Overview of Immunohistochemistry
(with a focus on wax-embedded sections)
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Diagram 1: Illustration of Indirect immunohistochemistry and Immunofluorescence methods.
“IHC is like cooking. There are many recipes out there, but some of them do not work out very well. However, when they do, they are great! You need the right ingredients, a dose of experience, a few tricks from old cooks, and a grain of common sense”.

There are several traps to avoid…. 

- sensitivity and specificity of the antibodies and technical procedure used are crucial to avoid false-positive and false-negative results.
False-positive and false-negative results can be caused by:

- Primary antibody failing to detect their target antigen, even if it is present in the tissue
- **Why?**
  - conformation changes induced by fixation or embedding
  - low affinity of the antibody for the target
  - failure of antibody to penetrate into tissue
- Antibodies binding non-specifically to other targets or tissue components (both primary and secondary antibodies)

**Blindly following an established protocol may prove insufficient. Each different antibody needs optimisation of the general protocol to ensure specific binding.**
FIXATIVES
Influence of tissue preparation - fixatives

Fixation changes the chemical properties of tissue constituents and alters 3D protein conformation by cross-linking. It has a major impact on affinity and selectivity of antibodies. Epitope masking can also occur when fixation alters penetration of antibodies into the tissue.
dependence of GABAA receptor $\alpha_3$ subunit-immunoreactivity on fixation
For fixatives consider:

Reducing or enhancing fixation:

- Type of fixative (eg, formalin vs paraformaldehyde)
- pH
- Concentration of fixative (eg, 4% vs 10%)
- Duration of fixation
- Use of additives (eg, picric acid)

Antigen retrieval
ANTIGEN RETRIEVAL
Antigen Retrieval

- Epitope masking can occur when fixation alters penetration of antibodies into the tissue.
- Antigen retrieval breaks down cross-links to expose the epitope and allow the primary antibody to bind.
- Several retrieval methods exist, designed to break different types of cross-link.

Eg:
- heating/boiling in acidic buffer
- enzyme digestion (eg, trypsin, Protease K)
- detergent (eg, triton, Tween20)

Choose carefully, depending on nuclear or cytoplasmic staining
repeated freeze/thaw

Beware false positive after Ag Retrieval
The type of retrieval matters…

(A) Formalin-fixed, paraffin-embedded osteosarcoma sample after CD31 staining with standard heat induced epitope retrieval at 98°C with optimized enzymatic epitope retrieval.

(B) Formalin-fixed, paraffin-embedded osteosarcoma sample after FOXP3 staining with standard heat induced epitope retrieval at 98°C with optimized epitope retrieval at 127°C.

The type of retrieval matters…

<table>
<thead>
<tr>
<th></th>
<th>Citrate Buffer</th>
<th>Tris-HCl+5%Urea</th>
<th>Citraconic Anhydride</th>
<th>Frozen Section</th>
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<tr>
<td><strong>CD4</strong></td>
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<td><strong>PAR4</strong></td>
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ANTIBODY SPECIFICITY
Antibody Specificity

Monoclonal Antibodies:

- Mouse challenged with antigen
- Spleen Cells
- Myeloma Cells
- Fusion
- Hybridomas
- Culture in HAT Medium
- Select for positive cells
- Harvest monoclonal antibodies

Polyclonal Antibodies:

- A solution containing a specific antigen is injected into a rabbit; the rabbit is immunized.
- Antiserum is taken from the rabbit; the supernatant contains the antibodies of interest.
- Antibodies are incubated with sepharose beads that is conjugated to the original antigen.

Antibodies that recognize the original antigens form a complex and are attached to the beads; unbound antibodies pass through the column, known as flow through.

The beads are washed by buffers in order to wash out the remaining unbound antibodies and weakly bound antibodies; antibodies that strongly bind to the antigen will stay.

Antibodies of interest are eluted at acidic pH by disrupting the interaction between antibodies and antigens; the eluent is required to neutralize afterwards.
Primary Antibody Specificity: controls

- Ideally, a tissue section should remain unstained after IHC processing if it is devoid of the target antigen.
- In practice, this is generally not the case - IgGs bind with low affinity to numerous (mostly unidentified) tissue constituents.
- Non-specific signals in tissues devoid of their target, such as a section from a knockout mouse, can also display non-specific staining.

How to know staining is real?

CONTROLS!

1. Knockout mice
2. Two antibodies raised against different epitopes of the antigen of interest (should show identical staining pattern)
3. Inactivate antibody with its antigen prior to use (does not control for several targets sharing a common epitope recognised by the antibody)
4. Positive control tissue - where staining pattern is known.

Also:
Use blocking solutions
Optimise temperature, concentration and duration of incubation, as well as duration of rinsing steps.
Secondary Antibody Specificity: Blocking background staining

- Secondary antibodies are raised against IgGs of the species in which the primary antibodies were raised.
- Used in fairly high concentration - non-specific binding to tissue components (e.g., ECM) is higher than for primary Abs.

![IgG KO mouse](image)

- May also cross-react with IgGs from other species (relevant for dual staining).
- Use highly adsorbed secondary antibodies.
- Use blocking solutions.
- Optimise temperature, concentration and duration of incubation, as well as duration of rinsing steps.
- Use controls: No primary antibody
Secondary Antibody Specificity: Blocking non-specific binding
ENDOGENOUS PEROXIDASE
False positive: Endogenous peroxidase

A. In tissue where endogenous peroxidase activity hasn’t been blocked, DAB will react with peroxidase naturally found in the tissue and give a false positive background result.

B. Blocking this peroxidase activity by incubation with hydrogen peroxide (H2O2) eliminates this problem.
**Remember:**

**Assess for false positives:**
- Does the tissue autofluoresce?
- Are there endogenous peroxidases?
- Do the secondary antibodies alone produce staining?
- Does the primary antibody label the expected structures?

**Assess for false negatives:**
*Failure to detect an antigen does not mean the antibody doesn’t work.*

- Is antigenicity lost during fixing?
- Does tissue processing have a deleterious effect? (try frozen sections)
- Does antigen retrieval give positive staining?
- Did you dilute the antibody too far in advance? (Abs stick to plastic!)
- Did you add peroxide to DAB substrate?
- Did you use the correct secondary?
- Did you make up ABC at least 30 minutes prior to use?
- Do you need to amplify the signal?
Test 1: Does the antibody stain without antigen retrieval?

Method: Follow general staining protocol, using dilutions of 1:50, 1:100 and 1:200 of primary antibody on test sections (or dilutions within range of suggested concentration to use on data sheet).

Result: no staining at all?
- Test 2: Try trypsin digestion (antigen retrieval)
  Method: General staining protocol with same range of primary Ab dilutions, but pre-treat with trypsin digestion (0.1% trypsin in TBS, 10 minutes, 37°C).
  - no stain: Test 5: Try heat Antigen retrieval
  - weak stain: Test 6: Try test 3, but with trypsin pre-treatment
  - overstained: Test 7: Try test 4, but with trypsin pre-treatment

Result: weak staining with lower dilutions?
- Test 3: Try overnight incubation of primary.
  Method: General staining protocol with same primary Ab dilutions, but incubate overnight at 4°C.
  - weak stain: Test 8: Try more conc. primary, test 2, 5, 12 or different primary
  - overstained: Test 9: Try test 4 with overnight incubation

Result: Overstained with lots of background?
- Test 4: Try more dilute primary.
  Method: Repeat general staining protocol but with more dilute antibody range (eg, 1:300, 1:500)
  - weak stain: Test 10: Try intermediate Ab dilutions
  - overstained: Test 11: Try lower dilutions, different blocking buffers, or test secondary not binding.

Method: General staining protocol with same range of primary Ab dilutions, but pre-treat with heat (95°C, 10 mins, 10mM citrate buffer, pH6).

- no stain: Test 12: Try heat Ag retrieval + triton X100
- weak stain: Test 13: Try test 3, with heat pre-treatment
- overstained: Test 14: Try test 4, with heat pre-treatment

Method: General staining protocol with same range of primary Ab dilutions, but pre-treat with heat followed by triton X100 (0.5% in PBS, 10 mins, RT).

- no stain: Test 16: Try different primary. Or proteinase K or urea pre-treatment
- weak stain: Test 17: Try test 3, with heat & triton treatment
- overstained: Test 18: Try test 4, with heat & triton treatment

Result at any stage: Clean, clear stain with one or more dilutions? Use this as working protocol.