

# Common Causes of Background in Immunocytochemistry

## ***Hydrophobic Interaction***

### What is it?

Hydrophobic interactions occur between macromolecules in aqueous media when their surface tension is lower than water. This attraction is the result of van der Waals forces. Hydrophobicity is a property shared by all proteins and is imparted through the side chains of neutral amino acids phenylalanine, tyrosine, and tryptophan. These have lower attraction for water molecules so they link together and force out water molecules. Hydrophobicity is one of the natural forces that confers stability on the tertiary structure of peptides and can also link different protein molecules together.

### Why is it a problem?

Proteins become more hydrophobic by fixation with aldehyde-containing reagents, such as formalin, as the reactive amino acids are cross linked within and between adjacent tissue proteins. The extent of hydrophobic cross-linking during fixation is a function of time, temperature, and fixative, so it will change if the fixation protocol changes. (Therefore, once optimized, the fixation protocol should be controlled.)

In addition, immunoglobulins are very hydrophobic. In general, subclass IgG3 and IgG1 are more hydrophobic than IgG2 and IgG4. Please remember that secondary antibodies are subject to this type of background as well. Other factors that increase immunoglobulin hydrophobicity:

1. Isolation techniques that promote formation of aggregates
2. Storage conditions that lead to aggregates

### What tissues and cells does it affect?

Connective tissue: collagen, laminin, elastin, proteoglycans

Squamous epithelium: keratins

Adipocytes: lipids

### How do I fix it?

1. Blocking protein in separate step and/or in antibody diluent.
  - Protein blocking should occur just prior to application of primary antibody
  - Bovine serum albumin, non-fat dry milk, casein, normal sera
2. Excessive background from formalin overfixation can be remedied by postfixation with Bouin's, Zenker's, or B5 fixative. (Caron BL and Banks. Lab Investig 1979; 40:244-45.)
3. Lower the ionic strength of the antibody diluent.
  - The closer the diluents' pH to the antibody isoelectric point (pI), the stronger the hydrophobic interaction.

- Biotinylation can change the pI of the antibody
4. Add detergent (Tween 20) or ethylene glycol to diluent
    - \* CAUTION – can damage some antibodies
  5. Raise pH of diluent for polyclonal antibodies
    - They will not have a single pI like monoclonal antibodies.

### ***Ionic and Electrostatic Interactions:***

#### What is it?

The isoelectric point (pI) is the pH at which a molecule carries no net electrical charge. In order to have a sharp pI, a molecule must be amphoteric, meaning that it has both acidic and basic functional groups. Proteins and amino acids meet these requirements.

At a pH below the pI, the protein will carry a net positive charge. Above the pI the protein will carry a net negative charge. For example, in a buffer with the pH of 7.4 a protein with a pI of 9 will have a positive charge. If the pI of the protein is 6, the protein will have a negative charge. Proteins with a positive charge will be attracted to proteins with a negative charge and visa versa.

#### Why is it a problem?

Immunoglobulins have a pI that range from approximately 5.8 to 8.5. At physiological pH and at the pH for most antibody diluents, antibodies can have a net positive or negative charge. If the tissue has the opposite charge, then the antibody will stick to it. For example, it has been reported that the negatively charged sites on the endothelium and collagen fibers can interact with the positively charged rabbit Fab fragments and horseradish peroxidase type VI (Pino RM. J Histochem 1985;33:55-58.)

It has been suggested that electrostatic charges were the cause for increased nuclear background seen occasionally with antigen retrieval (Wieczorek R et al. J Histochem 1997;20:139-143). Retrieval using 1% zinc sulfate, 0.01 M citrate (pH 6.0), or 0.01 M Tris (pH 9.0) had this nuclear background staining a small percentage of the time which was eliminated by increasing the antibody dilution.

Also consider that avidin is a glycoprotein containing 10% carbohydrates and a pI of 10. At physiological pH, it can non-specifically bind to negatively charged tissue compartments. Streptavidin contains no carbohydrates and has a pI of 5. It will not non-specifically bind to lectins or extracellular matrix proteins.

#### What tissues and cells does it affect?

All tissues

Charged extracellular matrix proteins

### How do I fix it?

1. Add NaCl to diluent buffer to neutralize charges
  - \* NOT recommended for monoclonal antibodies
2. The addition of proteins in blocking step and in antibody diluent can also neutralize charges
  - \* CAUTION – it can also increase hydrophobic and electrostatic interactions

### ***Endogenous Enzyme and Pseudoperoxidase Activity:***

#### What is it?

Peroxidase activity is the result of the decomposition of H<sub>2</sub>O<sub>2</sub> and is a common property of all heme containing proteins such as hemoglobin (red blood cells and hemorrhage), myoglobin (muscle cells), cytochrome (granulocytes, monocytes), and catalases (liver and kidney).

Alkaline phosphatase (AP) is an enzyme in the blood, intestines, liver, and bone cells. Its chemical structure varies (called isoenzymes) depending on where it is produced. This makes it possible to determine where a problem has originated. When bones are growing, liver cells are damaged, or a biliary obstruction occurs, alkaline phosphatase levels rise considerably.

#### Why is it a problem?

Horseradish peroxidase (HRP) is an enzyme isolated from the root of a horseradish plant. HRP can be linked to a secondary antibody or to a molecule of avidin and utilized in immunohistochemistry. HRP has an iron-containing heme group (hematin) as its active site and is brown colored in solution. The hematin forms a complex with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) causing it to decompose to water and atomic oxygen. The atomic oxygen is then free to oxidize several substances, including polyphenols and nitrates. There are several electron donors that when oxidized become colored, insoluble products called chromagens. These include 3,3'-Diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC).

Calf intestine alkaline phosphatase can also be linked to secondary antibodies or to avidin molecules. AP removes and transfers phosphate groups from organic esters via hydrolysis. In the case of IHC, AP hydrolyzes naphthol phosphate esters to phenolic compounds and phosphates. The phenols couple with colorless diazonium salts to produce insoluble, colored azo dyes. These include Fast Red and Fast Blue.

#### What tissues and cells does it affect?

- Peroxidases
  - Leukocytes, Red Blood Cells, Muscle, Liver, Kidney
- Alkaline Phosphatase

- Liver, Bone, Leukocytes, Kidney, Ovary, Bladder, Salivary Gland, Placenta, GI Tract

#### How do I fix it?

1. Inactivate endogenous peroxidase with H<sub>2</sub>O<sub>2</sub> solution
  - 3% for 5–10 minutes in paraffin sections
  - 0.3 % for 5-20 minutes in frozen tissue
  - May contain methanol
    - \* Not recommended for detection of cell surface markers or frozen sections
  - The complex formed between HRP and excess H<sub>2</sub>O<sub>2</sub> is catalytically inactive and in the absence of an electron donor (chromogenic substances), is reversibly inhibited.
2. Inactivate endogenous alkaline phosphatase with
  - a. Levamisole solution
  - b. 0.03N HCL for intestine
3. Inhibit enzyme activity with cyanide or azide
  - YIKES! Both very toxic

### ***Endogenous Biotin:***

#### What is it?

Biotin is a B vitamin (B7) that is widely distributed in tissue where it is bound to enzymes and other proteins. It has a very strong binding affinity for avidin.

#### Why is it a problem?

Avidin and streptavidin that is bound to the HRP or AP will bind to biotin in the tissue instead of the biotin on the secondary reagents.

#### What tissues and cells does it affect?

Liver (hepatic nodules), kidney (tubular epithelium), lymphoid tissue (paracortical histiocytes), central nervous tissue, and in adipose tissue.

Cytoplasmic

Worse in frozens

#### How do I fix it?

1. Sequential 10-20 minute incubation with 0.01%-0.1% avidin followed by 0.001%-0.01% biotin prior to staining procedure

## ***Fc Receptors:***

### What is it?

IgG molecules, specific for a certain antigen or surface component, bind to a pathogen or protein by the Fab region. The Fc portion of the antibody can point out and bind to the Fc receptor (FcR) on phagocytes and other cells. Different Fc receptors are expressed on different cell type with variable affinity. After the pathogen has been bound, the interaction of the Fc and the Fc receptor allows the engulfment of the pathogen by the now activated phagocyte.

### Why is it a problem?

As mentioned above, Fc receptors are expressed on a number of cell types and can be induced by inflammatory cytokine, radiation, or infectious agents. In addition, some viruses encode their own Fc receptors as a means to subvert the host immune response. These receptors can bind the primary and secondary antibodies and appear as positive staining in endothelium and white blood cells.

There is immunoglobulin class and species specificity for FcR. Some human FcR will bind mouse monoclonal IgG2a and IgG3, but not other subclasses. In addition, others have found that goat sera will not react with FcR on human leukocytes.

### What tissues and cells does it affect?

Macrophages, neutrophils, eosinophils, Dendritic cells, platelets, Langerhans cells, tissue macrophages, B cells, Mast cells, endothelial cells, and infected cells (Herpes viruses)

Some reports say that this does not happen in fixed tissue. In my experience, this is bogus! But it is much stronger in frozen tissue.

### How do I fix it?

1. Add normal sera containing immunoglobulin to protein blocking step
  - Because Fc receptors have different affinity for different species, use 5% of serum from the host of the tissue you are staining. For example, use 5 % of dog serum in protein block if you are staining dog tissue.
2. Use F(ab') fragments instead of intact Ig molecules
3. 0.025% Triton X-100 may dissolve Fc receptors
  - may also damage your antigen

## ***Endogenous Antibodies:***

### What is it?

Antibodies are present in the sera and bath over the tissue where they can interact with their specific target, such as a pathogen. As a result, they can become trapped in the tissue. In addition, antibody producing cells are located in organs such as the lymph nodes and spleen.

### Why is it a problem?

These antibodies can get trapped in your tissue and serve as a target for your IHC detection. For example, if you are staining mouse tissue with a mouse monoclonal antibody, your mouse specific secondary antibody will recognize both your monoclonal antibody and any endogenous mouse immunoglobulin trapped in the tissue.

Also, applies when using:

- Rabbit antibodies on rabbit tissue
- Goat antibodies on goat tissue
- Rat antibodies on rat tissue
- Dog antibodies on dog tissue
- Human antibodies on human tissue

In addition, the secondary antibody may have a low affinity for antibodies of other species. In this case they will also detect endogenous antibodies in the tissue through cross-reactivity.

### What tissues and cells does it affect?

All tissue, particularly spleen.

### How do I fix it?

1. Use another antibody
  - Use one made in another species
2. Use a directly labeled primary antibody
  - Won't work if you need to amplify the signal
    - Can amplify using antibodies directed against label
  - Techniques to directly label an antibody
    - Labeling antibody can be harsh and time consuming
3. Block endogenous antibodies
  - HAM block
    - Use Horse anti-Mouse to bind to all endogenous antibody
    - Make sure use unlabeled HAM antibody
  - Commercial kits
    - Chemicon Mouse-To-Mouse
    - Vector M.O.M.
    - Zymed Histomouse-SP
4. Complex the primary with the secondary first in a tube
  - Trick is to get good balance between concentration of primary and secondary
  - Block free secondary with mouse serum
  - Then apply to tissue
  - **MUST USE ISOTYPE CONTROL TO EVALUATE SPECIFIC STAINING!**

- Dako ARK kit

5. Use an isotype specific secondary

- Each IgG isotype only makes up 20-25% of the serum
- Most mouse monoclonal antibodies are IgG1
- Does not work very well

6. If secondary antibodies cross react:

- Block with serum from the animal the secondary was made in
- Use a species absorbed secondary antibody
- Reduce concentration of secondary
- Use an isotype specific secondary

### ***Antigen Diffusion:***

#### What is it?

The antigen you are staining may diffused from its site of synthesis or storage into the surrounding tissue. In addition, the antigen may be in high concentrations in the blood plasma and may perfuse the tissue before fixation. Antigens may also be internalized by phagocytes, resulting in staining not normally seen in such cells.

#### Why is it a problem?

This can give you spurious results in regard to the normal distribution of the antigen.

#### What tissues and cells does it affect?

Underfixed tissue.

Edematous tissue.

Tissue macrophages.

#### How do I fix it?

1. Properly fix tissue

### ***Antibody Cross-Reactivity:***

#### What is it?

Many amino acid motifs are shared among proteins. This is particularly true among proteins of like classes or similar function. A monoclonal or polyclonal antibody developed to detect one protein may bind to epitopes shared with other proteins.

This can be used to our advantage, however. Many times antibodies developed against a protein for use in one species will cross react with the same protein in another species.

#### Why is it a problem?

This can produce spurious results and make you think that a protein is expressed in a tissue, by a certain cell type, or during a particular process.

What tissues and cells does it affect?

All tissues, all cells.

How do I fix it?

1. Absorption of antibody against like proteins
2. Screen monoclonal antibody clones to eliminate cross reactive antibodies
3. Try a different antibody that recognized a different epitope.

### ***Natural and Contaminating Antibodies:***

What is it?

When you inject a protein into an animal, the animal reacts to that foreign protein using its adaptive immune response and makes antibodies against it. We have manipulated that system in the lab to make monoclonal and polyclonal antibodies.

Why is it a problem?

Low-level natural antibodies may be present in the sera of animals as the result of prior environmental exposure. These may increase in titer during immunization with the use of adjuvants and can give rise to an increased titer of nonspecific antibodies. It has been reported that rabbits and goats contain environmental antibodies to keratins.

In addition, it is difficult to have a pure antigen preparation when inoculating animals for antibody production. If the host reacts to the impurities, contaminating antibodies will occur. Ask me about the awesome antibody that I once made to glutathione S transferase (GST) instead of CMV US28!!

What tissues and cells does it affect?

All tissue, all cells – depending on contaminating antibody epitope recognition

How do I fix it?

1. Decrease concentration and/or incubation time of primary antibody
  - Natural and contaminating antibodies are usually in very low concentration and can easily be diluted out.
2. Affinity absorb primary antibody
3. Use a different antibody!

### ***Miscellaneous causes of background:***

1. Sections dry out at any time after deparafinization and rehydration can cause reagents to dry on slide

- a. Add detergent (Tween) to buffers to help “spread” solutions out of slide
  - b. Do longer incubations in humidity chamber
  - c. Cover slides with coverslip
2. Folding or lifting of tissue can trap reagents
3. Tissue sections too thick
4. Tissue damage due to antigen retrieval
  - a. Choose gentler solution
  - b. Decrease temperature
  - c. Decrease time
5. Inadequate washing in between steps can leave reagents behind
6. Tissue damage caused by drying before fixation, by incomplete fixation penetration, or from necrosis.
7. Inadequate deparafinization prior to staining
8. Bacterial or yeast contamination of IHC reagents or water bath used at sectioning
9. Additives such as gelatin, egg, or glue included in water bath to increase section adhesion.
10. Undissolved chromagen granules
11. Antibody too concentrated
  - a. Dilute
  - b. Incubate for shorter period
12. Control (isotype) immunoglobulin contaminated with other antibodies