IMMUNOHISTOCHEMISTRY BASICS

Ken Green IHC Laboratory
Division of Anatomic Pathology
Health Care Corporation of St. Johns
Eastern Health
Immunohistochemistry

Immunology + Histology + Chemistry

The Principles of IHC

The Good, The Bad and The Ugly

The Good

Appropriate block, well processed, well fixed excellent 3 mu section.
Excellent IHC demonstrating expected profile, and confirms original suspicion

The Bad

Appropriate block, well processed, well fixed excellent 3 mu section.
Excellent IHF staining with unexpected results.
Repeat, reassess, recheck and seek help through colleagues or outside consultation
The Ugly

Inappropriate block, not well processed or fixed
Cannot be sectioned properly
IHC results are unreliable or erratic
No diagnostic value
Waste of time and resources

In a Nutshell

- Formalin fixed, processed, paraffin embedded Tissue
- Cut at 3 Microns
- Baked in 42 degree oven
- Stained using 3 step indirect streptavidin method
- Sent to Pathologist for interpretation and diagnosis

Simply Stated

IHC is based on the principle of Antigen Antibody Reaction.

Immune System

The Immune system can be divided into two subsystems.
The innate immunity system recognizes microbes or foreign cells that do not belong in our bodies.
The acquired immunity system causes a production of antibodies against antigens.
Definitions

Antibody - protein used by the immune system to identify and neutralize foreign objects like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target.

Antigen - a molecule that stimulates the production of antibodies.

Avidin - protein that binds biotin strongly, forming an irreversible bond.

Biotin - Water soluble B complex vitamin.

Enzyme - a protein that catalyzed a chemical reaction.

Epitope - part of a foreign organism that is being recognized by antibodies.

Factors that influence IHC success

- The avidity of antibodies of the antigen
- The specificity of for the antigen
- How the structure of the epitopes on the antigen are altered during the technique
- How easily the antibody can reach the antigen
- The quality of the secondary reagent

Labelling Method

Three Step Indirect Streptavidin method

1. Primary Antibody against the tissue antigen.
2. Secondary (which will recognize the primary) will be tagged with biotin.
3. Third layer will be a streptavidin enzyme complex allowing the streptavidin to recognize the biotin.

Dab will be used to precipitate a colour reaction.

The benefits of this system is that the sandwich effect of layers gives a stronger and bulkier signal.
Rule 3
Don`t believe everything you read, hear, or see.

FORMALIN PARADOX
Formalin is our best fixative to date, but during the fixation process it forms methylene bridges which mask antigen sites. We have to unmask the antigen sites so that we can produce the antigen / antibody reaction necessary for IHC.

A Case in point
A series of technical developments in IHC have created sensitive detection systems. Among them is the enzymatic (Horseradish peroxidase) developed by Avrameas and colleagues which in the presence of a suitable colorogenic substrate system, allowing visualization of the labeled antibody by orthodox light microscopy. (Dabbs 2002)
OR
A brown colour (Dab) is added to the IHC system so that we can see the antigen antibody reaction under microscope. (Green 2007)

Paradox #2
Antigen/ Antibody reactions are heat sensitive and sections should not be incubated above 60 degrees Celsius. Due to the fact that we may loose Antigenicity, But during Antigen retrieval (AR) sections are boiled at 95 – 100 degrees Celsius to unmask Antigen sites.
Antigen Retrieval

AR is the concept of recovering lost immuno reactivity through exposure to heat or enzyme.

HIER

- Heat induced AR
- Buffers of various pH values used
- Sodium citrate pH 6.0
- EDTA pH 8.0
- Heated to 95 to 100 degrees Celsius
- Short 8 minutes
- Mild 30 minutes
- STD 60 minutes
- Extended 90 minutes

The object is to obtain optimal AR with minimum time as this process is very harsh on tissue, and too much will result in tissue damage.

Paradox #3

Not all Antibodies need AR.
Some Antibodies need Heat induced epitope retrieval (HEIR).
Some Antibodies need Proteolytic induced epitope retrieval (PEIR)

PIER

Proteolytic Induced AR

Enzymes are used to break down the cross link proteins formed during formalin fixation.
Examples:
- Pepsin
- Trypsin
- Pronase
- Protease
- Protease K

In our laboratory we use Protease and Protease K.
Not all antibodies need AR
All antibodies are different
Each antibody has to be assessed on an individual basis to determine the best AR.

Remember
Simply an Antigen / Antibody reaction

Monoclonal Antibodies
Advantages
More specific and less background staining

Disadvantages
More sensitive to tissue fixation

Polyclonal Antibodies
Advantages
Less sensitive to fixation

Disadvantages
Can cause higher background staining
Primary Antibody

- Antibodies are like people
- All are individual
- All share similar characteristics
- Some share many characteristics
- Some share few characteristics
- Some are totally different

Examples:
- CK7 - Cytoplasmic
- ER - Nuclear
- CD3 - Membraneous
- CD68 - Cytoplasmic and Membraneous
- Calretinin - Cytoplasmic and Nuclear

Primary Antibody Validation

When a new Antibody is introduced to the laboratory, it has to be validated. The validation process involves:
- Antibody dilution
- AR requirements
- Primary Antibody incubation time
- Positive controls
- Negative controls
- Comparison to known patient results

Primary Antibody Dilution

- Most are predilute by the manufacturer
- Some have to be diluted to suit the individual laboratory
- Use manufacturer’s recommended dilution and adjust for your laboratory

IHC Antibodies

There is no

ONE SIZE FITS ALL

There is no

ONE SIZE FITS MOST
Protocol

Every antibody has its own protocol
A protocol is a recipe – unique series of steps which are followed to achieve a desired result, each time and every time.

Once the parameters are determined the protocol is set and should not be altered unless the antibody or clone changes

Sample Protocol

Anti CD3 Antibody has been considered the best all-round T cell marker
Remember just an antigen – antibody reaction

As you can see from the extended version of the protocol, there is no such thing as a simple antigen - antibody reaction.

There are many factors which can affect the final result.
IHC GOAL

To ensure run to run reproducibility, evaluated against in-house controls, days, weeks, and months apart.

Factors affecting IHC detection threshold

- Biological variation
- Sample collection
- Fixation processing
- Section thickness
- Section pre-treatment (AR)
- Antibody type or clone
- Antibody dilution
- Antibody incubation time
- Antibody incubation temperature
- Sensitivity of detection kits
- Histochemical reaction

The greatest factor affecting IHC detection threshold is

Fixation and Processing

(90% of IHC staining problems)

Formalin Fixation - Processing

Results in

- Variable morphology and cell content
- Variable shrinkage and Hardening
- Variable masking - destruction of epitopes
- Variable porosity
- Variable basophilic - acidophilic reactions
- Variable intensity of stain

Can lead to variable success - failure of IHC techniques
Rapidly Changing Technology

- One step direct conjugate method
- Multi step detection PAP (Peroxidase Antiperoxidase)
- Avidin biotin conjugate ABC
- Biotin – Streptavidin (B-SA)
- Amplification – Tyramide
- Polymer based Labelling

These systems give greater sensitivity when combined with sensitive Antibodies
- Polyclonal Rabbit
- Monoclonal Mouse
- Rabbit Monoclonals

IHC is the future

- IHC has revolutionized the classification and diagnosis of tumors.
- No longer are we dependent on histology alone or on special stains.

Having just said that

- The most special stain/test still done today is a well fixed, well processed, well sectioned, thin, no knife tracks, H&E or (PAS) stained section to be viewed under the light microscope.

Basics

How to use IHC

- Morphology decides “what is the lesion”
- Benign vs. Malignant
- Differential diagnosis (DDX)
- IHC provides lineage confirmation
- IHC standard of care in surgical pathology
- Benign cells have characteristics distribution of protein epitopes which typically/usually are carried with them into neoplasia.
- Rules and guidelines have been developed
- Sometimes tumors do not by the rules
Morphologically similar tumors are not necessarily antigenically identical. Sometimes tumors do not play by the rules and sometimes tumors do not read the literature. IHC should confirm what you already know taking into consideration the patient history, H&E stain, along with special stains. IHC is only one piece of the puzzle.

### 10 Commandments of IHC

1. Start with a reasonable differential diagnosis (DDX)
2. Never use 1 antibody in a DDX
3. Use a panel
4. Sensitivity must be high
5. Use monoclonal antibodies when possible
6. Control should match slide
7. Use your own database
8. If results are weird investigate further
9. If a beginner or a problem arises GET HELP
10. Send uncommon/rare cases to an expert

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### Summary

**Points to Ponder**

IHC is a fantastic tool to aid in the diagnostic process. When used in conjunction with H & E Morphology, patient history, to confirm what you already know.

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**The End**