  - make internal and external structures of cell more visible by increasing contrast with background
  - have two common features
    - chromophore groups
      - chemical groups with conjugated double bonds
      - give dye its color
    - ability to bind cells

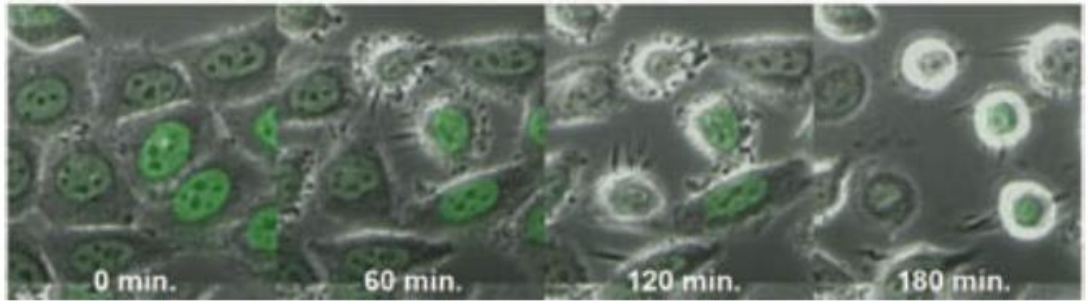
Photobleaching and Phototoxicity

Photobleaching: photochemical destruction of the fluorophore
In an excited triplet state, fluorophores may interact with another molecule to produce irreversible covalent modifications



Phototoxicity: illumination of a fluorophore causes damage to the cell expressing it, eventually leading to cell death
Common situation: the excited dye molecule passes its excess energy on to O_2, creating reactive oxygen species (ROS):

- ROS reacts with dye ⟶ dye bleaches
- ROS diffuses away and reacts with other dyes or cell components

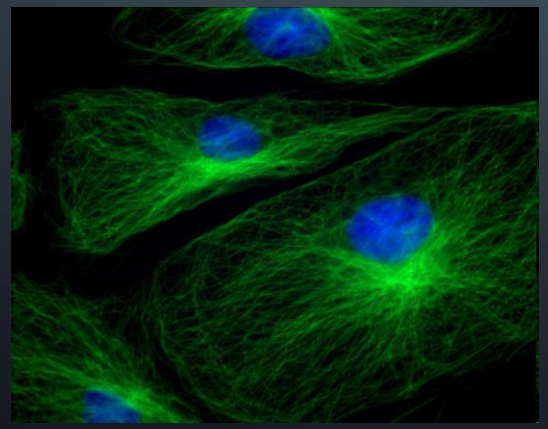


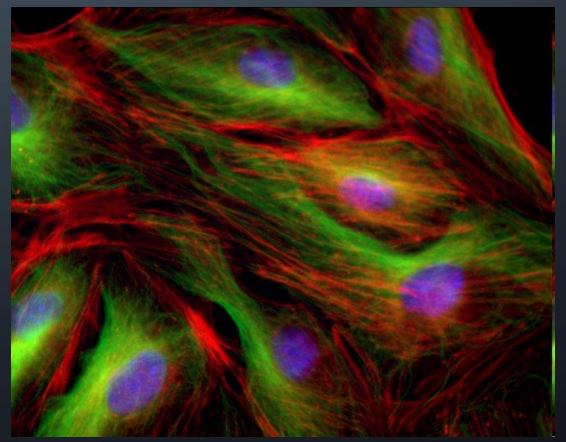
Solution:

- Reduce the intensity of the excitation light and frequency of illumination
- Close down the field aperture in order to restrict the illuminated area

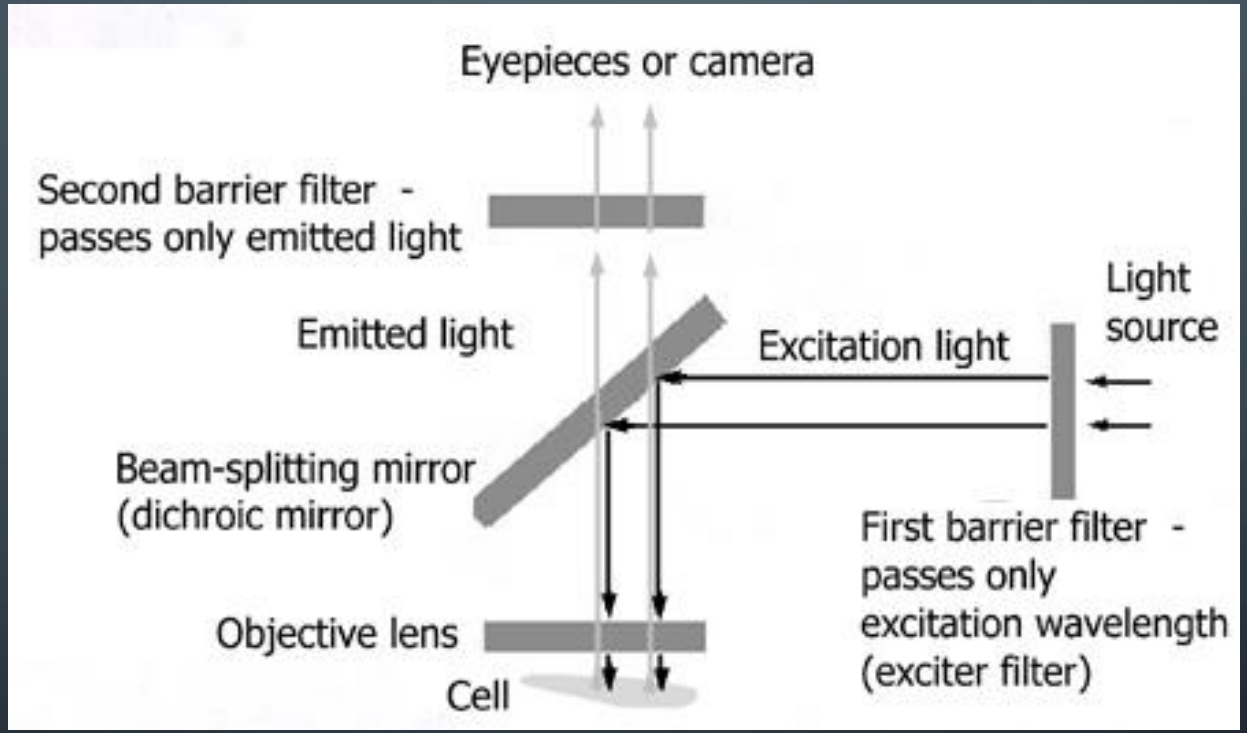
IMMUNOFLUORESCENCE MICROSCOPY:

- When an antibody, or the anti immunoglobulin antibody used to detect the antibody is labeled with a fluorescent dye
- This method is used when looking at the subcellular location of a protein of interest



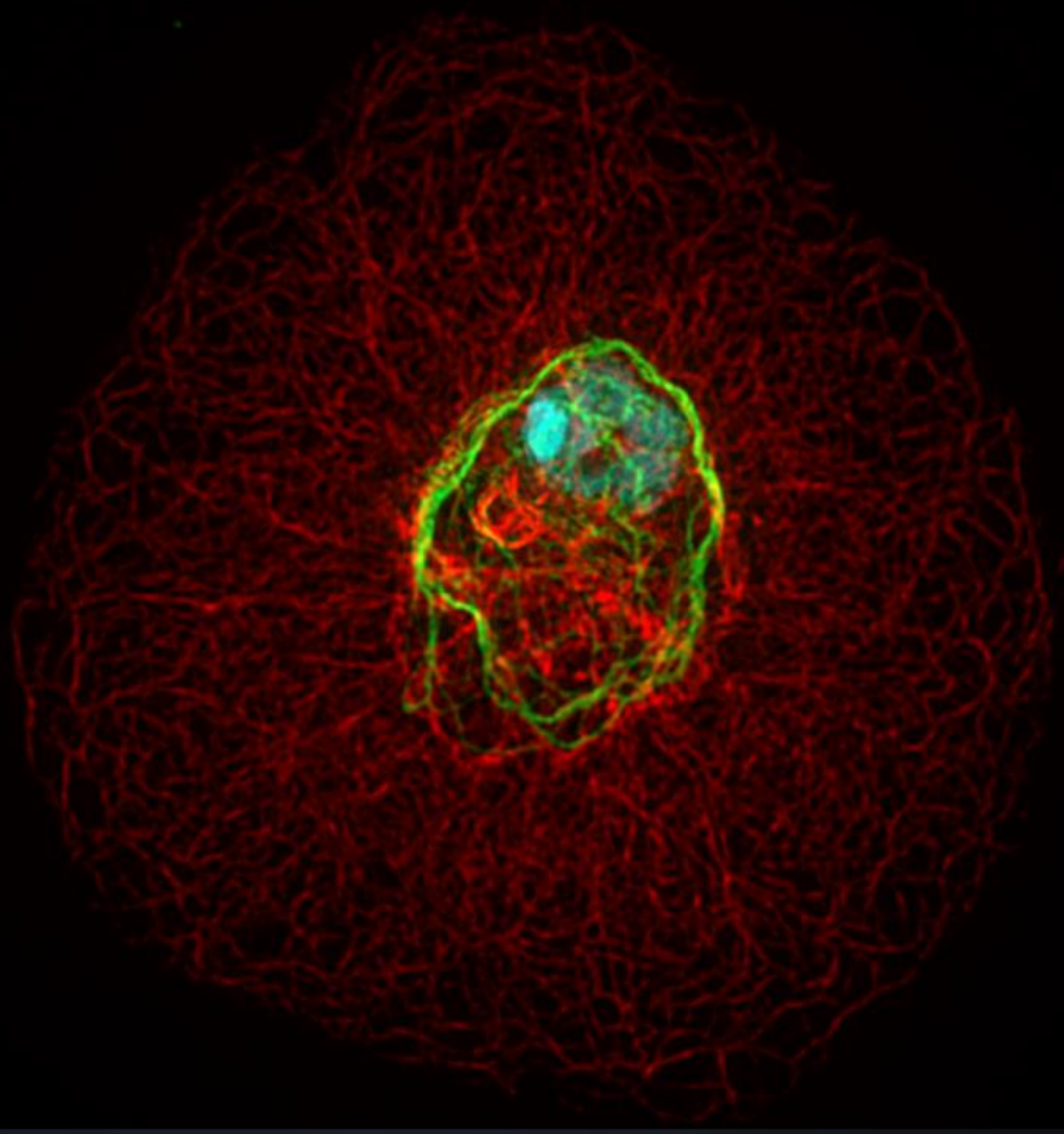


IMMUNOFLUORESCENCE MICROSCOPY:



Typical Immunocytochemistry Protocol

Fixation
Permeabilisation
Washes
Blocking
1° antibody
Washes
2° antibody
Washes
Mounting



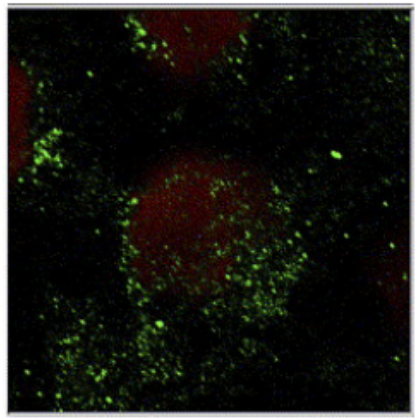
Two Types of Fixation

Denaturing fixation:

Cold methanol or cold acetone stored at -20 °C, samples submerged at -20 °C for 10 to 20 min

- destroys 3D protein structure
- dissolves lipids into micelles
- poor morphological preservation and poor protein retention
- makes some epitopes accessible
- best used after cross-linking fixation

MeOH

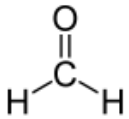


Cross-linking fixation:

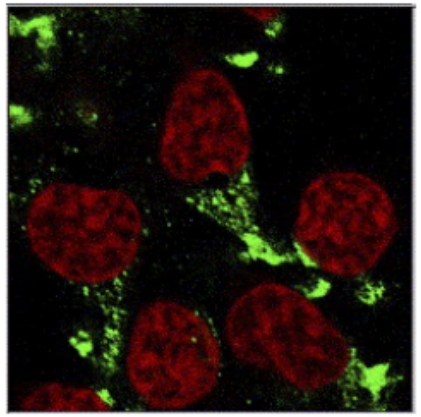
- aldehyde groups cross-link molecules in cells and tissues
- extensive cross-linking prevents antibody penetration

Formaldehyde used for immunocytochemistry in light microscopy

- cross-links 1° amines of Lys and Arg, sulfhydryl groups of Cys, OH groups, double bonds
- binds to amino acids, peptides, proteins and some lipids, but not RNA, DNA or most sugars
- retention of DNA and RNA due to protein cross-linking
- for cultured cells fixation usually for 20 min in 2 - 4% formaldehyde

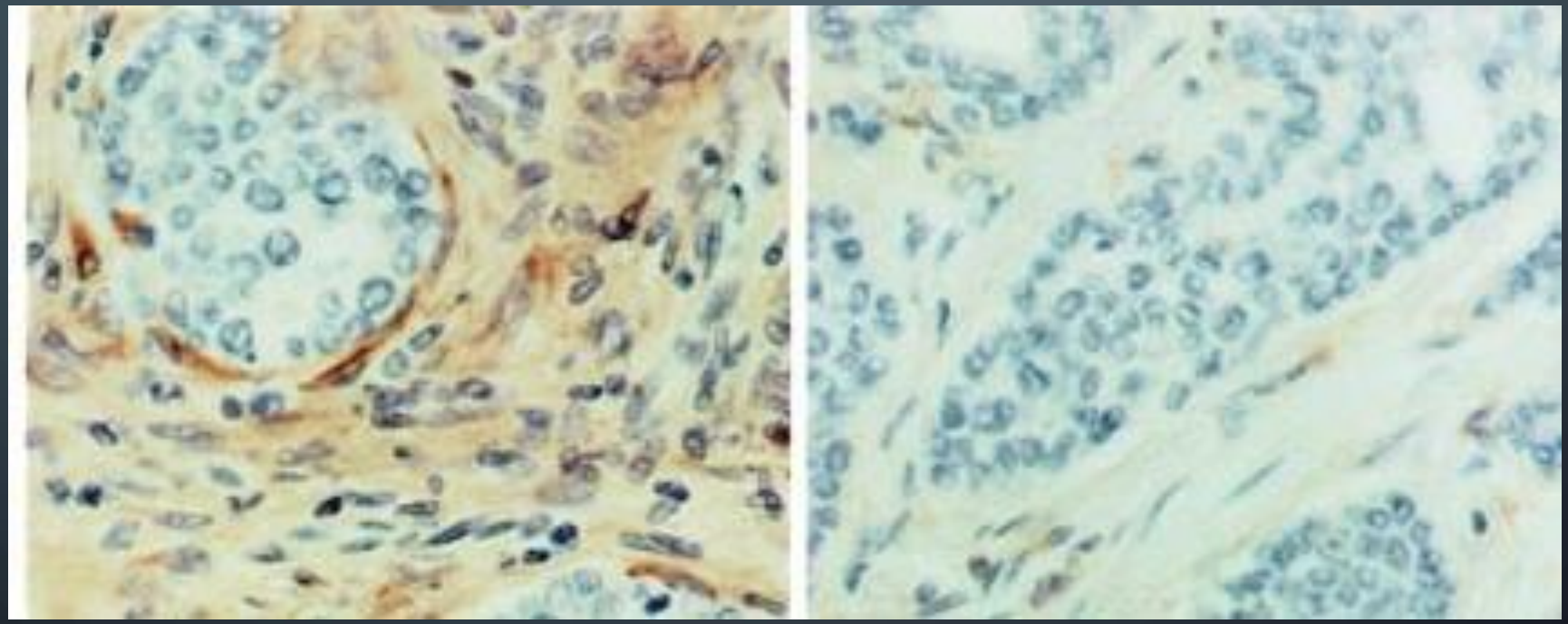


PFA



FIXATION

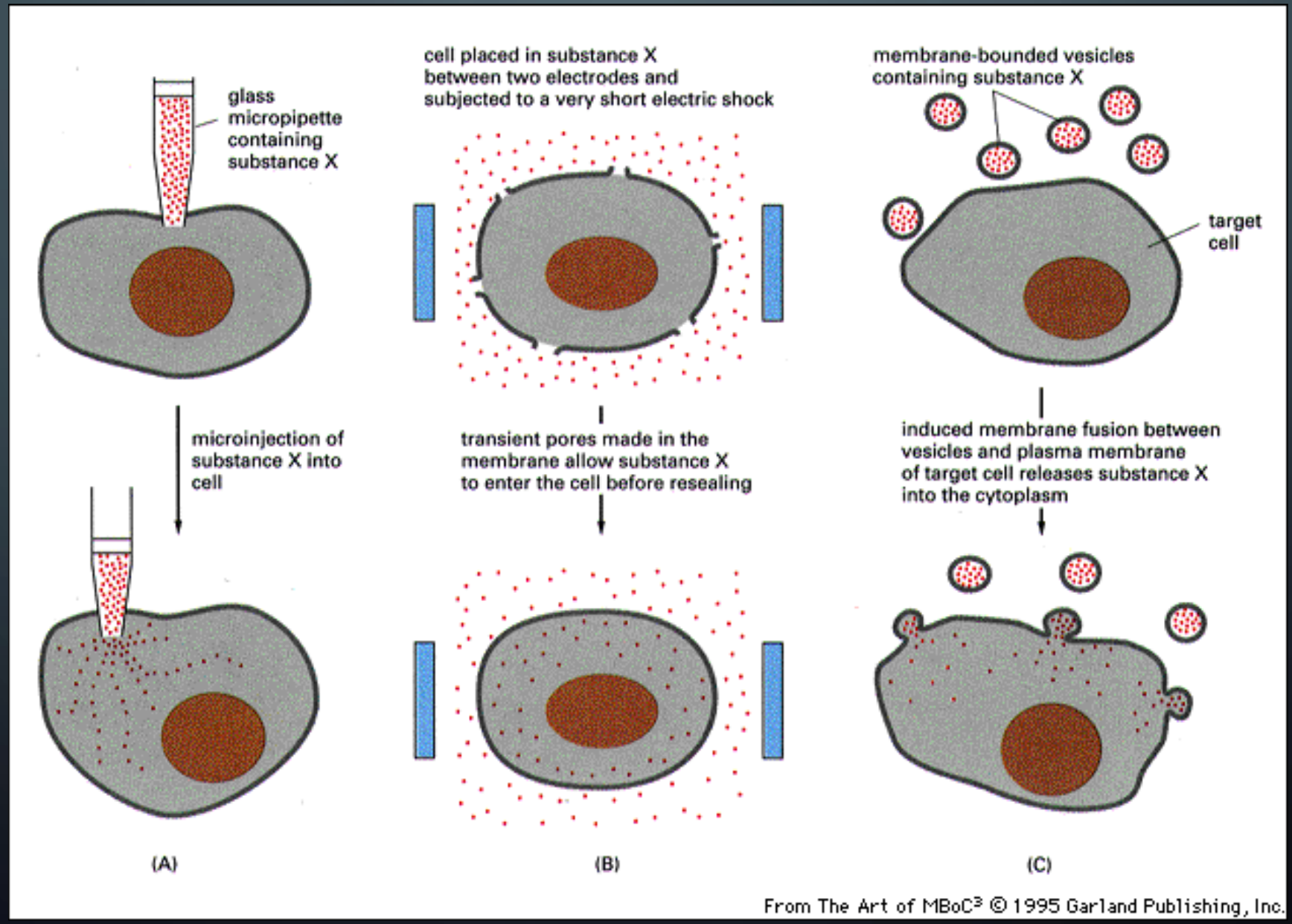
- Aldehyde
  - 10% NBF
  - 4% formaldehyde with PBS buffer
  - 2% formaldehyde with picric acid and PBS
  - The paraformaldehyde paradox
  - Immersion v. transcardial perfusion
  - 24-72 hours
  - Many others
  - Best for good architecture
- Frozen
  - LN2
  - With or without sucrose
  - OCT
  - Fix with acetone or methanol (fix by coagulation, also permeabilizes)
  - Best for cell membrane antigens, cytokines



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

HOW DO WE GET FLUORESCENT PROBES INTO CELLS

- Kill the cell and make the membrane permeable
- Live cells
  - Diffusion: some can cross membrane
  - Microinjection- stick and tiny needle through membrane
  - Trauma: rip transient holes in membrane by mechanical shear (scrape loading) or electrical pulse (electroporation)
  - Lipid vesicles that can fuse with membrane
  - Transfect with fluorescent protein vector


glass micropipette containing substance X
microinjection of substance X into cell
(A)
cell placed in substance X between two electrodes and subjected to a very short electric shock
transient pores made in the membrane allow substance X to enter the cell before resealing
(B)
membrane-bounded vesicles containing substance X
target cell
induced membrane fusion between vesicles and plasma membrane of target cell releases substance X into the cytoplasm
(C)
From The Art of MBoC³ © 1995 Garland Publishing, Inc.

Permeabilisation

Aim: to allow fixative to enter the cells/tissue more quickly if necessary
to allow antibodies to penetrate fixed cells/tissue
done by removing lipids with detergents

Detergents:

- polar lipids with a hydrophilic (water soluble) end and a hydrophobic end that binds the hydrophobic moieties of water insoluble compounds and renders them hydrophilic

Nonionic detergents:

- contain methyl groups that participate in hydrogen bonds and are able to solubilise membranes but do not destroy protein-protein interactions

Triton X-100: used to permeabilise unfixed or lightly fixed eukaryotic cell membranes (0.1% in PBS)
Tween 20: milder than Triton X-100, used to reduce surface tension in blocking, antibody incubation and wash steps (0.1%)
Nonidet P-40 (Igepal Ca-630 from Sigma-Aldrich): used to permebilise unfixed cells (0.1% in PBS for 5-10s)

Ionic detergents:

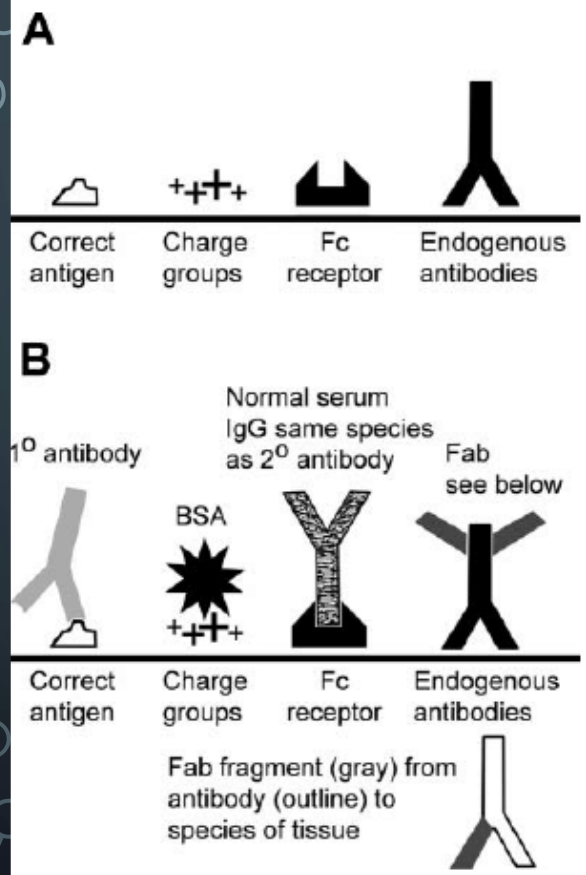
- have highly charged hydrophilic groups and are very effective at solubilising membranes, but also destroy native three dimensional protein structures

SDS, deoxycholate, CHAPS
Not used for immunocytochemistry

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

Blocking



Aim: to allow binding of antibodies only to appropriate sites

Sources of nonspecific binding:

Charged groups

Occur on proteins (esp. histones) or lipids

Also generated by fixation in formalin or glutaraldehyde

To block use bovine serum albumin at 10-30mg/mL (fraction V)

Fc receptors

On macrophages and other immune cells, which bind any antibody

To block whole IgG 1° and 2° antibodies from binding to Fc receptors, incubate cells in buffer containing 5-10% normal serum from the host species of the 2° antibody

Endogenous antibodies

Only a problem for 2° antibodies recognising the same species as your tissue/ cells and only at inflammation sites or in cell cultures of immune system cell types

To block use Fab fragments raised in the same species as the 2° antibody that recognise the species of your tissue/cells as part of the blocking procedure

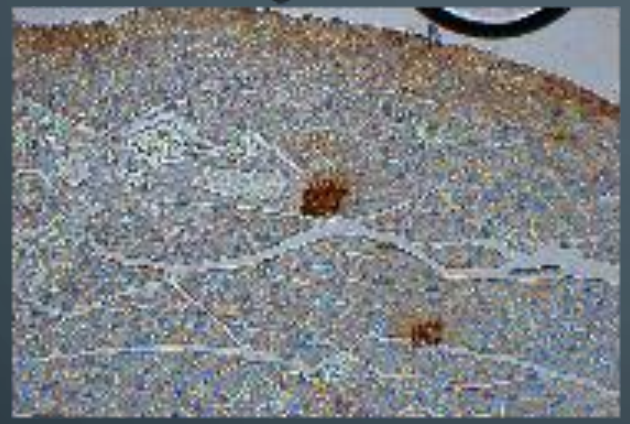
For general blocking can also try MAXblock (Active Motif): protein based, non-mammalian blocking agent, no cross-reactivity with 2° antibodies

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

NON-SPECIFIC STAINING

Before block



After block

Mouse-on-Mouse
Monoclonal mouse insulin/mouse pancreas

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

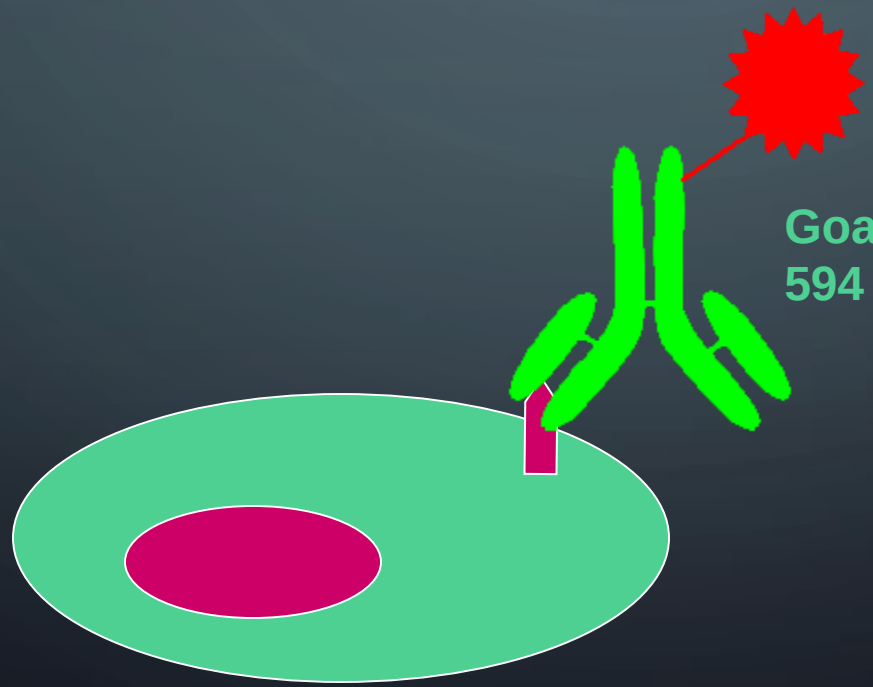
TYPES OF PROBES

- Some change intensity of fluorescence depending on pH or [Ca^{++}]
- Some bind specific structures
  - ER
  - actin
  - Golgi
  - Plasma membrane
  - Mitochondria
- Fluorescently labeled purified protein
- Antibodies

IMMUNOFLUORESCENCE LOCALIZATION OF PROTEINS IN DEAD/FIXED CELLS

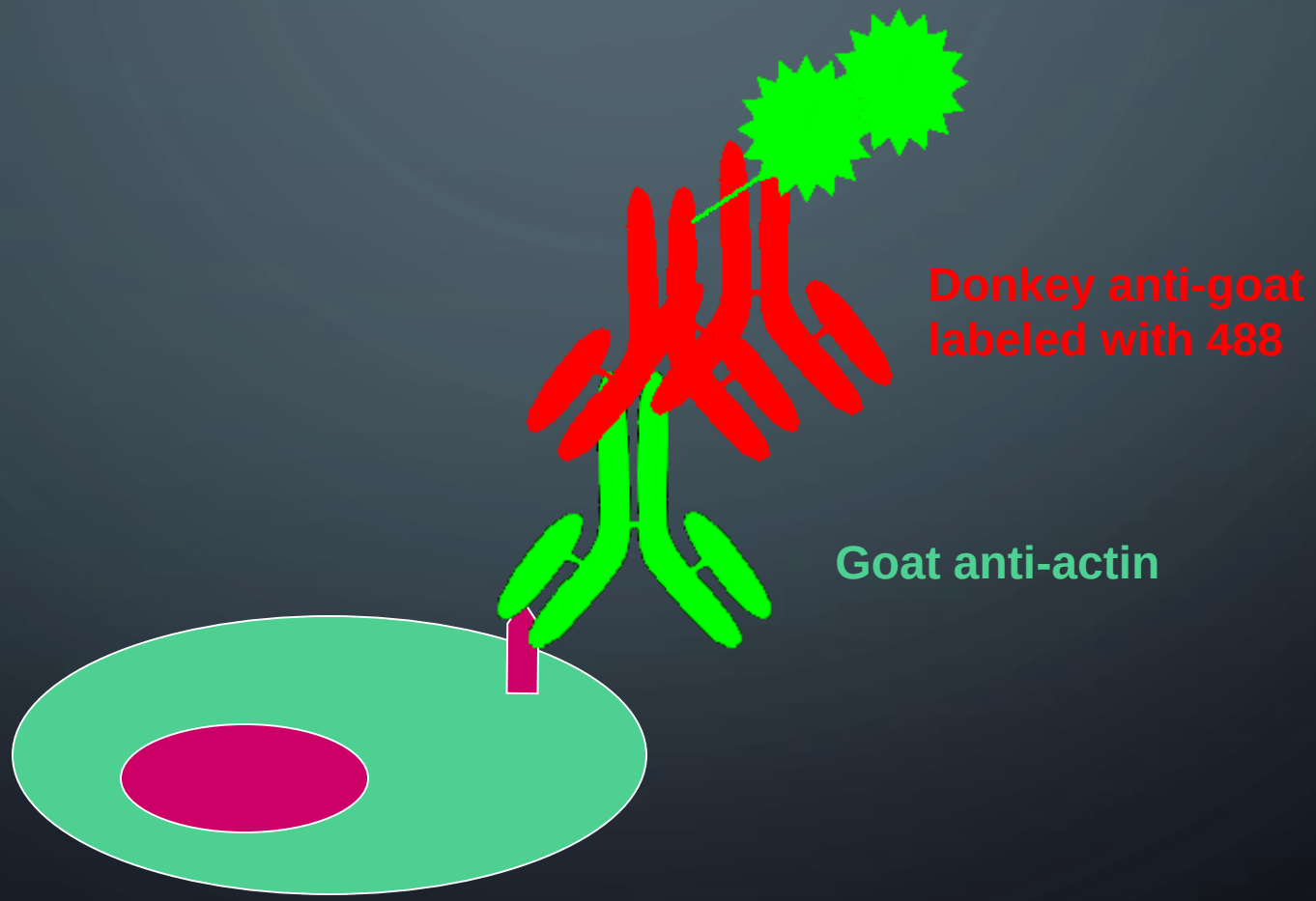
- You can purify almost any protein from the cell (Biochemistry)
- Make an antibody to it by injecting it into a rabbit or mouse (primary antibody)
- Use the antibody to bind to the protein in the fixed cell
- Fixed cells can be made permeable so antibodies can get into interior
- Use a fluorescent “secondary antibody” (anti-rabbit or mouse) to localize the primary antibody

DIRECT METHOD-
PRIMARY ANTIBODY ONLY

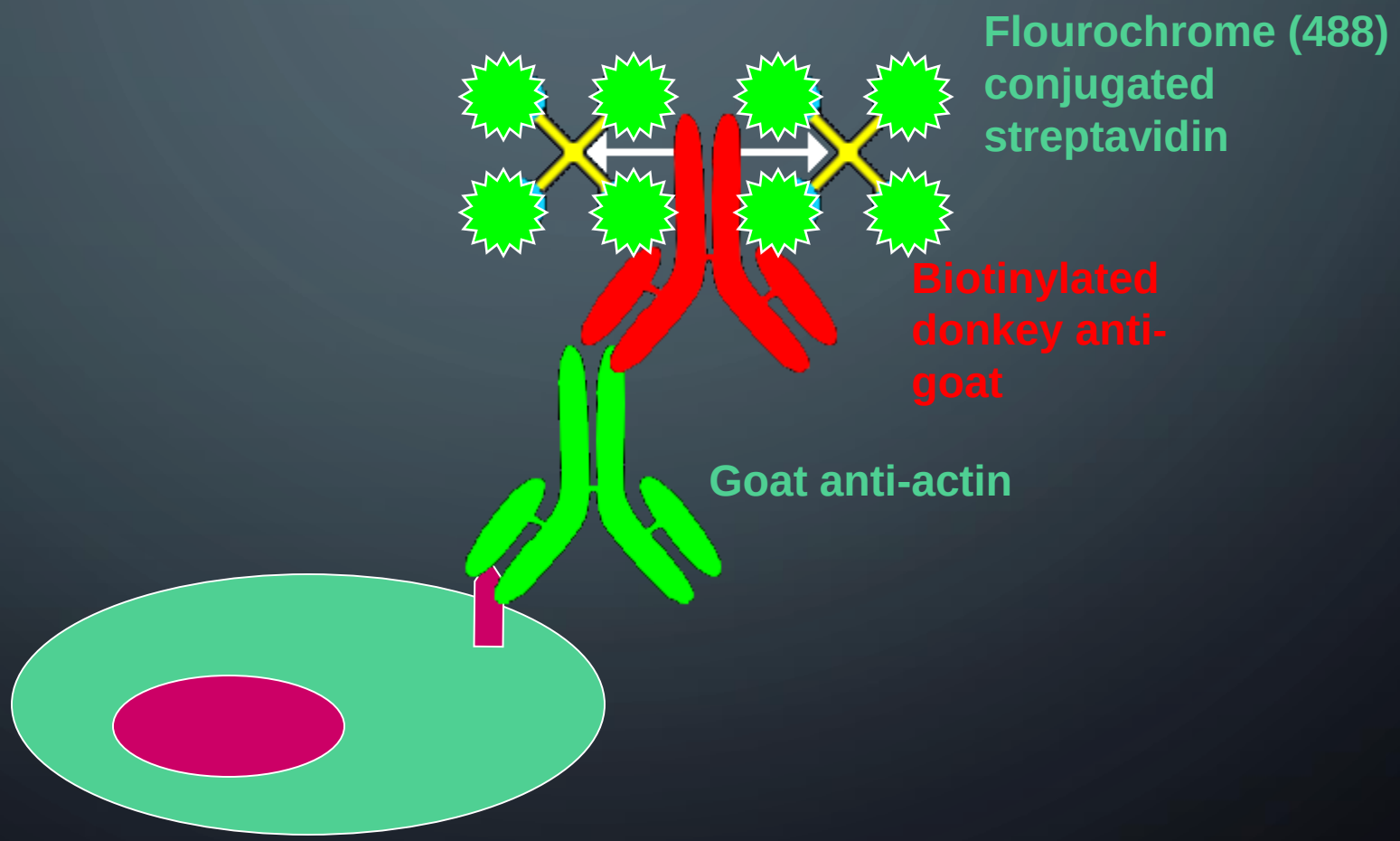


Goat anti-actin labeled with 594

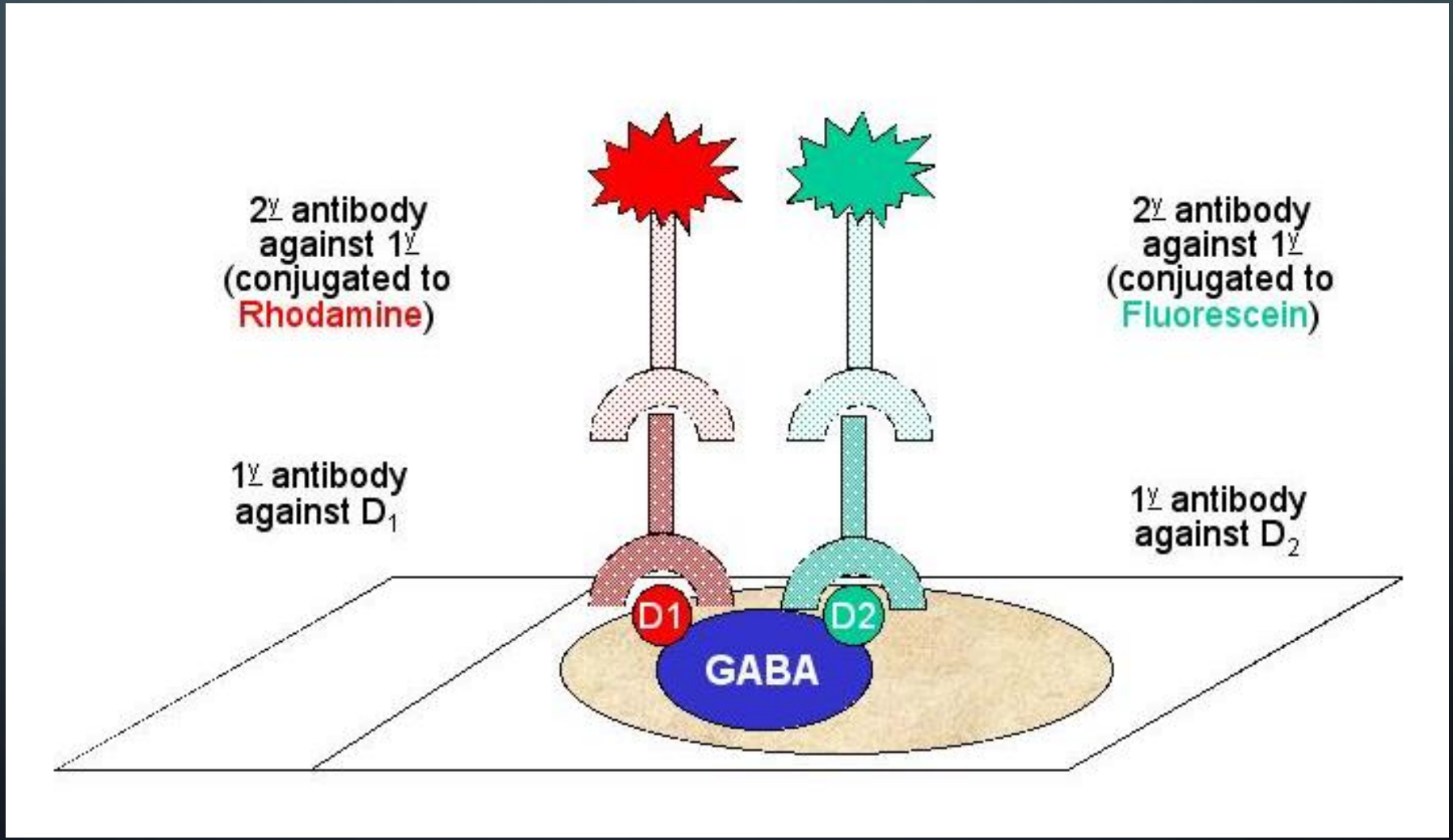
INDIRECT METHOD – PRIMARY AND SECONDARY ANTIBODIES



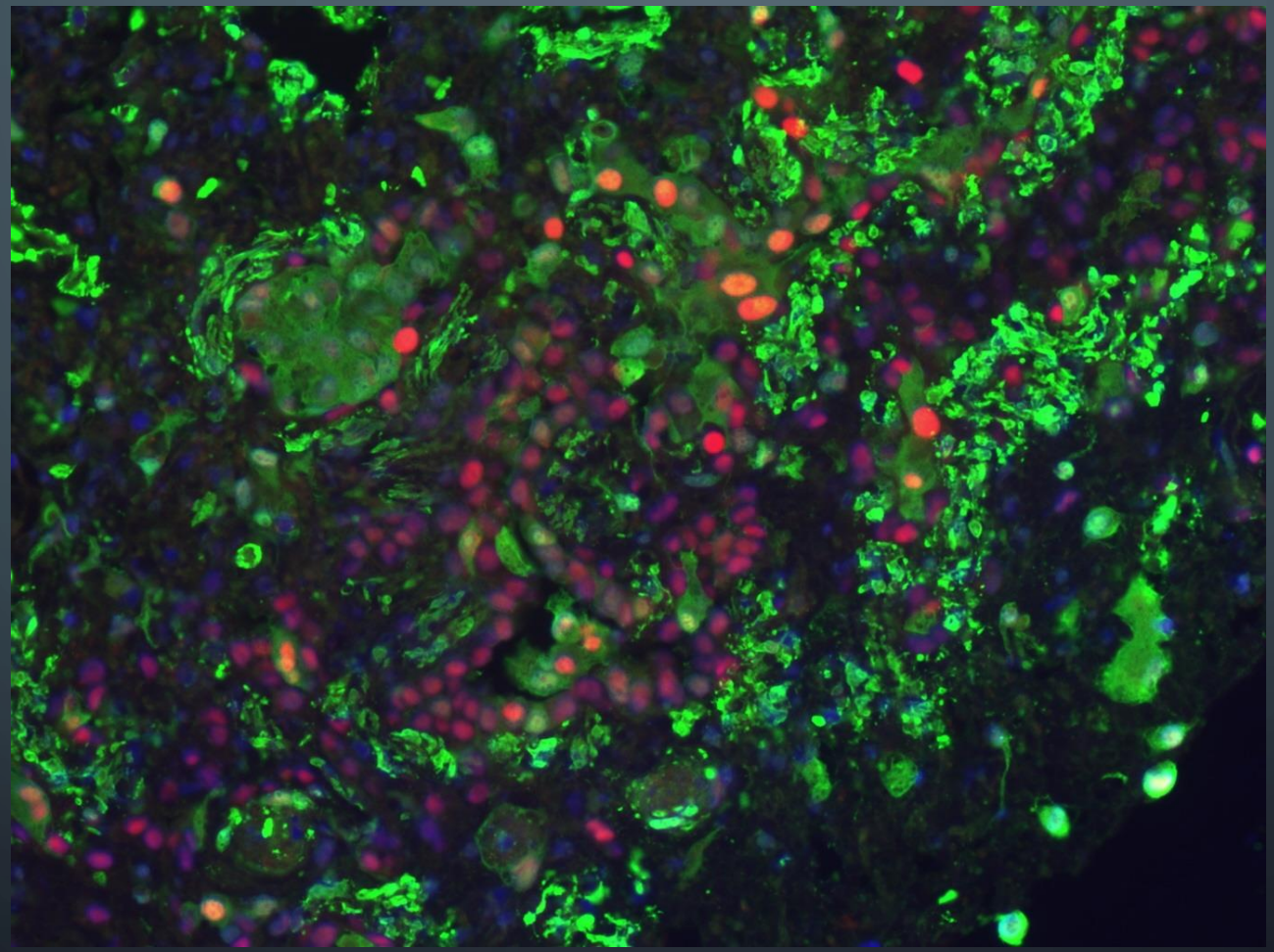
ENZYME LINKAGE INDIRECT METHOD



MULTIPLE IMMUNOFLUORESCENCE



MULTIPLE LABELLING OF A TISSUE SECTION



Polyclonal antibodies

Advantage:

- High levels of labelling because they bind several epitopes on the same protein

Disadvantages:

- Can label multiple proteins that share epitopes
- Different batches have different antibodies

Monoclonal antibodies

Advantages:

- Single epitope selected for high specificity
- Different clones can be generated to different epitopes on the same antigen
- Single clone can recognise post-transcriptionally modified protein (e.g. phosphorylation)
- Same clone can be generated indefinitely

Disadvantages:

- Low levels of labelling possible
- Mostly from mice

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

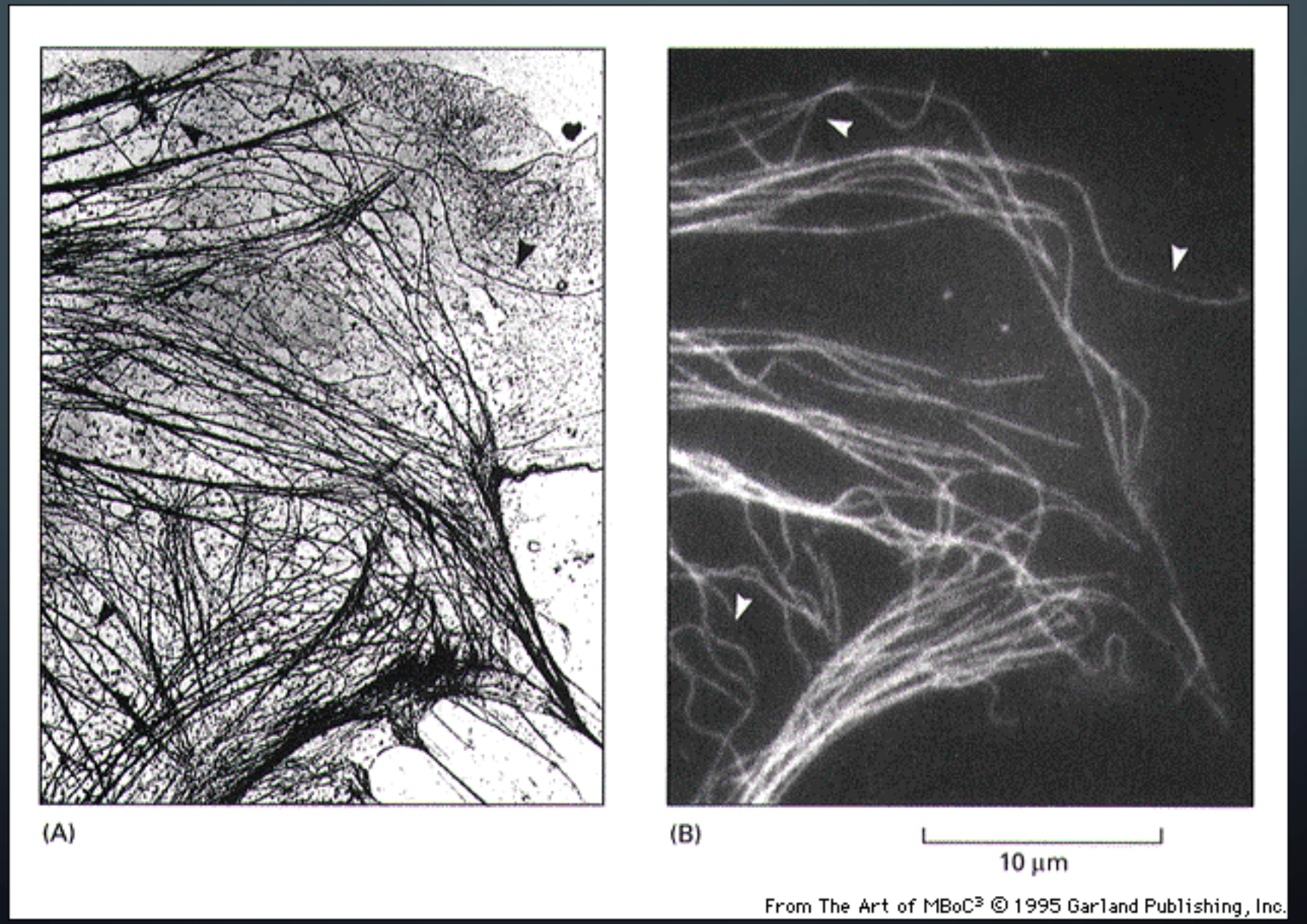
MAKE SURE YOUR ANTIBODY IS VALIDATED FOR YOUR APPLICATION!!!

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

ASCITES, SUPERNATANT, SERUM?

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

ANTI-TUBULIN IMMUNOFLUORESCENT LOCALIZATION OF MICROTUBULES

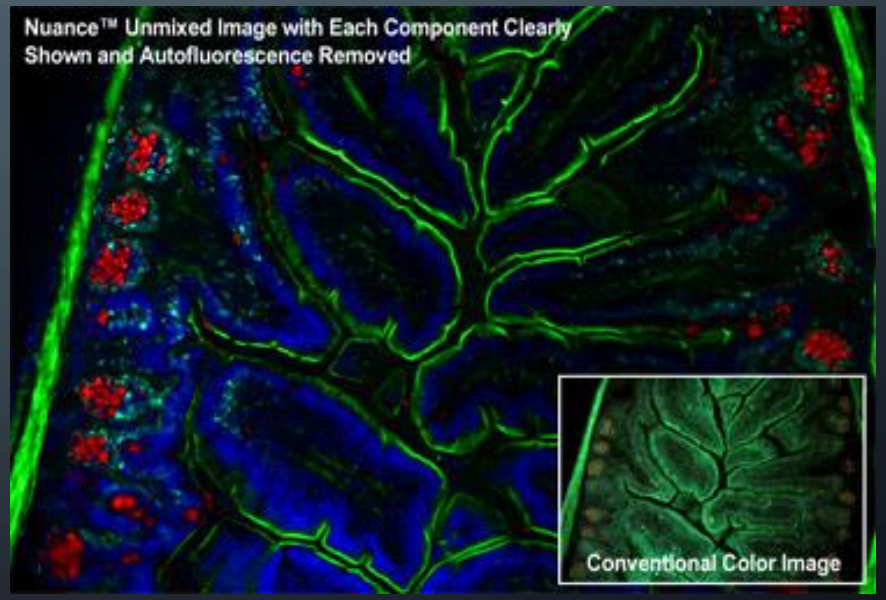


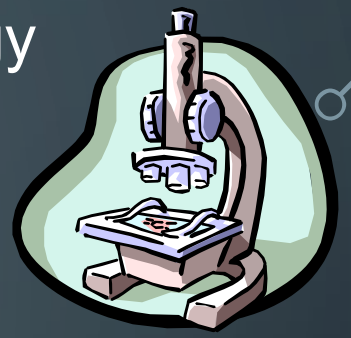
TECHNIQUE:

- Common dyes: fluorescein, rhodamine
- Dyes chosen are excited by a certain light wavelength, usually blue or green, and emit light of a different wavelength in the visible spectrum
  - Eg. Fluorescein emits green light
  - Eg. Rhodamine emits orange/red light
- By using selective filters in a fluorescence microscope only the light from the dye is detected
- Available fluorescent labels now include red, blue, cyan or yellow fluorescent proteins

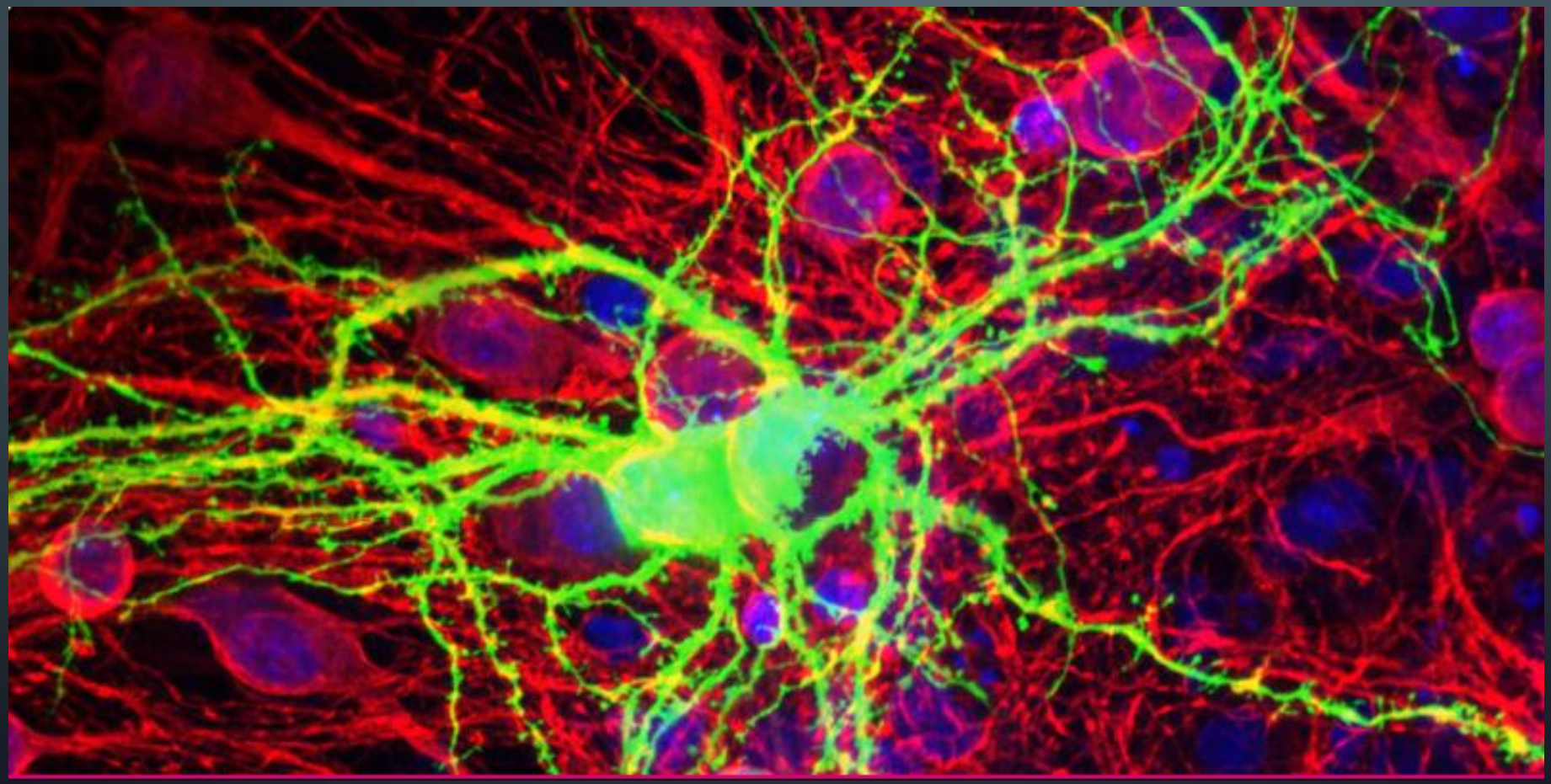
USES:

- This can be used to detect the distribution of any protein
- By attaching different dyes to different antibodies the distribution of two or more molecules can be determined in the same cell or tissue sample





Question?



Thanks for your attention!

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