Immunohistochemistry and related techniques

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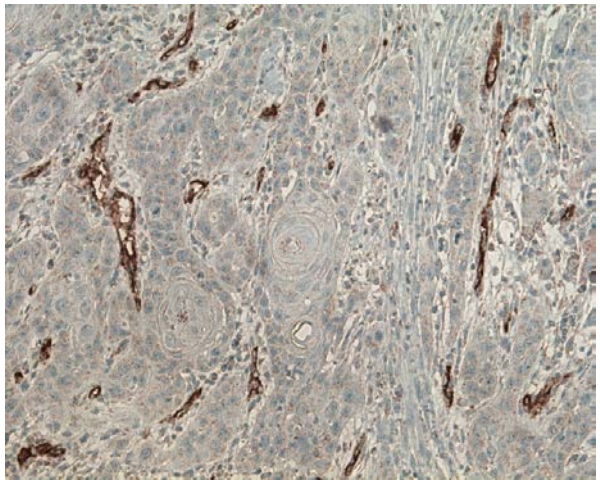
19th November, 2020

MRC Human Immunology Unit

Applications of immunohistochemistry

Examining histological samples

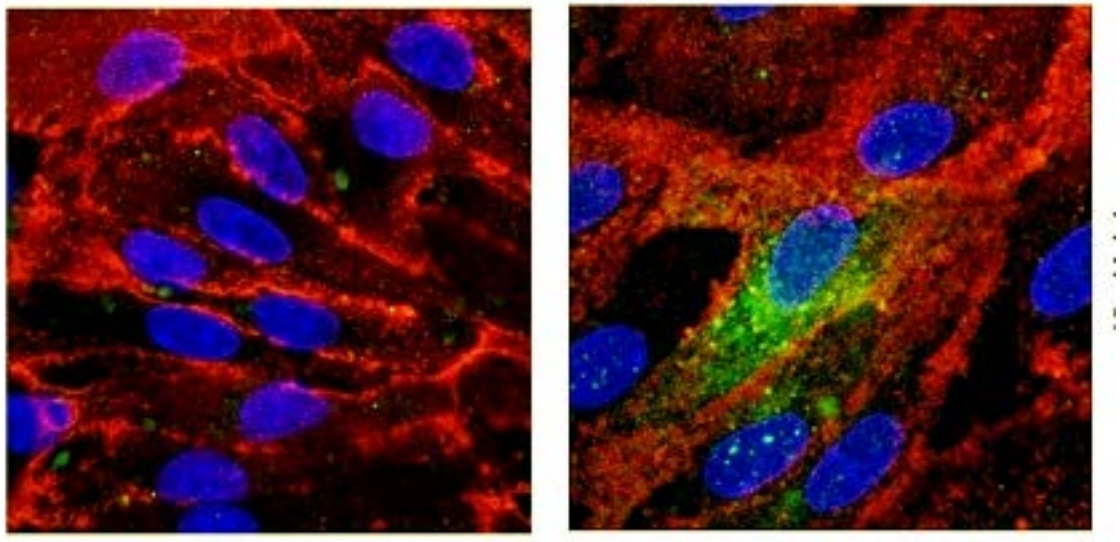
- Determining tissue distribution of an antigen
  - in health
  - in disease
- Looking at prognostic markers of disease
- Identification of microscopic structures according to proteins expressed



Applications of immunocytochemistry

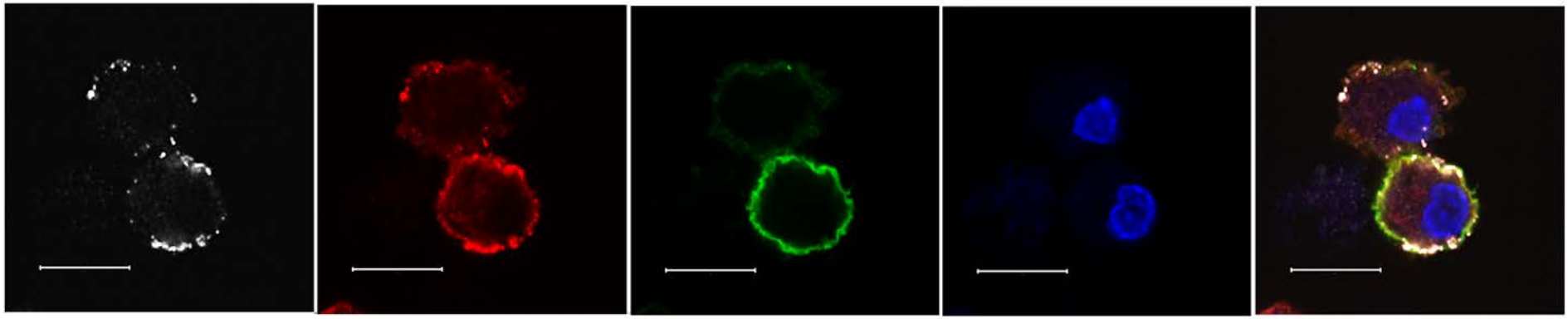
Examining cells

- Phenotyping cells
- Visualising subcellular localisation of antigens



Why do immunohistochemical or immunofluorescence microscopy?

- Gives you a different perspective, complementing other techniques (Western blotting, FACS, ELISA etc)
- Gives you pretty pictures

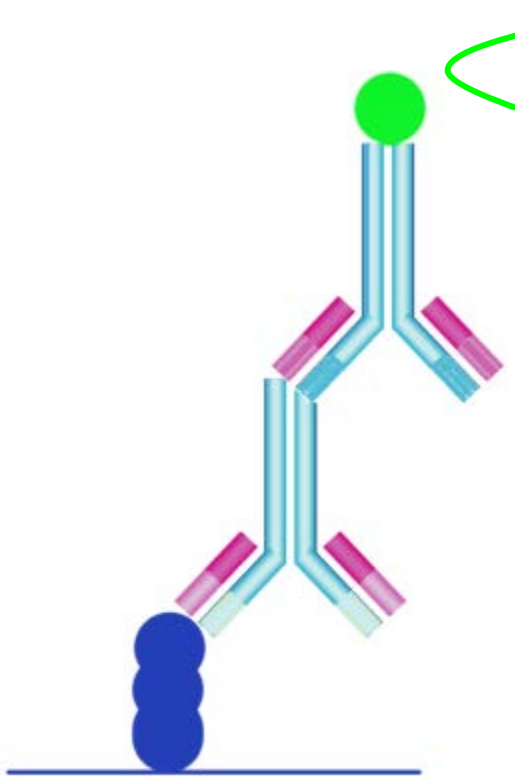


General Principal of Immunodetection

Secondary antibody, conjugated to a fluorophore (immunofluorescence) or HRP (immunohistochemistry or immunocytochemistry)

Primary antibody

Protein of interest



Tissue or cell

Excitation light

Emission light

Detection by microscope

WASH SAMPLE BETWEEN EACH INCUBATION STEP, USUALLY WITH PBS

Basic protocol for immunostaining

1. Tissue or cells: The nature of the sample

↓

2. Steps in between: To fix or not to fix?; is permeabilisation necessary?

↓

3. Blocking non-specific binding

↓

4. Primary antibody

↓

5. Secondary antibody

↓

6. Mounting

↓

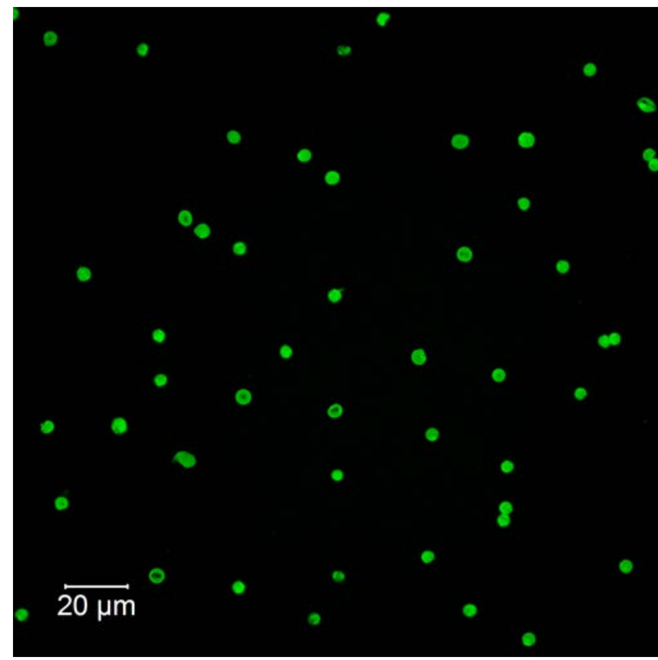
Microscopy

1. Nature of sample

- Cells
- Paraffin-fixed tissue
- Frozen tissue
- Whole-mount tissue staining

Cells

- If cells are adherent, check suitability of tissue culture ware for microscopy. (BD Falcon chamber slides usually good)
- Attach non-adherent cells to slides by cytospin



Cells from mouse dermis, fixed to slide by cytospin

Frozen tissue sections

- Tissue must be freshly frozen and cut into thin (<6μm) sections using a cryostat, then stored at -20 ºC or -80 ºC
- ***ADVANTAGE:*** No antigen-retrieval required, or signal amplification (usually)
- ***DISADVANTAGE:*** Some damage due to freezing may occur, especially with thicker tissue

Whole-mount tissue staining

- Pieces of tissue (<1mm thick) perfused or incubated with paraformaldehyde (or other fixative) then stained for immunofluorescence microscopy and viewed in confocal sections

- ***ADVANTAGE:***
  - Allows intact tissue architecture to be viewed

- ***DISADVANTAGES****:*
  - Time consuming and not all antibodies work on fixed tissue
  - Thicker tissue may require optical clearing

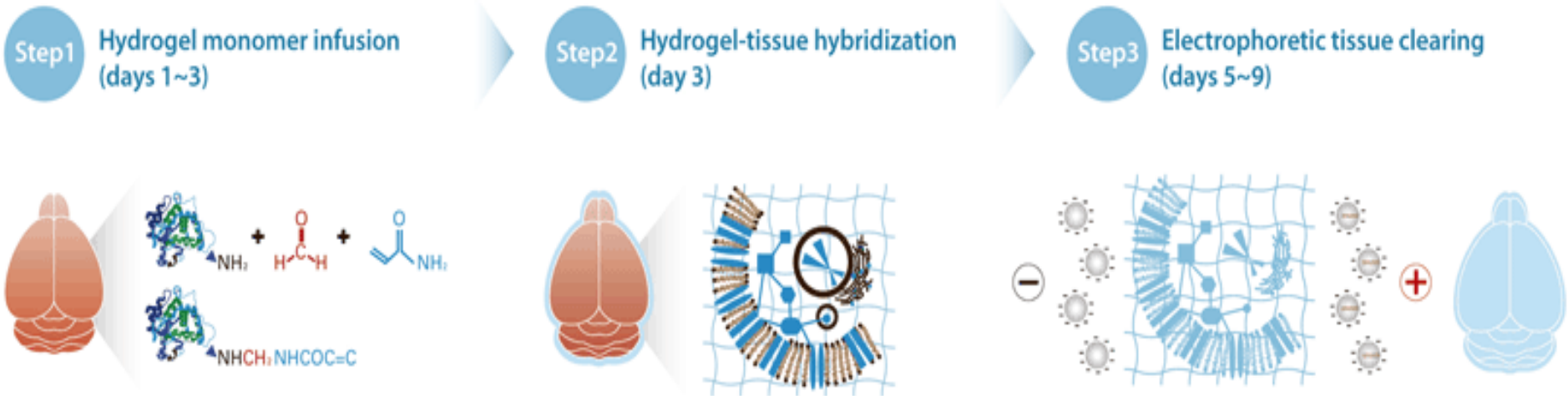
Tissue clearing

- Optically clearing tissue homogenises light scattering within biological samples so that individual components have approx the same RI.
- May be solvent based (removes intracellular fluids and lipids, leaving dehydrated proteins)
- Aqueous based (replaces intracellular fluids, lipids and proteins remain, hydrated)
- Richardson *et al* (Cell) 2015

Hama *et al* (Nat Neurosci) 2011

X-Clarity™ tissue clearing system

- Forms a hydrogel network to support the tissue ultrastructure, whilst lipids are removed by electrophoresis
- Tissue can then be stained using antibodies
- Viewed in 3-d



https://www.labtech.com/x-clarity-tissue-clearing-system

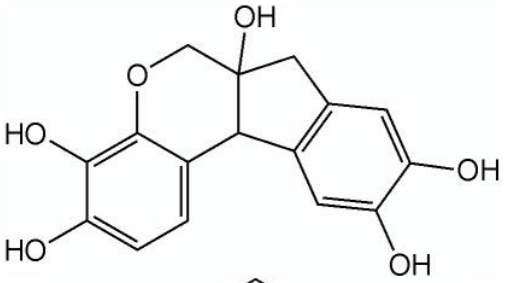
Paraffin-fixed tissue

- Cut from paraffin-embedded blocks using a microtome
- Allows tissue samples to be archived and stored for long time at room temperature in a paraffin block or cut into thin (< 4μm) sections
- ***ADVANTAGES***
  - Generally good preservation of tissue architecture
  - Serial sections may allow comparisons of staining for different antigens (or with H&E staining)
- ***DISADVANTAGES***
  - Antigen retrieval required
    - Remove paraffin embedding material
      - CitroClear (limonene-derivative)
      - Xylene

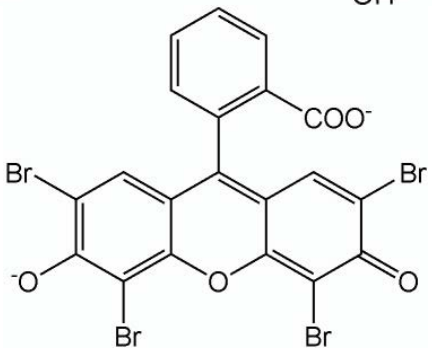
    - Rehydrate tissue by incubating tissue in ethanol-water, gradually decreasing ethanol concentration from 100%, to 50%, to pure water
    - Heat in aqueous antigen retrieval buffer
  - May require signal amplification
  - Some epitopes may be destroyed

Haematoxylin-Eosin staining (H&E)

- **Haematoxylin**
  - Basic, binding nucleic acid-rich regions e.g.nuclei
  - Stains structures blue-purple



- **Eosin**
  - Acidic, staining basic regions of the cell, e.g.cytoplasm
  - Stains structures pink and red blood cells bright red



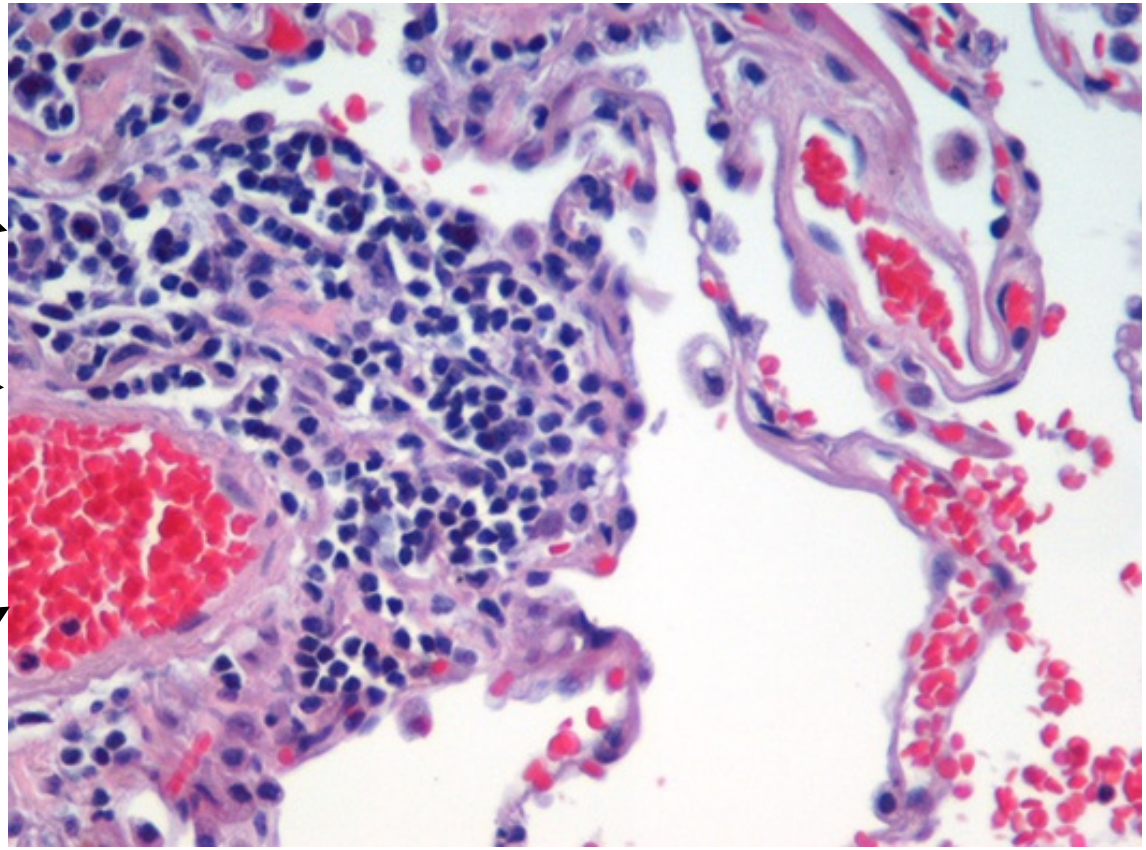
- H&E staining usually used to show overall tissue architecture
- Haematoxylin counterstaining alone often performed in immunohistochemistry, to avoid confusion between red eosin and brown HRP signal

Example of H&E staining (in inflamed lung tissue)

Haematoxylin-positive nuclei

Eosin-positive cytoplasm

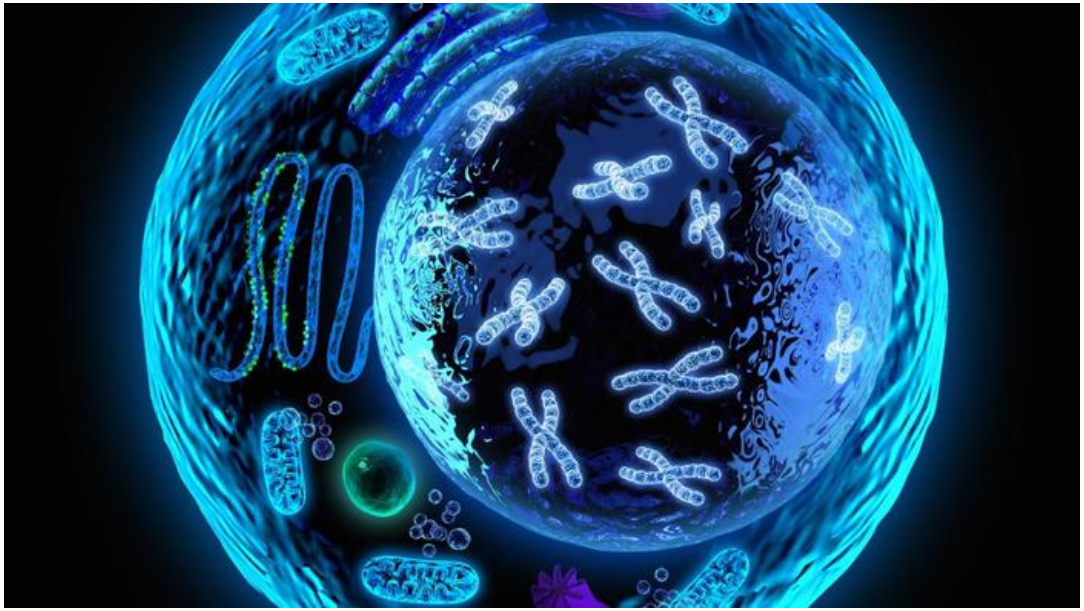
Eosin-positive erythrocytes



2. Protein/antigen of interest

- Is fixation necessary?

- Is permeabilization necessary?
  - Is antigen on the cell surface or intracellular?
  - Cytoplasmic or nuclear?



Fixation

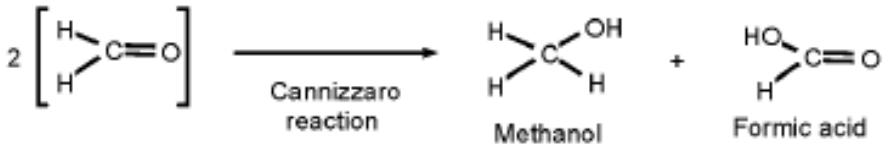
- To preserve cells and tissues as they naturally occur
- Fixatives denature proteins by
  - coagulation, or
  - cross-linking, or
  - both coagulation and cross-linking
- ***ADVANTAGES***
  - Prevents autolysis by inactivating lysosomal enzymes
  - Stabilizes fine intracellular and extracellular structures
  - Binds target antigens in their native locations
  - Prevents loss of cellular constituents
  - Inhibits growth of bacteria etc
- ***DISADVANTAGES***
  - Antibody might not recognize fixed antigen
- Osmotic pressure (to prevent tissue distortion) and pH must also be considered

Examples of chemical fixatives

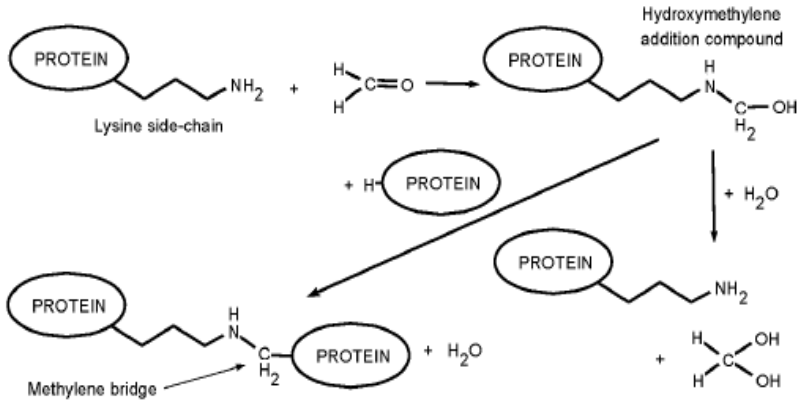
- **Aldehydes**
  - e.g. formaldehyde, glutaraldehyde 
  - Induce cross-linking but not coagulation
- **Protein denaturing agents**,
  - e.g. zinc salts, acetic acid, alcohol (ethanol, methanol)
  - Induce coagulation
- **Oxidizing agents** e.g. osmium tetroxide  $
- Miscellaneous

Examples of Fixatives - Formaldehyde

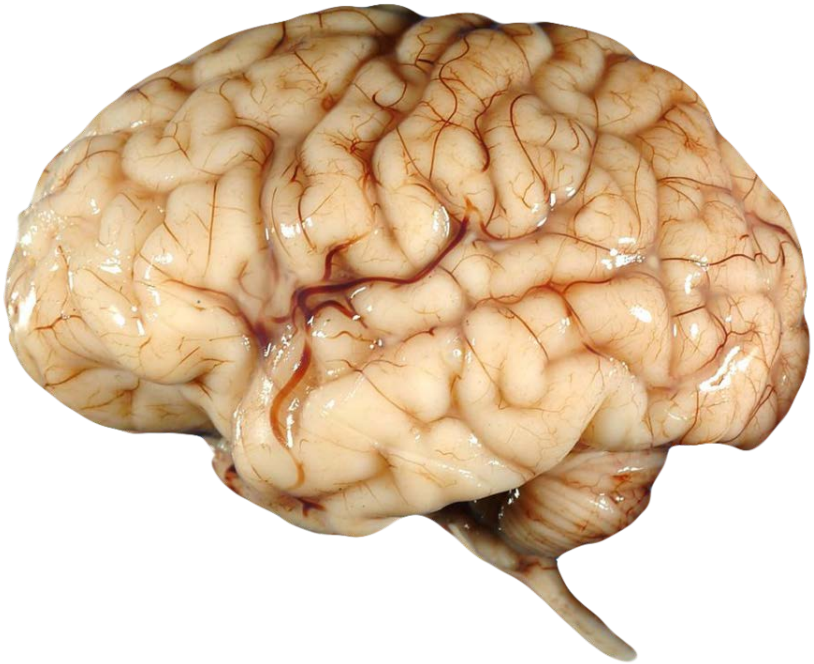
- White crystalline solid formed from polymerization of methanal gas (formaldehyde) by heating
- Dissolve in water to 2X desired concentration (0.5-4%) at 55 ^{o}C then dilute in 2X concentrate PBS and adjust pH to 7.4
- Beware of using old stocks e.g. old bottles of commercial formalin



- Adds to the side-chains of basic amino acids (especially lysine) and to amide N atoms of peptide linkage



- Formaldehyde penetrates rapidly into tissues (low MW) BUT chemical reactions (e.g. methylene bridge formation) occur slowly
- Beware under-fixing, especially large, soft specimens
- Beware. It's toxic



Examples of Fixatives – Zinc salts

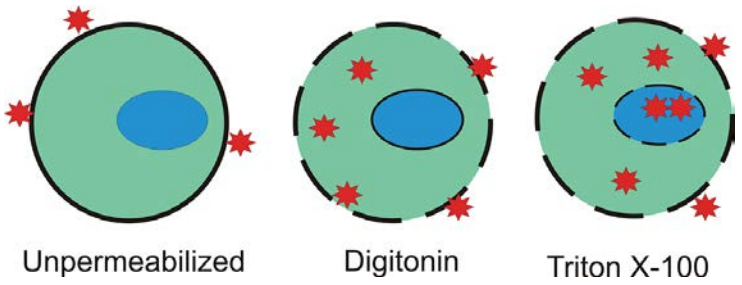
- React with amino, carboxyl and sulphydryl groups to produce cross-linking
- Coagulates and immobilizes proteins
- Can be added to formalin solutions, to enhance fixation and staining (especially nuclei)
- Less destructive for some epitopes

Examples of fixatives - Alcohols

- >80% methanol and 50-60% ethanol
- Coagulants that denature proteins
- Replace water in the tissue, disrupting hydrophobic and hydrogen bonding, exposing internal hydrophobic groups of proteins and altering tertiary structure and solubility in water. i.e. they dehydrate tissue
- Can cause distortion of tissue
- Dissolve/disrupt cell membrane lipids
  - Permeabilize cells BUT
  - Intracellular macromolecules may escape

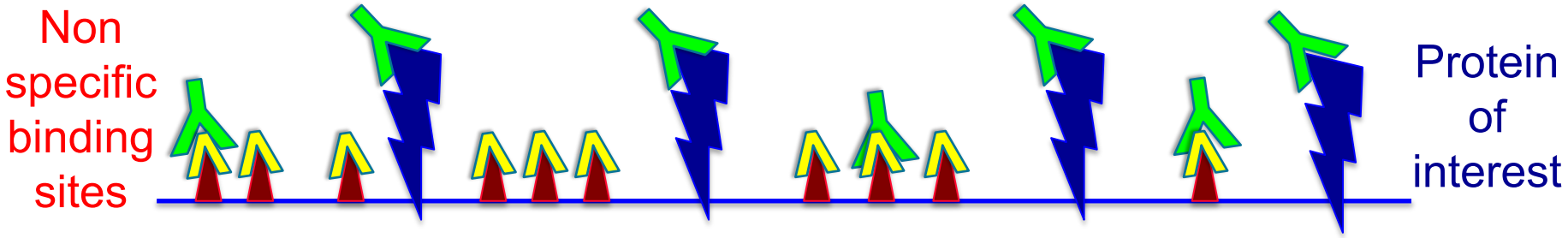
Permeabilisation

- Not usually necessary for cytoplasmic staining after alcohol fixation of cells or tissue sections, or for examining cell-surface antigens
- Non-ionic detergents used to permeabilize cell and nuclear membranes, usually after PFA fixation.
- **Mild detergents**
  - e.g. saponin, digitonin
  - Dissolve cholesterol and create pores that are large enough for antibodies to penetrate
  - Reversible, so keep this present throughout staining protocol
- **Stronger detergents**
  - e.g. Triton X100, Tween 20
  - Interacts with both proteins and lipids, forming pores and/or removing them from membrane
  - Long incubations may lyse cells



- Concentration (0.1-0.5%) and time (usually 5-10 mins) depends upon subcellular localisation of antigen and the individual antibody
- Detergent may be included as a separate step, or with the blocking step or at all antibody steps throughout.
- Whole-mount staining improved by detergent (usually triton X-100) present throughout, due to thickness of tissue

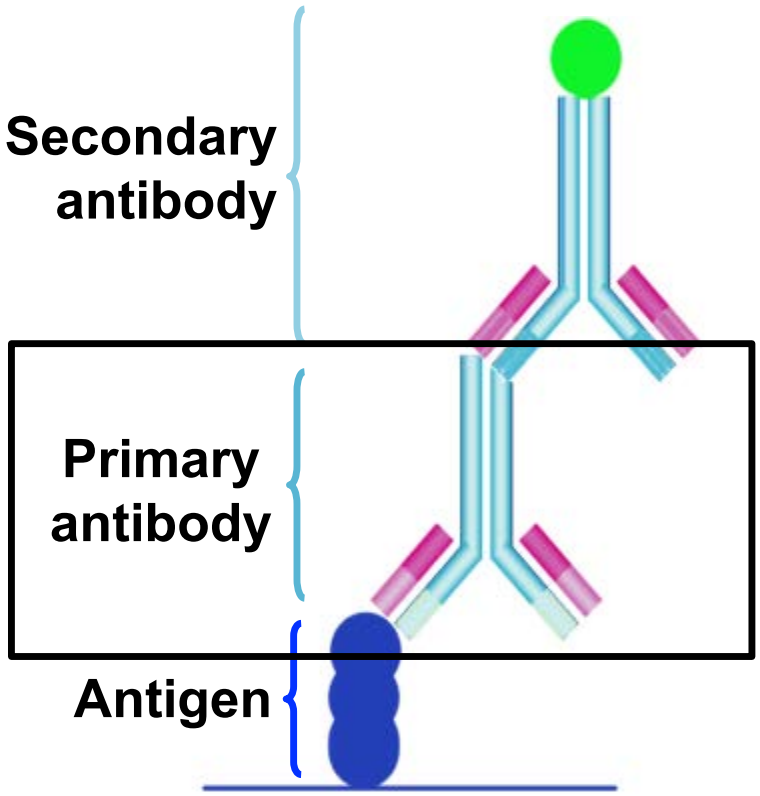
3. Blocking non-specific binding



- In addition to specific antibodies, apply higher concentrations of irrelevant proteins to fill up non-specific binding sites. For example:
  - BSA, 1%
  - Goat serum, 5-10%
  - Foetal calf serum, 5-10%
  - Milk powder, 1-3%
- Blocking agent applied in a preliminary incubation and with all subsequent antibody incubations
- Use secondary antibodies that have been cross-adsorbed against other species, to avoid cross-reactivity when doing double/triple staining.
- *Blocking is usually with species that secondary antibody is raised in*

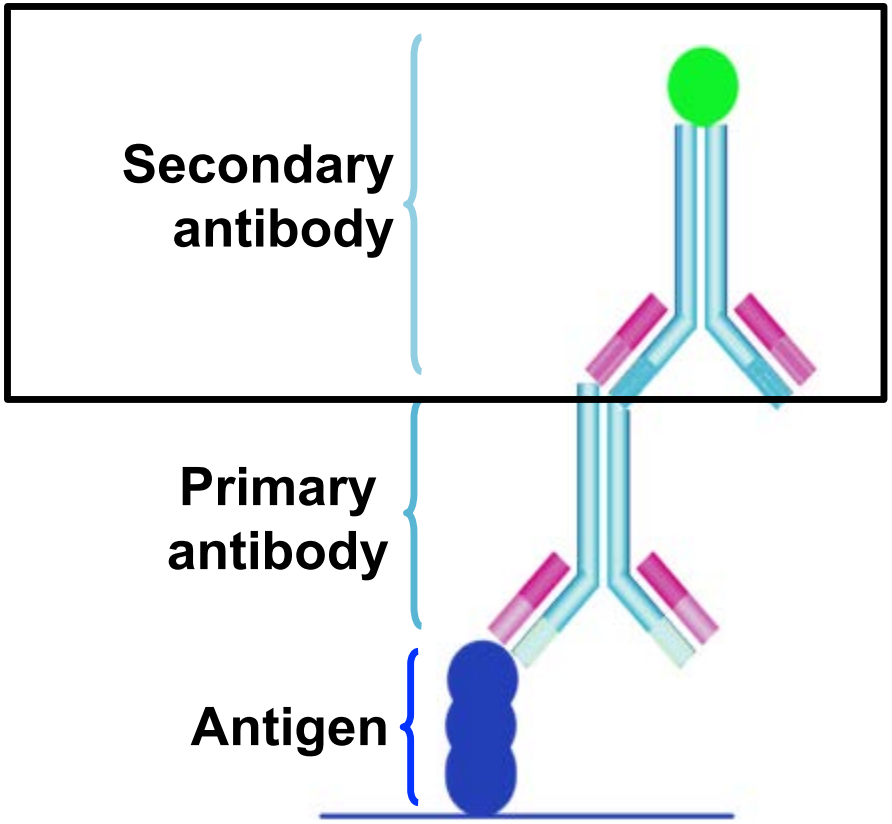
4. Primary Antibody

- Monoclonal or polyclonal?
- Binding may be dependent upon conformation of antigen or state of glycosylation
- Does it recognise only native antigen or fixed antigen?
- Class and isotype, if monoclonal.
  - Most are IgG (although some, against carbohydrate moieties may be IgM).
  - Isotype may be IgG1, IgG2a etc.
- Length of incubation: 30 mins room temperature-to-4^{o}C overnight



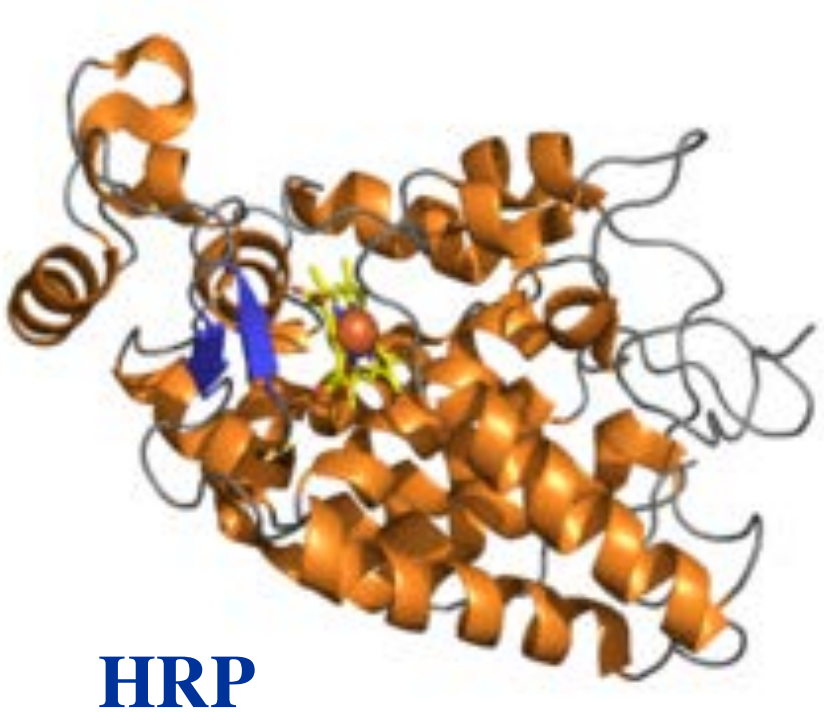
5. Secondary Antibody

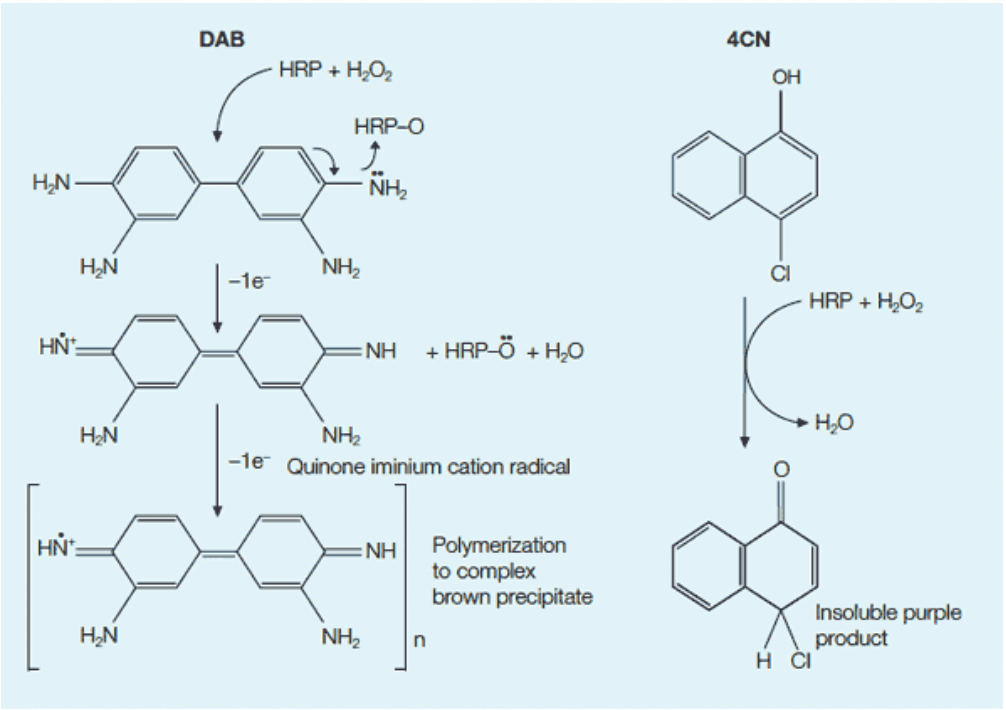
- Polyclonal antibody raised in a species different from the primary antibody
- Cross-adsorbed against other species to improve specificity
- Blocking buffer should include IgG of this species, to reduce non-specific binding
- For immunohistochemistry, conjugated with horseradish peroxidase (HRP)
- For immunofluorescence, conjugated with a fluorophore



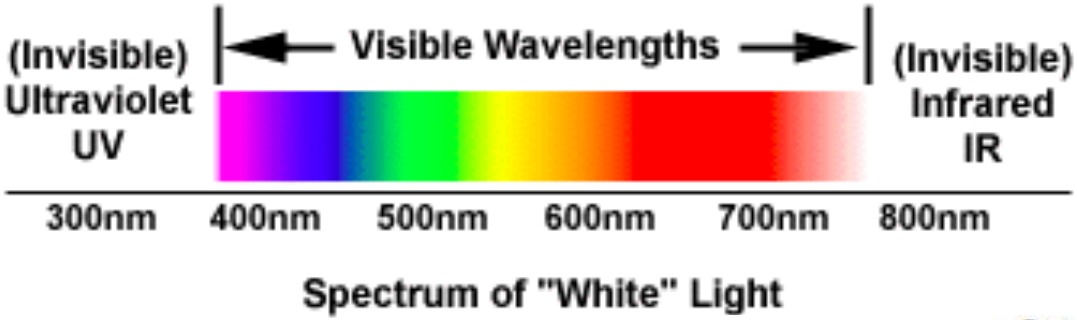
Secondary antibodies – Horseradish peroxidase conjugated

- Horseradish peroxidase reaction product depends upon substrate, usually DAB (3,3'-diaminobenzidine) which gives brown stain
- Block endogenous peroxidase activity beforehand

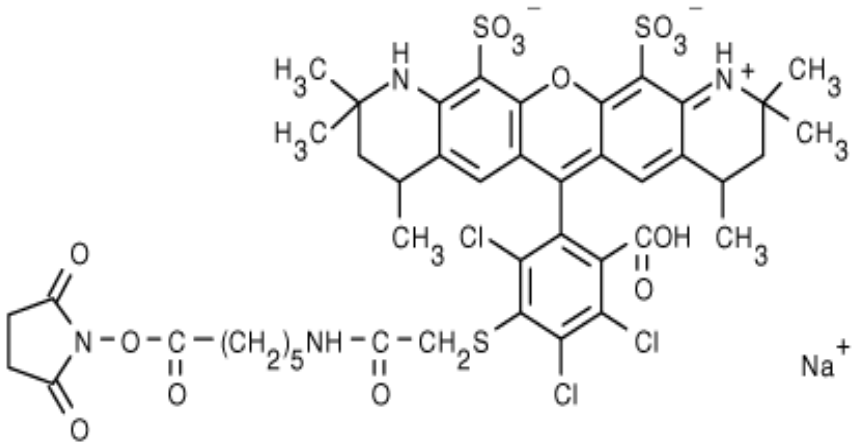




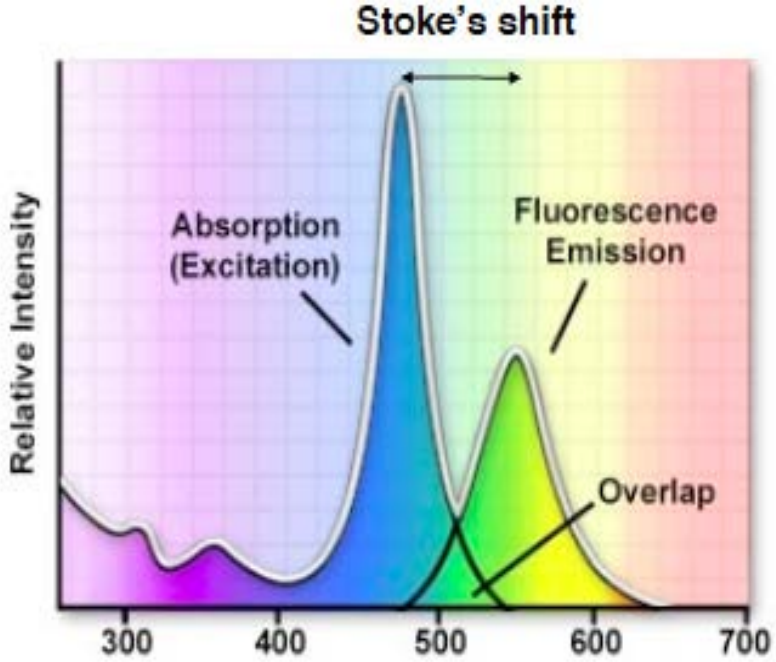
Secondary antibodies - Fluorescent dyes



Spectrum of "White" Light



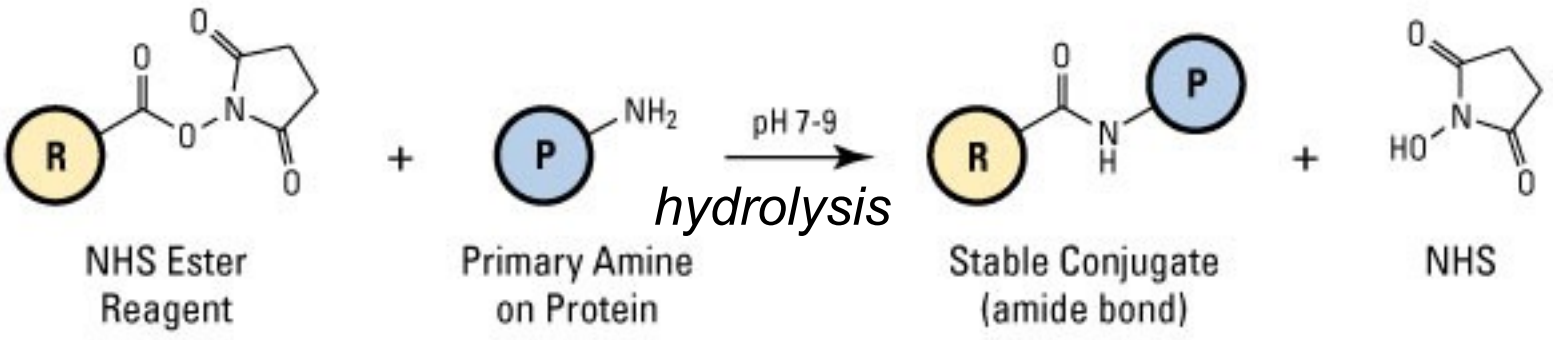
e.g. AlexaFluor 546



https://www.biolegend.com/spectraanalyzer

Directly conjugated antibodies

- Primary antibody is already covalently linked to a fluorophore or to HRP
- Useful in double staining with monoclonal antibodies from the same host species
- Conjugation can be performed in lab using a kit (e.g. Molecular Probes), provided primary antibody is abundant and at high concentration BUT overlabelling may reduce antigenicity



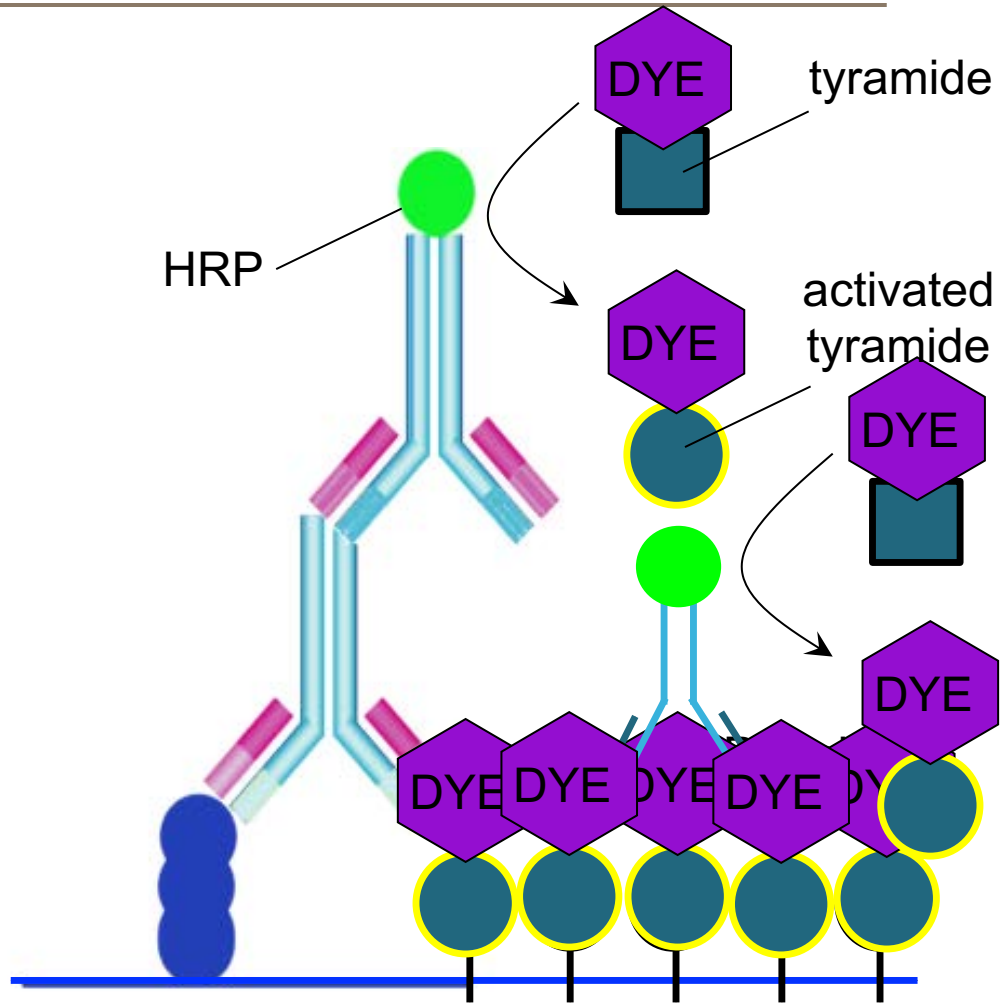
Fluorophore with ester linkage

Primary amine on antibody to be labeled

Labeled antibody

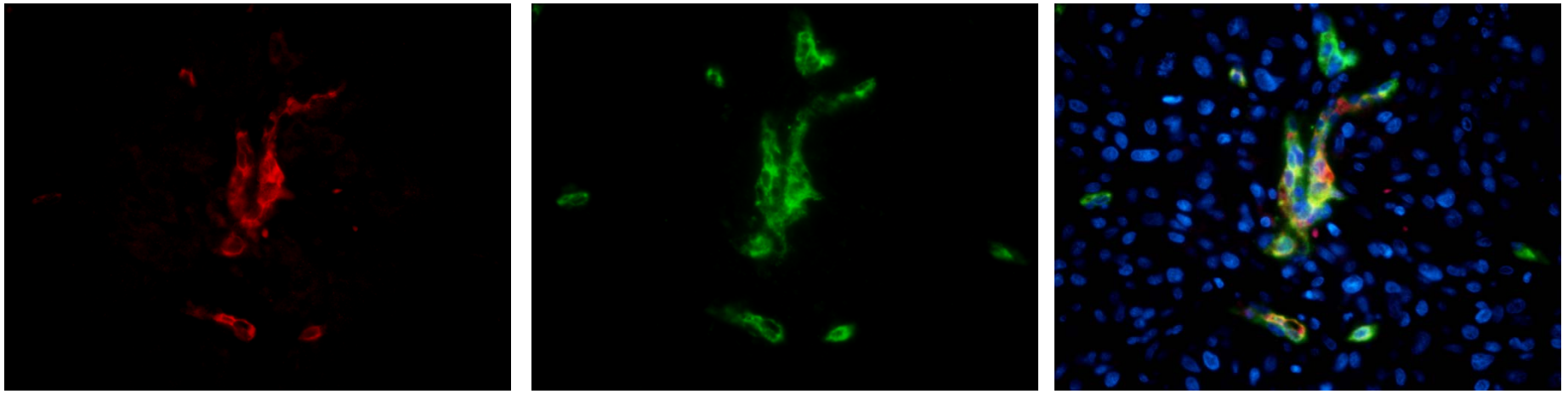
Signal Amplification
(e.g. Tyramine Signal Application)

- Used if signal is weak
- Several methods, usually rely on forming complexes that contain a large number of labelled molecules
- Primary antibody is detected by secondary antibody conjugated to HRP
- A dye-labelled tyramide is applied → local deposition of activated tyramide derivative
- Deposited dye is detected by an HRP-labelled anti-dye antibody
- More dye-labelled tyramide is applied → activated tyramide deposited
- More dye present on specimen



50x fold greater sensitivity than the standard HRP-labelled method

Signal amplification for immunofluorescence microscopy

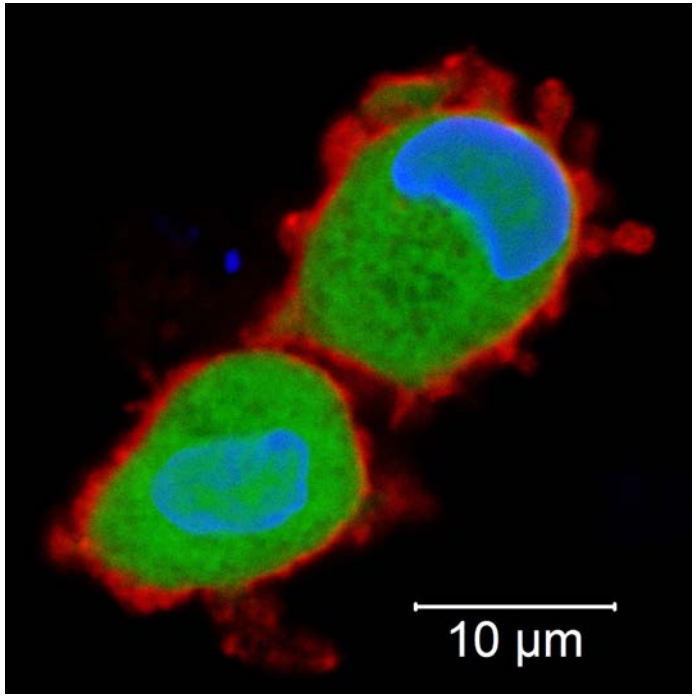


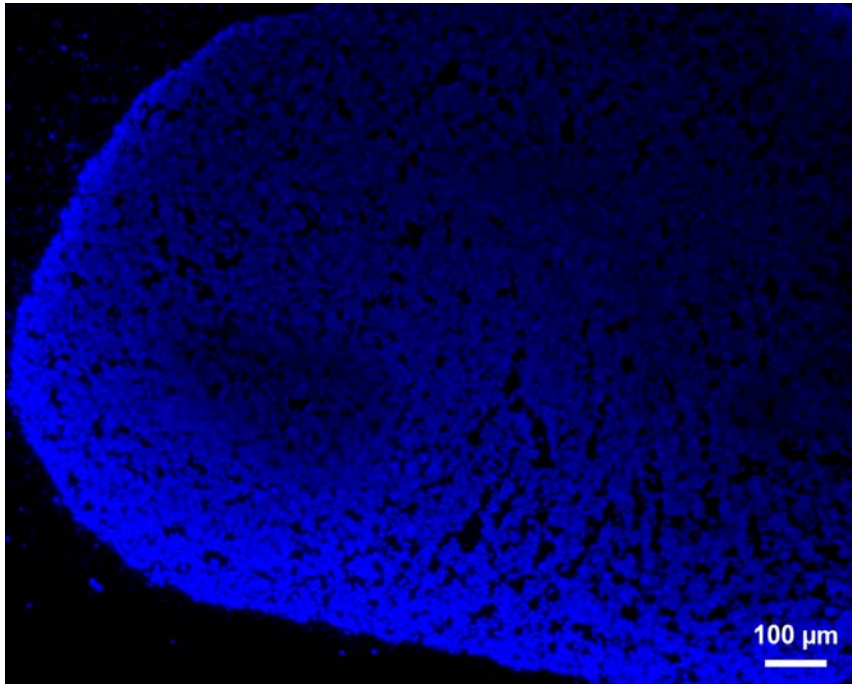
•Paraffin section of head and neck squamous cell carcinoma

•Tumour lymphatics stained for LYVE-1, detected by AlexaFluor568 (red) and ESAM, detected by amplification (CSA) with a streptavidin-488 (green), counterstained with DAPI (blue)

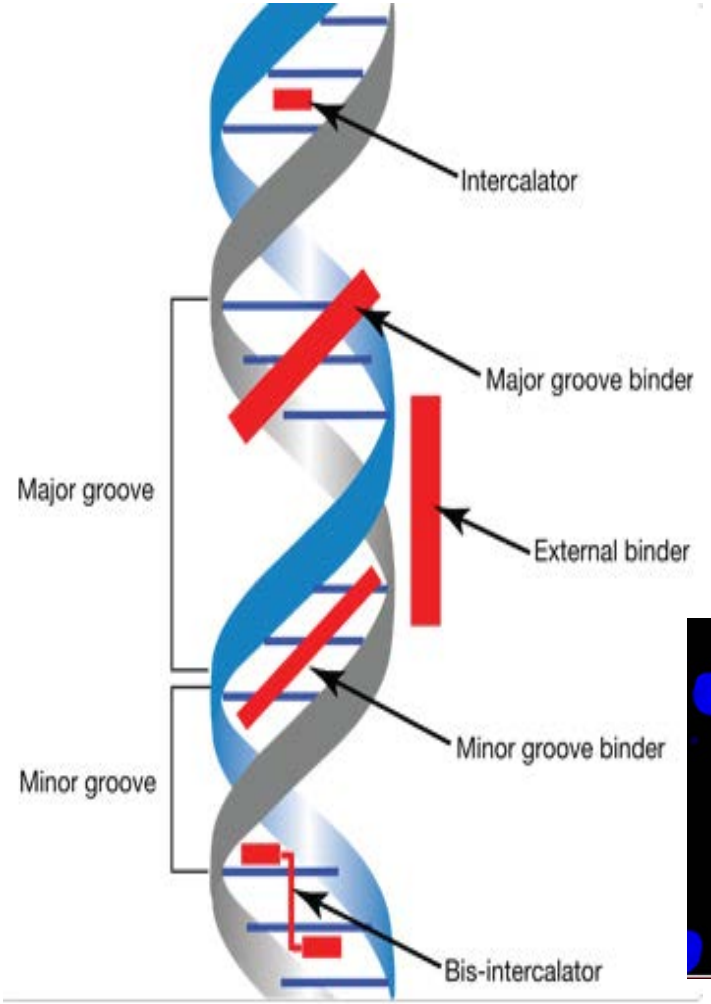
Counterstains

- Reduce background fluorescence
- Identify cellular organelles
- Finding your sample down the microscope

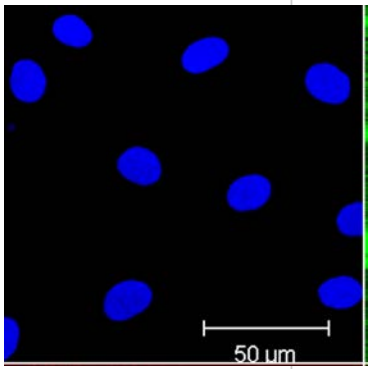


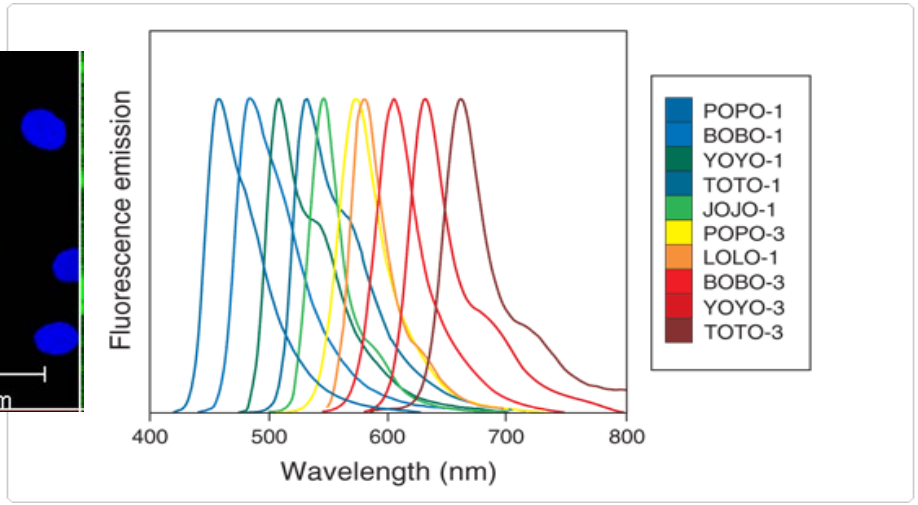


Nuclei Stains



- **DAPI** and **Hoechst** dyes are minor-groove binders
- **Ethidium bromide** and **propidium iodide** are intercalating dyes
- Molecular Probes® dyes **Cyanine monomers** (TOPRO family) and **Cyanine dimers** (TOTO family) provide dyes with a broad range of spectral characteristics to match most excitation sources.





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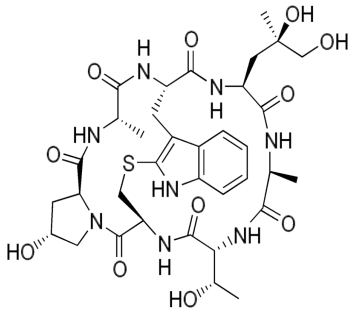
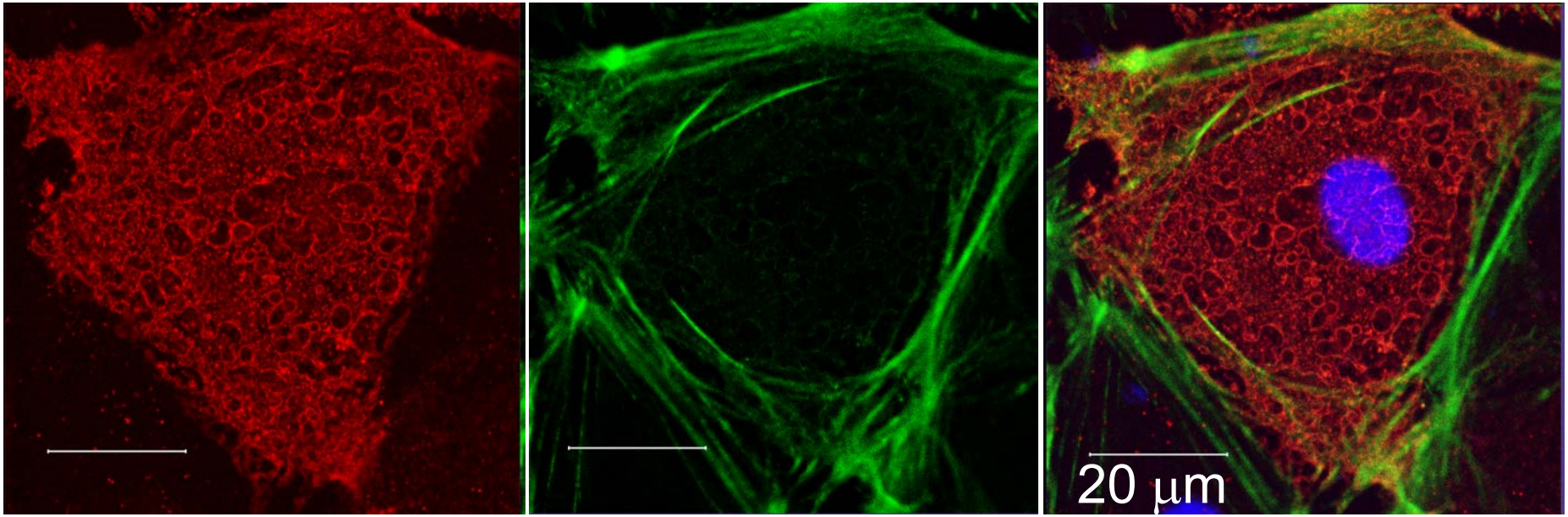
Counterstains - phalloidin

- Cyclic peptide, conjugated to a variety of fluorophores
- Binds F actin and prevents depolymerisation
- Cells must be fixed (use LiveAct to image live cells)

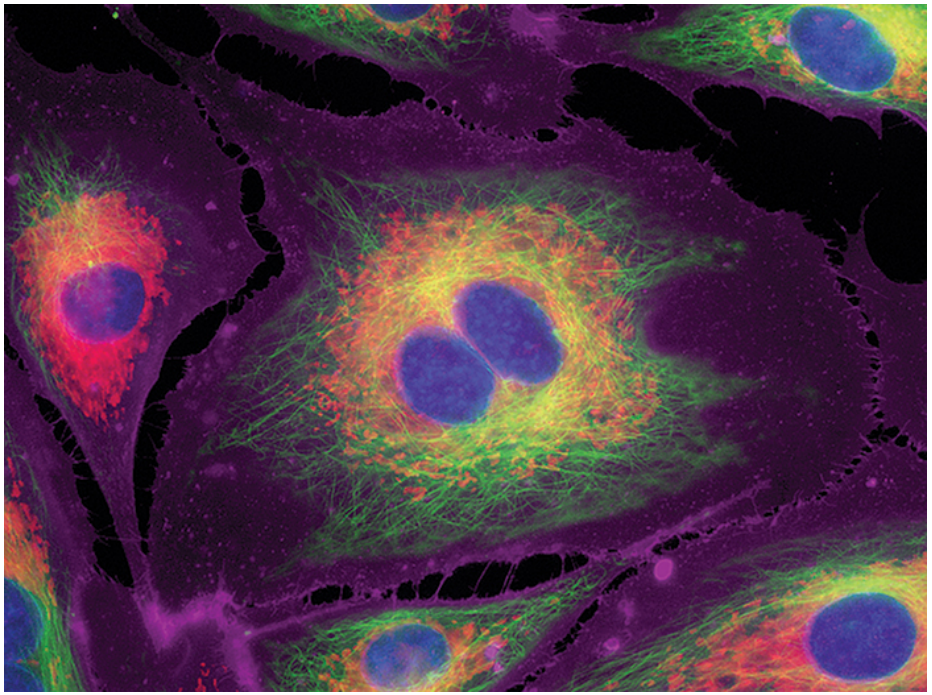




LYVE-1Phalloidin DAPI

Labeling plasma membranes

- CellLight®, CellMask®, Wheat germ agglutinin (WGA)
- WGA is a lectin, binding N-acetylglucosamine and N-acetylneuraminic acid



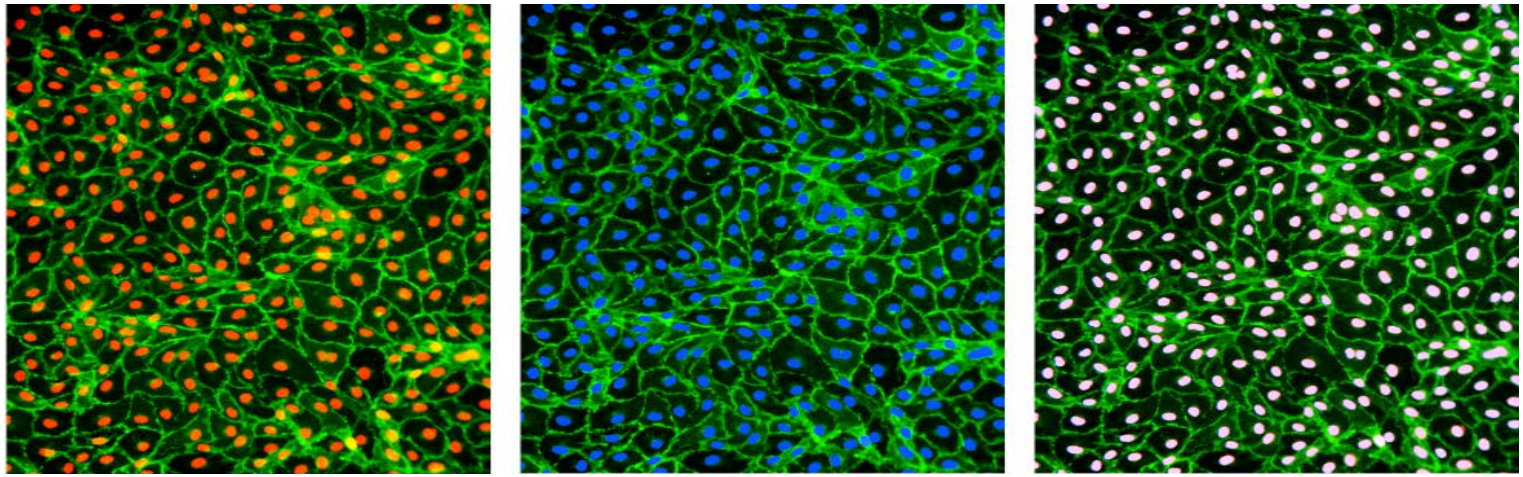
6. Mounting the sample

- Mounting medium
  - Reduces dehydration
  - Sticks coverslip to sample
  - May be organic solvent based or water-based (depending upon microscope objective)
  - For immunohistochemistry:
    - E.g. Aquamount Improved (BDH)
  - For immunofluorescence:
    - Vectashield mounting medium provides strong initial fluorescence and retards photobleaching during microscopic examination and storage)
    - DAPI may be included in mounting medium (4',6 diamidino-2-phenylindole), producing a blue fluorescence with excitation at approx. 360 nm and emission at approx. 460 nm when bound to DNA. No emission overlap with fluorescein, AlexaFluor488, 568, 594 etc.
- Coverslip, secured by CoverGrip Sealant (BEWARE if using oil for lens immersion) or hard-set mounting medium

Results. What can you expect to get?

Staining cells

- Primary human dermal endothelial cells
- Fixed with 4 % PFA, 5 mins r.t.
- Permeabilised with triton x100 0.5 %, 10 mins, r.t.
- Non-specific binding blocked with 1% BSA + 10% goat serum, 20 mins r.t.
- Stained for a transcription factor (Prox1) and a cell junctional protein (CD31), 4^{o} O/N in blocking buffer
- Secondary antibodies AlexaFluor568 and AlexaFluor488 used, 30 mins r.t.
- Counterstained with DAPI (blue); epifluorescence microscopy

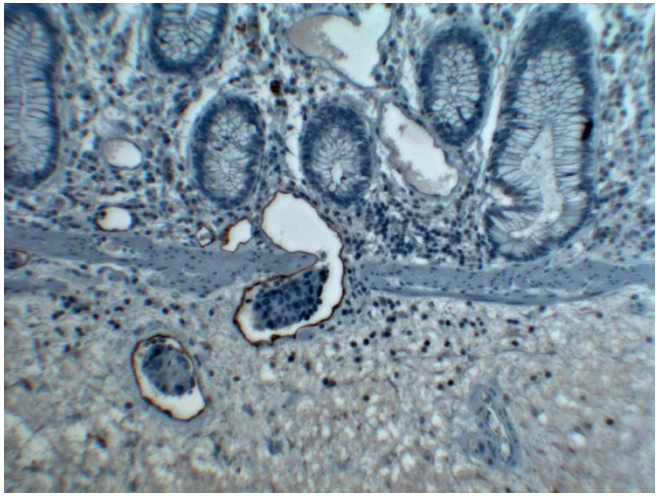


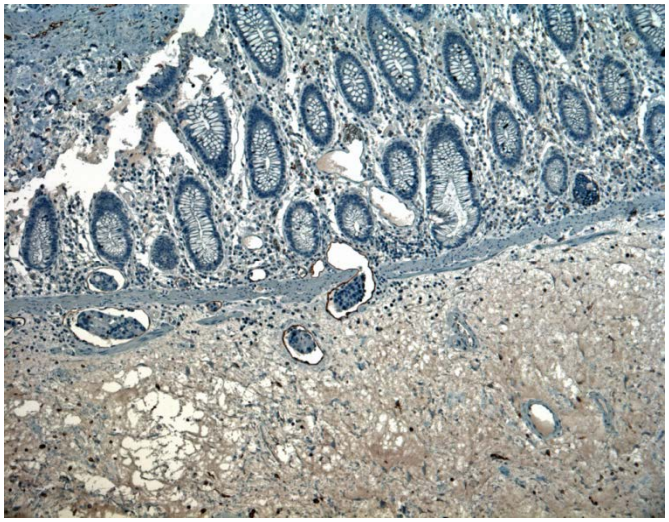
Prox1 CD31 DAPI CD31 Prox1 DAPI CD31

Immunohistochemical staining of paraffin-fixed sections

Human colonic mucosa and submucosa

- Antigen retrieval
- Stained for LYVE-1 expression (brown)
- Detected using goat anti-rabbit HRP and DAB substrate
- Counter-staining with hematoxylin (blue)
- Mounted with Aquamount
- Light microscopy

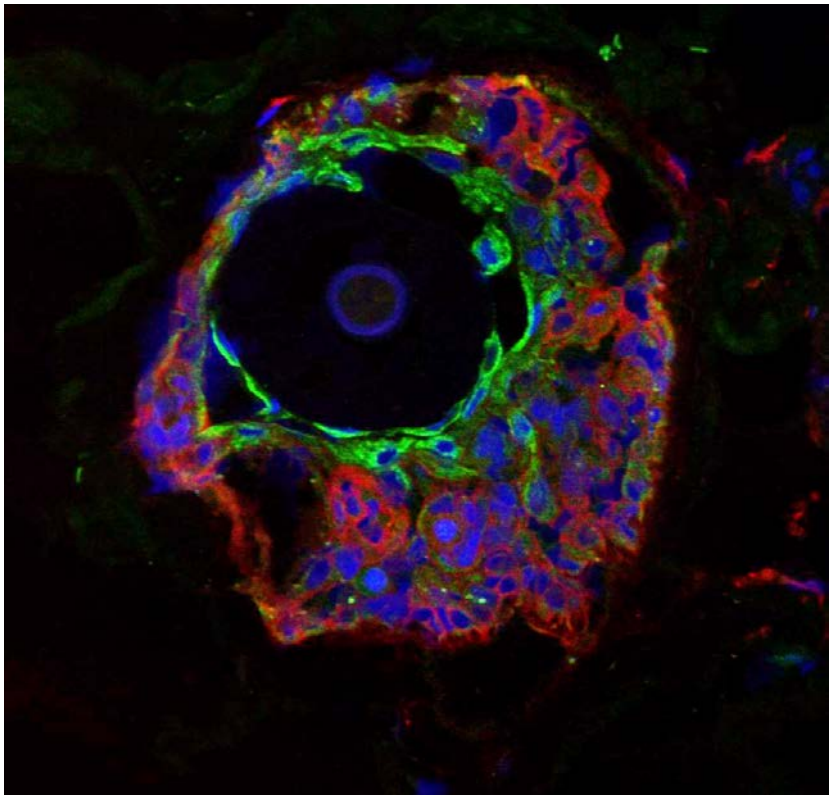




Immunofluorescence staining of frozen tissue

Frozen sections of human skin

- Fixed in PFA 1% 5 mins r.t.
- Blocked with BSA 1% + FCS 10 %
- Stained for CX3CL1 and podoplanin, 45 mins r.t. in blocking buffer
- Secondaries applied: AlexaFluor488 (green) and AlexaFluor568 (red), 30 ins r.t., in blocking buffer
- Mounted in Vectashield-DAPI
- Confocal microscopy

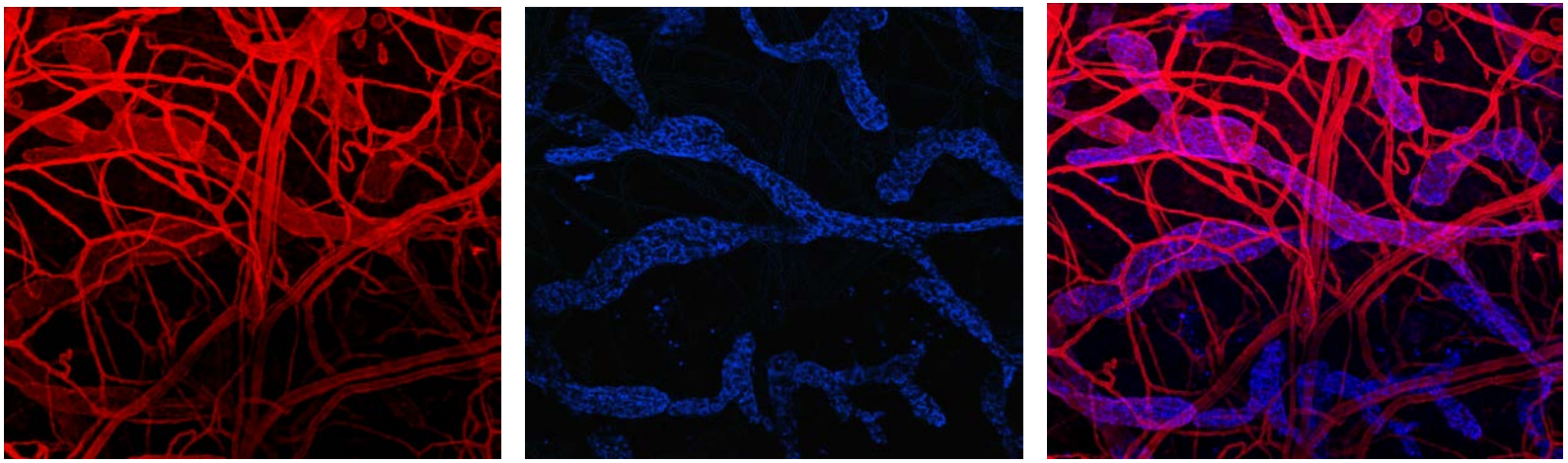


CX3CL1 Podoplanin DAPI

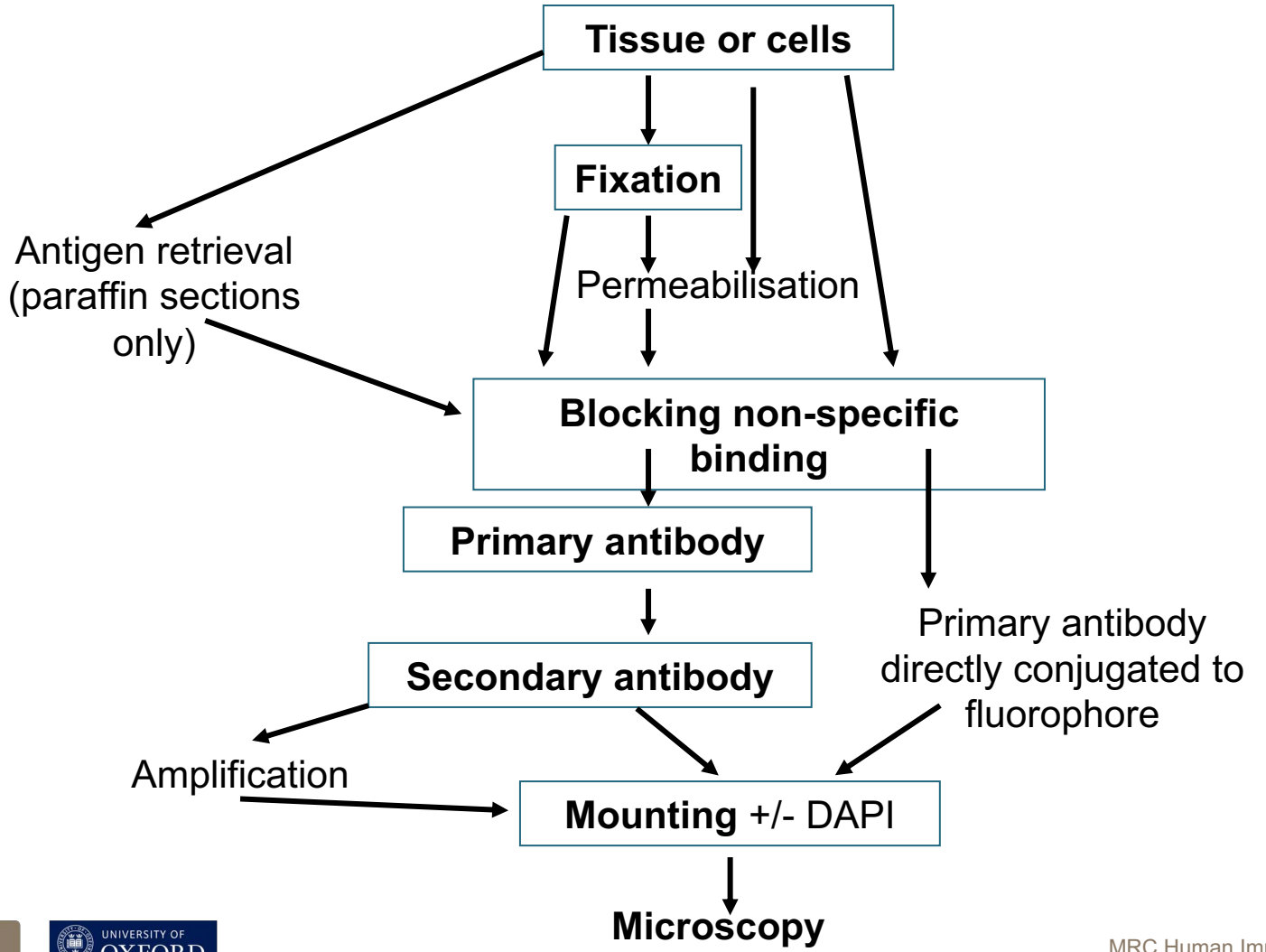
Whole-mount staining

Mouse dermis, < 1 mm thick

- Fixed with 1 % PFA, 4^{o} O/N
- Washed and permeabilised with triton X100 0.3 %, 3 h, r.t.
- Non-specific binding blocked with 1% BSA + 10% goat serum, 2 h r.t.
- Primary antibodies applied in blocking buffer with triton X100, 4^{o} O/N
- Washed in PBS-triton X100 0.3 %, 3 h, r.t.
- Secondary antibodies AlexaFluor568 and AlexaFluor647 applied, 2 h r.t.
- Washed in PBS-triton X100 0.3 %, 3 h, r.t.
- Mounted in Vectashield and viewed by confocal



Basic protocol for immunostaining



Sources of more information

- Molecular Probes (ThermoFisher Scientific)
- https://www.biolegend.com/spectraanalyzer
- DAKO cytomation (for immunohistochemistry techniques and amplification methods)
- BD Falcon (tissue cultureware)
- Vector Laboratories (mounting medium)
- Sigma (detergents, general lab reagents)
- Antibodies:
  - Bio-Rad (AbD Serotec)
  - Biotechne (R and D Systems)
  - BD Biosciences
  - Chemicon (Millipore)