Introduction

How does it look like?

Smooth muscle actin  Parvalbumin  Dystrophin

Signal versus background

When do we need IHC?

In clinic:

➢ To compare level of expression between
  • non-treated / treated
  • healthy / sick tissue
➢ To identify markers relative to disease status
  • diagnostic
  • prognostic

In research:

➢ To localize newly identified proteins in
  • Control / experimental tissue
Definition

Immunohistochemistry (IHC) technique allows the localisation of proteins (antigen) in tissue sections (Histology).

This is achieved by using antibodies (Immuno) specifically directed against the protein of interest.

Antigen-antibody interaction is visualized by a chemical reaction (Chemistry).

1941 Coons and coworkers first developed the techniques for antibody-mediated localization of tissue antigens.

How to generate an antibody against the protein of interest?

1. Inject the protein to a host (different animal from which the protein is issued), it will react to this foreign substance by synthesizing an antibody.

2. The protein will stimulate the production of cells and produce antibodies that recognize it.
How to generate an antibody against the protein of interest?

1. Host is injected with the protein of interest
2. Antibodies are generated
3. Antibodies can be collected in the serum. This serum contains antibodies to all antigens the host was exposed to.

Concepts:
- Polyclonal antibody
- Monoclonal antibody

Nature of antibodies:
- IgG, IgM, IgA, IgD, IgE
- Fab: antigen-binding fragment, variable fragment
- Fc: constant fragment (of a species), carries the specific antigenic sites for that particular IgG.

Immunohistochemistry
**Tissue sections**

- Paraffin sections • microtome
- Cryo-sections • cryostat
- Fresh sections • vibratome

**Detection system**

Chromogenic detection: an enzyme degrades a substrate (e.g., DAB)  
fluorescence
The method

STEP 1
Make the antigen visible to the antibody
- permeabilisation
- unmasking (when formaldehyde fixation)

Avoid antibody to bind somewhere else
- blocking non specific binding
- what is a good antibody?

STEP 2
Secondary antibody what for?
- same secondary for many primaries
- signal amplification

Avoid secondary antibody to bind somewhere else
- blocking non specific binding
- antibody species

Visualize antibody-antigen binding
- fluorophore (fluorescein, rhodamin, Alexa, etc.)
- biotin, horseradish peroxidase, alkaline phosphatase
**STEP 3**

Amplify signal

- (strept)avidin-biotin-HRP complex
- polymer
- TSA (fluor or other)

**STEP 4**

Color reaction: add substrate of the enzyme

- DAB (brown), NBT/BCIP (blue)

**Introduction**

**History**

- 1941 Coons and coworkers first developed the techniques for antibody-mediated localization of tissue antigens
- 2001-2002 advances in antigen retrieval
- 1999-2007 development of new reagents for antigen detection
- 2004 automation
- 2008 improvements in digital imaging microscopy and image analysis

**Application tips**
Before you kill your animal what do you have to think about?

1. What do I want to do with the stained section?
2. Should I fix the tissue or not?
3. Will I freeze the tissue or embed it in paraffin?

**Answer**
- Depends on the primary antibody!
- Depends on the tissue!
- Depends on what type of analyses I want to do

Where did I get my antibody from?

1. Commercially available
   - Look in the data sheet
2. I got it from somebody
   - Did this person performed IHC?
   - Which animal was used?
   - Ask for the information
3. Home made or no information
   - Test your antibody first!
   - Test all conditions

Take Home Message

If the antigen that you will be probing for in a tissue is destroyed during fixation and/or processing or if the antibodies are unable to penetrate a tissue to gain access to that antigen, nothing you do in latter steps will result in a successful outcome. Therefore, making sure that the study design includes the appropriate tissue fixation and processing for the antibodies you plan to use for IHC is important.
Reaction at a glance

Tissue section preparation → permeabilization, unmasking
  Blocking → endogenous molecules, unspecific binding of antibodies
  Primary antibody → binds to protein of interest
  Secondary antibody → binds to primary antibody, conjugated with marker (fluor, biotin, enzyme)
  Detection → chemical reaction with the marker to obtain a colored reaction that will stain the cell expressing the protein of interest

Unmasking, permeabilisation

1. Unmasking: give accessibility to the antigenic site (epitope) that was masked through fixation (cross-linking)

   ![Diagram](image1.png)

   - Aldehyde
   - Cross-Link
   - Antibody
   - Protein
   - HEATING

2. Permeabilisation: open cell membranes to allow antibodies to penetrate into cells

Unmasking: methods

1. Heat Induced Epitope Retrieval (HIER):
   - Heating provides the energy not only to rupture the hydroxyl bonds between amino acids due to the fixative, freeing some antigens, but also to release tissue-bond calcium ions which contribute to tighter bonds.
   - Buffer used can be acidic (citrate pH6) or basic (TRIS-EDTA pH8) depending on total charge of the protein.

2. Enzymatic treatment: trypsin, protease, pepsin (or cocktail)
   - It is thought that enzymes break the cross-linking bonds due to the fixative to reveal antigenic sites.

Permeabilisation: methods

1. Detergent: Triton X-100, Tween 20
   - Extraction of soluble cellular material

2. Enzymatic treatment: Proteinase K
   - Creates pores in the cell membrane
Blocking

1. Unspecific binding of antibodies

2. Endogenous enzymes (HRP, AP) or molecules (avidin, biotin, SA)

Blocking: methods

1. Blocking of proteins: normal goat serum or BSA
   - An excess of proteins (e.g. serum from species that secondary Ab was made in, other serum, BSA, casein,...) will cover the tissue and prevent primary and secondary Ab to bind non-specifically to tissue.

2. Blocking of endogenous enzymes: H₂O₂ / levamisole
   - H₂O₂ is a substrate for peroxidase and so will “occupy” all endogenous peroxidase (e.g. RBC) so that the only peroxidase able to convert the substrate DAB will be peroxidase linked to Ab.
   - For AP enzyme blocked with levamisole

3. Blocking of endogenous biotin:
   - High endogenous level of biotin can be found in frozen kidney, liver, and intestinal organs. In FFPE there should not be any background (only if HIER with Tris/EDTA). If adding avidin, this endogenous biotin will be saturated, so that labelled avidin will only bind to biotin conjugated to antibody. Instead of biotinylated antibody you can use a polymer.

Immunoreaction

1. Primary antibody: detects protein of interest (eventually labelled)

2. Secondary antibody: labelled antibody that detects primary antibody

3. Amplification: addition of a labelled third reagent

Labels:
- Fluorochrome
- Enzyme
**Immunoreaction: methods**

- **Direct method:**
  - Primary antibody labeled

- **Two steps indirect method:**
  - Secondary antibody labeled
  - Polymer

- **Three steps indirect method:**
  - (Strept)avidin-biotin technologies (ABC)

**Detection**

1. Fluorescent label: observation
2. Enzyme: add substrate to get a colored precipitate

**Detection: methods**

- **Chromogenic:**
  - Horseradish peroxidase (HRP) / DAB (brown), AEC (red), chloro-naphthol (blue-grey)
  - Alkaline phosphatase (AP) / Fast red, NBT/BCIP (blue)

- **Fluorescence:**
  - Alexa...
  - FITC
  - Rhodamin
  - Quantum dots
  - Other

**Antibodies species**

- **Polyclonal Ab**
  - goat anti-rabbit
  - rabbit anti-mouse
  - mouse anti-antigen

- **Monoclonal Ab**
  - goat anti-mouse
  - mouse anti-mouse

Detailed protocol: IHC for NeuN

**Tissue:** mouse brain sections FFPE (formalin fixed paraffin embedded)

**Antibody:** mouse anti-NeuN monoclonal

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dewaxing</td>
<td>60°C</td>
<td>45 min</td>
</tr>
<tr>
<td>Tissue: mouse brain sections FFPE (formalin fixed paraffin embedded)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody</td>
<td>1:2000 in antibody diluent</td>
<td></td>
</tr>
<tr>
<td>Rehydration</td>
<td>37°C</td>
<td>60 min</td>
</tr>
<tr>
<td>RT 30 min</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>PBS RT 5 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microwave in Tris-EDTA pH 8.0 90°C 20 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cooling RT 30 min</td>
<td>2 x short</td>
<td></td>
</tr>
<tr>
<td>Rinse in PBS RT 5 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blocking</td>
<td>10% donkey serum in PBS/T RT 10 min</td>
<td></td>
</tr>
<tr>
<td>Rinse in PBS RT 5 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% H2O2 in 10% methanol in water RT 10 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinse in PBS RT 5 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHC NeuN antibody 1:2000 in antibody diluent 37°C 60 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinse with PBS/T RT 2 x short</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinse with PBS/T RT 5 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotinylated Donkey anti-mouse 1:100 in PBS 37°C 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prepare A/B complex during this time (1/100 of A and B in PBS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinse with PBS/T RT 2 x short</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/B complex RT 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinse with PBS RT 2 x short</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAB, (1 drop of DAB sol.B in 1 ml of DAB sol.A) RT (3 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stop reaction in water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrate and mount</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Results**

IHC for NeuN on FFPE section of mouse brain (cerebellum)

*With HIER*  
*Without HIER*

**Looks straightforward but is it really so easy???
Example: unmasking

No pretreatment  Short HIER pH6  Long HIER pH6

3 different pretreatments: where is the truth?

Example: unmasking

Protease  HIER pH6

2 different pretreatments: where is the truth?

Controls
The top 6 slides

<table>
<thead>
<tr>
<th>Control</th>
<th>Slide treatment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Is the signal specific for this Ab?</td>
<td>No primary Ab</td>
<td>-</td>
</tr>
<tr>
<td>2. Is the signal due to that specific Ab or to any Ab of the host?</td>
<td>Host serum (non-immune serum)</td>
<td>-</td>
</tr>
<tr>
<td>3. Is the signal due to the binding of secondary Ab to primary Ab?</td>
<td>No secondary Ab</td>
<td>-</td>
</tr>
<tr>
<td>4. Is the signal due to endogenous enzymes (HRP) or biotin?</td>
<td>No detection</td>
<td>-</td>
</tr>
<tr>
<td>5. Is the signal specific for this Ab?</td>
<td>Negative tissue</td>
<td>-</td>
</tr>
<tr>
<td>6. Does the protocol work?</td>
<td>Positive tissue</td>
<td>+</td>
</tr>
</tbody>
</table>

Troubleshooting
1. No signal

- Did you test your antibody on a Western blot loaded with tissue extract?
- Test other tissue pretreatments
- Test other tissue preparation
- If it exist test another antibody
- If signal very faint amplify it
Troubleshooting

2. Background

- Background due to species of primary antibody (e.g. mouse)
  - Find an antibody from another species
  - Use an intermediate antibody (e.g. MAb rabbit-a-mouse)

- Background due to secondary antibody
  - Use affinity purified antibodies
  - Block with serum of the secondary antibody species

- Background due to detection system
  - Block endogenous enzyme (HRP or AP)
  - Use polymer system instead of biotinylated secondary antibodies
  - If studying skin avoid to use DAB detection

Troubleshooting

3. Multiple stain

- Use affinity purified antibodies from different species (be cautious with rat and mouse antibodies, they often cross-react or react with mouse Igs)

  - If antibodies from the same species (e.g. Rb-a-X and Y):
    1. Incubate with Rb-a-X
    2. Incubate with an excess of unconjugated Fab fragments directed against host of primary antibody (G-a-Rb)
    3. Detect with a labelled antibody directed against the host of your secondary antibody (Ms-a-G)
    4. Incubate with Rb-a-Y
    5. Detect with a labelled a-Rb antibody

Tests for unknown Ab and tissue

- Animal
  - PFA fix
  - No fix

- Paraffin
  - HIER pH6
  - HIER pH8
  - Protease

- Frozen
  - PFA fix
  - Acetone fix
  - HIER pH6
  - HIER pH8
  - Protease

But it is not finished!!!

- Test several proteases
- Test several proteases concentrations
- Test several primary Ab concentrations

Take home message

ALWAYS BE CRITICAL!
The End

Ref:
- "Lessons in immunohistochemistry". Dapson et al. 2005
- Workshops of the "Annual convention of NSH", Fort Lauderdale 2005
- "Introduction to immunocytochemistry" second Ed., Polak and Van Noorden 1997
- "The histochemical society annual short course", vol. 1 2009